HOESSLI • ILANGUMARAN

Daniel C. Hoessli • Subburaj Ilangumaran

GPI-Anchored Membrane Proteins and Carbohydrates

GPI-Anchored Membrane Proteins and Carbohydrates

R.G. LANDES COMPANY

Molecular Biology
Intelligence
Unit 7

GPI-Anchored Membrane Proteins and Carbohydrates

Daniel C. Hoessli, M.D.
Department of Pathology
University of Geneva
Geneva, Switzerland

Subburaj Ilangumaran, B.V.Sc., Ph.D
Department of Pathology
University of Geneva
Geneva, Switzerland

R.G. LANDES
COMPANY
Austin, Texas
U.S.A.

Molecular Biology Intelligence Unit

GPI-Anchored Membrane Proteins and Carbohydrates

R.G. LANDES COMPANY
Austin, Texas, U.S.A.

Copyright ©1999 R.G. Landes Company

All rights reserved.
No part of this book may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording, or any information storage and retrieval system, without permission in writing from the publisher.
Printed in the U.S.A.

Please address all inquiries to the Publishers:
R.G. Landes Company, 810 South Church Street, Georgetown, Texas, U.S.A. 78626
Phone: 512/ 863 7762; FAX: 512/ 863 0081

ISBN: 1-57059-557-7

While the authors, editors and publisher believe that drug selection and dosage and the specifications and usage of equipment and devices, as set forth in this book, are in accord with current recommendations and practice at the time of publication, they make no warranty, expressed or implied, with respect to material described in this book. In view of the ongoing research, equipment development, changes in governmental regulations and the rapid accumulation of information relating to the biomedical sciences, the reader is urged to carefully review and evaluate the information provided herein.

Library of Congress Cataloging-in-Publication Data

GPI-anchored membrane proteins and carbohydrates / edited by Daniel C. Hoessli, Subburaj Ilangumaran.
p. cm. -- (Molecular biology intelligence unit)
ISBN 1-57059-557-7 (alk. paper)
1. Membrane proteins. 2. Phosphoinositides. 3. Glycoconjugates.
I. Hoessli, Daniel. II. Ilangumaran, Subburaj. III. Series.
QP 552.M44G75 1999
571.6'.4--dc21 98-43470
CIP

Publisher's Note

Landes Bioscience produces books in six Intelligence Unit series: *Medical, Molecular Biology, Neuroscience, TissueE ngineering, Biotechnology* and *Environmental.* The authors of our books are acknowledged leaders in their fields. Topics are unique; almost without exception, no similar books exist on these topics.

Our goal is to publish books in important and rapidly changing areas of bioscience for sophisticated researchers and clinicians. To achieve this goal, we have accelerated our publishing program to conform to the fast pace at which information grows in bioscience. Most of our books are published within 90 to 120 days of receipt of the manuscript. We would like to thank our readers for their continuing interest and welcome any comments or suggestions they may have for future books.

Michelle Wamsley
Production Manager
R.G. Landes Company

CONTENTS

1. **GPI-Anchored Biomolecules—
An Overview 1**
Martin G. Low
Discovery of GPI Anchors 1
Distribution of GPI Anchors 3
Why Use a GPI Molecule for Membrane Anchoring? 5
Does the GPI Anchor Have Additional Functions
Besides Anchoring Proteins to the Cell Surface? 7
Summary 11

2. **Biosynthesis of GPIs in Mammalian Cells 15**
Anant K. Menon and Peter Bütikofer
GPI Anchor Assembly 15
GPI Lipid Remodeling 19
Inhibitors of GPI Biosynthesis 20
Attachment of GPI to Protein 20
Subcellular Location and Membrane Topology
of GPI Biosynthesis 21

3. **Organization of GPI-Anchored Proteins in Mammalian
Cell Membranes 29**
Satyajit Mayor and Teymuras V. Kurzchalia
Lateral Heterogeneity in Cell Membranes 29
Association of GPI-Anchored Proteins with Domains,
Detergent-Insoluble Complexes and Caveolae 30
Mechanisms of Detergent Insolubility 33
Direct Evidence for GPI-Anchored Protein Domains
in Living Cell Membranes 34
Functional Implications of GPI-Anchored Protein Domains 35
Summary 38

4. **Signaling through GPI-Anchored Receptors 43**
Subburaj Ilangumaran, Peter J. Robinson and Daniel C. Hoessli
Association of GPI-Linked Receptors with Transmembrane
Proteins into Receptor Complexes 43
Detergent-Resistant Sphingolipid Microdomains as Signaling
Platforms for GPI-Anchored Receptors 48
Sphingolipid Microdomains and Caveolae 51
Localization and Regulation of Src Family PTKs in Sphingolipid
Microdomains 52
Transmembrane Receptors Requiring the Participation
of Sphingolipid Microdomains for Signaling 55
Interactions of the Sphingolipid Microdomains
with Transmembrane Proteins: Implications
for Proximal
Signaling Events 56

Role of the GPI Anchor in Signaling 56
GPI Anchor-Derived Second Messengers 57
Signaling via GPI-Anchored Glycoproteins of the Lymphocytes 57
Summary 59

5. GPI-Anchored Proteins of Leucocytes: Inventory, Structural Properties and Functions 71
Vacláv Horejsí, Petr Dráber and Hannes Stockinger
CD14 71
CD16b 73
CD24 74
CD48 75
CD52 75
CD55 75
CD58 (LFA-3) 75
CD59 76
CD66 76
CD73 76
CD87 77
CD90 78
CDw108 79
CD109 79
CD157 (Mo5, BST-1) 79
RT6 79
Melanotransferrin 80
Ly-6 80
Other Leucocyte GPI-anchored Proteins 81

6. GPI-Anchored Proteins in the Nervous System 93
David A. Harris
Growth Factor Receptors 93
Molecules Involved in Axonal and Synaptic Adhesion 95
Caveolae and Detergent-Resistant Rafts 96
Possible Functions of Rafts in the Nervous System 97
Prions 99
Summary 104

7. GPI-Anchored Complement Regulatory Molecules 109
Carmen W. van den Berg and B. Paul Morgan
The Complement System 109
Decay Accelerating Factor (DAF, CD55) 110
CD59 116
Homologous Restriction Factor 121
Why Are So Many C-Regulatory Molecules Anchored through a GPI Anchor? 122

8. GPI-Containing Molecules of Pathogenic Mycobacteria and Protozoa **131**
Pascal Schneider and Daniel C. Hoessli
Mycobacterial GPI-Linked Molecules 131
GPI in Protozoa 133
Summary 149

9. Enzymes Cleaving the Phosphodiester Bond in the GPI Anchor **167**
Martin G. Low and Urs Brodbeck
GPI Anchor-Cleaving Phospholipases from Bacteria 167
GPI Anchor-Cleaving Phospholipase C (GPI-PLC) from Trypanosomes 169
GPI Anchor-Cleaving Activity in Plants 170
Mammalian GPI-PLC 171
Mammalian GPI-PLD 172
Summary 180

10. Intercellular Transfer of GPI-Anchored Molecules **187**
Isabelle A. Rooney
Possible Functions of the GPI Anchor 187
Evidence that GPI-Anchored Proteins can Transfer between Cell Membranes 188
Mechanisms by which Cells Acquire Exogenous GPI-Anchored Proteins 189
Mechanisms by which Cells Release GPI-Anchored Proteins 190
Importance of Intercellular GPI-Anchored Protein Transfer 192

11. Engineering GPI-Anchored Proteins **197**
Periasamy Selvaraj, Rebecca S. McHugh and Shanmugam Nagarajan
Naturally Occurring Membrane Isoforms of Cell Surface Glycoproteins 197
Transmembrane Proteins Engineered into GPI-Anchored Forms 198
Comparative Studies Using Naturally Occurring and Genetically Engineered Membrane Isoforms 199
GPI-Mediated Protein Transfer 201
Potential Application of GPI-Protein Transfer in Tumor Immunotherapy 203
Summary 205

Index **213**

EDITORS

Daniel C. Hoessli, M.D.
Department of Pathology
University of Geneva
Geneva, Switzerland
Chapters 4 and 8

Subburaj Ilangumaran, B.V.Sc., Ph.D.
Department of Pathology
University of Geneva
Geneva, Switzerland
Chapter 4

CONTRIBUTORS

Urs Brodbeck, Ph.D.
Institute of Biochemistry and Molecular Biology
University of Berne
Berne, Switzerland
Chapter 9

Peter Butikofer, Ph.D.
Institute of Biochemistry and Molecular Biology
University of Berne
Berne, Switzerland
Chapter 2

Petr Draber, Ph.D.
Department of Mammalian Gene Expression
Institute of Molecular Genetics
Academy of Sciences of the Czech Republic
Prague, Czech Republic
Chapter 5

David A. Harris, M.D., Ph.D.
Department of Cell Biology and Physiology
Washington University School of Medicine
St. Louis, Missouri, U.S.A.
Chapter 6

Vaclav Horejsi, Ph.D.
Institute of Molecular Genetics
Academy of Sciences of the Czech Republic
Prague, Czech Republic
Chapter 5

Teymuras V. Kurzchalia, Ph.D.
Max-Delbrueck-Centre for Molecular Medicine
Berlin, Germany
Chapter 3

Martin G. Low, B.Sc., Ph.D., FRS
Department of Physiology and Cellular Biophysics
Columbia University Health Sciences
New York, New York, U.S.A.
Chapters 1 and 9

Satyajit Mayor, Ph.D.
National Centre for Biological Sciences
TIFR Centre, IISC Campus
Bangalore, India
Chapter 3

Rebecca S. McHugh, Ph.D.
National Institute of Health
Bethesda, Maryland, U.S.A.
Chapter 11

Anant K. Menon, Ph.D.
Department of Biochemistry
University of Wisconsin
Madison, Wisconsin, U.S.A.
Chapter 2

B. Paul Morgan, M.D., Ph.D.
Department of Biochemistry
University of Wales College
of Medicine
Cardiff, United Kingdom
Chapter 7

Shanmugam Nagarajan, Ph.D.
Department of Pathology
Emory University
Atlanta, Georgia, U.S.A
Chapter 11

Peter J. Robinson, Ph.D.
MRC Clinical Sciences Centre
Transplantation Biology
ICSTM, Hammersmith Hospital
London, U.K.
Chapter 4

Isabelle A. Rooney, Ph.D.
The Scripps Research Institute
Department of Molecular Biology
La Jolla, California, U.S.A.
Chapter 10

Periasamy Selvaraj, Ph.D.
Department of Pathology
Emory University
Atlanta, Georgia, U.S.A.
Chapter 11

Pascal Schneider, Ph.D.
Institute of Biochemistry
University of Lausanne
Lausanne, Switzerland
Chapter 8

Hannes Stockinger, Ph.D.
Institute of Immunology
University of Vienna
Vienna, Austria
Chapter 5

Carmen W. van den Berg, Ph.D.
Department of Pharmacology
University of Wales College of
Medicine
Cardiff, U.K.
Chapter 7

PREFACE

This book is intended to provide the relevant and recent information about GPI-anchored molecules and hopefully clarify a number of issues regarding their involvement in biological and pathological situations. In both microbes and mammalian cells, such molecules have become intriguing plasma membrane constituents, and inquiries into why and how they accumulate and function in membranes have led to the elaboration of new concepts in membrane biology. For instance, membrane organization of proteins and lipids, transmembrane signaling and membrane recycling have been revisited to accommodate recent data on GPI-anchored molecules. Moreover, the capacity of microbial GPI-anchored molecules to exchange between different membranes and alter functional properties of host cells has highlighted unexpected ways for virulence expression on the part of those microorganisms. Research on the function and membrane organization of GPI-anchored biomolecules has grown quite extensively in the past five years, but a number of these observations remain inadequately explained. These issues were discussed in a recent meeting, "GPI Anchors and Biological Membranes" (organized by U. Brodbeck, P. Robinson and D. Hoessli; reported in Trends in Cell Biology 1998; 8:87-89) held at Splügen, Switzerland, September 14-17, 1997. This meeting also provided an opportunity to hear almost all of the contributors to this book and was quite useful in organizing the subjects presented in the following chapters.

In his introductory chapter, Martin Low reviews how GPI anchors were discovered and how "GPI research" has evolved from a purely biochemical problem to a fascinating issue in cell biology. In chapter 2, Peter Butikofer and Anant Menon describe the mammalian cell pathways for GPI anchor biosynthesis and discuss how GPI anchors are distributed in cellular membranes. Chapter 3 is devoted to GPI anchors and the organization of cellular membranes; Satyajit Mayor and Teymuras Kurzchalia discuss these important and sensitive issues. Subburaj Ilangumaran, Peter Robinson and Daniel Hoessli give, in chapter 4, an overview of the equally sensitive issue of signaling via GPI-anchored receptors. In chapter 5, Vaclav Horejsi, Petr Draber and Hannes Stockinger unfold the panorama of GPI-anchored surface proteins expressed by hematopoietic cells. David Harris reviews in chapter 6 the particular behavior of GPI-anchored prions and cell-surface proteins in the nervous system. The current state of the art concerning GPI-anchored complement defense surface molecules is illustrated in chapter 7 by Carmen van den Berg and Paul Morgan. Chapter 8 is an attempt by Pascal Schneider and Daniel Hoessli to highlight the baroque variety of GPI anchors in a selection of microbial pathogens. The properties of phospholipases that cleave the GPI anchor, and their possible in vivo functions, are discussed by Martin Low and Urs Brodbeck in chapter 9. The intercellular exchange of GPI-anchored molecules between cells in the test tube and body fluids is illustrated by Isabelle Rooney in chapter 10. Finally,

Periasamy Selvaraj, Rebecca McHugh and Shanmugam Nagarajan show in chapter 11 how GPI-anchored molecules can be made and utilized in experimental medicine.

The synthesis achieved at this point does not reflect a full consensus, but the multidisciplinary approach of “GPI research” has been useful in providing fresh insights into old issues and defining further experimental goals. The enthusiasm of all contributors, their friendly collaboration and serious efforts to provide this timely synthesis is gratefully acknowledged by the editors of this book.

Daniel C. Hoessli, M.D.
Department of Pathology
University of Geneva
Geneva, Switzerland

Subburaj Ilangumaran, B.V.Sc., Ph.D.
Department of Pathology
University of Geneva
Geneva, Switzerland

FOREWORD

The complex and novel chemistry of glycosylphosphatidylinositols (GPI)s has allowed evolution to develop a surprising diversity of cell membrane functions in a great range of cell types from mycobacteria, yeast and protozoa to man. Even for investigators who were already impressed with the diversity of cellular lipids, the discovery of these units and the elucidation of the first complete structure in 1988 added an unexpected dimension to membrane biology. The dolichol-linked oligosaccharides which are assembled and transferred to protein acceptors in the ER and the glycosphingolipids which mature in the Golgi and then move to the cell surface were no longer the only glycolipids of importance. Free GPIs and GPIs linked to either proteins or polysaccharides have now become the centerpiece for investigations by chemists, cell biologists, immunologists, protozoologists, cell signalling aficionados, and many others.

Is there a unifying characteristic of the enzymes, antigens and even prions which are anchored by a GPI rather than by a transmembrane anchor? One of the first generalizations to be formulated with regard to GPI anchors was that they endowed proteins with increased mobility in the plane of the lipid bilayer, by comparison to trans-membrane proteins. Perhaps because membrane proteins often are influenced by their molecular neighbors and because not all GPI anchors are identical, it has proven difficult to sustain this hypothesis. It has become evident, however, that GPI-anchored proteins,

- are primarily found at the cell surface, perhaps because they cannot be retained in the ER and Golgi,
- associate with compositionally distinctive domains of membrane lipids, in all likelihood because of strong lipid-lipid interactions,
- preferentially concentrate at the apical cell surface of many polarized cells, implying that they are recognized by components of the sorting machinery in the Golgi complex,
- can transduce signals upon cross-linking, implying that they interact with components which extend across the membrane, and
- appear to cause their protein moieties to have an "unusual" relation to the membrane bilayer, which is reflected in the observation that GPI anchored membrane fusogens cannot mediate full membrane-membrane fusion but instead participate in "hemifusion."

Among the relatively uncharted areas of GPI biology are the mechanisms of transfer of GPI-anchored membrane proteins between cell surfaces, both in vivo and in vitro. Moreover, in a related area with significant applied potential, lipid or GPI derivatization of proteins provides a convenient means for allowing their insertion into cell surfaces, thereby allowing radical alternation of the cell surface without transfection.

Apart from GPI biology of the cell surface, there has been major interest in GPI biosynthesis in the ER. The biosynthesis of GPIs makes use of precursors which are also used for synthesis of other lipids and glycans. By contrast, to the

extent that they have been identified, the transferases which link these components together, the enzymes responsible for lipid remodeling and the transamidase which joins the complete GPI to newly-synthesized proteins in the endoplasmic reticulum appear to be uniquely dedicated to GPI biosynthesis. Thus, a novel equipment was invented in order to generate GPI anchors. It is therefore surprising to realize that mammalian cell mutants can survive in culture even if they do not synthesize the simplest of GPI structures and have no GPI-anchored proteins on their surface. This comment also pertains to affected red and white blood cells in the hemolytic anemia, paroxysmal nocturnal hemoglobinuria, in which early steps of GPI biosynthesis are blocked. Moreover, since GPI units which are not linked to protein can normally be detected on the surface, they too cannot be needed for survival under these conditions.

Apart from the issues of identifying the enzymes which assemble GPIs, flip them to the lumenal surface of the ER membrane, and add them to proteins, several other aspects of anchor addition are relatively unexplored. For example, there is little characterization of the condition of acceptor proteins prior to GPI addition. Interestingly, in most cases these proteins do not leave the ER, thereby providing a conspicuous example of censorship of transport along the secretory path. A further unexplored area concerns the physical relation of the GPI biosynthetic enzymes to each other, their relation to the transamidase, and the relation (if any) between the transamidase and the translocon which delivers newly-synthesized acceptor protein to the lumenal space of the ER.

The present volume provides a unique resource of broad interest. By bringing together diverse areas of biology related to GPIs, it exemplifies the multiplicity of investigations which have flourished during the past ten years. It will serve as a landmark in this still evolving field.

Alan M. Tartakoff, Ph.D.
Case Western Reserve University
Institute of Pathology
Cleveland, Ohio, USA
July, 1998

Chapter 1

GPI-Anchored Biomolecules—An Overview

Martin G. Low

In 1985 it was shown that glycosylphosphatidylinositols (GPIs) were covalently attached to a diverse group of cell surface proteins and played an essential role in anchoring them to the plasma membrane.[1-5] This discovery provoked an explosion of research by numerous investigators into the distribution, structure, biosynthesis and function of GPI anchors. Over the last decade research in the first three of these areas has produced a fairly comprehensive picture of where GPIs are found, what they look like and how they are made. Although there are still many fascinating details to be resolved, particularly in the area of GPI biosynthesis, relatively little remains controversial. However, this sentiment certainly does not apply to the last of these research areas, the function of GPIs: Why do cells go to all the trouble of making GPIs? This is an obvious and important question, but it is also a much more open-ended one. Not surprisingly, many of the attempts to define functional roles for GPI anchors have resulted in inconclusive or controversial findings. At present it is difficult to tell if the GPI anchor is capable of conferring "unique" functional properties on some cell surface proteins that cannot be provided by other means of membrane anchoring. Are there special structure-dependent features of the GPI anchor, with its conserved Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol glycan, that have led to its widespread use for attaching proteins to cell surfaces, or is this just another example of biochemical redundancy in higher organisms? The purpose of this chapter is not to provide answers to this question but to indicate why the question remains important.

Discovery of GPI Anchors

The "fluid mosaic" model of membrane structure proposed in the early 1970s[6] envisaged a continuous, relatively disordered lipid bilayer traversed by hydrophobic regions of proteins. The fluid mosaic model essentially settled a long-running debate in which earlier models had attempted (in vain) to reconcile the relatively constant chemical composition of the cell membrane with its diverse functional properties. However, two important features of membrane structure were not predicted by the fluid mosaic model: covalent linkage between membrane lipids and proteins and "long range" organization of the lipids within the plane of the bilayer. As will be seen below, it has become apparent that these two aspects of membrane structure are intimately related.

The first indication that proteins could be attached to membranes via a covalent interaction with a phospholipid molecule came from investigations into the pathophysiology of anthrax. It had been noticed that in the terminal stages of this disease secondary toxic shock was accompanied by increased alkaline phosphatase in urine and plasma.[7] However, more detailed studies with soluble toxin preparations indicated that the major increase in alkaline

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

phosphatase circulating in the blood occurred a few hours after injection, several days before the appearance of other symptoms and, finally, death of the animal. Subsequent attempts to purify the toxin revealed that the factor responsible for phosphatase release was a contaminant of the toxin preparation.[8] Furthermore, the phosphatase-releasing factor was produced in much greater amounts by *B. cereus* (a relatively mild human pathogen) and *B. thuringiensis* (an insect pathogen!).[9] Fortunately, this interesting phenomenon was investigated further, even though it was clear by then that the phosphatase-releasing factor had little or no role in the pathogenesis of anthrax. It should be pointed out that this work was being carried out under the auspices of the US Army Chemical Corps at Fort Detrick and it could be argued that this discovery was one of the few demonstrable benefits (to cell biology) of the cold war! The demonstration that the factor could also release alkaline phosphatase from bone or kidney slices in vitro allowed the development of a relatively simple "bioassay" and partial purification of the factor from *B. cereus*. Surprisingly, the releasing factor was separated from the major, broad specificity phospholipase C and copurified instead with a minor "phosphatidylinositol-specific" phospholipase C activity.[10,11]

The work on phosphatase-releasing factor was apparently abandoned at this point and these important observations were essentially ignored for over a decade. Their potential significance was only appreciated when the releasing activity was observed in preparations of phosphatidylinositol-specific phospholipase C (PI-PLC) which had been purified to homogeneity from *B. cereus*[12] and the unrelated bacteria Staphylococcus aureus[13] and *Clostridium novyi*.[14] Furthermore, it was shown that alkaline phosphatase release induced by PI-PLC did not require intact tissues and could be reproduced with tissue homogenates and membrane fractions as well as isolated cells.[13] Under these conditions, essentially all of the alkaline phosphatase could be released from the membrane. A survey of other cell surface enzymes indicated that liver 5'-nucleotidase[15,16] and erythrocyte acetylcholinesterase[17] were releasable, but three others were not. The release of acetylcholinesterase from erythrocytes occurred in the absence of hemolysis, suggesting that the effects of PI-PLC were restricted to the cell surface and did not result in gross disruption of membrane structure.

Collectively this work demonstrated that individual molecules of some cell surface proteins were attached to the membrane via a relatively specific interaction with the polar headgroup of phosphatidylinositol.[16] The nature of this interaction could not be determined definitively, but three observations indicated that it was relatively strong and probably covalent:[18]

1. Alkaline phosphatase could not be released from membranes by manipulating the ionic conditions or by addition of a headgroup analog (inositol-glycerol phosphate);
2. Alkaline phosphatase released by PI-PLC and then repurified to remove free inositol phosphate was unable to rebind to liposomes even if they contained PI;
3. Alkaline phosphatase which had been delipidated by extraction with butanol was able to rebind to liposomes regardless of whether they contained PI or not.

Similar results were subsequently obtained with affinity-purified acetylcholinesterase.[19] However, the idea that these enzymes were anchored to the membrane via a covalent linkage with inositol was not taken seriously until chemical analyses revealed that the membrane-anchoring domain of acetylcholinesterase[4] and alkaline phosphatase[20] contained stoichiometric amounts of myo-inositol which was retained even after PI-PLC treatment.

Although the presence of inositol in purified acetylcholinesterase and alkaline phosphatase provided compelling evidence for a covalent interaction, the molecular details remained obscure. However, separate studies on two other proteins, the variant surface glycoprotein of *Trypanosoma brucei* and rat brain Thy-1, had suggested that they might utilize a glycophospholipid moiety for attachment to the membrane.[5,21,22] The connection between these three, quite independent lines of research became apparent when it was shown

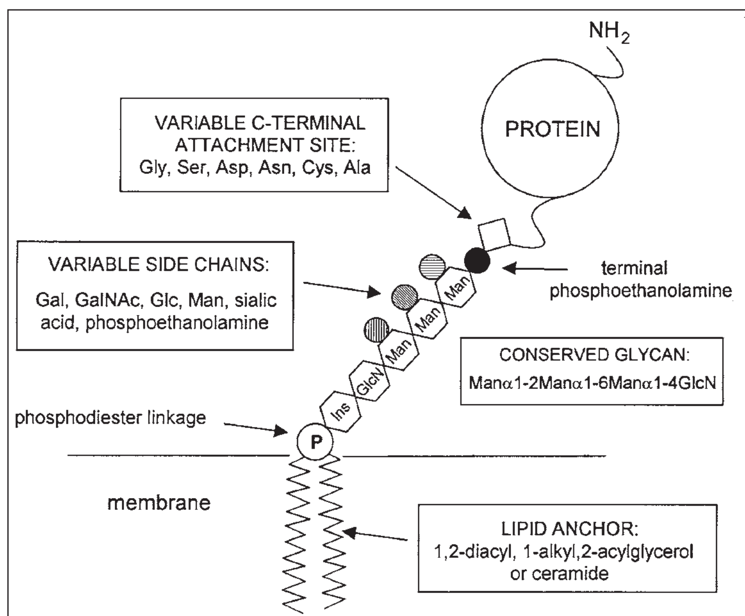

Fig. 1.1. GPI anchor structure. This cartoon summarizes the main structural features common to all GPI-anchored proteins and the range of structural variation. The lipid moiety of the phosphatidylinositol molecule can be a 1,2-diacylglycerol, a 1-alkyl,2-acylglycerol, a *lyso*, 1-alkylglycerol or a ceramide of varying chain length and degree of unsaturation. The type of sugar residues in the glycan backbone and the linkages between them are conserved in all GPI-anchored proteins characterized to date. The glycan is linked to the C-terminal amino acid through a phosphoethanolamine residue. The identity of the C-terminal amino acid has a limited range of variability between different proteins. The mannose residues in the glycan backbone can also be linked to a variety of side chains, ranging from single sugar or phosphoethanolamine residues to complex glycan chains. The 2-OH group on the inositol ring can be palmitoylated, rendering the GPI molecule insensitive to cleavage by PI-PLC. Some of the structural variations appear to be restricted to particular groups of organisms, but at present there is insufficient data to assess the functional or evolutionary significance of these variations.

that PI-PLC could remove the anchoring domain from VSG and Thy-1 and convert these proteins to a soluble form.[1-3] The chemical similarities between the anchors in these four proteins, and the availability of a highly selective reagent for degrading it (i.e., PI-PLC), laid the foundation for the rapid identification of many other GPI-anchored proteins, as well as the elucidation of GPI structures and biosynthetic pathways (Fig. 1.1).

Distribution of GPI Anchors

Distribution Amongst Organisms

GPI-anchored proteins are broadly distributed in eukaryotic organisms.[23-25] In mammals and other vertebrates they have been identified in every major tissue and cell type examined. GPI-anchored proteins have also been found in representatives from many other major groups of eukaryotic organisms, including protozoa, yeast, slime molds, nematodes, molluscs and insects. Evidence is emerging that they are also present in photosynthetic

organisms such as algae, as well as aquatic and terrestrial higher plants.[26,27] Although structural data is only available for a few well-characterized systems, it is reasonable to assume that all eukaryotes have the capability of producing GPIs and attaching them to proteins. The observation that there is absolute conservation in the core structure of the GPI anchor (Fig. 1.1) between protozoa, slime molds, yeast and mammals suggests that it appeared at least 1.2 billion years ago (when protozoa are believed to have diverged from all other eukaryotic organisms). GPI-anchored proteins appear to be relatively abundant in several protozoa (both free-living and parasitic) and at least one GPI-anchored protein has been detected in *Giardia lamblia*.[28] Since this organism is believed to represent the most primitive type of protozoa, GPI-like anchoring molecules may have existed even earlier. It is also noteworthy that several protozoa contain extreme variants of the GPI structure which only retain the Manα1-4GlcNα1-6-*myo*-inositol head group and are attached to a wide variety of glycan chains rather than protein.[25] These lipids may have appeared as a result of additions to the biosynthetic pathway because *Leishmania* also express GPI-anchored proteins containing the conserved glycan core. There is some preliminary evidence for GPI-like molecules linked to proteins in the thermoacidophile *Sulfolobus acidocaldarius*, but there is no detailed information on their composition or structure.[29] Archaetidylinositol (a diphytanylglycerol ether analog of 1-6-glucosaminyl phosphatidylinositol) has been found in *Methanosarcina barkeri*.[30] However, there are as yet no reports of it being attached to protein, and this lipid does not appear to be widely distributed, even amongst other methanogens.[31] It is relevant to note that *Sulfolobus acidocaldarius* and *Methanosarcina barkeri* represent the two major kingdoms of Archaea (Crenarchaeota and Euryarchaeota respectively) which suggests that "primitive" GPI-like molecules could have begun to emerge when archaebacteria and eukaryotes diverged about 1.8-2 billion years ago.[32]

Numerous variations have been found in the lipid moiety and the side chains attached to the conserved glycan core of the GPI anchor (see Fig. 1.1 and ref. 25). It is probable that some of these side chains (e.g., mannose) are widely distributed throughout eukaryotes, although systematic, comparative studies have not been done as yet. This broad distribution may not apply to the ceramide lipid moiety (found in yeast and *Dictyostelium* but not in higher eukaryotes) and the phosphoethanolamine side chain (found in higher eukaryotes and *Dictyostelium* but not yeast or protozoa[33-35]). This suggests that the acquisition of the phosphoethanolamine side chain and the loss of the ceramide in GPI of higher eukaryotes occurred about 1 billion years ago when fungi and *Dictyostelium* diverged from other major eukaryotic groups.[32] It will be interesting to see if any of these structural variations are present in GPI anchors of plants (which also diverged about 1 billion years ago).

Although free PI is found in several bacterial species and glycosylated forms of PI are present in *Mycobacteria* (see chapter 8), there is at present no evidence indicating that GPI-anchored proteins are present in eubacteria. As described below, bacteria utilize a different form of lipid anchor for attaching proteins to the cell surface and this may represent yet another fundamental biochemical difference between eubacteria and all other organisms.

Distribution Among Proteins

Over 100 distinct GPI-anchored proteins have been identified (listings of GPI-anchored proteins which include many of these proteins have appeared in review articles;[23-25] more recent additions may be found in the Medline database where "glycosylphosphatidylinositols" has been used as an indexing term since 1992). It is difficult to be more precise about the number of distinct GPI-anchored proteins because of the occurrence of multiple isoforms and species homologs which often acquire quite different names. Also, the techniques available for identifying a GPI anchor are quite diverse (e.g., chemical composition, radiolabeling with anchor components, release by PI-PLC, identification of GPI attachment signal from

genomic or cDNA sequence etc.) and have not always been applied uniformly and/or interpreted with equal rigor. CD antigens have been comprehensively studied with respect to PI-PLC sensitivity and currently 11 (i.e., about 10%) are believed to be GPI-anchored. It is likely that, in most cell types and organisms (except protozoa), GPI-anchored proteins represent a minority of cell surface proteins both in amount and number.

A striking feature of GPI-anchored proteins is that they are, from a structural point of view, a very diverse group.[23,24] They differ markedly in molecular size, quaternary structure and extent/type of glycosylation: e.g., alkaline phosphatase is a homodimer of 67 kDa subunits; CD52 is a heavily glycosylated dodecapeptide; glypican is a heparan sulfate proteolgycan. They also have quite distinct biochemical functions such as ectoenzymes (e.g., acetylcholinesterase), cell adhesion molecules, complement regulatory proteins and protective coats in parasites. In fact, they are found on all the major types of cell surface protein which do not require some part of the polypeptide chain to cross the phospholipid bilayer, i.e., everything except ion channels, solute transporters and most signaling receptors. The majority of GPI-anchored proteins were chosen for study because of their species/tissue-specific expression patterns, developmental regulation, association with disease or mere abundance, and many have not yet had any specific biochemical function assigned to them. Furthermore, all of these classes of GPI-anchored proteins are also found in polypeptide-anchored forms. For example, hydrolytic ectoenzymes or immunoglobulin superfamily adhesion molecules occur in both GPI-anchored and polypeptide-anchored forms. In a few cases the same protein (e.g., CD16 or LFA-3) can even be expressed naturally either in a GPI-anchored or a polypeptide-anchored form, depending on the cell type. There is also evidence that some proteins may utilize both a GPI and polypeptide anchor at the same time.[36]

Why Use a GPI Molecule for Membrane Anchoring?

The ability of bacterial PI-PLC to remove the lipid moiety and thereby release most GPI-anchored proteins from membranes suggests that, despite its conserved structure, the phosphoinositol-glycan is simply a linking moiety and may not be directly involved in the interaction with the membrane (Fig. 1.1). This raises the obvious question: Why not use a simpler lipid (i.e., without a glycan linker) as a membrane anchor? Presumably the glycan has some other role which is of functional value. It is well known that GPI molecules are not the only type of lipid that can be used to modify membrane proteins, thereby increasing their hydrophobicity and affinity for membranes. Direct N-myristoylation of N-terminal glycine residues or S-palmitoylation of cysteine residues (usually close to the N or C-terminus) had been demonstrated on several intracellular proteins in yeast and mammalian cells by the early 1980s, before the existence of the GPI anchor was widely accepted. However, it is only in the last five years that the important role of these other lipid groups has become appreciated. During that time it also became apparent that a novel type of lipid modification was occurring in which cysteine residues close to the C-terminus become modified by isoprenoids: a 15-carbon farnesyl or a 20-carbon geranylgeranyl group.[37]

Although these other types of lipid anchor only occur on intracellular proteins (or in some cases of S-palmitoylation on intracellular sites of transmembrane cell surface proteins) it is relevant to compare them to GPI anchors. Studies of the intracellular lipid anchors have provided a great deal of information on the relative effectiveness of different lipid groups for membrane attachment, as well as the contribution of other factors such as electrostatic interactions in determining the membrane binding affinity.[38,39] This information can be extrapolated, with caution, to GPI anchors. Biophysical studies, which have largely been confirmed by experiments in intact cells, indicate that myristate (14 carbon unbranched fatty acid) and farnesyl groups are by themselves very weak anchors and are unable to retain

proteins in a membrane. Indeed, some farnesylated (e.g., nuclear lamin) or N-myristoylated proteins (e.g., cAMP dependent protein kinase catalytic subunit) do not associate with membranes at all, and the lipid group may have a structural role or be involved solely in protein-protein interaction. Increasing the chain length by using palmitate (16 carbon unbranched fatty acid) or geranylgeranyl groups provides higher affinity association with the membrane, but these lipids, by themselves, are still unable to provide stable anchoring.

At the cell surface, a weak lipid anchor would be a serious liability because the protein would be continuously (and essentially irreversibly) lost into the extracellular fluid. Maybe this is why eukaryotic cells do not use a single lipid group for anchoring proteins at the cell surface, utilizing instead the GPI anchor with its ceramide, diacyl or alkyl, acyl lipid moiety. It is relevant to note here that a lipid anchor containing three fatty acyl groups is utilized by prokaryotes. In proteins which use this lipid anchor (designated by the unfortunate and confusing name 'bacterial lipoproteins') the N-terminal cysteine is modified by an amide-linked fatty acid and a thioether-linked diacylglycerol.[40,41] This structure anchors proteins to the periplasmic face of the inner or outer membrane or to the cell surface in Gram-negative or Gram-positive bacteria respectively. However, these structures do not occur in eukaryotes, and it seems that GPI anchoring is the only lipid-based anchoring mechanism available for stable high affinity attachment of proteins to the cell surface of eukaryotic cells.

In the contained environment inside the cell, weak, readily reversible binding to membranes via a lipid anchor is less problematic. Indeed, it may provide a highly versatile mechanism for regulating protein function, because relatively small changes in the binding affinity can have a very marked effect on the distribution of proteins between the cytoplasm and membranes.[38,39] There are several mechanisms which are utilized by the cell to modulate the binding affinity:

1. Additional lipid groups can be used to increase the binding, e.g., S-palmitoylation of a cysteine near the amino terminus of N-myristoylated tyrosine kinases Fyn and Lck, S-palmitoylation near the C-terminus of farnesylated H-Ras or N-Ras, use of an additional geranylgeranyl group or carboxymethylation of isoprenylated proteins;
2. Basic residues near the N-terminus of myristoylated Src or the C-terminus of farnesylated K-Ras increase the electrostatic attractiont o the negatively-charged membrane surface;
3. Conversely, the binding affinity can be reduced by removing the extra S-palmitoyl group, by "masking" the lipid group with a soluble protein containing a hydrophobic binding site or by phosphorylating the protein to increase electrostatic repulsion from the membrane.

A major question to be answered is whether similar mechanisms play a role in determining the membrane binding affinity of different GPI-anchored proteins. As noted above, the binding affinity of the GPI anchor is predicted (from the fatty acid composition) to be relatively high. Furthermore, the glycan chain connecting the protein to the lipid moiety is likely to be highly flexible, and as a consequence the high binding affinity of the lipid group is not offset by a loss of entropy due to restrictions in rotational and translational motion of the protein. Variations in binding affinity could result from electrostatic interactions with the bilayer surface, the attachment of additional lipid groups (e.g., inositol acylation of GPI) or even participation in microdomains (see below). Because of the high binding affinity of the lipid anchor, these secondary interactions might not be large enough to have a major effect on the distribution of the proteins between the cell surface and the extracellular fluid. However, such interactions could have a profound influence on the rate and direction of GPI transfer between different cells. Similar interactions have been proposed to regulate the

transfer of intracellular lipid-anchored proteins between different organelles, and their influence on GPI-anchored proteins at the cell surface should be taken into consideration.[42]

Does the GPI Anchor Have Additional Functions Besides Anchoring Proteins to the Cell Surface?

The complexity of the GPI anchor compared to polypeptide anchors or other types of lipid anchor strongly suggest that it has other functions besides anchoring proteins to the cell surface. In general, it is not possible to state what contribution a GPI anchor makes to the function of a particular protein beyond retaining it at the cell surface. This is for two main reasons:

1. As noted above, the biochemical function and/or physiological role of most GPI-anchored proteins is not known;
2. The biophysical properties of the GPI in the membrane are not thoroughly understood.

Furthermore, it seems extremely unlikely that the GPI anchor would have the same function in parasitic or free-living unicellular organisms such as protozoa and yeast, as well as multicellular organisms such as higher plants and vertebrates. Presumably, GPI-like molecules, which appeared at a very early stage of eukaryotic evolution, have acquired a diverse set of functions which differ not only between different proteins but also between different unicellular organisms and the different cell types of multicellular organisms. As described below, GPI anchors have several "unique" properties not found in other lipid anchors, which may have important functional consequences. However, it should be emphasized that for none of these "functions" is there compelling evidence that the conserved Mana1-2Mana1-6Mana1-4GlcNa1-6myo-inositol glycan core is required.

A variety of mammalian cell lines deficient in GPI biosynthesis do not appear to be seriously impaired in their ability to survive and grow in culture, even though they have lost the ability to express GPI-anchored proteins. This indicates that GPI-anchored proteins (or free GPI molecules) are not required for most "housekeeping" functions of mammalian cells. However, in budding or fission yeast, mutations in any of the three genes known to be required for GlcNAc-PI biosynthesis are conditionally lethal and mutant cells exhibit aberrant cell wall maturation and cell division.[43-45] GPI molecules have also been shown to play an essential role in mammalian development. A knockout of the mouse *PIG-A* gene (responsible for the first step in GPI biosynthesis) is lethal and *PIG-A*-deficient mice are not viable unless chimerism is >5%.[46] Similarly, GPI-deficient embryonic stem (ES) cells would only differentiate in vitro if they were mixed with normal ES cells.[47] A tissue-specific knockout of *PIG-A* in skin resulted in mice with aberrant skin development.[46] It was suggested that GPI-anchored proteins play an important role in the transport of ceramide into the extracellular space of the epidermal horny layer, although the mechanism for this effect is unclear.[46] The profound developmental effects of mutations that block GPI biosynthesis is not a surprising result given that numerous GPI-anchored proteins will have been mislocated/degraded within the cell, leading to reduced cell surface expression. However, interpretation of these results is complicated by the fact that protein-free GPI molecules may have essential functions distinct from protein anchoring (e.g., signaling) and it is conceivable that their absence (rather than impaired expression of GPI-anchored proteins) is responsible for the developmental disruptions resulting from mutation of GPI biosynthetic genes.

An alternative approach to the question of GPI anchor function has been to study, at the cellular level, distinctive properties shared by several GPI-anchored proteins which transcend their individual biochemical differences. Three general properties of GPI anchors have received the most attention in this respect:

1. Their ability to form a protective coat;

2. Their tendency to associate with other lipid molecules and proteins, resulting in the formation of discrete clusters or microdomains—this property could have many important consequences, including the ability of many GPI-anchored proteins to act as "receptors" for transmembrane signaling events as well as influencing their intracellular transport, localization to particular sites on the cell surface and intercellular transfer;
3. Their ability to be cleaved by endogenous "GPI-specific" phospholipases.

Protective Coat

It has been suggested that the relatively high frequency with which GPI anchors are utilized in parasitic protozoa (compared to mammalian cells) is because of the relatively harsh environment these organisms are exposed to in the bloodstream of the mammalian host or the gut of an insect vector.[25] The GPI anchor appears to be a highly versatile mechanism for anchoring molecules which have a protective role at the cell surface (see chapter 8). Thus, GPI-anchored proteins (e.g., VSG, the major surface protein in the bloodstream form of *T. brucei*) can be packed at relatively high density into the cell surface, thereby forming an effective barrier without affecting the integrity of the phospholipid bilayer. Such high densities might not be achievable with a polypeptide anchor. Alternatively, the glycan chain can provide a location for the introduction of side chains which can enhance the barrier function by filling space between the glycan chains in a less densely packed protein coat (e.g., PARP, the major surface protein in the insect form of *T. brucei*). A further adaptation of this strategy is the use of protein-free GPI-like molecules (which retain the Manα1-4GlcNα1-6*myo*-inositol portion) for the attachment of a variety of glycan side chains to the cell surface of *T. cruzi* and *Leishmania* spp.

Microdomains, Transmembrane Signaling and Intracellular Transport

Thy-1 and 5'-nucleotidase were proposed (on the basis of detergent insolubility) to form complexes with other plasma membrane components even before they were shown to have GPI anchors.[48,49] Interest in the nature of these complexes was renewed by the observation that Thy-1 and 5'-nucleotidase, in common with several other GPI-anchored proteins, participate in transmembrane signaling events (see chapter 4).[50] Since the GPI anchor does not cross the membrane, interaction of the GPI-anchored protein with one or more proteins containing a transmembrane polypeptide seems the most reasonable explanation for these effects. Furthermore, the formation of "specific" complexes with particular membrane components could also provide an explanation for the ability of the GPI anchor to act as an apical sorting signal in some polarized cells.[51] The existence of complexes is supported by some photobleaching studies which indicated that a proportion of the GPI-anchored protein molecules on the cell surface can have relatively low lateral mobility.[52] There is also evidence indicating that GPI-anchored proteins are clustered soon after they arrive at the cell surface.[53] However, the poor spatial resolution of these techniques precludes determination of the size, stability or composition of the complexes. Fluorescence and electron microscopy have also been used to determine if GPI-anchored proteins colocalize with morphological features and other markers on the cell surface.[54] Unfortunately, interpretation of these studies has been complicated by the tendency of GPI-anchored proteins to redistribute during fixation and staining with labeled antibodies.[55-57] Redistribution to caveolae following crosslinking with antibodies is particularly interesting, because these specialized regions of the cell surface may provide a site where extracellular signals recognized by GPI-anchored proteins can be passed to components of established signal transduction pathways.[58] It is relevant to note that, although the physiological stimuli

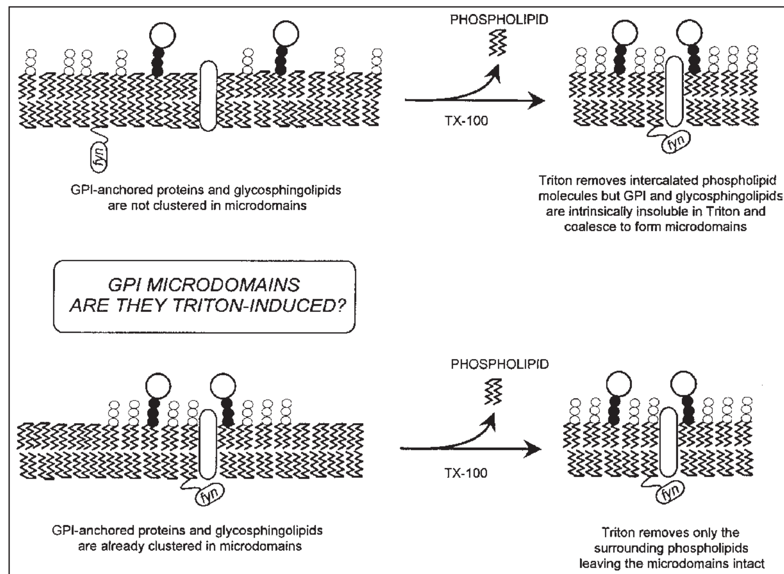

Fig. 1.2. GPI-anchored proteins and microdomains. This cartoon summarizes the controversy regarding the origin of Triton-insoluble microdomains that contain GPI-anchored proteins. In the upper part of the Figure, GPI-anchored proteins are randomly distributed and not clustered together with glycosphingolipids (and other membrane components) into microdomains. However, when the bulk of the phospholipids are extracted with Triton X-100, the GPI-anchored proteins and glycosphingolipid are forced into clusters because they have low solubility in Triton. The lower Figure shows how essentially the same result would be produced if the GPI-anchored proteins and glycosphingolipids were already clustered into microdomains. A major concern is whether other molecules (shown here by the lipid-anchored, Src family tyrosine kinase Fyn and a transmembrane protein) are also forced into the microdomains and a non-physiological association with GPI and glycosphingolipids simply as a result of their Triton insolubility.

recognized by GPI-anchored proteins are largely unknown, crosslinking by antibodies usually provides an alternative and highly effective activating stimulus.[50]

An additional problem identified by the electron microscopy studies is that the redistribution referred to above can also be produced by extraction in cold detergents such as Triton X-100.[56] This latter observation is both intriguing and worrisome because these are essentially the same conditions that are used to isolate the complexes for biochemical analysis. One interpretation of this result is that by removing the Triton-soluble bulk phospholipid, preexisting "GPI microdomains" simply aggregate into large rafts, permitting their isolation on the basis of relatively low buoyant density. However, it is also possible that "randomly"-distributed GPI-anchored proteins are forced into a non-physiological association with other insoluble components when bulk phospholipid is removed (Fig. 1.2). The final composition of these complexes could easily be influenced by extrinsic factors and therefore be potentially misleading.[59] Studies on natural and model membranes indicate that GPI anchors have low Triton solubility (probably as a result of a relatively high proportion of long, saturated hydrocarbon chains) and this may be the trivial explanation of why they appear to localize "specifically" in the complexes.[60,61] Obviously this phenomenon could

have a major influence on the reliability of composition analysis of microdomains, as well as tyrosine kinase assays performed on immunoprecipitates. At best, the potential for induction of complexes by Triton X-100 makes it difficult to distinguish genuine and preexisting interactions from spurious ones. Hopefully, more detailed studies of the size, stability and structure of microdomains in intact membranes and how these parameters are affected by detergent extraction will allow the biochemical analysis of microdomains and their role in signaling to be approached with more confidence in the future (see chapter 3).[62] As noted above, there is currently no evidence that features specific to the GPI anchor (such as the conserved Man_3GlcN-inositol glycan) or any of its side chains determine the ability of GPI-anchored proteins to participate in these complexes. The concept of a specific GPI "receptor" in the cell surface, although an attractive one, has little experimental support.

Anchor Degradation by Endogenous "GPI-Specific" Phospholipases

The initial identification of GPI-anchored proteins in both *T. brucei* and mammalian tissues was complicated by the presence of relatively large amounts of endogenous anchor-degrading phospholipases which removed the anchor during extraction.[18,63] At first this autolytic phenomenon was regarded mostly as a nuisance, but it was soon realized that the enzymes responsible were relatively specific for GPI structures and might be responsible for GPI degradation in vivo. As described in chapter 9, these two enzymes, the *T. brucei* (G)PI-specific phospholipase C and the mammalian GPI-specific phospholipase D, have been studied quite extensively at the molecular level over the last decade. However, in spite of this interest the function of these enzymes remains unknown. This apparent lack of progress is particularly evident for mammalian GPI-PLD. The occurrence of soluble forms of GPI-anchored proteins in culture media or body fluids has been recognized for many years and considerable evidence has accumulated that release from cells is due to GPI-PLD. However, as with other areas in the GPI field, the physiological significance of GPI-PLD mediated protein release remains uncertain.

Several potential functions of GPI-PLD have been suggested, although it should be emphasized that these possibilities are all highly speculative.

Intracellular Catabolism of GPIs

There is some data indicating that GPI-PLD can act on GPI-anchored proteins inside cells.[64,65] GPI degradation at an intracellular location might also provide a mechanism for removing excess free GPIs that were not used for attachment to proteins.

Rapid Downregulation of GPI-Anchored Proteins on Cell Surface

Rapid removal of the protein from the cell surface might be useful in immunological or developmental recognition processes which require transient adhesive cell contact. It would also provide a mechanism for terminating an activating signal mediated by a GPI-anchored protein.

Regulation of Intercellular Transfer

Recent studies indicate that intercellular transfer of a GPI-anchored form of CD4 between cocultured cells can be quite rapid (i.e., minutes).[66,67] The mechanism is not known, but efficient transfer seems to require prolonged cell contact and consequently would be restricted to neighboring cells in tissues. Transfer between blood cells and endothelial cells would also be limited because close contact of these cells is transient and relatively rare. However, the observation that transfer can be mediated by HDL in vitro also raises the possibility of continuous rapid and promiscuous transfer between different blood cells and endothelial cells without the necessity for direct contact.[68] A potentially important function

of GPI-PLD might be to restrict or regulate promiscuous transfer by cleaving the GPI-anchor when it is no longer protected by insertion into the lipid bilayer.

Summary

Many proteins are anchored to the cell surface via covalent linkage to a glycosylphosphatidylinositol (GPI) molecule located in the membrane lipid bilayer. GPI molecules are broadly distributed amongst eukaryotic organisms, and in multicellular organisms they are present in essentially all cell types. GPI-anchored proteins have diverse biochemical and physiological functions, and relatively little is known about the properties of GPI molecules which have led to their widespread use for cell surface protein anchoring.

References

1. Ferguson MAJ, Low MG, Cross GAM. Glycosyl-sn-1,2-dimyristylphosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. J Biol Chem 1985; 260:14547-14555.
2. Low MG, Kincade PW. Phosphatidylinositol is the membrane-anchoring domain of the Thy-1 glycoprotein. Nature 1985; 318:62-64.
3. Low MG, Ferguson MAJ, Futerman AH et al. Covalently attached phosphatidylinositol as a hydrophobic anchor for membrane proteins. TIBS 1986; 11:212-215.
4. Futerman AH, Low MG, Ackermann KE et al. Identification of covalently bound inositol in the hydrophobic membrane-anchoring domain of *Torpedo* acetylcholinesterase. Biochem Biophys Res Commun 1985; 129:312-317.
5. Tse AGD, Barclay AN, Watts A et al. A glycophospholipid tail at the caroxyl terminus of the Thy-1 glycoprotein of neurons and thymocytes. Science 1985; 230:1003-1008.
6. Singer SJ, Nicolson GL. The fluid mosaic model of the structure of cell membranes. Science 1972; 175:720-731.
7. Smith H, Keppie J, Stanley JL et al. The chemical basis of the virulence of *Bacillus anthracis*: IV. Secondary shock as the major factor in death of guinea pigs from anthrax. Br J Exp Path 1955; 36:323-335.
8. Slein MW, Logan GF. Mechanism of action of the toxin of *Bacillus anthracis*: Effect in vivo on some blood serum components. J Bacteriol 1960; 80:77-85.
9. Slein MW, Logan GF. Mechanism of action of the toxin of *Bacillus anthracis*: II. Alkaline phosphatasemia produced by culture filtrates of various bacilli. J Bacteriol 1962; 83:359-369.
10. Slein MW, Logan GF. Partial purification and properties of two phospholipases of *Bacillus cereus*. J Bacteriol 1963; 85:369-381.
11. Slein MW, Logan GF. Characterization of the phospholipases of *Bacillus cereus* and their effects on erythrocytes, bone, and kidney cells. J Bacteriol 1965; 90:69-81.
12. Ikezawa H, Yamanegi M, Taguchi R et al. Studies on phosphatidylinositol phosphodiesterase (phospholipase C type) of *Bacillus cereus*. Biochim Biophys Acta 1976; 450:154-164.
13. Low MG, Finean JB. Release of alkaline phosphatase from membranes by a phosphatidylinositol-specific phospholipase C. Biochem J 1977; 167:281-284.
14. Taguchi R, Ikezawa H. Phosphatidylinositol-specific phospholipase C from *Clostridium novyi* Type A. Arch Biochem Biophys 1978; 186:196-201.
15. Taguchi R, Yoshinari A, Ikezawa H. Purification and properties of phosphatidylinositol-specific phospholipase C of *Bacillus thuringensis*. Biochim Biophys Acta 1980; 619:48-57.
16. Low MG, Finean JB. Specific release of plasma membrane enzymes by a phosphatidylinositol-specific phospholipase C. Biochim Biophys Acta 1978; 508:565-570.
17. Low MG, Finean JB. Non-lytic release of acetylcholinesterase from erythrocytes by a phosphatidylinositol-specific phospholipase C. FEBS Lett 1977; 82:143-146.
18. Low MG, Zilversmit DB. Role of phosphatidylinositol in attachment of alkaline phosphatase to membranes. Biochemistry 1980; 19:3913-3918.

19. Futerman AH, Fiorini RM, Roth E et al. Physicochemical behavior and structural characteristics of membrane-bound acetylcholinesterase from *Torpedo* electric organ: Effect of phosphatidylinositol-specific phospholipase C. Biochem J 1985; 226:369-377.
20. Low MG, Futerman AH, Ackermann KE et al. Removal of covalently bound inositol from *Torpedo* acetylcholinesterase and mammalian alkaline phosphatases by deamination with nitrous acid: Evidence for a common anchoring structure. Biochem J 1987; 241:615-619.
21. Ferguson MAJ, Haldar K, Cross GAM. *Trypanosoma brucei* variant surface glycoprotein has a sn-1,2-dimyristyl glycerol membrane anchor at its COOH terminus. J Biol Chem 1985; 260:4963-4968.
22. Campbell DG, Gagnon J, Reid KBM et al. Rat brain Thy-1 glycoprotein: The amino acid sequence, disulphide bonds and an unusual hydrophobic region. Biochem J 1981; 195:15-30.
23. Low MG. The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim Biophys Acta 1989; 988:427-454.
24. Field MC, Menon AK. Glycolipid anchoring of cell surface proteins. In: Schlesinger MJ, ed. Lipid modifications of proteins. Boca Raton: CRC Press, 1993:83-134.
25. McConville MJ, Ferguson MAJ. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem J 1993; 294:305-324.
26. Morita N, Nakazato H, Okuyama H et al. Evidence for a glycosylinositolphospholipid-anchored alkaline phosphatase in the aquatic plant *Spirodela oligorrhiza*. Biochim Biophys Acta Gen Subj 1996; 1290:53-62.
27. Takos AM, Dry IB, Soole KL. Detection of glycosyl-phosphatidylinositol-anchored proteins on the surface of *Nicotiana tabacum* protoplasts. FEBS Lett 1997; 405:1-4.
28. Das S, Traynor-Kaplan A, Reiner DS et al. A surface antigen of *Giardia lamblia* with a glycosylphosphatidylinositol anchor. J Biol Chem 1991; 266:21318-21325.
29. Kobayashi T, Nishizaki R, Ikezawa H. The presence of GPI-linked protein(s) in an archaeobacterium, *Sulfolobus acidocaldarius*, closely related to eukaryotes. Biochim Biophys Acta Gen Subj 1997; 1334:1-4.
30. Nishihara M, Utagawa M, Akutsu H et al. Archaea contain a novel diether phospholipid with a polar head group identical to the conserved core of eucaryal glycosyl phosphatidylinositol. J Biol Chem 1992; 267:12432-12435.
31. Nishihara M, Koga Y. Two new phospholipids, hydroxyarchaetidylglycerol and hydroxyarchaetidylethanolamine, from the Archaea *Methanosarcina barkeri*. Biochim Biophys Acta 1995; 1254:155-160.
32. Doolittle RF, Feng D-F, Tsang S et al. Determining divergence times of the major kingdoms of living organisms with a protein clock. Science 1996; 271:470-477.
33. Haynes PA, Gooley AA, Ferguson MAJ et al. Post-translational modifications of the *Dictyostelium discoideum* glycoprotein PsA—Glycosylphosphatidylinositol membrane anchor and composition of O-linked oligosaccharides. Eur J Biochem 1993; 216:729-737.
34. Fankhauser C, Homans SW, Thomas-Oates JE et al. Structures of glycosylphosphatidylinositol membrane anchors from *Saccharomyces cerevisiae*. J Biol Chem 1993; 268:26365-26374.
35. Conzelmann A, Puoti A, Lester RL et al. Two different types of lipid moieties are present in glycophosphoinositol-anchored membrane proteins of *Saccharomyces cerevisiae*. EMBO J 1992; 11:457-466.
36. Howell S, Crine P. Type VI membrane proteins? Trends Biochem Sci 1996; 21:171-172.
37. Casey PJ. Protein lipidation in cell signalling. Science 1997; 268:221-224.
38. Bhatnagar RS, Gordon JI. Understanding covalent modification of proteins by lipid: Where cell biology and biophysics mingle. Trends Cell Biol 1997; 7:14-20.
39. McLaughlin S, Aderem A. The myrsitoyl-electrostatic switch: A modulator of reversible protein-membrane interactions. Trends Biochem Sci 1995; 20:272-276.
40. Wu HC, Tokunaga M. Biogenesis of lipoproteins in bacteria. Curr Top Microbiol Immunol 1986; 125:127-157.
41. Hayashi S, Wu HC. Lipoproteins in bacteria. J Bioenerg Biomemb 1990; 22:451-471.

42. Shahinian S, Silvius JR. Doubly-lipid-modified protein sequence motifs exhibit long-lived anchorage to lipid bilayer membranes. Biochemistry 1995; 34:3813-3822.
43. Leidich SD, Kostova Z, Latek RR et al. Temperature-sensitive yeast GPI anchoring mutants *gpi2* and *gpi3* are defective in the synthesis of N-acetylglucosaminyl phosphatidylinositol. Cloning of the *GPI2* gene. J Biol Chem 1995; 270:13029-13035.
44. Leidich SD, Orlean P. Gpi1, a *Saccharomyces cerevisiae* protein that participates in the first step in glycosylphosphatidylinositol anchor synthesis. J Biol Chem 1996; 271:27829-27837.
45. Colussi PA, Orlean P. The essential *Schizosaccharomyces pombe* gpil+ gene complements a bakers' yeast GPI anchoring mutant and is required for efficient cell separation. Yeast 1997; 13:139-150.
46. Tarutani M, Itami S, Okabe M et al. Tissue-specific knockout of the mouse *Pig-A* gene reveals important roles for GPI-anchored proteins in skin development. Proc Natl Acad Sci USA 1997; 94:7400-7405.
47. Dunn DE, Yu J, Nagarajan S et al. A knock-out model of paroxysmal nocturnal hemoglobinuria: *Pig-A* hematopoiesis is reconstituted following intercellular transfer of GPI-anchored proteins. Proc Natl Acad Sci USA 1996; 93:7938-7943.
48. Hoessli D, Rungger-Brandle E. Association of specific cell-surface glycoproteins with a Triton X-100-resistant complex of plasma membrane proteins isolated from T-lymphoma cells (P 1798). Exp Cell Res 1985; 156:239-250.
49. Mescher MF, Jose MJL, Balk SP. Actin-containing matrix associated with the plasma membrane of murine tumour and lymphoid cells. Nature 1981; 289:139-144.
50. Brown D. The tyrosine kinase connection: how GPI-anchored proteins activate T cells. Curr Opin Immunol 1993; 5:349-354.
51. Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 1992; 68:533-544.
52. Ishihara A, Hou Y, Jacobson K. The Thy-1 antigen exhibits rapid lateral diffusion in the plasma membrane of rodent lymphoid cells and fibroblasts. Proc Natl Acad Sci USA 1987; 84:1290-1293.
53. Hannan LA, Lisanti MP, Rodriguez-Boulan E et al. Correctly sorted molecules of a GPI-anchored protein are clustered and immobile when they arrive at the apical surface of MDCK cells. J Cell Biol 1993; 120:353-358.
54. Anderson RGW, Kamen BA, Rothberg KG et al. Potocytosis: Sequestration and transport of small molecules by caveolae. Science 1992; 255:410-411.
55. Mayor S, Rothberg KG, Maxfield FR. Sequestration of GPI-anchored proteins in caveolae is triggered by cross linking. Science 1994; 264:1948-1951.
56. Mayor S, Maxfield FR. Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. Mol Biol Cell 1995; 6:929-944.
57. Schnitzer JE, McIntosh DP, Dvorak AM et al. Separation of caveolae from associated microdomains of GPI-anchored proteins. Science 1995; 269:1435-1439.
58. Liu J, Oh P, Horner T et al. Organized endothelial cell surface signal transduction in caveolae distinct from glycosylphosphatidylinositol-anchored protein microdomains. J Biol Chem 1997; 272:7211-7222.
59. Parkin ET, Turner AJ, Hooper NM. Isolation and characterization of two distinct low-density, Triton-insoluble, complexes from porcine lung membranes. Biochem J 1996; 319:887-896.
60. Hooper NM, Turner AJ. Ectoenzymes of the kidney microvillar membrane: Differential solubilization by detergents can predict a glycosyl-phosphatidylinositol membrane anchor. Biochem J 1988; 250:865-869.
61. Schroeder R, London E, Brown D. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. Proc Natl Acad Sci USA 1994; 91:12130-12134.

62. Schroeder RJ, Ahmed SN, Zhu Y, London E, Brown DA. Cholesterol and sphingolipid enhance the Triton X-100 insolubility of glycosylphosphatidylinositol-anchored proteins by promoting the formation of detergent-insoluble ordered membrane domains. J Biol Chem 1997;(In Press)
63. Cardoso de Almeida ML, Turner MJ. The membrane form of variant surface glycoproteins of *Trypanosoma brucei*. Nature 1983; 302:349-352.
64. Scallon B, Fung W-J, Tsang C et al. Primary structure and functional activity of a phosphatidylinositol-glycan specific phospholipase D. Science 1991; 252:446-448.
65. Scallon BJ, Kado-Fong H, Nettleton MY et al. Novel strategy for secreting proteins: Use of phosphatidylinositol-glycan-specific phospholipase D to release chimeric phosphatidylinositol-glycan anchored proteins. Biotechnology 1992; 10:550-556.
66. Anderson SM, Yu G, Giattina M et al. Intercellular transfer of a glycosylphosphatidylinositol (GPI)-linked protein: Release and uptake of CD4-GPI from recombinant adeno-associated virus-transduced HeLa cells. Proc Natl Acad Sci USA 1996; 93:5894-5898.
67. Miller JL, Giattina M, Blanchette Mackie EJ, Dwyer NK. Variegated transfer of recombinant glycosylphosphatidylinositol-anchored CD4 among cultured cells: Correlation of flow cytometric and microscopic observations. J Lab Clin Med 1998; 131:215-221.
68. Väkevä A, Jauhiainen M, Ehnholm C et al. High-density lipoproteins can act as carriers of glycophosphoinositol lipid-anchored CD59 in human plasma. Immunology 1994; 82:28-33.

CHAPTER 2

Biosynthesis of GPIs in Mammalian Cells

Anant K. Menon and Peter Bütikofer

Glycosylphosphatidylinositol (GPI) structures are widely distributed among eukaryotic organisms in the form of GPI anchors covalently linked to select proteins, or as free glycophospholipids. GPIs have a common ethanolamine-phosphate-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol-phospholipid backbone structure that is assembled by stepwise transfer of components to phosphatidylinositol (Fig. 2.1). This review will focus on recent progress towards delineating the reactions involved in GPI biosynthesis in mammalian cells. Previous general reviews on GPI biosynthesis can be found in Englund,[1] Ferguson,[2] Stevens,[3] Takeda and Kinoshita,[4] Vidugiriene and Menon,[5] Udenfriend and Kodukula,[6] and Kinoshita et al.[7] More specific reviews on GPI biosynthesis in parasites include those of Turco and Descoteaux,[8] McConville and Ferguson,[9] Mengeling et al,[10] and Ferguson.[11]

GPI Anchor Assembly

General Outline of the GPI Biosynthetic Pathway

GPI biosynthesis is initiated by transferring *N*-acetylglucosaminyl (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI) to yield GlcNAc-PI. GlcNAc-PI is then de-*N*-acetylated, inositol acylated, triply mannosylated and decorated with phosphoethanolamine residues to generate a GPI molecule that can be attached to protein. Attachment occurs via the amino group of a phosphoethanolamine moiety linked to the 6-position of the terminal mannose in the GPI structure. Many aspects of the GPI biosynthetic pathway can be reproduced in cell-free systems using cell lysates or subcellular membrane fractions supplemented with appropriate soluble metabolites (e.g., UDP-GlcNAc, GDP-mannose). GPI biosynthesis is not essential for the viability of mammalian cells in culture (although mammals and yeast with defects in GPI synthesis are not viable), and a number of mutant cell lines are available that are unable to express GPI-anchored proteins at the cell surface because of defects in steps required for assembly of a complete GPI structure. These cell lines have been invaluable in identifying genes that most likely encode the biosynthetic enzymes required for GPI assembly. An outline of the GPI biosynthetic pathway is shown in Figure 2.1 and discussed in more detail below; the membrane topologies of gene products known to be involved in GPI assembly are shown in Figure 2.2.

Biosynthesis of GlcNAc-PI

The synthesis of GlcNAc-PI from UDP-GlcNAc and PI[12] occurs on the cytoplasmic face of the endoplasmic reticulum (see later for a more complete discussion of the subcellular

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

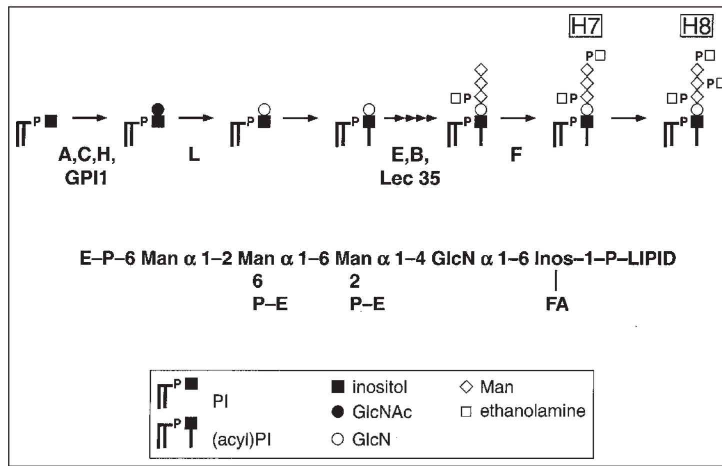

Fig. 2.1. GPI assembly in mammalian cells. The figure shows an outline of the GPI biosynthetic pathway in mammalian cells and the structure of the GPI molecule H8. GlcNAc-PI is synthesized from UDP-GlcNAc and PI in a reaction requiring the products of the *PIG-A*, *PIG-C*, *PIG-H* and *GPI1* genes. GlcNAc-PI is de-*N*-acetylated to form GlcN-PI. The PIG-L protein may be the de-*N*-acetylase. GlcN-PI is elaborated by inositol acylation, mannosylation and phosphoethanolamine addition. The 3 mannose residues are derived from dolichol-P-mannose, and the terminal phosphoethanolamine residue (attached to the mannose residue at the non-reducing terminus of the glycan) is derived from phosphatidylethanolamine. Mutants belonging to complementation class E are defective in dolichol-P-mannose synthesis and are consequently unable to mannosylate GPIs. The class B mutants are defective in the third mannosyltransferase, and the Lec35 mutation affects Dol-P-Man usage. Class F mutants lack the ethanolamine-phosphotransferase activity involved in attaching the terminal EtN-P residue.

location of GPI biosynthesis). At least four gene products (termed PIG-A, PIG-C, PIG-H, and GPI1) are known to be involved in GlcNAc-PI synthesis (Fig. 2.2)[2,7,13,14] and the current model is that a complex of these gene products yields the functional GlcNAc transferase enzyme.[15] The nature and stoichiometry of the complex is unknown, but PIG-A, which bears some resemblance to a bacterial GlcNAc transferase, has been proposed to be the catalytic unit. The other components of the complex may have other substrate binding or regulatory functions, or play a role in the assembly of the complex. The proposed hetero-oligomeric nature of the GPI GlcNAc transferase is unusual for a glycosyltransferase involved in the modification of secretory proteins, since ER and Golgi glycosyltransferases tend to be monomers or homo-oligomeric complexes (see e.g., ref. 16).

Somatically acquired deletions or mutations in the PIG-A gene of hematopoietic stem cells have been shown to cause paroxysmal nocturnal hemoglobinuria (PNH), an acquired hemolytic disease in humans characterized by abnormal activation of complement on blood cells due to a deficiency of GPI-anchored complement regulatory proteins.[17-19] The absence of decay accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) on PNH erythrocytes results in the increased sensitivity of these cells to lysis induced by complement, and in a characteristic intermittent intravascular hemolysis. Recent reviews on GPI deficiency in PNH cells can be found in Kinoshita et al[20,21] and Rosse.[22]

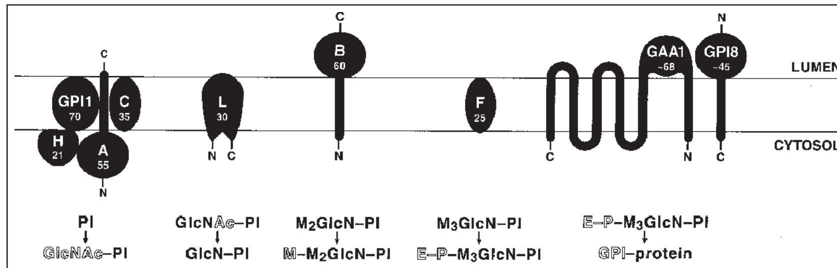

Fig. 2.2. Membrane topology of gene products involved in GPI biosynthesis. The proposed topological arrangements of gene products involved in GPI assembly are shown, along with the reactions in which they participate. The human PIG-A (phosphatidylinositol glycan of complementation class A) gene encodes an ER transmembrane protein of 484 amino acids[105] with a large cytoplasmic domain.[106] The sequence homology of the cytoplasmic domain with a bacterial GlcNAc transferase[107] suggests that PIG-A bears the catalytic site for GlcNAc transfer. Chromosomal localization studies have established that *PIG-A* is on the X chromosome.[17] Human *PIG-C* encodes a predicted ER protein of 297 amino acids with multiple potential transmembrane domains.[108] The human PIG-H gene which is localized on chromosome 1 encodes a protein of 188 amino acids[109] with its N- and C-termini facing the cytoplasm.[106] PIG-H has no significant sequence homology with other known proteins, and thus its function cannot be predicted from its primary structure. The genomic localization of *PIG-H* is on chromosome 12 in the mouse.[110] Since many of the genes on mouse chromosome 12 are located on human chromosome 14, human *PIG-H* may also be located on chromosome 14 (Ware et al, 1994).[110] There are no human diseases known to be caused by deletions or mutations in *PIG-C* or *PIG-H*. Human *GPI1* encodes a 581 amino acid protein that has at least one putative transmembrane domain and shows a 24% amino acid identity to the gene product of yeast *GPI1*.[15] *PIG-L* encodes a putative ER membrane protein of 252 amino acids with a large cytoplasmic domain.[27] It has no sequence homology to other known proteins. *PIG-B* encodes a 554 amino acid ER transmembrane protein with its N-terminus on the cytosolic side and C-terminus within the lumen of the ER.[54] Since deletion of the cytoplasmic portion of PIG-B does not affect complementation of the mutant, the active site of PIG-B is likely present on the lumenal side of the ER. The human *PIG-B* gene is located on chromosome 15.[54] *PIG-F* encodes a hydrophobic protein of 219 amino acids with no homology to other known proteins;[111] *PIG-F* is located on mouse chromosome 2. Human *GPI8* encodes a protein of 395 amino acids and is localized on chromosome 1.[94] A human homologue of yeast *GAA1* has recently been isolated and encodes a membrane protein of 621 amino acids.[112]

De-N-acetylation of GlcNAc-PI to GlcN-PI

In the second step of the GPI biosynthetic pathway, GlcNAc-PI is de-*N*-acetylated to yield GlcN-PI. The reaction has been reported to be specifically stimulated by GTP in vitro,[23] but the reasons for this effect remain unclear. Studies of the substrate specificity of the enzyme have been reported with respect to N-acyl groups (R) that can be released from GlcNR-PI substrates (R = acetyl, propionyl, etc.);[24] these studies show that GlcNAc-PI is, by far, the preferred substrate. Two cell lines have been generated that are defective in de-*N*-acetylase activity. The two lines have been tentatively assigned to different complementation classes, class J[25] and class L.[26] However, since no complementation analyses have been reported, it is unclear whether or not the two cell lines indeed belong to different complementation classes. Rescue of the class L mutant led to the cloning and characterization of the *PIG-L* gene;[27] *PIG-L* encodes a putative ER membrane protein (PIG-L) that may correspond to the GlcNAc-PI de-*N*-acetylase. It is possible that the putative PIG-J protein is

distinct from PIG-L and represents a second subunit (auxiliary or catalytic) of the de-*N*-acetylase.

Inositol Acylation of GlcN-PI to GlcN-(acyl)PI

Acylation of the inositol ring is an obligatory step prior to mannosylation in GPI biosynthesis in mammalian cells[28,29] and yeast,[30] but not in trypanosomes.[31] This modification renders the GPI structure insensitive to cleavage by GPI-hydrolyzing phospholipases C.[32] Results from several studies indicate that the acyl chain is attached at the 2-position of the inositol ring.[33-36] Compositional analyses of mammalian GPI structures have identified palmitic acid as the predominant[34] or only[32,35,37,38] acyl chain bound to the inositol moiety, although in vitro studies by Doerrler et al[29] show that a number of different fatty acids can be transferred to inositol (see also Sharma et al[24]). Interestingly, inositol acylated GPI structures in trypanosomes show heterogeneity in the composition of inositol-bound acyl chains.[36,39]

Inositol acylation in mammals and yeast involves fatty acyl-CoA,[29,40] but it is not clear if an acyl-CoA is the direct donor of the acyl chain for the acyltransferase or if another metabolite serves as an intermediate in acyl transfer to GlcN-PI.[29] In trypanosomes it appears that the inositol acyl donor is not acyl CoA, but quite possibly a phospholipid.[41] The differences in inositol acylation in mammals and yeast versus trypanosomes are reinforced by the observation that inositol acylation is inhibited by phenylmethylsulfonyl fluoride (PMSF) in trypanosomes but not in mammalian cells;[42] the mammalian and parasite inositol acyltransferase enzymes are likely to be substantially different.[31,42,43]

Mannosylation of GlcN-(acyl)PI

The elaboration of GlcN-(acyl)PI proceeds by the addition of mannose and phosphoethanolamine (EtN-P) residues to yield a mature GPI structure. Biochemical analyses using a trypanosome cell-free system indicate that the three core mannose residues are derived from dolichol-phosphomannose (Dol-P-Man).[44] Consistent with this observation, mammalian and yeast mutants[45-48] with a defect in Dol-P-Man synthesis or utilization are deficient in GPI biosynthesis. GPI biosynthesis in the class E and Lec15 mammalian mutants can be restored by transfecting the cells with a yeast cDNA, *DPM1*, encoding yeast Dol-P-Man synthase.[49,50] Curiously, a human homolog of *DPM1* restored Dol-P-Man synthesis, and hence GPI synthesis, in the class E mutant but not in the Lec15 mutant.[51] These results indicate that at least two genes are involved in Dol-P-Man synthesis in mammalian cells. The human homologue of *DPM1* encodes a protein of 260 amino acids with a 30% amino acid identity with the yeast translation product.[51] Human DPM1 lacks a transmembrane domain that is present in the yeast protein and it is possible that the gene mutated in the Lec15 mutant is necessary for membrane association of mammalian DPM1.[7]

The first mannosylated GPI intermediate, Manα1-4GlcN-(acyl)PI, is modified by an EtN-P residue on the 2-position of the mannose to generate (EtN-P)Manα1-4GlcN-(acyl)PI.[52,53] Transfer of two mannose residues to (EtN-P)Manα1-4GlcN-(acyl)PI then yields the completed core mannosylated structure Manα1-2Manα1-6(EtN-P)Manα1-4GlcN-(acyl)PI. Mutant cell lines defective in the transfer of the third mannose have been identified (class B mutants)[14,52] and the gene product PIG-B (Fig. 2.2) is presumed to encode the third mannosyltransferase.[54]

EtN-P Addition

The final step in GPI precursor biosynthesis involves the addition of EtN-P to the 6-position of the third mannose to form EtN-P-6Manα1-2Manα1-6(EtN-P)Manα1-4GlcN-(acyl)PI. The EtN-P donor in yeast[55] and trypanosomes[56] is phosphatidylethanolamine,

and in all likelihood the same is true in the mammalian system.[56] A GPI structure containing three mannoses accumulates in a class F lymphoma mutant, suggesting that the class F mutant cells are defective in attachment of the terminal phosphoethanolamine.[14] The *PIG-F* gene which complements this defect most likely encodes the terminal ethanolamine-phosphotransferase.

An additional EtN-P residue may be added to the second mannose of the GPI core to form EtN-P-6Manα1-2(EtN-P)Manα1-6(EtN-P)Manα1-4GlcN-(acyl)PI.[57] This EtN-P, as well as the other side-chain EtN-P (attached to the innermost mannose early in GPI biosynthesis), may be derived from phosphatidylethanolamine.[56]

GPI Lipid Remodeling

In almost all cases where the lipid composition of a GPI anchor has been determined, it was found that the fatty acyl or alcohol chains attached to the glycerol moiety, or the ceramide moiety, consisted of components that are not typically found in membrane lipids. These results suggest that:

1. Specific lipids may be selected for the initiation of GPI biosynthesis;
2. GPI biosynthesis may be initiated in membrane domains having a unique lipid composition;
3. The lipids on the GPI structures may selectively be remodeled during or after GPI biosynthesis; or
4. A combination of the above.

The GPI anchor of *T. brucei* variant surface glycoprotein (VSG) contains exclusively myristic acid attached in ester linkage to the glycerol backbone.[58] However, GPI anchor precursors in trypanosomes have stearic acid in the *sn*-1 position and a mixture of fatty acids in the *sn*-2 position of the glycerol, implying that the fatty acids must be removed and replaced with myristic acid during or after biosynthesis. The reaction sequence involved in the replacement of the fatty acids by myristate has been termed fatty acid remodeling.[59] The remodeling process occurs on the completed GPI precursor by sequential replacement of first the *sn*-2 fatty acid and then the *sn*-1 fatty acid with myristate. A process similar to fatty acid remodeling, called myristate exchange, serves to exchange the myristic acid of the VSG GPI anchor with myristate from myristoyl-CoA.[60,61] This exchange may function as a proof-reading mechanism to ensure the VSG GPI anchor's unique fatty acid composition.[62] To date, VSG represents the only protein with a GPI anchor consisting of two identical fatty acyl chains.

A different GPI lipid remodeling mechanism has been observed in *Trypanosoma cruzi* parasites. Both epimastigote and metacyclic trypomastigote forms of *T. cruzi* have GPI-anchored mucin-like glycoproteins on their cell surface (see chapter 8). Detailed structural analyses of the mucin GPI anchors from isolated metacyclic trypomastigotes and epimastigotes in culture show that while the carbohydrate structures are identical in both stages of the parasite, the GPI lipid moieties are different.[63] The PI moiety of the epimastigote mucins consists of alkylacylglycerol, while that of the metacyclic trypomastigote mucins is mostly ceramide-based. These results suggest that when epimastigotes transform into metacyclic trypomastigotes, the PI moiety of the mucin GPI anchors is modified from glycerol-based lipids to lipids having mostly a ceramide backbone.[63]

Yet another GPI lipid exchange process has been noted in the yeast *Saccharomyces cerevisiae*. Yeast GPI precursors are known to consist of completely alkaline-sensitive diacylglycerol moieties.[30,64] However, most of the protein GPI anchors in yeast are composed of an alkaline-resistant ceramide backbone.[65] This observation implies that the diacylglycerol moiety found in the GPI precursors is replaced with a sphingolipid moiety to

generate the profile of mature protein GPI anchors. The GPI lipid exchange reaction was shown to occur only after the transfer of the GPI anchor to protein.[65]

Although GPI lipid remodeling reactions have not been described in mammalian cells, it is possible that they occur. Studies on the lipid composition of human[32] and bovine[66] erythrocyte acetylcholinesterase have shown that only a limited selection of fatty acyl and alcohol chains is present in their GPI anchors. More recently, a similar GPI molecular species composition has also been found in human erythrocyte CD59, suggesting that at least in this cell, different proteins receive identical GPI anchors.[38] However, the molecular species composition of the PI pools in human and bovine erythrocytes is clearly different from that of the respective protein GPI anchors,[67] indicating that selection processes or specific remodeling reactions must be involved during GPI biosynthesis or after GPI attachment to protein. A similar situation has also been observed in acetylcholinesterase from the electric organ of *Torpedo marmorata*, which consists of a set of PI molecular species that is distinctly different from that of the bulk PI.[68] The reason that GPI structures consist of atypical lipid species is unknown.

Inhibitors of GPI Biosynthesis

A number of compounds have been identified that interfere with GPI biosynthesis in cells and/or cell-free systems. These compounds have not only been critical in delineating the reactions involved in GPI biosynthesis, but have also proved extraordinarily useful in highlighting differences between the GPI biosynthetic pathways in mammalian cells, yeast and protozoan parasites. Despite the conserved GPI core structure, significant species-specific variations exist in the biosynthesis of GPIs; these differences may eventually be exploited to provide treatments for yeast and protozoal infections of humans and animals. Smith et al[69] used cell-free systems to show that early GPI intermediates containing 2-O-methyl inositol, i.e., an inositol residue substituted on the 2-hydroxyl group, the position of the attachment of the inositol-bound fatty acid, can be used by the GPI biosynthetic machinery of parasites but not by that of a human cell line. The results from this study are consistent with earlier observations showing that inositol acylation of GlcN-PI is a pre-requisite for subsequent mannosylation in mammalian cells[51,70] but not in parasites. Sütterlin et al[71] identified and isolated a terpenoid lactone that specifically blocks addition of the third mannose residue in the GPI core in the mammalian and yeast GPI biosynthetic pathways. The compound was found to be ineffective in parasitic protozoa.

In earlier reports, several other compounds were identified that inhibited GPI biosynthesis. Although these compounds proved useful in elucidating the steps involved in GPI biosynthesis, they did not distinguish between the mammalian and parasite pathways and/or were relatively unspecific inhibitors. For example, mannosamine blocks GPI biosynthesis in mammalian cells and trypanosomes by inhibiting the addition of the third mannose to the respective GPI intermediates.[72-75] Also, mannosylation of mammalian and protozoan GPI intermediates is reduced in cells and cell-free preparations treated with 2-fluoro-2-deoxyglucose (cells) and amphomycin (cell-free systems), inhibitors of Dol-P-Man synthase.[44,76,77] However, since mannosamine, 2-fluoro-2-deoxyglucose and amphomycin also affect N-glycosylation, they cannot be used specifically against the GPI biosynthetic pathway. Other useful GPI biosynthesis inhibitors include the protease inhibitors PMSF and diisopropylfluorophosphate, which inhibit inositol acylation and deacylation, respectively, in trypanosomes; PMSF has no effect on GPI assembly in mammalian cells.[31,42]

Attachment of GPI to Protein

GPI anchors are attached to select proteins via the action of an ER enzyme, presumed to be a transamidase (GPI: protein transamidase, or GPT). The GPT-catalyzed reaction

involves removal of a C-terminal signal sequence from an ER-translocated polypeptide substrate and concomitant attachment of a GPI moiety to yield a GPI-anchored polypeptide. Nontransfer of GPIs to target proteins results in lack of surface expression of these proteins (see, e.g., refs. 78, 79) and thus GPI-anchoring may be viewed as a control for the exit of certain proteins from the ER.[80]

Genes encoding GPI-anchored proteins specify two signal sequences in the primary translation product—an N-terminal signal sequence for targeting the protein to the ER and a C-terminal signal sequence directing the attachment of the GPI anchor.[6] The hydrophobic N-terminal signal peptide is typical of proteins that are targeted to the ER and is removed during or after translocation across the ER membrane, although its cleavage is not strictly necessary for subsequent GPI addition.[81] The second hydrophobic signal peptide at the C-terminus of the polypeptide chain is also removed during processing, followed by the attachment of the completed GPI precursor, via the terminal EtN-P, to the newly exposed C-terminal amino acid. This final step occurs via a transamidase reaction.[82] The entire sequence of events—ER targeting, translocation, N-terminal signal sequence cleavage and GPI addition—can be reproduced in vitro.[83,84] In addition to the obvious requirements for the ER translocation machinery and GPT, soluble, lumenal ER proteins are required for successful GPI anchor addition.[85] These proteins may well be ER chaperones such as BIP/GRP78.

The cleavage site (ω site) between the C-terminal signal peptide and the mature GPI-anchored protein has been determined experimentally and also by mutational studies (see, e.g., refs. 86-89). Extensive analyses and testing of possible C-terminal signal peptides suggest that GPI attachment to protein only occurs when mainly small amino acids (Gly, Ala, Cys, Ser, Asp, Asn) are present at the ω and ω+1 sites, with cleavage occurring between the ω and ω+1 site. At the ω+2 site, most mammalian GPI-anchored proteins contain either Ala or Gly. These studies indicate that GPI addition occurs in a small amino acid domain at the amino end of a hydrophobic C-terminal signal peptide. A detailed compilation of the available data on the ω site and the hydrophobic signal peptide has been published by Udenfriend and Kodukula.[6]

Mutants in mammalian cells (class K)[25,90] and yeast (gaa1 and gpi8)[91,92] have been identified that appear to synthesize the complete GPI precursor yet lack GPI-anchored proteins. These cells are most likely defective in GPT. A mouse homologue of *GAA1* has recently been cloned.[93] Disruption of *GAA1* in a mouse cell line results in lack of surface expression of GPI-anchored proteins, demonstrating its involvement in GPI anchor attachment.[93] The human class K mutant, which has a reported defect in transamidase activity,[90] could be complemented with human *GPI8*[93,94] but not *GAA1*,[93] confirming that at least two genes are involved in GPI transfer to proteins in mammalian cells (Fig. 2.2). The GPI8 protein may represent the catalytic component of the transamidase complex, since the yeast GPI8 gene was found to encode an ER membrane protein with a large lumenal domain (Fig. 2.2) and homology to a plant endopeptidase which has been shown to have transamidase activity.[92] In addition, it has recently been shown that reconstitution of class K cells with human *GPI8* restored the ability of isolated microsomes to form an active carbonyl in the presence of a suitable substrate,[94] which is regarded as an intermediate in catalysis by a transamidase.[82] Together, these data indicate that GPT is, at the very least, a complex of GAA1 and GPI8; it is possible that GPT contains other hitherto unidentified components.

Subcellular Location and Membrane Topology of GPI Biosynthesis

GPIs are synthesized in the endoplasmic reticulum (ER). However, the ER is a heterogeneous organelle with compositionally distinct subregions (see, e.g., ref. 95) and recent data suggest that GPI biosynthetic activities are not uniformly distributed in the ER

membrane. For example, early biosynthetic steps (up to the addition of the first mannose residue) appear to be unusually concentrated in a rapidly sedimenting subregion of the ER.[96,97] Unlike conventionally isolated 'microsomal' ER, this GPI-active ER subregion cofractionates with mitochondria in simple differential centrifugation analyses of cell homogenates, and may correspond to a previously described ER fraction shown to be active in lipid synthesis[98] and possibly also involved in phospholipid transfer between the ER and mitochondria (see, e.g., ref. 99). Later steps in GPI assembly appear to be less compartmentalized and occur with similar efficiencies in the 'mitochondrion-associated' ER subregion as well as in 'microsomal' ER.[97] The significance of this compartmentation of the GPI biosynthetic pathway is unclear.

The initial steps of GPI synthesis are not only laterally compartmentalized in the ER membrane,[97] but also appear to be transversely confined. Early GPI biosynthetic intermediates (GlcNAc-PI and GlcN-PI) can be hydrolyzed by PI-specific phospholipase C in intact ER vesicles, indicating that these lipids are located in the outer (cytoplasmic) leaflet of ER vesicles.[100] The membrane topologies of the GPI GlcNAc transferase complex as well as that of the candidate de-*N*-acetylase, PIG-L, are compatible with this proposal since the bulk of the proteins appear to be cytoplasmically disposed (Fig. 2.2). Thus GlcNAc-PI and GlcN-PI are most likely synthesized on the cytoplasmic face of the ER. Experiments with trypanosomes and yeast indicated that even the later GPI intermediates were accessible to membrane topological probes, suggesting that these more elaborate structures are also located on the cytoplasmic face of the ER.[101-103] Based on these data, and the knowledge that GPI-anchored proteins are confined to the exoplasmic membrane leaflet, it was proposed that the entire GPI biosynthetic pathway is confined to the cytoplasmic face of the ER membrane bilayer with the mature, triply mannosylated, phosphoethanolamine-containing GPIs being flipped into the exoplasmic leaflet (via a GPI translocator/flippase?) for transfer to protein.[101] However, it is conceivable that at least some part of the biosynthetic pathway is located in the lumenal leaflet of the ER (the lumenally-biased membrane topology of mammalian PIG-B (Fig. 2.2), a candidate GPI mannosyltransferase, suggests that the third GPI mannosylation reaction may occur in the lumenal leaflet of the ER in mammalian cells), and that GPI intermediates take advantage of numerous and/or promiscuous lipid translocators in the ER[104] to find their way to the cytoplasmic leaflet, where they are detected by membrane topological probes.

Acknowledgments

A.K.M. was supported by National Institutes of Health grant GM55427 and the University of Wisconsin, and P.B. acknowledges the support in part by Swiss National Science Foundation grants 3100-050587.97 (to P.B.) and 3100-049458.96 (to Urs Brodbeck, University of Bern). We thank David Rancour, Deepak Sharma, and Jolanta Vidugiriene for comments on the manuscript, Taroh Kinoshita for providing preprints of unpublished manuscripts, Adam Steinberg and Laura van der Ploeg (Department of Biochemistry, University of Wisconsin-Madison) for the figures, and K.M.M. and M.S.B. for much appreciated stimulation.

References

1. Englund PT. The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. Annu Rev Biochem 1993; 62:121-138.
2. Ferguson MAJ. What can GPI do for you? Parasitol Today 1994; 10:48-52.
3. Stevens VL. Biosynthesis of glycosylphosphatidylinositol membrane anchors. Biochem J 1995; 310:361-370.
4. Takeda J, Kinoshita T. GPI-anchor biosynthesis. Trends Biochem Sci 1995; 20:367-371.

5. Vidugiriene J, Menon AK. Biosynthesis of glycosylphosphatidylinositol anchors. Methods Enzymol 1995; 250:513-535.
6. Udenfriend S, Kodukula K. How glycosylphosphatidylinositol-anchored membrane proteins are made. Annu Rev Biochem 1995; 64:563-591.
7. Kinoshita T, Ohishi K, Takeda J. GPI-anchor synthesis in mammalian cells: Genes, their products, and a deficiency. J Biochem 1997; 122:251-257.
8. Turco SJ, Descoteaux A. The lipophosphoglycan of Leishmania parasites. Annu Rev Microbiol 1992; 46:65-94.
9. McConville MJ, Ferguson MAJ. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem J 1993; 294:305-324.
10. Mengeling BJ, Beverly SM, Turco SJ. Designing glycoconjugate biosynthesis for an insidious intent: Phosphoglycan assembly in Leishmania parasites. Glycobiol 1997; 7:873-880.
11. Ferguson MAJ. The surface glycoconjugates of trypanosomatid parasites. Phil Trans R Soc Lond 1997; 352:1295-1302.
12. Doering TL, Masterson WJ, Englund PT et al. Biosynthesis of the GPI membrane anchor of the trypanosome variant surface glycoprotein: Origin of the non-acetylated glucosamine. J Biol Chem 1989; 264:11168-11173.
13. Stevens VL, Raetz CRH. Defective glycosyl phosphatidylinositol biosynthesis in extracts of three Thy-1 negative lymphoma cell mutants. J Biol Chem 1991; 266:10039-10042.
14. Sugiyama E, DeGasperi R, Urakaze M et al. Identification of defects in glycosylphosphatidylinositol anchor biosynthesis in the Thy-1 expression mutants. J Biol Chem 1991; 266:12119-12122.
15. Watanabe R, Inoue N, Westfall B et al. The first step of glycosylphosphatidylinositol biosynthesis is mediated by a complex of PIG-A, PIG-H, PIG-C and GPI1. EMBO J 1998; 17:877-885.
16. Fleischer B, McIntyre JO, and Kempner ES. Target sizes of galactosyltransferase, sialyltransferase, and uridine diphosphatase in Golgi apparatus of rat liver. Biochemistry 1993; 32:2076-2081.
17. Takeda J, Miyata T, Kawagoe K et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. Cell 1993; 73:703-711.
18. Bessler M, Mason PJ, Hillmen P et al. Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene. EMBO J 1994; 13:110-117.
19. Miyata T, Yamada N, Iida Y et al. Abnormalities of PIG-A transcripts in granulocytes from patients with paroxysmal nocturnal hemoglobinuria. N Engl J Med 1994; 330:249-255.
20. Kinoshita T, Inoue N, Takeda J. Defective glycosyl phosphatidylinositol anchor synthesis and paroxysmal nocturnal hemoglobinuria. Adv Immunol 1995; 60:57-101.
21. Kinoshita T, Inoue N, Takeda J. Role of phosphatidylinositol-linked proteins in paroxysmal nocturnal hemoglobinuria pathogenesis. Annu Rev Med 1996; 47:1-10.
22. Rosse WF. Paroxysmal nocturnal hemoglobinuria as a molecular disease. Medicine 1997; 76:63-93.
23. Stevens VL. Regulation of glycosylphosphatidylinositol biosynthesis by GTP. Stimulation of *N*-acetylglucosamine-phosphatidylinositol deacetylation. J Biol Chem 1993; 268:9718-9724.
24. Sharma DK, Smith TK, Crossman A et al. Substrate specificity of the N-acetylglucosaminyl-phosphatidylinositol de-*N*-acetylase of glycosylphosphatidylinositol membrane anchor biosynthesis in African trypanosomes and human cells. Biochem J 1997; 328:171-177.
25. Mohney RP, Knez JJ, Lakshmeswari R et al. Glycoinositol phospholipid anchor-defective K562 mutants with biochemical lesions distinct from those in Thy-1-murine lymphoma mutants. J Biol Chem 1994; 269:6536-6542.
26. Stevens VL, Zhang H, Harreman M. Isolation and characterization of a Chinese hamster ovary (CHO) mutant defective in the second step of glycosylphosphatidylinositol biosynthesis. Biochem J 1996; 313:253-258.
27. Nakamura N, Inoue N, Watanabe R et al. Expression cloning of PIG-L, a candidate *N*-acetylglucosaminyl-phosphatidylinositol deacetylase. J Biol Chem 1997; 272:15834-15840.

28. Urakaze M, Kamitani T, DeGasperi R et al. Identification of a missing link in glycosylphosphatidylinositol anchor biosynthesis in mammalian cells. J Biol Chem 1992; 267:6459-6462.
29. Doerrler WT, Ye J, Falck JR et al. Acylation of glucosaminyl phosphatidylinositol revisited. J Biol Chem 1996; 271:27031-27038.
30. Costello LC, Orlean P. Inositol acylation of a potential glycosyl phosphoinositol anchor precursor from yeast requires acyl coenzyme A. J Biol Chem 1992; 267:8599-8603.
31. Güther MLS, Ferguson MAJ. The role of inositol acylation and inositol deacylation in GPI biosynthesis in *Trypanosoma brucei*. EMBO J 1995; 14:3080-3093.
32. Roberts WL, Myher JJ, Kuksis A et al. Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase. J Biol Chem 1988; 263:18766-18775.
33. Ferguson MAJ. Site of palmitoylation of a phospholipase C-resistant glycosylphosphatidylinositol membrane anchor. Biochem J 1992; 284:297-300.
34. Xia MQ, Hale G, Lifley MR et al. Structure of the CAMPATH-1 antigen, a glycosylphosphatidylinositol-anchored glycoprotein which is an exceptionally good target for complement lysis. Biochem J 1993; 293:633-640.
35. Treumann A, Lifely MR, Schneider P et al. Primary structure of CD52. J Biol Chem 1995; 270:6088-6099.
36. Treumann A, Zitzmann N, Hülsmeier A et al. Structural characterization of two forms of procyclic acidic repetitive protein expressed by procyclic forms of *Trypanosoma brucei*. J Mol Biol 1997; 269:529-547.
37. Sevlever D, Humphry DR, Rosenberry TL. Compositional analysis of glucosaminyl(acyl)phosphatidylinositol accumulated in HeLa S3 cells. Eur J Biochem 1995; 233:384-394.
38. Rudd PM, Morgan BP, Wormald MR et al. The glycosylation of the complement regulatory protein, human erythrocyte CD59. J Biol Chem 1997; 272:7229-7244.
39. Güther MLS, Treumann A, Ferguson MAJ. Molecular species analysis and quantification of the glycosylphosphatidylinositol intermediate glycolipid C from *Trypanosoma brucei*. Mol Biochem Parasitol 1996; 77:137-145.
40. Stevens VL, Zhang H. Coenzyme A dependence of glycosylphosphatidylinositol biosynthesis in a mammalian cell-free system. J Biol Chem 1994; 269:31397-31403.
41. Field MC, Menon AK, Cross GAM. A glycosylphosphatidylinositol protein anchor from procyclic stage *Trypanosoma brucei*: Lipid structure and biosynthesis. EMBO J 1991; 10:2731-2739.
42. Güther MLS, Masterson WJ, Ferguson MAJ. The effects of PMSF on inositol-acylation and fatty acid remodeling in African trypanosomes. J Biol Chem 1994; 269:18694-18701.
43. Smith TK, Sharma DK, Crossman A et al. Parasite and mammalian GPI biosynthetic pathways can be distinguished using synthetic substrate analogues. EMBO J 1997; 16:6667-6675.
44. Menon AK, Mayor S, Schwarz RT. Biosynthesis of glycosyl-phosphatidylinositol lipids in *Trypanosoma brucei*: Involvement of mannosyl-phosphoryldolichol as the mannose donor. EMBO J 1990; 9:4249-4258.
45. Chapman A, Fujimoto K, Kornfeld S. The primary glycosylation defect in class E Thy-1-negative mutant mouse lymphoma cells is an inability to synthesize dolichol-P-mannose. J Biol Chem 1980; 255:4441-4446.
46. Orlean P. Dolichol phosphate mannose synthase is required in vivo for glycosyl phosphatidylinositol membrane anchoring, O mannosylation, and N glycosylation of protein in *Saccharomyces cerevisiae*. Mol Cell Biol 1990; 10:5796-5805.
47. Singh N, Tartakoff AM. Two different mutants blocked in synthesis of dolichol-phosphoryl-mannose do not add glycophospholipid anchors to membrane proteins: Quantitative correction of the phenotype of a CHO cell mutant with tunicamycin. Mol Cell Biol 1991; 11:391-400.

48. Camp LA, Chauhan P, Farrar JD et al. Defective mannosylation of glycosylphosphatidylinositol in Lec35 Chinese hamster ovary cells. J Biol Chem 1993; 268:6721-6728.
49. Beck PJ, Orlean P, Albright C et al. The *Saccharomyces cerevisiae* DPM1 gene encoding dolichol-phosphate-mannose synthase is able to complement a glycosylation-defective mammalian cell line. Mol Cell Biol 1990; 10:4612-4622.
50. DeGasperi R, Thomas LJ, Sugiyama E et al. Correction of a defect in mammalian GPI anchor biosynthesis by a transfected yeast gene. Science 1990; 250:988-991.
51. Tomita S, Inoue N, Maeda Y et al. A homologue of *Saccharomyces cerevisiae* Dpm1p is not sufficient for synthesis of dolichol-phosphate-mannose in mammalian cells. J Biol Chem 1998; 273:9249-9254.
52. Puoti A, Desponds C, Fankhauser C et al. Characterization of glycophospholipid intermediates in the biosynthesis of glycophosphatidylinositol anchors accumulating in the Thy-1-negative lymphoma line SIA-B. J Biol Chem 1991; 266:21051-21059.
53. Hirose S, Prince GM, Sevlever D et al. Characterization of putative glycoinositol phospholipid anchor precursors in mammalian cells. J Biol Chem 1992; 267:16968-16974.
54. Takahashi M, Inoue N, Ohishi K et al. PIG-B, a membrane protein of the endoplasmic reticulum with a large lumenal domain, is involved in transferring the third mannose of the GPI anchor. EMBO J 1996; 15:4254-4261.
55. Menon AK, Stevens VL. Phosphatidylethanolamine is the donor of the ethanolamine residue linking a glycosylphosphatidylinositol anchor to protein. J Biol Chem 1992; 267:15277-15280.
56. Menon AK, Eppinger M, Mayor S et al. Phosphatidylethanolamine is the donor of the terminal phosphoethanolamine group in trypanosome glycosylphosphatidylinositols. EMBO J 1993; 12:1907-1914.
57. Hirose S, Knez JJ, Medof ME. Mammalian glycosylphosphatidylinositol-anchored proteins and intracellular precursors. Methods Enzymol 1995; 250:582-614.
58. Ferguson MAJ, Haldar K, Cross GAM. Trypanosoma brucei variant surface glycoprotein has a sn-1,2-dimyristoyl glycerol membrane anchor at its COOH terminus. J Biol Chem 1985; 260:4963-4968.
59. Masterson WJ, Raper J, Doering TL et al. Fatty acid remodeling: A novel reaction sequence in the biosynthesis of trypanosome glycosyl phosphatidylinositol membrane anchors. Cell 1990; 62:73-80.
60. Buxbaum LU, Raper J, Opperdoes FR et al. Myristate exchange. A second glycosyl phosphatidylinositol myristoylation reaction in African trypanosomes. J Biol Chem 1994; 269:30212-30220.
61. Buxbaum LU, Milne KG, Werbowetz KA et al. Myristate exchange on the *Trypanosoma brucei* variant surface glycoprotein. Proc Natl Acad Sci USA 1996; 93:1178-1183.
62. Werbowetz KA, Englund PT. Glycosyl phosphatidylinositol myristoylation in African trypanosomes. Mol Biochem Parasitol 1997; 85:1-7.
63. Acosta Serrano A, Schenkman S, Yoshida N et al. The lipid structure of the glycosylphosphatidylinositol-anchored mucin-like sialic acid acceptors of *Trypanosoma cruzi* changes during parasite differentiation from epimastigotes to infective metacyclic trypomastigote forms. J Biol Chem 1995; 270:27244-27253.
64. Sipos G, Puoti A, Conzelmann A. Glycosylphosphatidylinositol membrane anchors in *Saccharomyces cerevisiae*: Absence of ceramides from complete precursor glycolipids. EMBO J 1994; 13:2789-2796.
65. Conzelmann A, Puoti A, Lester RL et al. Two different types of lipid moieties are present in glycophosphoinositol-anchored membrane proteins of *Saccharomyces cerevisiae*. EMBO J 1992; 11:457-466.
66. Roberts WL, Myher JJ, Kuksis A et al. Alkylacylglycerol molecular species in the glycosylinositol phospholipid membrane anchor of bovine erythrocyte acetylcholinesterase. Biochem Biophys Res Commun 1988; 150:271-277.

67. Bütikofer P, Zollinger M, Brodbeck U. Alkylacyl glycerophosphoinositol in human and bovine erythrocytes. Molecular species composition and comparison with glycosyl-inositolphospholipid anchors of erythrocyte acetylcholinesterases. Eur J Biochem 1992; 208:677-683.
68. Bütikofer P, Kuypers FA, Shackleton C et al. Molecular species analysis of the glycosylphosphatidylinositol anchor of *Torpedo marmorata* acetylcholinesterase. J Biol Chem 1990; 265:18983-18987.
69. Smith TK, Cottaz S, Brimacombe JS et al. Substrate specificity of the dolichol phosphate mannose: Glucosaminyl phosphatidylinositol alpha1-4-mannosyltransferase of the glycosylphosphatidylinositol biosynthetic pathway of African trypanosomes. J Biol Chem 1996; 271:6476-6482.
70. Puoti A, Conzelmann A. Characterization of abnormal free glycophosphatidylinositols accumulating in mutant lymphoma cells of classes B, E, F, and H. J Biol Chem 1993; 268:7215-7224.
71. Sütterlin C, Horvath A, Gerold P et al. Identification of a species-specific inhibitor of glycosylphosphatidylinositol synthesis. EMBO J 1997; 16:6374-6383.
72. Lisanti MP, Field MC, Caras IW et al.. Mannosamine, a novel inhibitor of glycosylphosphatidylinositol incorporation into proteins. EMBO J 1991; 10:1969-1977.
73. Pan Y-T, Kamitani T, Bhuvaneswaran C et al. Inhibition of glycosylphosphatidylinositol anchor formation by mannosamine. J Biol Chem 1992; 267:21250-21255.
74. Ralton JE, Milne KG, Güther MLS et al. The mechanism of inhibition of glycosylphosphatidylinositol anchor biosynthesis in *Trypanosoma brucei* by mannosamine. J Biol Chem 1993; 268:24183-24189.
75. Sevelver D, Rosenberry TL. Mannosamine inhibits the synthesis of putative glycoinsitol phospholipid anchor precursors in mammalian cells without incorporating into an accumulated intermediate. J Biol Chem 1993; 268:10938-10945.
76. Schwarz RT, Mayor S, Menon AK et al. Biosynthesis of the glycolipid membrane anchor of *Trypanosoma brucei* variant surface glycoproteins: Involvement of Dol-P-Man. Biochem Soc Trans (London) 1989; 17:746-748.
77. Takami N, Oda K, Ikehara Y. Aberrant processing of alkaline phosphatase precursor caused by blocking the synthesis of glycosylphosphatidylinositol. J Biol Chem 1992; 267:1042-1047.
78. Delahunty, MD, Stafford, FJ, Yuan, LC, et al. Uncleaved signals for glycosylphos-phatidylinositol anchoring cause retention of precursor proteins in the endoplasmic reticulum. J Biol Chem 1993; 268:12017-12027.
79. Field MC, Moran P, Li W et al. Retention and degradation of proteins containing an uncleaved glycosylphosphatidylinositol signal. J Biol Chem 1994; 269:10830-10837.
80. Doering, TL, Schekman, R. GPI anchor attachment is required for Gas1p transport from the endoplasmic reticulum in COPII vesicles. EMBO J 1996; 15:182-191.
81. Howell S, Lanctot C, Boileau G et al. A cleavable N-terminal signal peptide is not a prerequisite for the biosynthesis of glycosylphosphatidylinositol-anchored proteins. J Biol Chem 1994; 269:16993-16996.
82. Maxwell SE, Ramalingam S, Gerber LD et al. An active carbonyl formed during glycosylphosphatidylinositol addition to a protein is evidence of catalysis by a transamidase. J Biol Chem 1995; 270:19576-19582.
83. Kodukula K, Amthauer R, Cines D et al. Biosynthesis of phosphatidylinositol-glycan (PI-G)-anchored membrane proteins in cell-free systems: PI-G is an obligatory cosubstrate for COOH-terminal processing of nascent proteins. Proc Natl Acad Sci USA 1992; 89:4982-4985.
84. Doering TL, Schekman R. Glycosyl-phosphatidylinositol anchor attachment in a yeast in vitro system. Biochem J 1997; 328,669-675.
85. Vidugiriene J, Menon AK. Soluble constituents of the ER lumen are required for GPI anchoring of a model protein. EMBO J 1995; 14:4686-4694.
86. Moran P, Raab H, Kohr WJ, Caras IW. Glycophospholipid membrane anchor attachment; molecular analysis of the cleavage/attachment site. J Biol Chem 1991; 266:1250-1257.

87. Nuoffer, C, Horvath, A, Riezman, H. Analysis of the sequence requirements for glycosylphosphatidylinositol anchoring of Saccharomyces cerevisiae Gas1 protein. J Biol Chem 1993; 268:10558-10563.
88. Coyne, KE, Crisci, A, Lublin, DM. Construction of synthetic signals for glycosylphosphatidylinositol anchor attachment. Analysis of amino acid sequence requirements for anchoring. J Biol Chem 1993; 268:6689-6693.
89. Kodukula K, Gerber, L, Amthauer R et al. Biosynthesis of glycosylphosphatidylinositol (GPI)-anchored membrane proteins in intact cells: Specific amino acid requirements adjacent to the site of cleavage and GPI attachment. J Cell Biol 1993; 120:657-664.
90. Chen R, Udenfriend S, Prince GM et al. A defect in glycosylphosphatidylinositol (GPI) transamidase activity in mutant K cells is responsible for their inability to display GPI surface proteins. Proc Natl Acad Sci USA 1996; 93:2280-2284.
91. Hamburger D, Egerton M, Riezman H. Yeast Gaa1p is required for attachment of a complete GPI anchor onto proteins. J Cell Biol 1995; 129:629-639.
92. Benghezal M, Benachour A, Rusconi S et al. Yeast Gpi8p is essential for GPI anchor attachment onto proteins. EMBO J 1996; 15:6575-6583.
93. Ohishi K, Inoue N, Takeda J et al. Mouse GAA1 homologue is involved in transfer of the GPI anchor to proteins. FASEB J 1997; 11:A1085.
94. Yu J, Nagarajan S, Knez JJ et al., The affected gene underlying the class K glycosylphosphatidylinositol surface protein defect codes for the GPI transamidase. Proc. Natl. Acad. Sci. USA 1997; 94:12580-12585.
95. Sitia R, Meldolesi J. Endoplasmic reticulum: A dynamic patchwork of specialized subregions. Mol Biol Cell. 1992; 3:1067-1072.
96. Menon AK, Baumann NA, van't Hof W et al. Glycosylphosphatidylinositols: Biosynthesis and intracellular transport. Biochem Soc Trans (London) 1997; 25:861-865.
97. Vidugiriene J, Sharma DK, Baumann NA et al. Initial steps of GPI biosynthesis are concentrated in a rapidly sedimenting subregion of the endoplasmic reticulum membrane. J Biol Chem 1999, in press.
98. Vance J. Phospholipid synthesis in a membrane fraction associated with mitochondria. J Biol Chem 1990; 265:7248-7256.
99. Shiao Y-J, Lupo G, Vance J. Evidence that phosphatidylserine is imported into mitochondria via a mitochondria-associated membrane and that the majority of mitochondrial phosphatidylethanolamine is derived from decarboxylation of phosphatidylserine. J Biol Chem 1995; 270:11190-11198.
100. Vidugiriene J, Menon AK. Early lipid intermediates in glycosyl-phosphatidylinositol anchor assembly are synthesized in the ER and located in the cytoplasmic leaflet of the ER membrane bilayer. J Cell Biol 1993; 121:987-996.
101. Vidugiriene J, Menon AK. The GPI anchor of cell-surface proteins is synthesized on the cytoplasmic face of the endoplasmic reticulum. J Cell Biol 1994; 127:333-341.
102. Mensa-Wilmot K, LeBowitz JH, Chang K et al. A glycosylphosphatidylinositol (GPI)-negative phenotype produced in Leishmania major by GPI phospholipase C from *Trypanosoma brucei*: Topography of two GPI pathways. J Cell Biol 1994; 124:935-947.
103. Djafarzadeh S, Conzelmann A. Sidedness of biosynthesis of glycosylphosphatidylinositol anchors in the endoplasmic reticulum of *Saccharomyces cerevisiae*. Chimia 1994; 48:401-404.
104. Menon AK. Flippases. Trends Cell Biol 1995; 5:355-360.
105. Miyata T, Takeda J, Iida Y et al. The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. Science 1993; 259:1318-1320.
106. Watanabe R, Kinoshita T, Masaki R et al. PIG-A and PIG-H, which participate in glycosylphosphatidylinositol anchor biosynthesis, form a protein complex in the endoplasmic reticulum. J Biol Chem 1996; 271:26868-26875.
107. MacLachlan PR, Kadam SK, Sanderson KE. Cloning, characterization, and DNA sequence of the rfaLK region for lipopolysacchararide synthesis in Salmonella typhimurium LT2. J Bacteriol 1991; 173:7151-7163.

108. Inoue N, Watanabe R, Takeda J et al. PIG-C, One of the three human genes involved in the first step of glycosylphosphatidylinositol biosynthesis is a homologue of *Saccharomyces cerevisiae* GPI2. Biochem Biophys Res Commun 1996; 226:193-199.
109. Kamitani T, Chang H-M, Rollins C et al. Correction of the Class H defect in glycosylphosphatidylinositol anchor biosynthesis in Ltk- cells by a human cDNA clone. J Biol Chem 1993; 268:20733-20736.
110. Ware RE, Howard TA, Kamitami T et al. Chromosomal assignment of genes involved in glycosylphosphatidylinositol anchor biosynthesis: Implications for the pathogenesis of paroxysmal nocturnal hemoglobinuria. Blood 1994; 83:3753-3757.
111. Inoue N, Kinoshita T, Orii T et al. Cloning of a human gene, PIG-F, a component of glycosylphosphatidylinositol anchor biosynthesis, by a novel expression cloning strategy. J Biol Chem 1993; 268:6882-6885.
112. Hiroi Y, Komuro I, Chen R et al. Molecular cloning of human homolog of yeast GAA1 which is required for attachment of glycosylphosphatidylinositols to proteins. FEBS Lett. 1998; 421:252-258.

CHAPTER 3

Organization of GPI-Anchored Proteins in Mammalian Cell Membranes

Satyajit Mayor and Teymuras V. Kurzchalia

A long-standing debate in the area of glycosylphosphatidylinositol (GPI)-anchored protein structure and function concerns their organization in plasma membranes of living cells. From a functional perspective, understanding how GPI-anchored proteins are distributed in cell membranes has implications for many of the putative roles ascribed to the GPI anchor. GPI-anchored proteins have been proposed to be associated with 'domains' at the cell surface; therefore at the outset we will define the use of the term 'domain' in its different contexts. In cells, there are domains that constitute morphological specializations of the plasma membrane, such as the apical and basolateral domains in polarized epithelia or axonal and dendritic surfaces in neurons. These domains arise due to active processes mediated and maintained by complex cellular machinery. Another type of domain arises due to lateral heterogeneities in relatively undifferentiated areas of the plasma membrane (e.g., areas devoid of morphologically defined specializations) in polarized and nonpolarized cells.[3,4] Studies on the trafficking of GPI-anchored proteins and glycosphingolipids in polarized epithelia (reviewed in refs. 3, 4), and the involvement of GPI-anchored proteins in intracellular signaling processes (reviewed in ref. 5), have led Simons and colleagues[8] to propose a mechanism that envisages domains or 'rafts' in the cell membranes, enriched in (glyco)sphingolipids, cholesterol and specific membrane proteins including GPI-anchored proteins. These domains dictate the sorting of associated proteins and may provide sites for assembling cytoplasmic signaling molecules.[8] In this chapter we will critically examine the available data on the organization of GPI-anchored proteins in the plasma membrane in the light of various biochemical and morphological techniques utilized to study the association of GPI-anchored proteins with specialized membrane domains.

Lateral Heterogeneity in Cell Membranes

Our concept of the structure and organization of the membrane of cells has undergone major revisions from the time of the fluid mosaic model proposed by Singer and Nicolson.[9] Instead of considering the membrane as 'two-dimensional oriented solution of integral proteins … in a viscous phospholipid bilayer', it is increasingly apparent that both protein and lipid components of the bilayer are anisotropically arranged in the lateral and vertical directions. These arrangements may be due to protein-protein, lipid-protein or lipid-lipid interactions or, as in the case of transbilayer asymmetry of membrane lipids,[4,10] due to active processes such as the asymmetric synthesis and flip-flop of some lipid classes.[11]

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

The study of lateral heterogeneity of proteins in cell membranes has been greatly aided by the development of microscopic techniques such as fluorescence recovery after photobleaching (FRAP), single particle tracking and optical laser traps. These studies have provided a much more complex picture of the cell membrane.[3] Analyses of protein diffusion at the cell surface has shown that most proteins do not undergo exclusively unrestricted lateral diffusion characteristic of a relatively dense, two-dimensional fluid; a considerable fraction of membrane proteins is immobile on long timescales as measured by FRAP experiments and single particle tracking analyses. In addition, they are transiently confined for average times of 6-8 seconds to small domains (200-400 nm in diameter) in relatively undifferentiated areas of the membrane.[12] However, the size and lifetimes of transient confinement do not appear to be dependent on the nature of the membrane anchor of the protein: GPI-anchored or transmembrane-anchored neural cell adhesion molecules and a ganglioside, GM1, exhibit similar diffusive motions at the surface of plasma membrane.[12-14] This, instead, may reflect considerable lateral heterogeneity in the membrane at the nanometer scale. Kusumi and coworkers[15] have proposed the idea of 'the membrane skeleton fence' as a restraint on lateral motion, whereas Jacobson and coworkers[16] have envisaged the existence of dense obstacle fields. Currently, the underlying bases for these lateral constraints are being vigorously investigated, and it is likely that these ideas are not mutually exclusive.

Most of the understanding of the lateral heterogeneity in the organization of membrane lipids has come from studies in artificial membranes. A clear indication of lateral heterogeneity is the coexistence of phase-separated regions in the membrane bilayer. This has been validated by theoretical considerations as well as experimental observations (reviewed in ref. 15). Many parameters appear to play a role in the maintenance of segregated domains in artificial bilayers (reviewed in refs. 2, 8, 15, 16). In a bilayer of lipids with mismatched acyl chains, small differences in interaction energies will lead to the segregation of liquid-ordered regions (rigid domains) consisting of long saturated acyl chain lipids, whereas the shorter acyl chain or unsaturated acyl chain lipids prefer the liquid-disordered, less rigid domains. Preferential lateral interactions between hydrophilic head groups are also an obvious mechanism for the formation of domains. There is considerable evidence for strong lateral interactions between cholesterol and sphingolipids, resulting in domain formation in liposomes (reviewed in refs. 2, 8, 17, 18). Cholesterol (and possibly other sterols) play a major role in modulating the size and extent of these domains due to their planar 'rigid' structure and potentiate the organization of liquid-ordered domains in membranes.[21,22] Although the plasma membrane domains may differ in size and structure from the phase-segregated regions or 'self-organized' domains observed in artificial systems, similar physical principles will apply to both. However, many physical measurements, including differential scanning calorimetry, spectroscopy, and NMR, have been too inconclusive to detect these 'self-organized' domains in living cells. This is probably due to the presence of a relatively large number of lipid species, and to the presence of proteins that have specific lipid annuli associated with their hydrophobic transmembrane domains.

In the following sections we will analyze the morphological and biochemical evidence for the presence of GPI-anchored proteins in membrane domains, and discuss the functional implications in some specific instances.

Association of GPI-Anchored Proteins with Domains, Detergent-Insoluble Complexes and Caveolae

Some clues regarding the existence of domains or 'rafts' in living cells rests on the observation that specific membrane proteins and lipids, including GPI-anchored proteins and lipid-linked nonreceptor tyrosine kinases, (glyco)sphingolipids and cholesterol form detergent-insoluble complexes in cold Triton X-100 (TX-100).[8,23] Although this is a

convenient methodology, copurification with such detergent-resistant complexes cannot constitute proof of preexisting domains in cells; the detergent resistant complexes may be different from the domains or 'rafts' that are involved in functional processes inside cells.[2,24,25] This is a poorly appreciated point and several researchers at different times, possibly due to the lack of alternative methodology, have equated caveolae, signaling complexes, or sorting domains with these biochemically generated entities (see below and refs. 24, 25).

A considerable interest in detergent-resistant complexes has been generated by the correlation of morphological observations that GPI-anchored proteins are clustered at significantly higher density in caveolae than on other areas of the plasma membrane,[27,28] and that caveolin/VIP-21, along with GPI-anchored proteins and a subset of membrane lipids (sphingomyelin, acidic and neutral glycolipids, and cholesterol), are largely insoluble in nonionic detergents, mainly cold Triton X-100.[29-32] Caveolae are small, 60-80 nm cell surface inpocketings with a characteristic protein coat of caveolin.[27] Together, these properties have been used to suggest that domains or 'rafts' enriched in these detergent insoluble components are associated with caveolae or 'caveolin/VIP-21-rich membranes' (reviewed in refs. 29,33). Furthermore, many cytoplasmically-oriented signaling molecules, including heterotrimeric GTP-ases, small GTP-ases, and nonreceptor type protein-tyrosine kinases (PTKs), have been localized to caveolae, primarily in aggregates derived with Triton X-100 (reviewed in ref. 21). Based on this 'copurification', GPI-anchored proteins have been proposed to mediate intracellular signaling in caveolae due to their association with PTKs in these structures.[23,33,34]

However, using fluorescently-labeled monoclonal antibodies to different GPI-anchored proteins, these proteins were shown not to be constitutively concentrated in caveolae; they are enriched in these structures only after crosslinking with polyclonal secondary antibodies.[35] Similar results have been obtained by using a fluorescein-labeled analogue of folic acid as a monovalent ligand to visualize the distribution of GPI-anchored folate receptor at the plasma membrane. The diffuse distribution of these proteins is, therefore, not a consequence of antibody binding.[25] Electron microscopic (EM) analyses of the cell surface distribution of GPI-anchored folate receptor, Thy-1 antigen, and alkaline phosphatase also confirmed that these proteins are not constitutively enriched in caveolae.[35-37] A careful quantitative EM analysis has shown that at the level of the sensitivity of the technique, GPI-anchored proteins are randomly distributed over the cell surface; they are neither concentrated in caveolae or clathrin coated pits nor excluded from them.[25,35] Thus, it appears that multimerization of GPI-anchored proteins regulates their sequestration in caveolae, but in the absence of agents that promote clustering they are uniformly distributed over the plasma membrane.[25,35-37]

These studies raise questions about how GPI-anchored proteins become enriched in the caveolae preparations obtained from detergent-extracted cells. Analysis of the effect of Triton X-100 on the surface distribution of GPI-anchored proteins show that GPI-anchored proteins at the cell surface are almost completely insoluble in Triton X-100 and become quantitatively incorporated in low density complexes, although only 4% of the surface is occupied by caveolae in fibroblast cells.[25] There also appears to be an increase in the extent of GPI-anchored protein clustering in the detergent-insoluble membranes. Under these conditions, transmembrane proteins such as the transferrin receptor and most other polypeptide-anchored proteins are readily solubilized.[25,31,38,39] These observations are contrary to the widespread interpretation that the low density Triton X-100 insoluble membranes represent a purified caveolae preparation (or a caveolin/VIP-21-rich preparation: 50- to 100-fold enriched) thereby accounting for the 100- to 200-fold enrichment of the GPI- anchored proteins contained in them.[33,34,38,40]

Using entirely different methods, Schnitzer and colleagues have shown that GPI-anchored proteins and caveolae may be separated from each other using a colloidal silica coating technique;[41] the caveolae may be separated from the colloidal silica coated membrane areas of cells because colloidal silica fails to penetrate the narrow necks of the caveolae. A further immuno-isolation of caveolin-coated membranes in this preparation resulted in the separation of many components initially thought to be part of 'purified' caveolae.[42] Another biochemical procedure involving sequential density sedimentation punctuated by a vigorous sonication procedure has been utilized to show that GPI-anchored proteins are enriched in caveolae/caveolin-containing fractions.[43] These studies are apparently paradoxical, and as yet there appears to be no clear resolution of the extent of concentration of GPI-anchored proteins in caveolae-coated membranes as determined by biochemical means.

It is possible that different biochemical procedures may provide contradictory results because the methods utilized alter lateral membrane organization differently. In this regard, treatment with colloidal silica may result in the immobilization of GPI-anchored and other proteins outside the caveolae and prevent their access to the narrow-necked structures. On the other hand, vigorous sonication may generate low density membrane fragments with altered composition leading to the copurification of caveolae and GPI-anchored proteins. A controversy also exists about the enrichment of heterotrimeric G-proteins in these detergent-insoluble complexes or 'purified caveolae' preparations.[43-46] Collectively, these observations show that biochemical procedures currently available to isolate specialized regions of the plasma membrane are yet to be refined to provide an unambiguous separation of its lipid-linked constituents.

Enrichment of several GPI-anchored proteins and glycolipids in detergent resistant complexes[33,38,40,47,48] has been interpreted to suggest that multiple GPI-anchored proteins are associated with each other (or with a common receptor) in these complexes. In apparent contradiction of these observations, Mayor and coworkers have shown that multiple GPI-anchored proteins on the same cell did not cocluster nor get recruited to caveolae unless they were independently crosslinked, showing that GPI-anchored proteins may not be stably associated with each other at the cell surface.[35] Similar observations have been made in lymphocytes and in keratinocytes where Thy-1 and GM-1 did not cocluster after crosslinking with multivalent agents.[37,48] In a mouse keratinocyte cell line, Fujimoto has shown that the GPI-anchored protein Thy-1, sphingomyelin, glycosphingolipids and β_2-microglobulin (possibly in conjunction with MHC class I) were independently recruited to caveolae only after crosslinking via primary and secondary antibodies, whereas another membrane protein, the transferrin receptor, was not sequestered in caveolae.[37] On the other hand, independently crosslinked GPI-anchored proteins did show a propensity to colocalize to the same small punctate areas of the plasma membrane.[35] Recently, Simons and coworkers have observed that crosslinked GPI-anchored proteins and sphingolipids colocalize to common patches outside caveolae and in cells that lack morphologically identifiable caveolae (Harder et al, *J Cell Biol* 1998:929-942). A transmembrane protein, MHC class I, is also recruited to caveolae only after crosslinking.[49] Thus, there must exist mechanisms for recruiting GPI-anchored proteins, sphingolipids and certain other membrane proteins to caveolae.

It is possible that there may be common 'receptors' that are capable of being recruited to caveolae and stabilize GPI-anchor- and sphingolipid-rich domains. One potential candidate is VIP-36, which is recruited to caveolae only after crosslinking and has lectin binding motifs.[50-52] Molecular analyses of the protein domains involved in recruiting transmembrane proteins to caveolae will provide an understanding of this process. Alternatively, GPI-anchored proteins may exist as small, dynamic domains which are stabilized by crosslinking (see below). These stabilized structures are then able to interact with each other and caveolae.

In the next section we discuss how detergent insoluble complexes may arise due to physical lipid-lipid interaction.

Mechanisms of Detergent Insolubility

The almost complete insolubility of the GPI-anchored proteins, along with their lack of enrichment in the relatively small punctate areas occupied by caveolae after detergent-treatment, showed that these proteins are detergent-insoluble independently of their association with caveolae. The lack of association with caveolin/VIP-21 is consistent with recent studies in lymphocytes where GPI-anchored Thy-1 and the ganglioside GM1 were detergent-insoluble and almost quantitatively formed 'low-density complexes', even though the cells lack detectable caveolin/VIP-21 or caveolae.[48] This could be due to the formation of specific detergent-insoluble glycolipid domains.[48] Alternatively, these proteins may be intrinsically detergent-insoluble in the milieu of the plasma membrane.

Brown and colleagues have demonstrated that the insolubility of GPI-anchored proteins and membrane lipids in Triton X-100 can be reconstituted in the absence of any special structures or protein(s).[53] The primary requirement for detergent insolubility is the presence of a significant mole fraction of high melting temperature lipids such as saturated acyl chain-containing phospholipids. Increasing concentration of cholesterol or neutral glycolipids positively influences the extent of insolubility. GPI-anchored proteins incorporated into such liposomes are insoluble in cold nonionic detergents, and they can be solubilized by increasing the temperature. In contrast, the lipid component is equally insoluble at high and low temperatures, suggesting that the GPI-anchored proteins are only loosely associated with the detergent-resistant membranes. Furthermore, detergent insolubility of GPI-anchored proteins requires the presence of the appropriate lipid milieu in the same bilayer as these proteins (D. Brown, personal communication and ref. 52). Thus, the insolubility of GPI-anchored proteins in Triton X-100 depends mainly on the acyl or alkyl chain composition of the membrane lipids, cholesterol or neutral glycolipid content, and probably the degree of saturation of the acyl or alkyl moiety of the GPI-anchor. Brown and colleagues have further shown that the requirements for promoting liquid-ordered phases or domains in artificial membranes and for generating detergent-insoluble complexes are similar. They observed that cholesterol and sphingolipids are not essential for insolubility in artificial liposomes, but potentiate the Triton X-100-insolubility of the incorporated GPI-anchored proteins.[22,54] In most GPI-anchored proteins, saturated alkyl/acyl chains appear to be the predominant components of the lipid portion of GPI-anchors,[55] which appears to contribute to their detergent insolubility.

Modulating cholesterol levels in vivo by treatment with agents that inhibit cholesterol synthesis (e.g., compactin, see refs. 56, 57) or alter its distribution (e.g., saponin, see ref. 58) have shown that GPI-anchored proteins become more soluble in detergent after reducing the available cholesterol in membranes. Utilizing a sphingolipid deficient cell line, Hanada and coworkers have shown that the insolubility of GPI-anchored proteins is considerably reduced when sphingolipid levels are lowered.[56] Furthermore, the extent of insolubility of the GPI-anchored protein examined could be restored to the control levels by metabolic compensation via exogenously added sphingolipids. These data are consistent with the hypothesis that detergent insoluble domains may require cholesterol and sphingolipids. However, in none of these studies was the composition of the detergent-resistant membranes determined. As mentioned above, cholesterol and sphingolipids enhance the detergent insolubility of GPI-anchored proteins by promoting the formation of ordered membrane domains which are likely to be insoluble in TX-100.[22,53,54] These domains are relatively stable on the time scales of fluorescence quenching experiments.[22]

Other lipid-linked proteins are also insoluble in nonionic detergents; many cytoplasmically oriented proteins such as PTKs and heterotrimeric G proteins have been found in detergent insoluble complexes.[33,40,47,59-61] As pointed out by Lisanti and coworkers, these proteins share a common lipid modification motif, Met-Gly-Cys, which is the site for N-myristylation (on the N-terminal Gly after cleavage of Met) and palmitoylation (on the Cys) at the N-terminus and may direct association with the detergent-insoluble complexes.[62] In confirmation of this hypothesis, the association of two PTKs, $p56^{lck}$ and $p59^{fyn}$, with the detergent-insoluble complexes was found to be due to the N-terminal myristoylation at the Gly residue and palmitoylation at the Cys3 residue.[63] Furthermore, another PTK, $p60^{src}$, which is N-myristoylated but not palmitoylated (since it has the sequence Met-Gly-Ser at the N-terminus) was found not to be associated with these detergent-insoluble complexes.[63] However, a mutant version of $p60^{src}$ with the Met-Gly-Cys motif at its N-terminus was found to be associated with the detergent-resistant complex.

These data are consistent with the idea that association with the detergent-insoluble membranes requires closely juxtaposed saturated fatty acyl chains and the presence in the same bilayer of the various saturated acyl/alkyl chain-containing proteins and lipids. Cytoplasmic lipid-modified proteins share the property of detergent insolubility with GPI-anchored proteins and association with the low-density complexes. However, in the case of the GPI-anchored proteins, this does not reflect an initial concentration in any specialized structures.[25]

Direct Evidence for GPI-Anchored Protein Domains in Living Cell Membranes

The failure to observe domains of GPI-anchored proteins by conventional imaging methods may be because the domains are dynamic on the time scales of imaging, and the resolution of current microscopic techniques is limited. Fluorescence microscopy is limited by the wavelength of the emitted light (>300 nm),[64] and electron microscopic techniques are too insensitive to detect small aggregates of lipidic species.[65] Therefore, these domains or 'rafts' must be small (much less than 300 nm in size), dynamic, and contain only a few protein species.

One method for proving the existence of domains would be to measure the molecular proximity between GPI-anchored proteins and determine whether they represent a nonrandom organization. To measure distances between GPI-anchored species, several investigators have utilized the technique of fluorescence resonance energy transfer (FRET).[66] FRET experiments that measured the aggregation state of chimeric GPI-anchored gD1 (a viral spike glycoprotein) also confirm that there is no significant clustering of these proteins at steady state.[67] However, newly arrived GPI-anchored gD1 at the plasma membrane appear to be clustered.[67] In these studies it was not established whether the clusters were due to the GPI-anchoring of the virus glycoprotein or to an intrinsic property of virus glycoprotein oligomerization during biosynthetic transport. In recent studies using imaging FRET measurements, Edidin and coworkers have shown that while the ganglioside GM1 appears to be present as clusters at the cell surface, a GPI-anchored protein exhibits FRET efficiencies consistent with random distributions at the cell surface.[68] It is possible that the precise arrangement of fluorophores on the antibodies and their relatively large sizes (5-8 nm in diameter) may limit the ability to observe significant FRET between individual GPI-anchored species which are organized in domains.

Recent studies using a small fluorescent ligand to study the distribution of the GPI-anchored folate receptor, coupled with a sensitive measure of the extent of energy transfer, have shown that GPI-anchored proteins occur in cholesterol-dependent submicron domains (less than 70 nm in diameter) at the surface of living cells (Varma and Mayor,

Nature 1998; 394:798-801). Consistent with this finding, Friedrichson and Kurzchalia have utilized classical chemical crosslinking techniques to show that GPI-anchored proteins are present as molecular aggregates consisting of at least 15 molecules (manuscript submitted). They show that the extent of chemical crosslinking does not depend on the expression levels of the GPI-anchored proteins in membranes but is critically dependent on the presence of a GPI anchor and cholesterol in membranes. In fact, detergent treatment appears to dramatically increase the extent of crosslinking (Friedrichson and Kurzchalia, *Nature* 1998; 394:802-805). These studies using fundamentally different methodologies provide direct evidence for the organization of GPI-anchored proteins in 'rafts' or domains in living cell membranes.

As discussed above the development of microscopic techniques such as fluorescence recovery after photobleaching (FRAP), single particle tracking and optical laser traps have provided a much more complex picture of the cell membrane.[3] Analyses of protein diffusion at the cell surface has shown that proteins are transiently confined for average times of 6-8 seconds to small domains in relatively undifferentiated areas of the membrane.[12] Although the size and life times of transient confinement do not appear to be dependent on the nature of the membrane anchor of the protein,[12-14] it would be interesting to correlate the domains identified by the different techniques. These new approaches should aid in the understanding the function of rafts, implicated in diverse processes such as protein sorting and signal transduction.

The model shown in Figure 3.1 is consistent with all available information on the organization of GPI-anchored proteins in cell membranes (wherein artifacts due to the methodology of detection have been taken into consideration). These submicron domains consisting of only a few GPI-anchored proteins are likely to be randomly distributed in membranes, as shown in Figure 3.2.

Functional Implications of GPI-Anchored Protein Domains

The most compelling evidence for domains in the undifferentiated plasma membrane of cells is functional. Several cell biological processes as diverse as signal transduction and sorting of membrane proteins towards morphologically distinct regions of the cell may be understood in terms of the presence of (glyco)sphingolipid, and cholesterol-enriched domains of lipid-linked proteins.[7,69-72] These domains or 'rafts' are postulated to recruit a specific set of membrane proteins and exclude others, thereby sorting membrane proteins or concentrating signaling molecules where they may interact more efficiently with their effectors. In this regard, the ability of the GPI-anchor to act as an apical sorting signal in polarized epithelia and to transduce cellular activation signals may be rationalized if GPI-anchored proteins are integral components of domains or 'rafts' in cell membranes.[8] Here, we will discuss the role of membrane domains in proteins sorting; their involvement in signaling is discussed in chapter 4.

Membrane Domains in Biosynthetic and Endocytic Sorting

Sorting of proteins to different routes during biosynthetic transport is mediated by specific protein sequence or glycosylation based motifs (e.g., the targeting of lysosomal membrane proteins to lysosomes from the trans-Golgi network[73] or the delivery of specific sets of proteins to the basolateral and apical domains of epithelial cells[1,2]). The relatively recent realization that lipid-based sorting motifs are also involved in sorting of membrane components comes from the observation that glycosphingolipids and GPI-anchored proteins are preferentially sorted to apical domains in epithelial cells.[5,74] The observation that GPI-anchored protein, glycosphingolipids and cholesterol are found in detergent-resistant complexes during biosynthetic transport to the apical membrane along with a canonical apical protein, the HA protein of influenza virus, has provided support for the

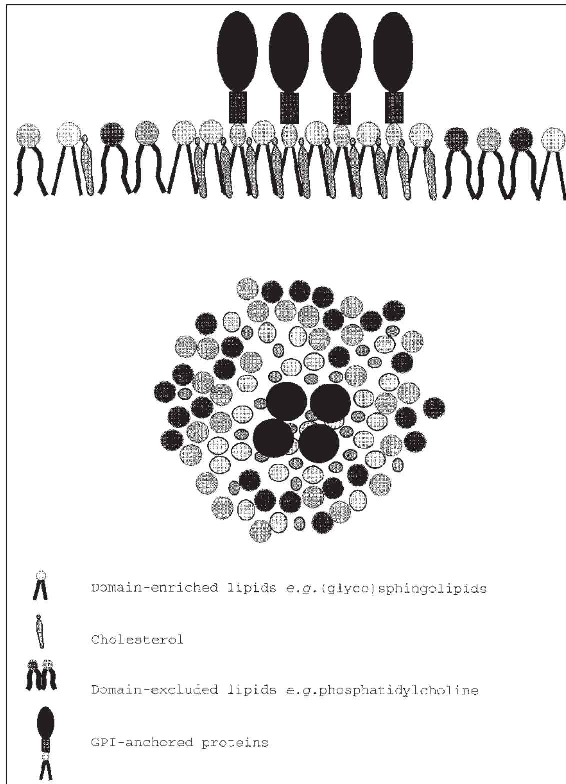

Fig. 3.1. Domain organization of GPI-anchored proteins. GPI-anchored proteins have been depicted in small, sub-micron sized domains or rafts in association with other raft components, cholesterol and (glyco)sphingolipids, consistent with the available information on the organization of these proteins. These domains or rafts are likely to be self-organizing and enriched in specific proteins and lipids, while excluding others, as proposed by the raft hypothesis.[8,23]

involvement of membrane rafts or DIGs in sorting.[8,19,29,75] However, there are several reasons to question the proposal that the detergent-insoluble complex or 'raft' is a 'sorting platform'. Sphingomyelin, although a major component of the 'detergent-resistant' lipids, is not enriched at the apical surface.[76] Replenishment of lipids incapable of being incorporated into detergent resistant membranes are capable of rescuing the impaired sorting of GPI-anchored proteins in sphingolipid-depleted cells.[77,78] Furthermore, although cholesterol depletion appears to result in the loss of the ability of GPI-anchored proteins to associate with detergent-insoluble complexes, it is still sorted to the apical domain.[57]

Recent work has also shown that there are different biosynthetic routes for 'apical' and 'basolateral' proteins in apparently morphologically 'nonpolarized' cells.[79,80] Therefore, understanding the basis of lipid-based sorting is a fundamental issue in membrane trafficking in all cell types. The organization of GPI-anchored proteins is clearly a handle to unravel the mechanism of domain structure, formation and maintenance in cells.

The internalization of membrane proteins and fluid into eukaryotic cells is mediated by clathrin-coated pits, nonclathrin coated pits and uncoated pits at the cell surface.[81-83] Rapid endocytosis of membrane proteins is mediated by specific, cytoplasmically-oriented, peptide signal-dependent enrichment of these proteins in clathrin-coated pits.[84] Membrane proteins lacking functional endocytosis signals are internalized at rates which are five- to twenty-fold slower and are similar to the rate of bulk membrane lipid internalization.[85-87] While clathrin-independent means of internalization have been demonstrated for many years, the specific mechanism for these processes have yet to be elucidated. Caveolae are

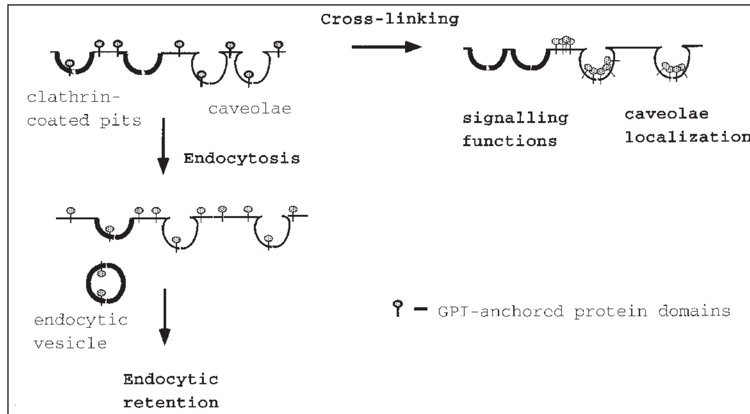

Fig. 3.2. Cell surface dynamics of GPI-anchored proteins. A schematic of the cell surface dynamics of GPI-anchored proteins wherein, in their native state, GPI-anchored proteins (organized as sub-micron domains) are free to diffuse in the plane of the bilayer. Consequently, they are neither enriched nor depleted in coated or noncoated pits and are taken into the cell by mechanisms of bulk membrane endocytosis. These proteins are then retained in endocytic compartments traversed by recycling membrane components. Upon crosslinking by antibodies or by physiological agents, these proteins will be clustered and preferentially localize to caveolae. Crosslinking in many instances is a prerequisite for activating the signaling function of GPI-anchored proteins (reviewed in ref. 5).

examples of nonclathrin mediated invaginations at the plasma membrane, but in most cell types, caveolae per se appear not to be internalized to a great extent.[36]

Based on the observations that cholesterol depletion disrupts caveolar morphology and inhibits folate uptake, and the requirement for a GPI anchor for efficient folate uptake, Anderson and coworkers proposed that GPI-anchored proteins such as the folate receptor take up small molecules via caveolae by a process called potocytosis.[88] Once again, the observation that the folate receptors (or any other GPI-anchored proteins) are not constitutively clustered in caveolae, questions the role of caveolae in folate uptake and the mechanism of GPI anchor-dependent folate uptake.

In the absence of crosslinking, glycolipid-containing areas of plasma membrane and GPI-anchored proteins are internalized via noncoated vesicles and clathrin-coated pathway at rates corresponding to overall membrane internalization rates[72,89,90] (Fig. 3.2). Regardless of their means of internalization, we have recently found that GPI-anchored proteins, including the folate receptor, are able to enter bona fide endosomes which contain recycling transferrin receptors and fluid phase markers and are quantitatively recycled back to the cell surface.[71,72,91] However, as compared to recycling receptors and overall membrane components, GPI-anchored proteins are extensively retained in endosomes.[71,72,91,92] This retention in endosomes is dependent on the lipid levels in cellular membranes: Cholesterol and sphingolipid depletion appears to relieve retention relative to other recycling receptors.[72,78] This lipid-dependent retention in endosomes will expose GPI-anchored proteins (specifically the folate receptor) to the acidic milieu of both sorting and recycling endosomes for longer times than recycling receptors with conventional transmembrane anchor. This is

likely to be the reason for the GPI anchor- and cholesterol and sphingolipid-dependent uptake of folates via the folate receptor.[93,94]

Another GPI-anchored protein, the cellular scrapie protein, is processed to the infectious proteinase K-resistant prion form more efficiently than its transmembrane anchored form[95] (also see chapter 6). This processing is dependent on acidic pH in endosomes and is inhibited by the depletion of cholesterol in membranes.[95-97] These observations are consistent with the observation that the presence of the GPI-anchor causes extensive retention of the cellular scrapie protein in acidic endosomes. If the infectious (prion) form of the scrapie protein were also present in these endosomes, then the cholesterol-dependent retention mechanism could facilitate an efficient conversion of the native scrapie protein into prions, possibly in distinct membrane domains or aggregates.[95] These observations suggest that cholesterol and possibly other lipids play a role in GPI-anchored protein sorting in endosomes.

Recent data supportive of the role of specific lipid domains (rigid or liquid-ordered domains) being segregated from other types of membrane (liquid-disordered or fluid areas) comes from studies on membrane organization and trafficking properties of the lipophilic dialkylcarbocyanines, the rigid domain-preferring DiIC16 and the fluid domain-preferring DiIC12, in mammalian cells.[98-100] These studies have shown that the two kinds of lipid analogues are sorted from each other at the surface of cells[98,99] and in endosomes,[100] consistent with the hypothesis that the rigid domain-preferring GPI-anchored proteins may segregate into specialized microdomains which are rich in cholesterol in endosomes and at the cell surface.

Summary

In this review we have examined the organization of GPI-anchored proteins in the plasma membrane in the context of the current understanding of lateral heterogeneity in cell membranes. We have also provided a critical evaluation of the association of GPI-anchored proteins with membrane domains of various types, including their association with caveolae, and have discussed the role of the GPI-anchor in protein sorting, which may have broader implications for the biology of many of the GPI-anchored proteins described to date.

References

1. Jacobson K, Sheets ED, Simson R. Revisiting the fluid mosaic model of membranes. Science 1995; 268:1441-2.
2. Edidin M. Lipid microdomains in cell surface membranes. Curr Opin Struct Biol 1997; 7:528-32.
3. Simons K, van Meer G. Lipid sorting in epithelial cells. Biochemistry 1988; 27:6197-202.
4. Rodriguez BE, Zurzolo C. Polarity signals in epithelial cells. J Cell Sci Suppl 1993; 17:9-12.
5. Robinson PJ. Phosphatidylinositol membrane anchors and T-cell activation. Immunol. Today 1991; 12:35-41.
6. Simons K, Ikonen E. Functional rafts in membranes. Nature 1997; 387:569-570.
7. Singer SJ, Nicolson GL. The fluid mosaic model of the structure of cell membranes. Science 1972; 175:720-731.
8. Welti R, Glaser M. Lipid domains in model and biological membranes. Chem Phys Lipids 1994; 73:121-137.
9. Menon AK. Flippases. Trends Cell Biol 1995; 5:355-360.
10. Sheets ED, Lee GM, Simson R et al. Transient confinement of a glycosylphosphatidylinositol-anchored protein in the plasma membrane. Biochemistry 1997; 36:12449-12458.
11. Simson R, Sheets ED, Jacobson K. Detection of temporary lateral confinement of membrane proteins using single-particle tracking analysis. Biophys J 1995; 69:989-93.

12. Simson R, Yang B, Moore SE et al. Structural mosaicism on the submicron scale in the plasma membrane. Biophys J 1998; 74:297-308.
13. Kusumi A, Sako Y. Cell surface organization by the membrane skeleton. Curr Opin Cell Biol 1996; 8:566-74.
14. Sheets ED, Simson R, Jacobson K. New insights into membrane dynamics from the analysis of cell surface interactions by physical methods. Curr Opin Cell Biol 1995; 7:707-14.
15. Brown RE. Sphingolipid organization in biomembranes: What physical studies of model membranes reveal. J Cell Science 1998; 111:1-9.
16. Schroeder F, Jefferson JR, Kier AB et al. Recent advances in membrane cholesterol domain dynamics: And intracellular cholesterol trafficking. Proc Soc Exp Biol Med 1996; 213:150-177.
17. Harder T, Simons K. Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. Curr Opin Cell Biol 1997; 9:534-42.
18. Schroeder F, Jefferson JR, Kier AB et al. Membrane cholesterol dynamics: Cholesterol domains and kinetic pools. Proc Soc Exp Biol Med 1991; 196:235-252.
19. Silvius JR, del Giudice D, Lafleur M. Cholesterol at different bilayer concentrations can promote or antagonize lateral segregation of phospholipids of differing acyl chain length. Biochemistry 1996; 35:15198-208.
20. Ahmed SN, Brown DA, London E. On the origin of sphingolipid/cholesterol rich detergent-insoluble cell membranes: Physiological concentrations of cholesterol and sphingolipid induce formation of detergent-insoluble, liquid-ordered lipid phase in model membranes. Biochemistry 1997; 36:10944-10953.
21. Brown D. The tyrosine kinase connection: How GPI-anchored proteins activate T cells. Curr Opin Immunol 1993; 5:349-54.
22. Kurzchalia TV, Hartmann E, Dupree P. Guilt by insolubility—does a protein's detergent insolubility reflect a caveolar location? Trends Cell Biol 1995; 5:187-189.
23. Mayor S, Maxfield FR. Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. Mol Biol Cell 1995; 6:929-44.
24. Weimbs T, Hui-Low S, Chapin SJ et al. Apical targeting in polarized cells: There's more afloat than rafts. Trends Cell Biol 1997; 7:393-399.
25. Parton RG, Simons K. Digging into caveolae. Science 1995; 269:1398-9.
26. Rothberg KG, Heuser JE, Donzell WC et al. Caveolin, a protein component of caveolae membrane coats. Cell 1992; 68:673-682.
27. Dupree P, Parton RG, Raposo G et al. Caveolae and sorting in the trans-Golgi network of epithelial cells. EMBO J 1993; 12:1597-605.
28. Brown D. Interactions between GPI-anchored proteins and membrane lipids. Trends Cell Biol 1992; 2:338-343.
29. Kurzchalia TV, Dupree P, Parton RG et al. VIP21, a 21-kD membrane protein is an integral component of trans-Golgi-network-derived transport vesicles. J Cell Biol 1992; 118:1003-14.
30. Hooper NM, Turner AJ. Ectoenzymes of the kidney microvillar membrane: Differential solubilization by detergents can predict a glycosyl-phosphatidylinositol membrane anchor. Biochem J 1988; 250:865-869.
31. Glenney JR, Zokas L. Novel tyrosine kinase substrates from rous sarcoma virus transformed cells are present in the membrane skeleton. J Cell Biol 1989; 108:2401-2408.
32. Lisanti MP, Tang ZL, Sargiacomo M. Caveolin forms a hetero-oligomeric protein complex that interacts with an apical GPI-linked protein: Implications for the biogenesis of caveolae. J Cell Biol 1993; 123:595-604.
33. Anderson RG. Caveolae: Where incoming and outgoing messengers meet. Proc Natl Acad Sci U S A 1993; 90:10909-13.
34. Mayor S, Rothberg KG, Maxfield FR. Sequestration of GPI-anchored proteins in caveolae triggered by crosslinking. Science 1994; 264:1948-51.
35. Parton RG, Joggerst B, Simons K. Regulated internalization of caveolae. J Cell Biol 1994; 127:1199-1215.
36. Fujimoto T. GPI-anchored proteins, glycosphingolipids, and sphingomyelin are sequestered to caveolae only after crosslinking. J Histochem Cytochem 1996; 44:929-41.

37. Sargiacomo M, Sudol M, Tang Z et al. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J Cell Biol 1993; 122:789-807.
38. Hooper NM, Bashir A. Glycosyl-phosphatidylinositol-anchored membrane proteins can be distinguished from transmembrane polypeptide-anchored proteins by differential solubilization and temperature-induced phase separation in Triton X-114. Biochem J 1991; 280:745-751.
39. Chang WJ, Ying YS, Rothberg KG et al. Purification and characterization of smooth muscle cell caveolae. J Cell Biol 1994; 126:127-38.
40. Schnitzer JE, McIntosh DP, Dvorak AM et al. Separation of caveolae from associated microdomains of GPI-anchored proteins. Science 1995; 269:1435-9.
41. Stan RV, Roberts WG, Predescu D et al. Immunoisolation and partial characterization of endothelial plasmalemmal vesicles (caveolae). Mol Biol Cell 1997; 8:595-605.
42. Smart EJ, Ying YS, Mineo C et al. A detergent-free method for purifying caveolae membrane from tissue culture cells. Proc Natl Acad Sci U S A 1995; 92:10104-8.
43. Huang C, Hepler JR, Chen LT et al. Organization of G proteins and adenylyl cyclase at the plasma membrane. Mol Biol Cell 1997; 8:2365-2378.
44. Schnitzer JE, Liu J, Oh P. Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including VAMP, NSF, SNAP, annexins, and GTPases. J Biol Chem 1995; 270:14399-404.
45. Chun M, Liyanage UK, Lisanti MP et al. Signal transduction of a G protein-coupled receptor in caveolae: Colocalization of endothelin and its receptor with caveolin. Proc Natl Acad of Sci USA 1994; 91:11728-32.
46. Lisanti MP, Scherer PE, Vidugiriene J et al. Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: Implications for human disease. J Cell Biol 1994; 126:111-26.
47. Fra AM, Williamson E, Simons K et al. Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. J Biol Chem 1994; 269:30745-30748.
48. Stang E, Kartenbeck J, Parton RG. Major histocompatibility complex class I molecules mediate association of SV40 with caveolae. Mol Biol Cell 1997; 8:47-57.
49. Fiedler K, Simons K. A putative novel class of animal lectins in the secretory pathway homologous to leguminous lectins. Cell 1994; 77:625-6.
50. Fiedler K, Parton RG, Kellner R et al. VIP36, a novel component of glycolipid rafts and exocytic carrier vesicles in epithelial cells. EMBO J 1994; 13:1729-40.
51. Fiedler K, Simons K. Characterization of VIP36, an animal lectin homologous to leguminous lectins. J Cell Sci 1996; 109:271-6.
52. Schroeder R, London E, Brown DA. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. Proc Natl Acad Sci USA 1994; 91:12130-12134.
53. Schroeder RJ, Ahmed SN, Zhu Y et al. Cholesterol and sphingolipid enhance the Triton X-100-insolubility of GPI-anchored proteins by promoting the formation of detergent-insoluble ordered membrane domains. J Biol Chem 1998; 273: 1150-1157.
54. McConville MJ, Ferguson MA. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem J 1993; 294:30-204.
55. Hanada K, Nishijima M, Akamatsu Y et al. Both sphingolipids and cholesterol participate in the detergent insolubility of alkaline phosphatase, a glycosylphosphatidylinositol-anchored protein, in mammalian membranes. J Biol Chem 1995; 270:6254-60.
56. Hannan LA, Edidin M. Traffic, polarity, and detergent solubility of a glycosylphosphatidylinositol-anchored protein after LDL-deprivation of MDCK cells. J Cell Biol 1996; 133:1265-76.
57. Cerneus DP, Ueffing E, Posthuma G et al. Detergent insolubility of alkaline phosphatase during biosynthetic transport and endocytosis. Role of cholesterol. J Biol Chem 1993; 268:3150-5.

58. Stefanova I, Horejsi V, Ansotegui IJ et al. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science 1991; 254:1016-1019.
59. Cinek T, Horejsi V. The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. J Immunol 1992; 149:2262-70.
60. Bohuslav J, Cinek T, Horejsi V. Large, detergent-resistant complexes containing murine antigens Thy-1 and Ly-6 and protein tyrosine kinase p56lck. Eur J Immunol 1993; 23:825-31.
61. Lisanti MP, Scherer PE, Tang ZL et al. Caveolae, caveolin and caveolin-rich membrane domains: A signalling hypothesis. Trends Cell Biol 1994; 4:231-235.
62. Shenoy SA, Dietzen DJ, Kwong J et al. Cysteine3 of Src family protein tyrosine kinase determines palmitoylation and localization in caveolae. J Cell Biol 1994; 126:353-63.
63. Inoue S. Imaging of unresolved objects, superresolution, and precision of distance measurements, with video microscopy. In: Lansing Taylor D, Wang Y-L, eds. Fluorescence Microscopy of living cells in culture. Part B Quantitative fluorescence microscopy-imaging and spectroscopy. San Diego: Academic Press, 1989: (Wilson L, ed. Methods in Cell Biology; vol 30).
64. Griffiths G. Fine structure immunochemistry. Heidelberg: Springer-Verlag, 1993:459.
65. Matko J, Edidin M. Energy transfer methods for detecting molecular clusters on cell surfaces. Methods Enzymol 1997; 278:444-62.
66. Hannan LA, Lisanti MP, Rodriguez-Boulan E et al. Correctly sorted molecules of a GPI-anchored protein are clustered and immobile when they arrive at the apical surface of MDCK cells. J Cell Biol 1993; 120:353-358.
67. Kenworthy AK, Edidin M. Ganglioside GM1 molecules cluster with one another, but not with a GPI-anchored protein, 5' NT, on the apical surface of MDCK cells. Mol Biol Cell 1997; 8:86a.
68. Lisanti MP, Rodriguez-Boulan E, Saltiel AR. Emerging functional roles for the glycosyl-phosphatidylinositol membrane protein anchor. J Membrane Biol 1990; 117:1-10.
69. Stulnig TM, Berger M, Sigmund T et al. Signal transduction via glycosyl phosphatidylinositol-anchored proteins in T cells is inhibited by lowering cellular cholesterol. J Biol Chem 1997; 272:19242-7.
70. Mayor S, Maxfield FR. Cholesterol-dependent sorting of GPI-anchored proteins in endosomes. Mol Biol Cell 1995; 6:231a.
71. Mayor S, Sabharanjak S, Maxfield FR. Cholesterol regulates the retention of GPI-anchored proteins in endosomes. EMBO J 1998; 17:4626-4638.
72. Peters C, Braun M, Weber B et al. Targeting of a lysosomal membrane protein: A tyrosine-containing endocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes. EMBO J 1990; 9:3633-3640.
73. Keller P, Simons K. Post-Golgi biosynthetic trafficking. J Cell Sci 1997; 110:3001-3009.
74. Lisanti MP, Rodriguez-Boulan E. Glycophospholipid membrane anchoring provides clues to the mechanism of protein sorting in polarized epithelial cells. Trends Biochem Sci 1990; 15:113-118.
75. Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 1992; 68:533-44.
76. van Meer G. Transport and sorting of membrane lipids. Curr Op in Cell Biol 1993; 5:661-73.
77. Mays RW, Siemers KA, Fritz BA et al. Hierarchy of mechanisms involved in generating Na/K-ATPase polarity in MDCK epithelial cells. J Cell Biol 1995; 130:1105-15.
78. Chatterjee S, Stevens VL, Mayor S. Lipid dependent retention of GPI-anchored proteins in the endocytic pathway of mammalian cells. Mol Biol Cell 1997; 8:302a.
79. Yoshimori T, Keller P, Roth MG et al. Different biosynthetic transport routes to the plasma membrane in BHK and CHO cells. J Cell Biol 1996; 133:247-56.
80. Musch A, Xu H, Shields D et al. Transport of vesicular stomatitis virus G protein to the cell surface is signal mediated in polarized and nonpolarized cells. J Cell Biol 1996; 133:543-58.
81. van Deurs B, Petersen OW, Olsnes S et al. The ways of endocytosis. Int Rev Cytol 1989; 117:131-177.

82. van Deurs B, Holm PK, Sandvig K et al. Are caveolae involved in clathrin-independent endocytosis? Trends Cell Biol 1993; 3:249-251.
83. Lamaze C, Schmid SL. The emergence of clathrin-independent pinocytic pathways. Curr Op Cell Biol 1995; 7:573-580.
84. Trowbridge IS, Collawn JF, Hopkins CR. Signal-dependent membrane protein trafficking in the endocytic pathway. Ann Rev Cell Biol 1993; 9:129-61.
85. McGraw TE, Pytowski B, Arzt J et al. Mutagenesis of the human transferrin receptor: Two cytoplasmic phenylalanines are required for efficient internalization and a second-site mutation is capable of reverting an internalization-defective phenotype. J Cell Biol 1991; 112:853-61.
86. Griffiths G, Back R, Marsh M. A quantitative analysis of the endocytic pathway in baby hamster kidney cells. J Cell Biol 1989; 109:2703-2720.
87. Cupers P, Veithen A, Kiss A et al. Clathrin polymerization is not required for bulk-phase endocytosis in rat fetal fibroblasts. J Cell Biol 1994; 127:725-735.
88. Anderson RGW. Potocytosis of small molecules and ions by caveolae. Trends Cell Biol 1993; 3:69-72.
89. Keller G-A, Siegel MW, Caras IW. Endocytosis of glycophospholipid-anchored and transmembrane forms of CD4 by different endocytic pathways. EMBO J 1991; 11:863-874.
90. Maxfield FR, Mayor S. Cell surface dynamics of GPI-anchored proteins. In: Haag F, Koch-Nolte F, ed. ADP-Ribosylation in animal tissues: Structure function and biology of mono(ADP-ribosyl) transferases and related enzymes. New York: Plenum Press, 1997: 355-364. Advances in Experimental Medicine & Biology; Vol 419.
91. Mayor S, Chatterjee S, Tang J et al. Functional sorting of GPI-anchored proteins in endosomes. Mol Biol Cell 1996; 6:452a.
92. Rijnboutt S, Jansen G, Posthuma G et al. Endocytosis of GPI-linked membrane folate receptor-α. J Cell Biol 1996; 132:35-47.
93. Chang W-J, Rothberg KG, Kamen BA et al. Lowering cholesterol content of MA104 cells inhibits receptor-mediated transport of folate. J Cell Biol 1992; 118:63-69.
94. Stevens VL, Tang J. Fumonisin B1 inhibition of folate receptor-mediated vitamin uptake: A possible cause of neural tube defects. J Biol Chem 1997; 272:18020-18025.
95. Taraboulos A, Scott M, Semenov A et al. Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. J Cell Biol 1995; 129:121-32.
96. Taraboulos A, Scott M, Semenov A et al. Biosynthesis of the prion proteins in scrapie-infected cells in culture. Brazilian J Med Biol Res 1994; 27:303-7.
97. Borchelt DR, Taraboulos A, Prusiner SB. Evidence for synthesis of scrapie prion proteins in the endocytic pathway. J Biol Chem 1992; 267:16188-99.
98. Thomas JL, Holowka D, Baird B et al. Large scale coaggregation of fluorescent lipid probes with cell-surface proteins. J Cell Biol 1994; 125:795-802.
99. Pierini L, Holowka D, Baird B. FcεRI-mediated association of 6-μm beads with RBL-2H3 cells results in the exclusion of signaling proteins from the forming phagosome and abrogation of normal downstream signaling. J Cell Biol 1996; 134:1427-1439.
100. Mukherjee S, Maxfield FR. Cellular cholesterol depletion alters the trafficking of lipid analogs differing solely in the degree of unsaturation of their hydrophobic tails. Mol Cell Biol 1997; 8:302a.

CHAPTER 4

Signaling through GPI-Anchored Receptors

Subburaj Ilangumaran, Peter J. Robinson and Daniel C. Hoessli

The ability of antibodies directed against GPI-anchored cell surface molecules to cause changes in cell physiology has been known for some time. Despite being confined to the extracellular leaflet, several GPI-anchored proteins, when crosslinked, can transduce cellular activation or inhibition signals, resulting in Ca^{2+} fluxes, protein tyrosine phosphorylation, cytokine secretion and proliferation or inhibition of defined responses[1,2] (Table 4.1). This prompted the search for an explanation of how the aggregation of receptors located exclusively on the outer face of the membrane could initiate intracellular signaling events. Parallel investigations into the organization of GPI-anchored proteins in plasma membranes have given us important new insights into membrane structure and organization. In this chapter, we shall describe the present state of the art in this fertile field and attempt to integrate the many new, and sometimes conflicting, data into a working model. We shall first review the recent work on extracellular and lateral interactions of GPI-anchored receptors with transmembrane signaling receptors. We shall then consider the composition of sphingolipid microdomains, the preferred habitat of GPI-anchored receptors in the plasma membrane, and discuss to what extent the GPI anchor itself participates in transmembrane signaling. Finally, we shall return to the lymphocyte where GPI-mediated signaling was originally observed and attempt to interpret the wealth of data within the framework of these new concepts of membrane organization.

Association of GPI-Linked Receptors with Transmembrane Proteins into Receptor Complexes

The simplest and most direct way for GPI-anchored receptors to participate in signaling is through physical association with transmembrane proteins that are already linked to an intracellular signaling machinery. In this cooperative interaction the transmembrane protein partner will either have intrinsic tyrosine kinase activity, or the capacity to initiate a signaling cascade upon ligand binding. Three GPI-anchored neurotrophic factor receptors have recently been assigned transmembrane signaling partners, and certain other GPI-anchored receptors were shown to interact with integrins.

GPI-Anchored Neurotrophic Factor Receptors

The GPI-anchored receptors for the glial-cell derived neurotrophic factor (GDNFR-α)[70,71] and neurturin (NTNR-α),[72,73] when bound to their respective ligands, are recognized by the Ret transmembrane receptor protein tyrosine kinase. Ret actually recognizes the GDNF-GDNFRα or NTN-NTNRα complexes and undergoes conformational changes that result in the activation of its protein tyrosine kinase (PTK) activity (see chapter 6, Fig. 6.1).

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

Table 4.1 Signal transduction via GPI-anchored glycoproteins

Molecule	Cells	Response	Ref.
Human			
CD14	Monocytes	**h** oxidative burst **h** IL-1 secretion **h** $[Ca^{2+}]_i$ **h** oxidative burst	3-6
	Transfected murine pre-B cell line	NF-κB activation **h** Tyr phosphorylation	7
	Monocytes	**h** PKC, MAPK, PI-3K activities **h** PI-(3,4,5)P3	8,9
CD16B	Monocytes Neutrophils	**h** $[Ca^{2+}]_i$ **h** oxidative burst	6,10
CD24	Tonsilar B cells Granulocytes	**h** $[Ca^{2+}]_i$ **h** oxidative burst	6,11
CD48	Neutrophils T-cell line	**h** $[Ca^{2+}]_i$	10,12
CD52	T-cells	**h** proliferation **h** cytokine production	13
CD55	T-cells Transfected EL4 mouse thymoma	**h** proliferation **h** IL-1 production	14,15
	Neutrophils Jurkat T-cell line	**h** $[Ca^{2+}]_i$ **h** Tyr phosphorylation	10 16
CD58	Neutrophils	**h** $[Ca^{2+}]_i$	10
	Monocytes	**h** IL-1 secretion	17
CD59	T-cell lines	**h** $[Ca^{2+}]_i$ **h** PI turnover **h** IL-1 secretion **h** proliferation	18
	Neutrophils	**h** $[Ca^{2+}]_i$ **h** Tyr phosphorylation	6,10
	Monocyte line	**h** $[Ca^{2+}]_i$	19
CD66b, c	Neutrophils	**h** CD11b expression	20
CD67	Neutrophils	**h** $[Ca^{2+}]_i$	6,10
CD73	T-cells	**h** IL-2 secretion **h** IL-2R expression **h** proliferation **h** Tyr phosphorylation	21-23
	B cells	**h** proliferation	24
CD87	Monocytes	**h** Tyr phosphorylation	25

Table 4.1 Signal transduction via GPI-anchored glycoproteins (con't)

Molecule	Cells	Response	Ref.
Human			
CD157 (Bst-1)	Monocyte cell lines	**h** and **i** Tyr phosphorylation	26
PrPc	T-cells	**h** proliferation	27
Mouse, Rat, Guinea pig, Chicken			
Thy-1	Spleen cells Peripheral T-cells T-cells clones T-cell line T-cell hybridoma	**h** proliferation **h** IL-2 secretion **h** $[Ca^{2+}]_i$ **h** IL-2R expression	28-33
	T-cell hybridoma	**h** Tyr phosphorylation	34
	Transfected human T-cells, murine B lymphoma	**h** $[Ca^{2+}]_i$ **h** PI turnover **h** IL-2 secretion	35-37
	Transfected neural cell line	**i** neurite outgrowth	38
	Thymocytes	Apoptosis	39
Ly-6	T-cells, T-cell clones, cell hybridoma	**h** or **i** proliferation* **h** or **i** secretion* **h** $[Ca^{2+}]_i$ **h** IL-2R expression	40-44
	B cells	**h** proliferation	45
	Transfected mouse T-cells, human T-cells	**h** or **i** secretion, NF-κB, AP-1, NF-AT activities	46, 47
Qa-2	T-cells, transgenic mouse T-cells	**h** proliferation **h** $[Ca^{2+}]_i$ **h** IL-2 secretion **h** IL-2R expression	48-50
sgp-60	T-cells	**h** IL-2 secretion, proliferation **h** IL-2 secretion, proliferation, $[Ca^{2+}]_i$, IP turnover*	51-53
Heat stable antigen	Purified CD4+ cells	Costimulation	54
	B lymphocytes	**h** $[Ca^{2+}]_i$	55
	B cell precursors	Apoptosis	56
TSA-1/Sca-2	T-cells, T-cell hybridoma, transfected human T-cells	**i** IL-2 secretion CD3 ζ Try phosphorylation*	57, 58
	T-cells hybridoma, in vivo	**i** anti-TCR-induced apoptosis	59
BST-1/BP3	Pre T-cells	**h** proliferation	60
Mouse F3	Transfected CHO cells	**h** Try phosphorylation	61

Table 4.1 Signal transduction via GPI-anchored glycoproteins (con't)

Molecule	Cells	Response	Ref.
Mouse, Rat, Guinea Pig, Chicken (con't)			
Rat RT6	T-cells	**h** proliferation	62
Rat Thy-1	mast cells	**h** $[Ca^{2+}]_i$ **h** Tyr phosphorylation **h** histamine release	63-66
Rat gp42	NK-like cell line	**h** $[Ca^{2+}]_i$ **h** PI turnover	67
Rat GDNFRα	embryonic spinal cord motor neurons	**h** Tyr phosphorylation of RET RPTK	68
Guinea Pig gpTAA	T-cells	h proliferation	69

* depending on the antibody used and the mode of crosslinking

A similar situation is encountered with the GPI-anchored receptor for the ciliary neurotrophic factor (CNTFR-α), which forms a tripartite receptor complex with CNTF and the LIF (leukemia inhibitory factor) binding protein (see Fig. 6.1). This complex then encounters gp130, the transmembrane signal transducing subunit of the interleukin 6 receptor, and signaling is initiated by the JAK kinases associated with gp130.[74] In all these instances, the ligand binding subunits apparently serve to limit ligand diffusion, in addition to their primary role in specificity determination. Whether the presence of a GPI anchor, which endows these neurotrophic factor receptors with higher mobility in the plane of the membrane, enhances capture and concentration of ligands is not known. However, it will become apparent in the next sections of this chapter that GPI-anchored receptors acquire signaling potential while residing in sphingolipid microdomains, so that GDNF and NTN-induced signals should be viewed within the context of other signaling molecules which reside in these domains.

Interactions of GPI-Linked Receptors with Integrins

In myeloid cells, the three GPI-linked receptors CD14 (LPS receptor), FcγRIIIB (CD16b) and CD87 (uPAR, or urokinase-type plasminogen activator receptor) have been shown to undergo lateral interactions with the β2 integrin CR3[25] (complement receptor type 3) (reviewed in ref. 75). Resonance energy transfer measurements and cocapping experiments suggest specific interactions between CD87-CR3, CD14-CR3 and CD16b-CR3 pairs (see ref. 76 for a review). In addition, coprecipitation of CD87 and CR3 in detergent-resistant membrane complexes containing Src family kinases (see below) suggests an additional transmembrane cooperation in signaling.[25] The association of CD16 and CD87 with CR3 is mediated by lectin-like properties of CR3 which are inhibitable by the appropriate monosaccharides.[76]

CD87 has also been reported to localize to caveolae in endothelial cells,[77] and to associate with β1 integrins in membrane complexes containing caveolin.[78] Caveolae are morphologically distinct plasma membrane invaginations present in all cell types that express the

Table 4.2 Association of GPI-linked molecules with Src family protein tyrosine kinases

Molecule	Src-kinase	Cell/tissue	Assay	Ref.
Human				
CD14	Lyn	Peripheral blood monocytes	Reprecipitation from IC kinase assay	84
CD48	Lck	T-cell line (HPB-ALL)	WB of IP from total lysate	85
CD55	Lck	T-cell line (HPB-ALL)	WB of IP from total lysate	85
	Lck, Fyn	HeLa Transfectants	Reprecipitation from IC kinase assay	15
CD59	Lck	T-cell line	Disappearance from the sphingolipid microdomain after clearing with anti-Thy-1 WB of IP from total lysate	85, 86
CD66b, c	Lyn, Hck	Peripheral blood	Reprecipitation from IC kinase assay	87
Mouse, Rat				
Thy-1	Lck	Rat and mouse thymocytes	Reprecipitation from IC kinase assay	88
		EL4 thymoma	Disappearance from the sphingolipid microdomain after clearing with anti-Thy-1	89
	Fyn	Rat and mouse thymocytes	Reprecipitation from IC kinase assay	88, 90
	Lyn	Rat basophilic leukemia	Reprecipitation from IC kinase assay	65
Ly6	Lck	EL4 thymoma	Disappearance from the sphingolipid microdomain after clearing with anti-Ly6	89
Ly-6C	Fgr	J774 macrophage cell line	IC kinase assay, comparison by peptide mapping	91
F3	Fyn	Adult mouse brain	WB of IP from GPI-rich microdomains	61, 92
CD24	c-Fgr	Small cell lung carcinoma	WB of IP from total lysate	93
	Lyn	Erythroleukemia (K562)	WB of IP from total lysate	93
	Lck, Fyn	ESb lymphoma	Reprecipitation from IC kinase assay	94
CD48	Lck, Fyn	Rat and mouse thymocytes	Reprecipitation from IC kinase assay	88

Table 4.2 Association of GPI-linked molecules with Src family protein tyrosine kinases (con't)

Molecule	Src-kinase	Cell/tissue	Assay	Ref.
Chicken				
F11	Fyn	Embryonic chick brain	In vitro kinase assay on IC from sphingolipid microdomains Microscopy (colocalization)	95
Axonin-1	Fyn	Cultured dorsal root ganglia	Reprecipitation from IC kinase assay	96

IC, Immune complex; IP, Immunoprecipitate; WB, Western blot

caveolar coat protein caveolin[79] (see below). Although CD87 may modulate β1 integrin signaling,[80] the functional relevance, if any, of the caveolar localization of the CD87-β1 integrin complex is not known. Unlike the CD87-β2 interaction,[81] which involves carbohydrate recognition and is labile, the relatively stable CD87- β1 integrin complexes are the result of protein-protein interactions and can be inhibited by peptides corresponding to specific motifs in the interacting proteins. CD87 may thus engage in distinct interactions with different integrin molecules, and could therefore signal through different pathways. Both β1 and β2 integrins are capable of mediating activation signals via multiple pathways (Ca^{2+} mobilization, protein tyrosine phosphorylations and $p21^{ras}$ activation).[82,83]

Detergent-Resistant Sphingolipid Microdomains as Signaling Platforms for GPI-Anchored Receptors

In contrast to the few GPI-anchored proteins described above which transduce signals by associating with known transmembrane signal transducer molecules, the signaling properties of the vast majority of GPI-linked receptors are linked to their localization in sphingolipid-rich microdomains. Involvement of Src family PTKs in signaling via GPI-anchored proteins was suggested because:

1. Crosslinking the GPI-anchored receptors at the cell surface leads to a rapid increase in tyrosine phosphorylation of several intracellular protein substrates (Table 4.1); and
2. PTKs coprecipitate with GPI-anchored receptors in mild detergent lysates of whole cells (Table 4.2).

Whether Src family PTKs participate in signaling through GPI-anchored receptors and sphingolipid microdomains has not been directly demonstrated, but much progress has been made towards understanding the relationship of Src PTKs to the plasma membrane, particularly to sphingolipid microdomains. The Src family PTKs associate with the inner leaflet of the plasma membrane through their amino-terminal acyl chains (see ref. 97 for a review) and thus do not communicate directly with the outer leaflet. An integral membrane protein that could bridge GPI-anchored receptors in the outer leaflet with a Src kinase in the inner leaflet was proposed,[1,2,98] but no candidate for the role of “adaptor” has been identified so far. A 100 kDa protein, reported to be required for signaling via GPI-anchored Thy-1 and which associates with Src PTKs, appears to be restricted to a subset of $CD4^+$ cells[99] and thus cannot qualify as a general adaptor linking GPI-anchored receptors to Src PTKs.

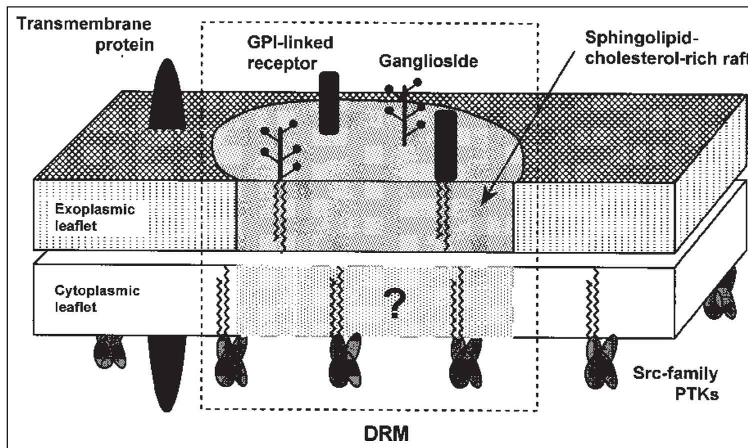

Fig. 4.1. A model for the organization of sphingolipid microdomains in the plasma membrane. Sphingolipids and cholesterol in the exoplasmic leaflet organize themselves into 'liquid ordered domains' or 'rafts' which segregate from the phospholipid milieu of the plasma membrane. Glycosphingolipids (gangliosides) and GPI-anchored proteins preferentially associate with these rafts, whilst most transmembrane proteins are excluded from them. These rafts, or microdomains, can be isolated as 'detergent resistant membranes' (DRMs) by isopycnic density gradient centrifugation of nonionic detergent lysates. Acylated signaling proteins, such as Src family PTKs, are also enriched in DRMs. The cytoplasmic face of the microdomains is also thought to differ in lipid composition from the rest of the plasma membrane, but whether the lipid composition is identical to that of the exoplasmic leaflet is not known.

While the search for transmembrane connecting mechanisms was going on, it became evident that GPI-anchored proteins are not randomly distributed in the plasma membrane but tend to accumulate in regions of the plasma membrane enriched in sphingolipids and cholesterol.[100] These 'sphingolipid microdomains' could be isolated because of their insolubility in nonionic detergents and buoyancy in isopynic density gradients[86,100-102] (see chapter 3). The biophysical basis for microdomain formation and detergent insolubility was shown to lie in the ability of sphingolipids to form a liquid ordered phase within the milieu of glycerophospholipids[103,104] (Fig. 4.1). Independent support for the localization of GPI-anchored proteins to sphingolipid microdomains has come from recent biophysical studies using the single particle tracking technique, which showed transient confinement of GPI-anchored proteins and gangliosides to "obstacle zones" of approximately 300 nm in diameter.[105,106] The concept of sphingolipid microdomains as signaling platforms is further supported by the fact that a number of glycosphingolipids (gangliosides) are also potent signal transducers that function by activating Src family PTKs (Table 4.3). Interestingly, the phosphoprotein profiles associated with the crosslinking of GPI-linked receptors and gangliosides in in vitro kinase assays are quite similar, suggesting that a common 'scaffold' of kinases and substrates may be involved in both instances (see references in Table 4.2 and 4.3). In addition to Src kinases, similarly acylated G protein α subunits also associate with GPI-anchored proteins in lymphocytes.[120]

Aggregation of GPI-anchored receptors, by antibody crosslinking for instance, may alter the distribution of sphingolipid microdomains on the cell membrane. What effect this may have on the distribution and activity of the Src family PTKs associated with the microdomains is only now becoming clear. At least three studies using different approaches

Table 4.3 Signal transduction via gangliosides

Ganglioside	Activation by	Cells	Response	Ref.
GM1	Polyclonal Ab	Rat thymocytes	hDNA synthesis	107
	Cholera toxin	Rat thymocytes	hDNA synthesis h$[Ca^{2+}]_i$	108,109
	Cholera toxin	Mouse N18 neuroblastoma	hdifferentiation	110
	Cholera toxin	Stimulated mouse B cells	iproliferation	111
	Cholera toxin	Rat mast cells	hIL-6 secretion iTNF-α secretion	112
GD1b	AA4 mAb	Rat basophilic leukemia	iTyr-P of cellular substrates	113
GD3	R24 mAb	Human T-cells	hDNA synthesis	114
	R24 mAb	Human T-cells	hcytokine synthesis hTyr-P of PLCγ hRas activation h$[Ca^{2+}]_i$	115,116
	R24 mAb	Rat cerebellar cultures	iTyr-P of cellular substrates	117

Association of Src family protein tyrosine kinases with gangliosides

Ganglioside	Src kinase	Cells	Ref.
GM3	c-Src	B16 melanoma	118
GD1b	Lyn	Rat basophilic leukemia	119
GD3	Lyn	Rat brain	117

provide support for the involvement of sphingolipid microdomains in signaling via GPI-anchored glycoproteins. First, purified GPI-anchored molecules such as CD59 that readily integrate into the plasma membrane (reviewed in ref. 121; also see chapters 7 and 10) become signaling competent only after they have become incorporated into sphingolipid microdomains.[19] The ability to induce a Ca^{2+} influx after crosslinking with anti-CD59 antibodies coincided temporally with the coprecipitation of CD59 and Src family kinases in detergent-resistant membrane complexes. Second, lowering the cellular cholesterol content (and consequently that of sphingolipid microdomains) markedly diminished the Ca^{2+} signaling via the endogenous GPI-anchored proteins CD59 and CD48 in lymphocytes.[12] The surface expression and association of CD59 and CD48 with microdomains were unaffected by cholesterol depletion, suggesting that signaling is a more sensitive indicator of subtle changes in lipid composition. Third, the Src family PTK Fyn coaggregates at the plasma membrane inner leaflet upon surface crosslinking of the GPI-linked proteins[95] or other sphingolipid microdomain components (Harder et al. J Cell Biol 1998; 141:929-942). These studies favor the idea that sphingolipid microdomains act as functional signaling

platforms, and that linking together of several individual domains by antibodies enhances their functional activity.

Sphingolipid Microdomains and Caveolae

The use of Triton X-100 and isopycnic centrifugation as a means of isolating GPI-rich membrane domains was criticized on the grounds that the removal of phospholipids by the detergent would automatically increase the buoyancy of proteins remaining associated with the detergent-insoluble fraction, raising doubts that 'microdomains' could simply be extraction artefacts and not natural features (see ref. 122 for a review). Further complexity arose when caveolae from endothelial cells and fibroblasts were shown to behave similarly upon TX-100 extraction and could also be recovered as buoyant membrane complexes.[123,124] Moreover, some experiments showing enrichment of GPI-anchored proteins and the ganglioside GM1 in caveolae[125,126] suggested that sphingolipid microdomains and caveolae were identical.

Despite these striking similarities, it was possible to distinguish caveolae from sphingolipid microdomains by a number of criteria. For instance, isolation of GPI-rich sphingolipid microdomains in cells that do not express caveolin pointed out that sphingolipid microdomains exist in the absence of caveolin expression or morphologically detectable caveolae.[127,128] Further, GPI-anchored molecules, sphingolipids and gangliosides were sequestered into caveolae only upon surface crosslinking,[129,130] and it was cautioned that coisolation of caveolin and GPI-anchored proteins after TX-100 extraction does not reflect the native distribution of the latter.[131] This would also explain why photocrosslinking of GM1 with caveolin was more extensive after crosslinking with cholera toxin.[132] Subsequent technical refinements for the isolation of caveolae without use of detergents allowed the physical separation of caveolae from the 'GPI-rich annulus', but still showed an enrichment for GM1, sphingomyelin and many signaling proteins in caveolar fractions.[133,134] More recently, 'affinity' purified caveolae were shown not to contain any signaling molecules[135] and morphological studies showed Gα proteins located not exclusively in caveolae, but also in noncaveolar membrane regions.[136]

In spite of these findings, there is still evidence that detergent-resistant, buoyant membranes fractions enriched in caveolin (hence referred to as caveolar membranes) contain a wide variety of signaling molecules (see Table 4.4). Individual signaling molecules show different degrees of enrichment in caveolar membranes. Direct interactions between caveolin and Src family PTKs, Gα protein subunits and PKC isozymes[78,142,143,148] through their putative 'caveolin binding motif',[149] and regulation of eNOS activity by reversible association with caveolae[150] fostered the view that caveolin could provide a framework for the caveolar signaling platform.[151] This view gave rise to a model where caveolae are surrounded by an annulus of sphingolipid microdomains, GPI-anchored proteins and sphingomyelin in the outer leaflet, and kinases in the inner leaflet. Signaling molecules on the inner leaflet bind to caveolin through distinct motifs[151] (Fig. 4.2). GPI-linked molecules do not seem to enter caveolae unless aggregated by antibodies or ligands. One problem with this model is that direct intercalation of the acylated signaling molecules into caveolar membrane is unlikely, as caveolin and its palmitate chains already occupy most of the caveolar membrane area.[122] Many observations remain difficult to resolve in any model, such as the exclusion of GPI-anchored proteins,[133] but inclusion of signaling molecules,[134] in caveolae isolated after cationized silica coating of the plasma membrane. It is interesting to note that low density membrane complexes of neuronal cells which do not contain caveolae contained a similar array of signaling molecules reported to be present in caveolae[152] (see chapter 6). Precise definition of the organization of the membrane associated signaling

Table 4.4 Signaling molecules enriched in caveolae

Molecule	Ref.
Src family PTKs	134, 137
Phosphoinositide phosphatase PI (4,5) P2	138
P13K, PLCγ	134
IP3 receptor	139, 140
Gα protein subunits	141
PKC isozymes	142
Ha-Ras	143
MAP kinase pathway components	144
Endothelial nitric oxide synthase	145
Receptor PTKs (PDGFR, EFGR)	146, 147

molecules in caveolae and their relationship to sphingolipid microdomains will definitely require further investigations and technical refinements.

Localization and Regulation of Src Family PTKs in Sphingolipid Microdomains

The localization of Src kinases to the plasma membrane inner leaflet[153,154] and their enrichment in sphingolipid microdomains[153,155] are critically dependent upon double acylation at the amino-terminal glycine and cysteines with myristate and palmitate, respectively. Doubly acylated Lck uniformly localizes to the plasma membrane inner leaflet in cells devoid of CD4 and CD8.[154] Similarly, doubly acylated Fyn first associates with membranes and then equilibrates with detergent-resistant membrane fractions.[153] An increase in the local concentration of PTKs following crosslinking of GPI-anchored receptors remains a possible outcome, although this has not been convincingly shown. Recent observations of Fyn cocapping with GPI-anchored receptors and other sphingolipid microdomain components (ref. 95, and Harder et al. J Cell Biol 1998; 141:929-942) suggest that movement and coalescence of sphingolipid microdomains may induce a concomitant redistribution of inner leaflet constituents. The plasma membrane inner leaflet sphingomyelin and cholesterol may also form domains similar to the outer leaflet domains.[156] Like most of the GPI-anchored proteins, the acyl chains of Src kinases are saturated and thus may preferentially associate with these putative inner leaflet domains. In support of this, about 50% of the lymphocyte Lck and Fyn kinases are recovered in the buoyant membrane fractions.[157,158] It is therefore particularly important to know whether:

1. Inner leaflet domains with detergent-resistant properties match the outer domains spatially; and
2. The inner and outer domains differ in their lipid composition. If so, then antibody-induced clustering could increase the size and potency of these preexisting signaling platforms.

The enzymatic activity of Src family PTKs is regulated primarily by phosphorylation of two key tyrosine residues that allow modular interactions within the kinase molecule

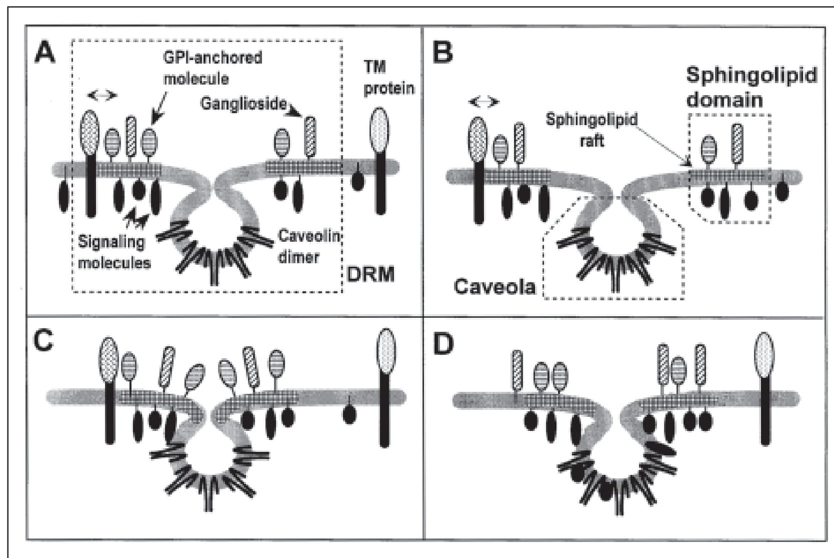

Fig. 4.2. A working model for the organization and regulatory interactions of sphingolipid rich microdomains and caveolae. (A) DRMs are, in practice, crude isolates consisting of sphingolipid-rich microdomains and caveolar components. Caveolae are nonclathrin coated plasma membrane invaginations formed by oligomerization of caveolin dimers and which include membrane cholesterol. Preparation of DRMs cannot distinguish between components of microdomain origin (e.g. GPI-anchored proteins, gangliosides and acylated, membrane-associated signaling molecules) and caveolae. Only a very few transmembrane proteins are included in DRMs, possibly due to association with GPI-anchored proteins or gangliosides through their extracellular domains. (B) Mechanical and affinity methods can separate caveolae from sphingolipid domains, with the signaling molecules (e.g., PTKs) associated exclusively with the latter. (C) Antibody-, ligand- or mild detergent-induced perturbation of microdomain integrity may cause GPI-anchored proteins, gangliosides and sphingomyelin to coalesce with caveolae, thus explaining their coisolation in DRMs. (D) In intact membranes, close proximity of the sphingolipid rafts to caveolae induced by crosslinking could result in the transfer of signaling molecules from microdomains into caveolae.

itself (see ref. 159 for review; Fig. 4.3). In Lck, phosphorylation of regulatory tyrosine residue Y505 (by the cytoplasmic PTK Csk) results in the formation of an intramolecular loop with the SH2 domain that locks the kinase in an inactive conformation. Dephosphorylation of Y505 by the CD45 protein tyrosine phosphatase may render Lck catalytically active by opening the molecule and exposing the kinase domain Y394.[160] Phosphorylation of Y394 by autophosphorylation (in trans) enhances the catalytic activity. This occurs selectively in the membrane bound forms of Lck and is critically dependent on N-terminal myristoylation and palmitoylation of the kinase.[161]

There is still some controversy about whether Lck and Fyn kinases are more or less enzymatically active when associated with sphingolipid microdomains. One report[161] suggests that Lck in sphingolipid microdomains is less active than elsewhere in the plasma membrane due to exclusion of the receptor tyrosine phosphatase CD45 from these microdomains, which leaves Lck in the inactive configuration. This explanation is not consistent, however, with the apparent increase in signaling ability exhibited by crosslinking

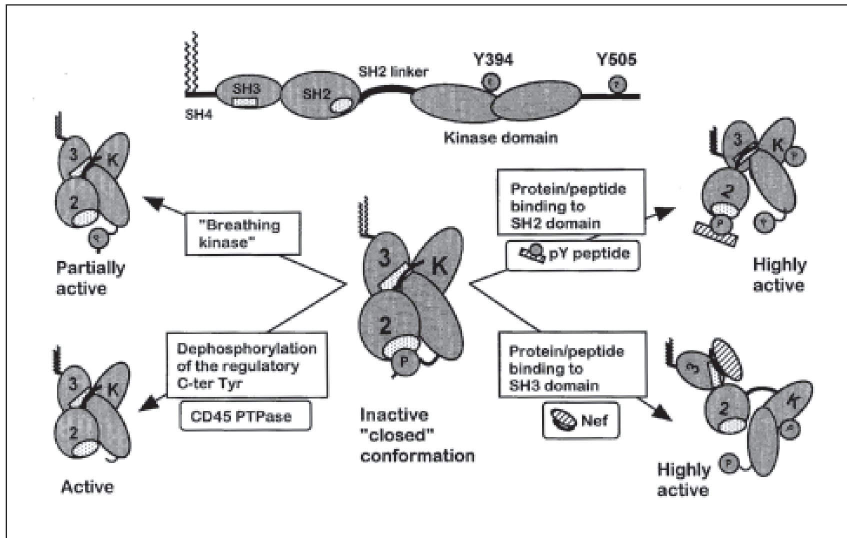

Fig. 4.3. Regulation of Src family PTKs. Src family PTKs such as Lck and Fyn are organized into distinct modules comprising kinase, SH2 and SH3 domains, and a consensus site for double acylation at the amino terminus (SH4 domain). The SH2 and SH3 domains contain binding pockets for phosphotyrosine (pY, P-Tyr) and a proline rich repeat, respectively. Two tyrosine residues, a regulatory one at the C-terminus and another within the kinase domain (activator Tyr) control the activity of the kinase. In Lck, phosphorylation of the regulatory Y505 results in binding of the C-terminus to the SH2 domain, thus locking the kinase in an inactive, closed conformation. This is stabilized by the SH2 linker binding to the SH3 domain. In contrast, phosphorylation of Y394 in the kinase domain enhances the catalytic activity of the enzyme. In its membrane-bound form, the kinase undergoes a conformational change allowing transient opening ("breathing") of the kinase domain and phosphorylation of the Y394, which can lead to its partial activation. The kinase is activated physiologically by dephosphorylation of Y505 by tyrosine phosphatases such as CD45. Other regulatory effects, e.g., binding of a P-Tyr competitor peptide, or allosteric displacement, e.g., HIV Nef protein binding to Hck, of the regulatory tyrosine from the SH2 domain can result in hyperactivation of the kinase.

GPI-linked molecules. Studies in the authors' laboratory showed that PTKs in lymphocyte sphingolipid microdomains are more active than in membranes containing less lipids,[162] but PTK activity of the latter could be dramatically increased by detergents while that of the former was minimally perturbed (Ilangumaran et al. Mol Biol Cell 1999:in press). This suggests that the membrane lipid environment may regulate PTKs activity, an idea which is consistent with molecular modelling of Src kinases wherein conformational interactions with noncatalytic domains of the kinase could modulate the catalytic activity without dephosphorylating the regulatory tyrosine[163] (Fig. 4.3). A recent study has indeed directly demonstrated that one active form of Lck can have both the active site (Y394) and the regulatory tyrosine (Y505) phosphorylated.[164] In another study, c-Src but not the highly active v-Src was shown to undergo a negative regulatory interactions with caveolin,[78] but it remains to be seen whether other PTKs of the Src family also undergo regulatory interactions with caveolin.

Lck activity is also modulated by exogenously added gangliosides[165] that probably insert into the plasma membrane,[166] and can alter the structure of the inner leaflet. Similarly, PKC activity in liposomes is influenced by GPI-anchored leishmanial lipophosphoglycans (see chapter 8) inserted into the outer leaflet of the bilayer.[167] Sphingolipid microdomains therefore appear to confer on Src family PTKs a unique form of regulation which may be perturbed either by crosslinking or by addition of exogenous gangliosides or GPI-anchored molecules.[121]

Transmembrane Receptors Requiring the Participation of Sphingolipid Microdomains for Signaling

A few transmembrane receptors also utilize the signaling machinery of sphingolipid microdomains. Studies on signaling through the FcεRI, the receptor for the Fc fragment of immunoglobulin E, have been especially informative, and preliminary evidence suggests that the B cell surface molecule CD20 and the hyaluronan receptor CD44 function similarly, as they both seem to utilize the signaling machinery of the sphingolipid microdomains.

The multichain FcεRI receptor is expressed on mast cells and serves to bind IgE to the cell surface in such a way that the antigen-binding site of the IgE remains available for crosslinking by multivalent antigens (see ref. 168 for a review). When appropriately crosslinked, FcεRI associates with sphingolipid microdomains where the Lyn tyrosine kinase phosphorylates the β and γ chains of the FcεRI.[169] Association of FcεRI with sphingolipid microdomains is critically dependent upon crosslinking of the IgE molecules. SH2-dependent recruitment of the downstream signaling molecules Syk and PLCγ1 to specific tyrosine phosphotyrosylated motifs in the cytoplasmic domain of FcεRI β subunits leads to signal propagation.[170] This study is actually the first to show colocalization of a ganglioside (GM1), the FcεRI receptor and intracellular signaling molecules (Syk and PLCγ1) to sphingolipid microdomains during cellular activation by a physiological stimulus. Interestingly, colocalization of SH2-containing polypeptides with FcεRI was found to be more transient than the GM1-FcεRI association of surface molecules, indicating the dynamic nature of these interactions.

CD20, a transmembrane protein which traverses the membrane bilayer four times, is involved in B lymphocyte maturation. Strikingly, almost all CD20 molecules are rapidly included in detergent-resistant membrane complexes after surface crosslinking with certain antibodies. These complexes contain Src family PTKs that are thought to relay the CD20 signal.[171] CD44, the ubiquitous and polymorphic hyaluronan receptor expressed both at the surface of cells that display (endothelial cells) or do not display (leukocytes) caveolae, is required for the extravasation of activated T cells in inflammatory sites.[172] It is the transmembrane portion of CD44 that confers detergent resistance properties to a subset of CD44 molecules,[173] although detergent-insoluble CD44 can also associate with the cytoskeleton, presumably via its intracellular domains (reviewed in ref. 174). The signaling capacity of CD44 and its association with Lck[175] and Fyn kinases are restricted to a small but significant proportion of the CD44 molecules that are recovered in sphingolipid-rich, buoyant membrane fractions.[176] Clearly, the signaling machinery of the sphingolipid microdomains is not exclusively used by GPI-anchored proteins, but also by a number of nonkinase, transmembrane receptors as well. Large membrane complexes containing a variety of transmembrane proteins and PTKs, distinct from the GPI-rich domains, have been demonstrated in lymphocytes[177] and whether there exists any communication between these two, especially upon stimulation via the transmembrane proteins, is a point worth investigating. It is also interesting to note that the m2 muscarinic acetylcholine receptor,

upon binding to an agonist but not an antagonist, is targeted to caveolar membrane fractions in cardiac myocytes.[178]

Interactions of the Sphingolipid Microdomains with Transmembrane Proteins: Implications for Proximal Signaling Events

As discussed above, the interactions between sphingolipid microdomains and the transmembrane proteins are reciprocal, as certain GPI anchored proteins utilize the signaling machinery of the transmembrane proteins, and certain transmembrane proteins utilize the signaling components of the sphingolipid microdomains. For instance, the ganglioside GM1 tightly binds to the high affinity NGF receptor Trk (with intrinsic tyrosine kinase activity) and modulates the responses to NGF.[179] This interaction requires glycosylated Trk not only for association with GM1 but also for expression of the Trk tyrosine kinase activity. Similarly, the recovery of EGFR, PDGFR and insulin receptor[146,147,152] in buoyant membrane complexes raises the interesting possibility that growth factor receptor tyrosine kinases localized next to the sphingolipid microdomains might undergo regulation by Src-family PTKs from the sphingolipid microdomains. Since PDGFR activity is modulated by Src family PTKs,[180] similar regulation could occur for other receptor PTKs when positioned near the Src family PTKs associated with the sphingolipid microdomains. One can envisage that association with GPI-anchored proteins or gangliosides may serve to bring the transmembrane receptors in contact with the sphingolipid microdomains, which in turn would regulate their signaling behavior. In cells that express caveolae, caveolin can be one such regulator, as the sphingolipid microdomains surrounding the caveolae may position the signaling molecules in close proximity to caveolin (Fig. 4.2).

Transmembrane proteins interact with the sphingolipid microdomains (e.g., CD44) or become detergent-insoluble upon stimulation (FcεRI, CD20) through different mechanisms. These interactions are weak and only demonstrable under mild lysis conditions.[169,176] In the case of CD44, the transmembrane segment confers TX-100 insolubility, although this does not explain why only a small fraction is recovered in TX-100-resistant complexes. It is possible that the transmembrane segment permits dynamic lateral interactions with sphingolipid microdomain components, and that only a small proportion of CD44 molecules are involved in these interactions. Reversible palmitoylation of Cys residues in the transmembrane segment of CD44 and dimerization may influence sphingolipid microdomain association, as it does modulate hyaluronan binding by CD44.[181] In contrast, the ligand-induced association of FcεRI with sphingolipid microdomains is induced by crosslinking and is very transient, and the pre-assembled signaling machinery of the sphingolipid microdomains appears to provide FcεRI a temporally and spatially regulated signaling platform.[170]

Role of the GPI Anchor in Signaling

A number of studies have questioned the requirement for a GPI-anchor in signaling. Typically, these studies involved engineering transmembrane forms of GPI-anchored receptors (see chapter 11) and compared their ability to transduce signals with that of the native GPI-anchored forms. For example, transmembrane forms of Qa-2 and Ly-6A/E were unable to stimulate cell proliferation after antibody-mediated crosslinking.[49,182] This is probably due to their inability to associate with sphingolipid microdomains, as was the case for the transmembrane form of placental alkaline phosphatase.[183] Importantly, the transmembrane form of CD55 (DAF, see chapter 7) no longer coprecipitated with the Src family PTKs, stressing the strict dependence of CD55 on sphingolipid microdomains for Src kinase association.[15] Comparison of the signaling pathways utilized by GPI-anchored and

transmembrane forms of CD14 shows that only the GPI-anchored form is able to induce tyrosine phosphorylation-dependent Ca^{2+} flux upon antibody mediated crosslinking.[184] Similarly to CD14, CD73 in transmembrane form still functions as a costimulator, albeit less efficiently.[185] However, the transmembrane form of CD14 binds to endotoxin (bacterial lipopolysaccharide, LPS) to the same extent as the GPI-anchored CD14 and mediates NF-κβ activation and protein tyrosine phosphorylation.[7] CD14 binds LPS complexed to serum LPS binding protein (LBP) and transfers LPS to a distinct membrane phospholipid environment; the anchor-free soluble CD14 is equally efficient in this process[186] (see also chapter 5). It appears that LPS can use alternate signal transduction mechanisms, as it can integrate phospholipid bilayers in a CD14-independent fashion,[187] stimulate cells that do not express Src family PTKs[188] and stimulate the CD14-negative cells via integrins.[189]

The signaling properties of engineered proteins consisting of transmembrane protein ectodomains linked to a GPI-anchor were also investigated. GPI-modified MHC class I ectodomains expressed in transgenic mouse T cells were functional,[49] while GPI-modified human CD4 expressed in murine T cells was not.[190] Surprisingly, GPI-modified human complement receptor 1 (transmembrane) and human tissue inhibitor (secreted) proteins expressed in murine thymoma did not associate with Src family PTKs.[191] Although association with sphingolipid microdomains has not been investigated in these very few cases, extracellular interactions could also influence the functionality of these heterologous proteins. Therefore, on one hand the GPI-linked proteins modified with the transmembrane anchor may still interact with sphingolipid microdomains through extracellular interactions, while on the other hand similar interactions may interfere with stable association of GPI-modified protein ectodomains with the same sphingolipid microdomains. Actually, the anchor type may have an influence on the conformation of the extracellular microdomain, with consequences on the signaling capacity.[192] Therefore, although the GPI-anchored proteins are usually targeted to sphingolipid microdomains, utilization of the signaling machinery of the latter appears to be modulated by the participation of other cell surface molecules, resulting in different outcomes.

GPI Anchor-Derived Second Messengers

Early observations by Saltiel and colleagues[193] suggested that insulin receptor signaling stimulated the production of lipidic second messengers derived from GPI anchors, possibly through the action of an endogenous PI-PLC (see chapter 9 for GPI specific phospholipases). At least some of the insulin-mediated effects were attributable to these GPI-derived inositol phosphoglycan and diacylglycerol moieties[194] (see ref. 195 for a review). Subsequently, these GPI-derived second messengers were shown to accumulate intracellularly following IL-2 receptor engagement.[196,197] It is quite possible that such messengers could also be derived from the 'free GPIs' reported to be 4-5 times more abundant than GPI-anchored proteins on the cell surface.[198] In any event, the once entertained possibility that signaling via GPI-anchored receptors could be mediated by inositol phosphoglycans derived from hydrolysis of the GPI anchor itself[2,197] has not received experimental confirmation so far.

Signaling via GPI-Anchored Glycoproteins of the Lymphocytes

Lymphocytes express a large number of signaling-competent GPI-anchored proteins (see chapter 5). It is apparent from the earlier sections that many GPI-anchored proteins in lymphoid cells depend upon localization to sphingolipid microdomains for their signaling properties. Although the use of antibody-mediated crosslinking as a means of cellular activation is not physiological, positive or negative signaling responses depending on the antibody used and the mode of crosslinking (Table 4.1) suggest interactions of GPI-anchored molecules with other membrane components. This section is intended to provide

a conceptual framework for such regulatory interactions in signaling via GPI-anchored proteins using the lymphocyte model.

One example of cooperative interaction is the requirement for GPI-anchored Ly-6/TAP for efficient signaling via the CD3-T-cell receptor complex (CD3/TCR). T-cell clones with reduced Ly-6 surface expression (after in vitro mutagenesis or antisense oligonucleotide treatment) showed a marked reduction in proliferation after stimulation via the antigen receptor complex.[199,200] Interestingly, the defect seemed to be specific to Ly-6, as the mutants expressed normal levels of GPI-anchored Thy-1. Removal of the GPI-anchored molecules by PI-PLC treatment,[199] and use of mutants deficient in GPI-anchor biosynthesis,[201] had similar effects on TCR signaling. These results are at variance with those of Presky et al who reported only a reduction in Con A, but not anti-CD3 responses in PI-PLC-treated murine splenic T lymphocytes.[202] Subsequent studies revealed that the signaling defect in Ly-6 deficient cells is due to a concomitant reduction in surface TCR expression, and a defect in Fyn kinase activity.[203]

Components of the CD3/TCR complex are also required for signaling via GPI-anchored glycoproteins, which again points to a close cooperation between the two types of receptor. For instance, stimulation via Thy-1, Ly-6 or human CD55 failed to induce T-cell proliferation or interleukin 2 secretion in the absence of TCR expression, although elevation of intracellular Ca^{2+} was not affected.[16,36,204] Qualitative differences in the signals transduced by Thy-1 and TCR suggest that they follow independent pathways, but do not preclude the possibility of a synergy between the two for generating optimal activation signals. While Thy-1 proliferative responses require Fyn kinase activity, Fyn was not required for CD3/TCR-induced proliferation and interleukin production.[37,205] Signaling via Ly-6 was also found to differ from CD3/TCR-mediated events.[47] The ability of Thy-1 to mobilize Ca^{2+} also occurred in transfected B cells.[35] Similarly, in human T cells activated via GPI-linked CD73, CD2 could replace the TCR as a provider of help.[22] Although signaling via GPI-anchored proteins is not expected to take precedence over TCR signals under physiological conditions, they usually synergize with CD3/TCR-mediated signals, possibly by modulating Lck and Fyn[206] activity in the membrane contact area where CD3/TCR accumulate.

One last type of interaction between GPI-anchored proteins and TCR is the negative regulation of TCR-mediated responses by signals generated via the GPI-anchored proteins. Crosslinking of the murine Ly-6A/E, and related sgp60 molecules inhibited anti-CD3-mediated T-cell activation by interfering with IL-2 production.[43,46,51] In all these cases the nature of crosslinking was important for eliciting the inhibitory response, as a different mode of crosslinking led to activation. The sgp60-mediated inhibition appeared to proceed at the level of Ca^{2+} mobilization.[53] Another GPI-anchored protein, the TSA differentiation antigen on murine T cells (thymic shared antigen, TSA-1/Stem cell antigen-2, Sca-2) was also shown to inhibit stimulation via anti-CD3 through interference with IL-2 production and tyrosine phosphorylation of the CD3 ζ chain.[58] Apparently, these inhibitory effects stem from extracellular interactions, as the GPI anchor was not essential for mediating inhibition.[46,58] TSA-1 has also been implicated in preventing anti-CD3-induced apoptosis of immature thymocytes,[59] while Thy-1 was shown to trigger thymocyte apoptosis by a TCR independent mechanism.[39]

During T-cell activation by antigen, the TCR recognizes peptide-MHC complexes on the antigen presenting cell (APC) (for a review see ref. 207). The cell-cell contact which ensues considerably reduces the intercellular space, so that larger molecules, such as CD44 and CD45, are forced away from the contact site. It is expected that the CD3/TCR complex undergoes numerous lateral interactions with GPI-anchored (Thy-1, Ly6) and transmembrane receptors (CD45, CD2), but which of those interactions remain operative after clearing the T cell/APC contact area of bulky surface molecules is still not known. If the normal

homeostatic equilibrium at the cell surface is suddenly disturbed, and small GPI-anchored costimulatory receptors and larger molecules such as CD44 and CD45 localize to different regions of the plasma membrane, the intracellular phosphorylation events are likely to be disturbed. However, testing such hypotheses will not be easy, as it will require techniques to follow cell surface topography without the use of antibodies which themselves cause redistribution.

Summary

In this chapter we have examined several concepts with the aim of achieving a better understanding of how GPI anchors participate in signaling. We propose that sphingolipid-rich domains provide a unique and special environment for GPI anchors. In these regions of the membrane, there is evidence for active cooperation of GPI-anchored molecules with the established signaling pathways, and particularly signal transducers such as the β integrins and gp130 (the common signal transducing chain of the cytokine receptor complex), which may relay messages from a range of different GPI-anchored proteins. The unique topographical distribution of GPI anchors in sphingolipid microdomains does not itself cause signal transduction but may bring potential tranducers in proximity to signaling molecules such as PTKs. Crosslinking of their GPI-linked passenger receptors may collect the Src protein tyrosine kinases and other signaling molecules at the inner leaflet to generate a localized threshold level of activation signals. Recruitment of cytoplasmic signaling molecules to such platforms is also expected to integrate other signaling pathways. Clearly, the concept that sphingolipid microdomains are preexisting signaling platforms which may coalesce and provide a scaffold for the recruitment, assembly and regulation of other signaling intermediates is gaining support. However, direct demonstration of a number of these propositions under physiological means of stimulation remains a challenging task.

References

1. Robinson PJ. Phosphatidylinositol membrane anchors and T-cell activation. Immunol Today 1991; 12:35-41.
2. Malek TR, Fleming TJ, Codias EK. Regulation of T lymphocyte function by glycosyl-phosphatidylinositol (GPI)-anchored proteins. Semin Immunol 1994; 6:105-113.
3. Schütt C, Ringel B, Nausch M et al. Human monocyte activation induced by anti-CD14 monoclonal antibody. Immunol Lett 1988; 19:321-328.
4. MacIntyre EA, Roberts PJ, Jones M et al. Activation of human monocytes occurs on crosslinking monocyte antigens to an Fc receptor. J Immunol 1989; 142:2377-2383.
5. Lund-Johansen F, Olweus J, Aarli A et al. Signal transduction in human monocytes and granulocytes through the PI-linked antigen CD14. FEBS Lett 1990; 273:55-58.
6. Lund-Johansen F, Olweus J, Symington FW et al. Activation of human monocytes and granulocytes by monoclonal antibodies to glycosylphosphatidylinositol-anchored antigens. Eur J Immunol 1993; 23:2782-2791.
7. Lee JD, Kravchenko V, Kirkland TN et al. Glycosyl-phosphatidylinositol-anchored or integral membranes forms of CD14 mediate identical cellular responses to endotoxin. Proc Natl Acad Sci USA 1993; 90:9930-9934.
8. Liu MK, Herrera-Velit P, Brownsey RW et al. CD14-dependent activation of protein kinase C and mitogen-activated protein kinases (p42 and p44) in human monocytes treated with bacterial lipopolysaccharide. J Immunol 1994; 153:2642-2652.
9. Herrera-Velit P, Reiner NE. Bacterial lipopolysaccharide induces the association and coordinate activation of p53/p56 lyn and phosphatidylinositol 3 kinase in human monocytes. J Immunol 1996; 156:1157-1165.
10. Morgan BP, Vandenberg CW, Davies EV et al. Crosslinking of CD59 and of other glycosyl phosphatidylinositol-anchored molecules on neutrophils triggers cell activation via tyrosine kinase. Eur J Immunol 1993; 23:2841-2850.

11. Fischer GF, Majdic O, Gadd S et al. Signal transduction in lymphocytic and myeloid cells via CD24, a new member of phosphoinositol-anchored membrane molecules. J Immunol 1990; 144:638-641.
12. Stulnig TM, Berger M, Sigmund T et al. Signal transduction via glycosyl phosphatidylinositol-anchored proteins in T cells is inhibited by lowering cellular cholesterol. J Biol Chem 1997; 272:19242-19247.
13. Rowan WC, Hale G, Tite JP et al. Crosslinking of the CAMPATH-1 antigen (CD52) triggers activation of normal human T lymphocytes. Int Immunol 1994; 7:69-77.
14. Davis LS, Patel SS, Atkinson JP et al. Decay-accelerating factor functions as a signal transducing molecule for human T cells. J Immunol 1988; 141:2246-2252.
15. Shenoy-Scaria AM, Kwong J, Fujita T et al. Signal transduction through decay-accelerating factor—Interaction of glycosyl-phophatidylinositol anchor and protein tyrosine kinases p56lck and p59fyn. J Immunol 1992; 149:3535-3541.
16. Tosello A-C, Mary F, Amiot M et al. Activation of T cells via CD55: Requirement of early components of the CD3-TCR pathway is required for IL-2 secretion. J Inflamm 1998; 48:13-27.
17. Le P, Denning S, Springer T et al. Anti-LFA-3 monoclonal antibody induces interleukin-1 (IL-1) release by thymic epithelial (TE) cells and monocytes. Fed Proc (FASEB J) 1987; 46:447.
18. Korty PE, Brando C, Shevach EM. CD59 functions as a signal-transducing molecule for human T cell activation. J Immunol 1991; 146:4092-4098.
19. van den Berg CW, Cinek T, Hallet MB et al. Exogenous glycosyl phosphatidylinositol-anchored CD59 associates with kinases in membrane clusters on U937 cells and becomes Ca++-signaling competent. J Cell Biol 1995; 131:669-677.
20. Skubitz KM, Campbell KD, Skubitz APN. CD66a, CD66b, CD66c, and CD66d each independently stimulate neutrophils. J Leuk Biol 1996; 60:106-117.
21. Thompson LH, Ruedi JM, Glass A et al. Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T cells to proliferate. J Immunol 1989; 143:1815-1821.
22. Massaia M, Perrin L, Bianchi A et al. Human T cell activation: Synergy between CD73 (ecto-5'-nucleotidase) and signals delivered through CD3 and CD2 molecules. J Immunol 1990; 145:1664-1674.
23. Airas L, Niemela J, Salmi M et al. Differential regulation and function of CD73, a glycosyl-phosphatidylinositol-linked 70-kD adhesion molecule, on lymphocytes and endothelial cells. J Cell Biol 1997; 136:421-431.
24. Pezzutto A, Thompson LF, Dorken B et al. The plasma membrane enzyme 5'-ectonucleotidase (CD73) regulates B cell activation. J Cell Biochem 1990; 14D:263.
25. Bohuslav J, Horejsi V, Hansmann C et al. Urokinase plasminogen activator receptor, beta 2-integrins, and Src-kinases within a single receptor complex of human monocytes. J Exp Med 1995; 181:1381-1390.
26. Okuyama Y, Ishihara K, Kimura N et al. Human BST-1 expressed on myeloid cells functions as a receptor molecule. Biochem Biophys Res Commun 1996; 228:838-845.
27. Cashman NR, Loertscher R, Nalbantoglu J et al. Cellular isoform of the scrapie agent protein participates in lymphocytes activation. Cell 1990; 61:185-192.
28. Maino VC, Norcross MA, Perkins MS et al. Mechanism of Thy-1-mediated T cell activation: Roles of Fc receptors, T200, Ia and H-2 glycoproteins in accessory cell function. J Immunol 1981; 126:1829-1836.
29. Gunter KC, Malek TR, Shevach EM. T-cell activating properties of an anti-Thy-1 monoclonal antibody. Possible analogy to OKT3/Leu4. J Exp Med 1984; 159:716-730.
30. MacDonald HR, Bron C, Rousseaux M et al. Production and characterization of monoclonal anti-Thy-1 antibodies that stimulate lymphokine production by cytolytic T cell clones. Eur J Immunol 1985; 15:495-501.
31. Kroczek RA, Gunter KC, Seligmann B et al. Induction of T cell activation by monoclonal anti-Thy-1 antibodies. J Immunol 1986; 136:4379-4384.

32. Bamezai A, Goldmacher VS, Reiser H et al. Internalization of phosphatidylinositol-anchored lymphocyte proteins: I. Documentation and potential significance for T cell stimulation. J Immunol 1989; 143:3107-3116.
33. Kummer U, Haunschild J, Reisbach G et al. Mitogenicity of anti-Thy-1 monoclonal antibodies attributable to an Fc-dependent mechanism. Eur J Immunol 1993; 23:2649-2654.
34. Hsi ED, Siegel JN, Minami Y et al. T cell activation induces rapid tyrosine phosphorylation of a limited number of cellular substrates. J Biol Chem 1989; 264:10836-10842.
35. Kroczek RA, Gunter KC, Germain RN et al. Thy-1 functions as a signal transduction molecule in T lymphocytes and transfected B lymphocytes. Nature 1986; 322:181-184.
36. Gunter KC, Germain RN, Kroczek RA et al. Thy-1-mediated T-cell activation requires coexpression of CD3/Ti complex. Nature 1987; 326:505-507.
37. Barboni E, Gormley AM, Pliego Rivero FB et al. Activation of T Lymphocytes by crosslinking of glycosylphospholipid-anchored Thy-1 mobilizes separate pools of intracellular second messengers to those induced by the antigen-receptor/CD3 complex. Immunology 1991; 72:457-463.
38. Tiveron M-C, Barboni E, Pliego Rivero F et al. Selective inhibition of neurite outgrowth on mature astrocytes by Thy-1 glycoprotein. Nature 1992; 355:745-748.
39. Hueber A-O, Raposo G, Pierres M et al. Thy-1 triggers mouse thymocyte apoptosis through a bcl-2-resistant mechanism. J Exp Med 1994; 179:785-796.
40. Malek TR, Ortega G, Chan C et al. Role of Ly-6 in lymphocyte activation. J Exp Med 1986; 164:709-722.
41. Rock KL, Yeh ETH, Gramm CF et al. TAP, a novel T cell-activating protein involved in the stimulation of MHC-restricted T lymphocytes. J Exp Med 1986; 168:315-333.
42. Yeh ETH, Reiser H, Daley J et al. Stimulation of T cells via the TAP molecule, a member in a family of activating proteins encoded in the Ly-6 locus. J Immunol 1987; 138:91-97.
43. Codias EK, Rutter JE, Fleming TJ et al. Downregulation of IL-2 production by activation of T cells through Ly-6A/E. J Immunol 1990; 145:1407-1414.
44. Yamanouchi S, Kuwahara K, Sakata A et al. A T cell activation antigen, Ly6C, induced on CD4(+) Th1 cells, mediates an inhibitory signal for secretion of IL-2 and proliferation in peripheral immune responses. Eur J Immunol 1998; 28:696-707.
45. Codias EK, Malek TR. Regulation of B lymphocyte responses to IL-4 and IFN-γ by activation through Ly-6A/E molecules. J Immunol 1990; 144:2197-2204.
46. Fleming TJ, Malek TR. Multiple glycosylphosphatidylinositol-anchored Ly-6 molecules and transmembrane Ly-6E mediate inhibition of IL-2 production. J Immunol 1994; 153:1955-1962.
47. Ivanov V, Fleming TJ, Malek TR. Regulation of nuclear factor-κB and activator protein-1 activities after stimulation of T cells via glycosylphosphatidylinositol-anchored Ly-6A/E. J Immunol 1994; 153:2394-2406.
48. Hahn AB, Soloski MJ. Anti-Qa-2-induced T cell activation. J Immunol 1989; 143:407-413.
49. Robinson PJ, Millrain M, Antoniou J et al. A glycolipid anchor is required for Qa-2-mediated T cell activation. Nature 1989; 342:85-87.
50. Cook RG, Leone B, Leone JW et al. Characterization of T cell proliferative responses induced by anti-Qa-2 monoclonal antibodies. Cell Immunol 1992; 144:367-381.
51. Reiser H. Sgp-60, a signal transducing glycoprotein concerned with T cell activation through the T cell receptor/CD3 complex. J Immunol 1990; 145:2077-2086.
52. Kato K, Koyanagi M, Okada H et al. CD48 is a counter-receptor for mouse Cd2 and is involved in T cell activation. J Exp Med 1992; 176:1241-1249.
53. Maschek BJ, Zhang W, Rosoff PM et al. Modulation of the intracellular Ca and inositol triphosphate concentrations in murine T lymphocytes by the glycosylphosphatidylinositol-anchored protein sgp-60. J Immunol 1993; 150:3198-3206.
54. Hubbe M, Altevogt P. Heat-stable antigen/CD24 on mouse T lymphocytes: Evidence for a costimulatory function. Eur J Immunol 1994; 24:731-737.
55. Kadmon G, von Bohlen und Halbach F, Schachner M et al. Differential, LFA-1-sensitive effects of antibodies to nectadrin, the heat-stable antigen, on B lymphoblast aggregation and signal transduction. Biochem Biophys Res Commun 1994; 198:1209-1215.

56. Chappel MS, Hough MR, Mittel A et al. Crosslinking the murine heat-stable antigen induces apoptosis in B cell precursors and suppresses the anti-CD40-induced proliferation of mature resting B lymphocytes. J Exp Med 1996; 184:1639-1649.
57. Kosugi A, Saitoh S-I, Narumiya S et al. Activation-induced expression of thymic shared antigen-1 on T lymphocytes and its inhibitory role for TCR-mediated IL-2 production. Int Immunol 1994; 6:1967-1796.
58. Saitoh S-I, Kosugi A, Noda S et al. Modulation of TCR-mediated signaling pathway by thymic shared antigen-1 (TSA-1)/Stem cell antigen-2 (Sca-2). J Immunol 1995; 155:5574-5581.
59. Noda S, Kosugi A, Saitoh S-I et al. Protection from anti-TCR/CD3-induced apoptosis in immature thymocytes by a signal through thymic shared antigen-1/stem cell antigen-2. J Exp Med 1996; 183:2355-2360.
60. Vicari AP, Bean AGD, Zlotnik A. A role for BP-3/BST-1 antigen in early T cell development. Int Immunol 1996; 8:183-191.
61. Cervello M, Matranga V, Durbec P et al. The GPI-anchored adhesion molecule F3 induces tyrosine phosphorylation: Involvement of the FNIII repeats. J Cell Sci 1996; 109:699-704.
62. Wonigeit K, Schwinzer R. Polyclonal activation of rat T lymphocytes by RT6 alloantisera. Transplant Proc 1987; 19:296-299.
63. Draberova L. Involvement of Thy-1 antigen in the activation of rat mast cells. Eur J Immunol 1989; 19:1715-1720.
64. Draberova L, Draber P. Functional expression of the endogenous Thy-1 gene and the transfected murine Thy-1.2 gene in rat basophilic leukemia cells. Eur J Immunol 1991; 21:1583-1590.
65. Draberova L, Draber P. Thy-1 glycoprotein and Src-like protein-tyrosine kinase p53/p56Lyn are associated in large detergent-resistant complexes in rat basophilic leukemia cells. Proc Natl Acad Sci USA 1993; 90:3611-3615.
66. Draberova L, Amoui M, Draber P. Thy-1-mediated activation of rat mast cells: The role of Thy-1 membrane microdomains. Immunology 1996; 87:141-148.
67. Seaman WE, Niemi EC, Stark MR et al. Molecular cloning of gp42, a cell-surface molecule that is selectively induced on rat natural killer cells by interleukin 2: Glycolipid membrane anchoring and capacity for transmembrane signaling. J Exp Med 1991; 173:251-260.
68. Jing S, Wen D, Yu Y et al. GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell 1996; 85:1113-1124.
69. Schäfer H, Scheper RJ, Healey DG et al. T cell proliferation induced by monoclonal antibodies to a phosphatidylinositol-linked differentiation antigen of guinea pig lymphocytes. Eur J Immunol 1991; 21:701-705.
70. Treanor JJS, Goodman L, Desauvage F et al. Characterization of a multicomponent receptor for GDNF. Nature 1996; 382:80-83.
71. Jing S, Wen D, Yu Y et al. GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNF-alpha, a novel receptor for GDNF. Cell 1996; 85:1113-1124.
72. Klein RD, Sherman D, Ho W-H et al. A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature 1997; 387:717-721.
73. Buj-Bello A, Adu J, Pinon LGP et al. Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. Nature 1997; 387:721-724.
74. Stahl N, Yancopoulos GD. The alphas, betas, and kinases of cytokine receptor complexes. Cell 1993; 74:587-590.
75. Petty H, R, and Todd, R.F. Integrins as promiscuous signal transduction devices. Immunol Today 1996; 17:209-212.
76. Kindzelskii AL, Laska ZO, Todd RF et al. Urokinase-type plasminogen activator receptor reversibly dissociates from complement receptor type 3 (amb2, CD11b/CD18) during neutrophil polarization. J Immunol 1996; 156:297-309.
77. Stahl A, Mueller BM. The urokinase-type plasminogen activator receptor, a GPI-linked protein, is localized in caveolae. J Cell Biol 1995; 129:335-344.

78. Li SW, Couet J, Lisanti MP. Src tyrosine kinases, G(alpha) subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin—Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. J Biol Chem 1996; 271:29182-29190.
79. Rothberg KG, Heuser JE, Donzell WC et al. Caveolin, a protein component of caveolae membrane coats. Cell 1992; 68:673-682.
80. Wei Y, Lukashev M, Simon DI et al. Regulation of integrin function by the urokinase receptor. Science 1996; 273:1551-1554.
81. Chapman HA. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. Curr Opin Cell Biol 1997; 9:714-724.
82. Hellberg C, Molony L, Zheng L et al. Ca2+ signalling mechanisms of the betas integrin on neutrophils: Involvement of phospholipase Cgamma2 and Ins(1,4,5)P3. Biochem J 1996; 317:403-409.
83. Schwartz MA, Schaller MD, Ginsberg MH. Integrins: Emerging paradigms of signal transduction. Ann Rev Cell Dev Biol 1995; 11:549-599.
84. Stefanova I, Corcoran ML, Horak EM et al. Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56Lyn. J Biol Chem 1993; 268:20725-20728.
85. Stefanova I, Horejsi V, Ansotegui IJ et al. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science 1991; 254:1016-1018.
86. Cinek T, Horejsi V. The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. J Immunol 1992; 149:2262-2270.
87. Skubitz KM, Campbell KD, Ahmed K et al. CD66 family members are associated with tyrosine kinase activity in human neutrophils. J Immunol 1995; 155:5382-5390.
88. Garnett D, Barclay AN, Carmo AM et al. The association of the protein tyrosine kinases p56lck and p60fyn with the glycosyl phosphatidylinositol-anchored proteins Thy-1 and CD48 in rat thymocytes is dependent on the state of cellular activation. Eur J Immunol 1993; 23:2540-2544.
89. Bohuslav J, Cinek T, Horejsi V. Large, detergent-resistant complexes containing murine antigens Thy-1 and Ly-6 and protein tyrosine kinase p56lck. Eur J Immunol 1993; 23:825-831.
90. Thomas PM, Samelson LE. The glycophosphatidylinositol-anchored Thy-1 molecule interacts with the p60fyn protein tyrosine kinase in T cells. J Biol Chem 1992; 267:12317-12322.
91. Hatakeyama S, Kazuya I, Kazumasa O et al. The murine c-fgr gene product associated with Ly6c and p70 integral membrane protein is expressed in cells of a monocyte/macrophage lineage. Proc Natl Acad Sci USA 1994; 91:3458-3462.
92. Olive S, Dubois C, Schachner M et al. The F3 neuronal glycosylphosphatidylinositol-linked molecule is localized to glycolipid-enriched membrane subdomains and interacts with L1 and Fyn kinase in cerebellum. J Neurochem 1995; 65:2307-2317.
93. Zarn JA, Zimmermann SM, Pass MK et al. Association of CD24 with the kinase c-fgr in a small cell lung cancer cell line and with the kinase Lyn in an erythroleukemia cell line. Biochem Biophys Res Commun 1996; 225:384-391.
94. Sammar M, Gulbins E, Hilbert K et al. Mouse CD24 as a signalling molecule for integrin-mediated cell binding: Functional and physical association with src-kinases. Biochem Biophys Res Commun 1997; 234:330-334.
95. Zisch AH, Dalessandri L, Amrein K et al. The glypiated neuronal cell adhesion molecule contactin/F11 complexes with src-family protein tyrosine kinase Fyn. Mol Cell Neurosci 1995; 6:263-279.
96. Kunz S, Ziegler U, Kunz B et al. Intracellular signaling is changed after clustering of the neural cell adhesion molecules axonin-1 and NgCAM during neurite fasciculation. J Cell Biol 1996; 135:253-267.
97. Resh M. Regulation of cellular signaling by fatty acid acylation and prenylation of signal transduction proteins. Cell Signal 1996; 8:403-412.
98. Brown DA. The tyrosine kinase connection: How GPI-anchored proteins activate T cells. Curr Opin Immunol 1993; 5:349-354.

99. Lehuen A, Beaudoin L, Bernard M et al. T cell activation through Thy-1 is associated with the expression of a surface protein (p100) on a subset of CD4 cells. Int Immunol 1995; 7:607-616.
100. Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 1992; 68:533-544.
101. Hoessli DC, Rungger-Brandle E. Isolation of plasma membrane domains from murine T lymphocytes. Proc Natl Acad Sci USA 1983; 80:439-443.
102. Hoessli DC, Rungger-Brandle E. Association of specific cell-surface glycoproteins with a Triton X-100-resistant complex of plasma membrane proteins isolated from T-lymphoma cells (P1798). Exp Cell Res 1985; 156:239-250.
103. Schroeder R, London E, Brown DA. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)- anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. Proc Natl Acad Sci USA 1994; 91:12130-12134.
104. Schroeder RJ, Ahmed SN, Zhu Y et al. Cholesterol and sphingolipid enhance the Triton X-100 insolubility of glycosylphosphatidylinositol-anchored proteins by promoting the formation of detergent-insoluble ordered membrane domains. J Biol Chem 1998; 273:1150-1157.
105. Sheets ER, Lee GM, Simson R et al. Transient confinement of a glycosylphosphatidylinositol-anchored protein in the plasma membrane. Biochemistry 1997; 34:12449-12459.
106. Simson R, Yang B, Moore SE et al. Structural mosaicism on the submicron scale in the plasma membrane. Biophy J 1998; 74:297-308.
107. Sela BA, Raz A, Geiger B. Antibodies to ganglioside GM1 induce mitogenic stimulation and cap formation in rat thymocytes. Eur J Immunol 1978; 8:268-274.
108. Spiegel S, Fishman PH, Weber RJ. Direct evidence that endogeneous GM1 gangliosides can mediate thymocyte proliferation. Science 1985; 230:1285-1287.
109. Dixon SJ, Stewart D, Grinstein S et al. Transmembrane signalling by the B subunit of cholera toxin: Increased cytoplasmic free calcium in rat lymphocytes. J Cell Biol 1987; 105:1153-1161.
110. Masco D, van de Walle M, Spiegel S. Interaction of ganglioside GM1 with the B subunit of cholera toxin modulates growth and differentiation of neuroblastoma N18 cells. J Neurosci 1991; 11:2443-2452.
111. Francis ML, Ryan J, Jobling MG et al. Cyclic AMP-independent effects of cholera toxin on B cell activation. J Immunol 1992; 148:1999-2005.
112. Leal-berumen I, Snider DP, Barajas-Jopez C et al. Cholera toxin increases IL-6 synthesis and decreases TNF-α production by rat peritoneal mast cells. J Immunol 1996; 156:316-321.
113. Swaim WD, Minoguchi K, Oliver C et al. The anti-ganglioside monoclonal antibody AA4 induces protein tyrosine phosphorylations, but not degranulation, in rat basophilic leukemia cells. J Biol Chem 1994; 269:19466-19473.
114. Welte K, Miller G, Chapman PB et al. Stimulation of T lymphocyte proliferation by monoclonal antibodies against GD3 ganglioside. J Immunol 1987; 139:1763-1771.
115. Norihisa Y, McVicar DW, Ghosh P et al. Increased proliferation, cytotoxicity, and gene expression after stimulation of human peripheral blood T lymphocytes through a surface ganglioside (GD3). J Immunol 1994; 152:485-495.
116. Ortaldo JR, Mason AT, Longo DL et al. T cell activation via the disialoganglioside GD3: Analysis of signal transduction. J Leukocyte Biol 1996; 60:533-539.
117. Kasahara K, Watanabe Y, Yamamoto T et al. Association of Src family tyrosine kinase Lyn with ganglioside GD3 in rat brain. Possible regulation of Lyn by glycosphingolipid in caveolae-like domains. J Biol Chem 1997; 272:29947-29963.
118. Yamamura S, Handa K, Hakomori S. A close association of GM3 with c-Src and rho in GM3-enriched microdomains at the B16 melanoma cell surface membrane: A preliminary note. Biochem Biophys Res Commun 1997; 236:218-222.
119. Minoguchi K, Swaim WD, Berenstein EH et al. Src family tyrosine kinase p53/56Lyn, a serine kinase and Fc epsilon RI associate with alpha-galactosyl derivatives of ganglioside GD1b in rat basophilic leukemia RBL-2H3 cells. J Biol Chem 1994; 269:5249-5254.

120. Solomon KR, Rudd CE, Finberg RW. The association between glycosylphosphatidylinositol-anchored proteins and heterotrimeric G protein alpha subunits in lymphocytes. Proc Natl Acad Sci USA 1996; 93:6053-6058.
121. Ilangumaran S, Robinson PJ, Hoessli DC. Transfer of exogenous glycosylphosphatidylinositol (GPI)-linked molecules to plasma membranes. Trends Cell Biol 1996; 6:163-167.
122. Brown DA, London E. Structure of detergent-resistant membrane domains: Does phase separation occur in biological membranes? Biochem Biophys Res Commun 1997; 240:1-7.
123. Sargiacomo M, Sudol M, Tang ZL et al. Signal transducing molecules and glycosylphosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J Cell Biol 1993; 122:789-807.
124. Smart EJ, Ying YS, Mineo C et al. A detergent-free method for purifying caveolae membrane from tissue culture cells. Proc Natl Acad Sci USA 1995; 92:10104-10108.
125. Anderson RGW. Plasmalemmal caveolae and GPI-anchored membrane proteins. Curr Opin Cell Biol 1993; 5:647-652.
126. Parton RG. Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. J Histochem Cytochem 1994; 42:155-166.
127. Fra AM, Williamson E, Simons K et al. Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. J Biol Chem 1994; 269:30745-30748.
128. Gorodinsky A, Harris DA. Glycolipid-anchored proteins in neuroblastoma cells form detergent-resistant complexes without caveolin. J Cell Biol 1995; 129:619-627.
129. Fujimoto T. GPI-anchored proteins, glycosphingolipids, and sphingomyelin are sequestered to caveolae only after crosslinking. J Histochem Cytochem 1996; 44:929-941.
130. Mayor S, Rothberg KG, Maxfield FR. Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. Science 1994; 264:1948-1951.
131. Mayor S, Maxfield FR. Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. Mol Biol Cell 1995; 6:929-944.
132. Fra AM, Masserini M, Palestini P et al. A photo-reactive derivative of ganglioside GM1 specifically cross-links VIP21-caveolin on the cell surface. FEBS Lett 1995; 375:11-14.
133. Schnitzer JE, McIntosh DP, Dvorak AM et al. Separation of caveolae from associated microdomains of GPI- anchored proteins. Science 1995; 269:1435-1439.
134. Liu J, Oh P, Horner T et al. Organized endothelial cell surface signal transduction in caveolae distinct from glycosylphosphatidylinositol-anchored protein microdomains. J Biol Chem 1997; 272:7211-7222.
135. Stan RV, Roberts WG, Predescu D et al. Immunoisolation and partial characterization of endothelial plasmalemmal vesicles (caveolae). Mol Biol Cell 1997; 8:595-605.
136. Huang CF, Hepler JR, Chen LT et al. Organization of G proteins and adenylyl cyclase at the plasma membrane. Mol Biol Cell 1997; 8:2365-2378.
137. Lisanti MP, Scherer PE, Vidugiriene J et al. Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: Implications for human disease. J Cell Biol 1994; 126:111-126.
138. Hope H, R, Pike, L,J. Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains. Mol Biol Cell 1996; 7:843-851.
139. Fujimoto T, Nakade S, Miyawaki A et al. Localization of inositol 1,4,5-trisphosphate receptor-like protein in plasmalemmal caveolae. J Cell Biol 1992; 119:1507-1513.
140. Schnitzer JE, Oh P, Jacobson BS et al. Caveolae from luminal plasmalemma of rat lung endothelium: Microdomains enriched in caveolin, Ca2+-ATPase, and inositol trisphosphate receptor. Proc Natl Acad Sci USA 1995; 92:1759-1763.
141. Chang WJ, Ying YS, Rothberg KG et al. Purification and characterization of smooth muscle cell caveolae. J Cell Biol 1994; 126:127-138.
142. Oka N, Yamamoto M, Schwencke C et al. Caveolin interaction with protein kinase C—Isoenzyme-dependent regulation of kinase activity by the caveolin scaffolding domain peptide. J Biol Chem 1997; 272:33416-33421.
143. Song KS, Li SW, Okamoto T et al. Copurification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains—Detergent-free purification of caveolae membranes. J Biol Chem 1996; 271:9690-9697.

144. Liu PS, Ying YS, Anderson RGW. Platelet-derived growth factor activates mitogen-activated protein kinase in isolated caveolae. Proc Natl Acad Sci USA 1997; 94:13666-13670.
145. Shaul PW, Smart EJ, Robinson LJ et al. Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. J Biol Chem 1996; 271:6518-6522.
146. Liu PS, Ying YS, Ko YG et al. Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. J Biol Chem 1996; 271:10299-10303.
147. Couet J, Sargiacomo M, Lisanti MP. Interaction of a receptor tyrosine kinase, EGF-R, with caveolins—Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities. J Biol Chem 1997; 272:30429-30438.
148. Li S, Okamoto T, Chun M et al. Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. J Biol Chem 1995; 270:15693-15701.
149. Couet J, Li SW, Okamoto T et al. Identification of peptide and protein ligands for the caveolin-scaffolding domain—Implications for the interaction of caveolin with caveolae-associated proteins. J Biol Chem 1997; 272:6525-6533.
150. Feron O, Saldana F, Michel JB et al. The endothelial nitric-oxide synthase-caveolin regulatory cycle. J Biol Chem 1998; 273:3125-3128.
151. Okamoto T, Schlegel A, Scherer PE et al. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. J Biol Chem 1998; 273:5419-5422.
152. Wu CB, Butz S, Ying YS et al. Tyrosine kinase receptors concentrated in caveolae-like domains from neuronal plasma membrane. J Biol Chem 1997; 272:3554-3559.
153. Vanthof W, Resh MD. Rapid plasma membrane anchoring of newly synthesized p59(fyn): Selective requirement for NH2-terminal myristoylation and palmitoylation at cysteine-3. J Cell Biol 1997; 136:1023-1035.
154. Bijlmakers MJJE, Isobenakamura M, Ruddock LJ et al. Intrinsic signals in the unique domain target p56(lck) to the plasma membrane independently of CD4. J Cell Biol 1997; 137:1029-1040.
155. Shenoy-Scaria AM, Gauen LKT, Kwong J et al. Palmitylation of an amino-terminal cysteine motif of protein tyrosine kinases p56lck and p59fyn mediates interaction with glycosyl-phosphatidylinositol-anchored proteins. Mol Cell Biol 1993; 13:6385-6392.
156. Brown RE. Sphingolipid organization in biomembranes: What physical studies of model membranes reveal. J Cell Sci 1998; 111:1-9.
157. Rodgers W, Rose JK. Exclusion of CD45 inhibits activity of p56(lck) associated with glycolipid-enriched membrane domains. J Cell Biol 1996; 135:1515-1523.
158. Ilangumaran S, Arni S, Chicheportiche Y et al. Evaluation by dot-immunoassay of the differential distribution of cell surface and intracellular proteins in glycosyl-phosphatidylinositol-rich plasma membrane domains. Anal Biochem 1996; 235:49-56.
159. Brown MT, Cooper JA. Regulation, substrates and functions of src. Biochim Biophys Acta 1996; 1287:121-149.
160. Sieh M, Bolen JB, Weiss A. CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck. EMBO J 1993; 12:315-321.
161. Yurchak LK, Sefton BM. Palmitoylation of either Cys-3 or Cys-5 is required for the biological activity of the lck tyrosine protein kinase. Mol Cell Biol 1995; 15:6914-6922.
162. Arni S, Ilangumaran S, van Echten-Deckert G, Sandhoff K, Poincelet M, Briol A, Rungger-Brandle E, Hoessli DC. Differential regulation of Src-family protein tyrosine kinases in GPI domains of T lymphocyte plasma membranes. Biochem Biophys Res Commun 1996; 225:801-807.
163. Moarefi I, Lafevrebernt M, Sicheri F et al. Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. Nature 1997; 385:650-653.
164. Hardwick JS, Sefton BM. The activated form of the Lck tyrosine protein kinase in cells exposed to hydrogen peroxide is phosphorylated at both Tyr-394 and Tyr-505. J Biol Chem 1997; 272:25429-25432.
165. Gouy H, Debr, P, Bismuth G. Triggering of a sustained calcium response through a p56lck-dependent pathway by exogenous ganglioside GM1 in human T lymphocytes. J Immunol 1995; 155:5160-5166.

166. Saqr HE, Pearl DK, Yates AJ. A review and predictive models of ganglioside uptake by biological membranes. J Neurochem 1993; 61:395-411.
167. Giorgione J, Turco SJ, Epand RM. Transbilayer inhibition of protein kinase C by the lipophosphoglycan from Leishmania donovani. Proc Natl Acad Sci USA 1996; 93:11634-11639.
168. Holowka D, Baird B. Antigen-mediated IgE receptor aggregation and signaling: A window on cell surface structure and dynamics. Ann Rev Biophys Biomol Struct 1996; 25:79-112.
169. Field KA, Holowka D, Baird B. Compartmentalized activation of the high affinity immunoglobulin E receptor within membrane domains. J Biol Chem 1997; 272:4276-4280.
170. Stauffer TP, Meyer T. Compartmentalized IgE receptor-mediated signal transduction in living cells. J Cell Biol 1997; 139:1447-1454.
171. Deans JP, Robbins SM, Polyak MJ et al. Rapid redistribution of CD20 to a low density detergent-insoluble membrane compartment. J Biol Chem 1998; 273:344-348.
172. DeGrendele HC, Estess P, Siegelman MH. Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science 1997; 278:672-675.
173. Perschl A, Lesley J, English N et al. Transmembrane domain of CD44 is required for its detergent insolubility in fibroblasts. J Cell Sci 1995; 108:1033-1041.
174. Bourguignon LYW. Interactions between the membrane-cytoskeleton and CD44 during lymphocyte signal transduction and cell adhesion. Curr Top Memb 1996; 43:293-312.
175. Taher TE, Smit L, Griffieon AW et al. Signaling through CD44 is mediated by tyrosine kinases: Association with p56lck in lymphocytes. J Biol Chem 1996; 271:2863-2867.
176. Ilangumaran S, Briol A, Hoessli DC. CD44 selectively associates with active Src-family kinases Lck and Fyn in glycosphingolipid-rich plasma membrane domains of human peripheral blood lymphocytes. Blood 1998; 91:3901-3908.
177. Cerny J, Stockinger H, Horejsi V. Noncovalent associations of T lymphocyte surface proteins. Eur J Immunol 1996; 26:2335-2343.
178. Feron O, Smith TW, Michel T et al. Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. J Biol Chem 1997; 272:17744-17748.
179. Mutoh T, Tokuda A, Miyadai T et al. Ganglioside GM1 binds to the Trk protein and regulates receptor function. Proc Natl Acad Sci USA 1995; 92:5087-5091.
180. Kypta RM, Goldberg Y, Ulug ET et al. Association between the PDGF receptor and members of the Src family of tyrosine kinases. Cell 1990; 62:481-492.
181. Liu DC, Sy MS. Phorbol myristate acetate stimulates the dimerization of CD44 involving a cysteine in the transmembrane domain. J Immunol 1997; 159:2702-2711.
182. Su B, Waneck GL, Flavell RA et al. The glycosylphosphatidylinositol anchor is critical for LY-6A/E-mediated T cell activation. J Cell Biol 1991; 112:377-384.
183. Rodgers W, Crise B, Rose JK. Signals determining protein tyrosine kinase and glycosyl-phosphatidylinositol-anchored protein targeting to a glycolipid-enriched membrane fraction. Mol Cell Biol 1994; 14:5384-5391.
184. Pugin J, Kravchenko VV, Lee JD et al. Cell activation mediated by glycosylphos-phatidylinositol-anchored or transmembrane forms of CD14. Infect Immun 1998; 66:1174-671180.
185. Resta R, Thompson LF. T cell signalling through CD73. Cell Signal 1997; 9:131-139.
186. Wurfel MM, Wright SD. Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers—Preferential interaction with particular classes of lipid. J Immunol 1997; 158:3925-3934.
187. Schromm AB, Brandenburg K, Rietschel ET et al. Lipopolysaccharide-binding protein mediates CD14-independent intercalation of lipopolysaccharide into phospholipid membranes. FEBS Lett 1996; 399:267-271.
188. Meng FY, Lowell CA. Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. J Exp Med 1997; 185:1661-1670.
189. Ingalls RR, Golenbock DT. CD11c/CD18, a transmembrane signaling receptor for lipopolysaccharide. J Exp Med 1995; 181:1473-1479.

190. Sleckman BP, Rosenstein Y, Igras VE et al. Glycolipid-anchored form of CD4 increases intracellular adhesion but is unable to enhance T cell activation. J Immunol 1991; 147:428-431.
191. Clissold PM. Recombinant glycosyl-phosphatidylinositol-anchored proteins are not associated with protein kinases in transfected thymoma cells. Biochem J 1994; 304:853-859.
192. Barboni E, Pilego Rivero B, George AJT et al. The glycosylphosphatidylinositol anchor affects the conformation of Thy-1 protein. J Cell Sci 1995; 108:487-497.
193. Saltiel AR, Sherline P, Fox JA. Insulin-stimulated diacylglycerol production results from the hydrolysis of a novel phosphatidylinositol glycan. J Biol Chem 1987; 262:1116-1121.
194. Susuki S, Sugawara K, Satoh Y et al. Insulin stimulates the generation of two putative insulin mediators, inositol-glycan and diacylglycerol in BC3H-1 myocytes. J Biol Chem 1991; 266:8115-8121.
195. Field MC. Is there evidence for phospho-oligosaccharides as insulin mediators? Glycobiology 1997; 7:161-168.
196. Merida I, Pratt JC, Gaulton GN. Regulation of interleukin 2-dependent growth responses by glycosylphosphatidylinositol molecules. Proc Natl Acad Sci USA 1990; 87:9421-9425.
197. Gaulton GN, Pratt JC. Glycosylated phosphatidylinositol molecules as second messengers. Semin Immunol 1994; 6:97-104.
198. Singh N, Liang L, Tykocinski ML et al. A novel class of cell surface glycolipids of mammalian cells: Free glycosyl phosphatidylinositols. J Biol Chem 1996; 271:12879-12884.
199. Yeh ETH, Reiser H, Bamezai A et al. TAP transcription and phosphatidylinositol linkage mutants are defective in activation through the T cell Receptor. Cell 1988; 52:665-674.
200. Flood PM, Dougherty JP, Ron Y. Inhibition of Ly-6A antigen expression prevents T cell activation. J Exp Med 1990; 172:115-120.
201. Romagnoli P, Bron C. Phosphatidylinositol-based glycolipid-anchored proteins enhance proximal TCR signalling events. J Immunol 1997; 158:5757-5764.
202. Presky DH, Low MG, Shevach EM. Role of phosphatidylinositol-anchored proteins in T cell activation. J Immunol 1990; 144:860-866.
203. Lee SK, Su B, Maher SE et al. Ly-6A is required for T cell receptor expression and protein tyrosine kinase fyn activity. EMBO J 1994; 13:2167-2176.
204. Sussman JJ, Saito T, Shevach EM et al. Thy-1 and Ly-6 mediated lymphokine production and growth inhibition of a T cell hybridoma require coexpression of the T cell antigen receptor complex. J Immunol 1988; 140:2520-2526.
205. Lancki DW, Qian DP, Fields P et al. Differential requirement for protein tyrosine kinase Fyn in the functional activation of antigen-specific T lymphocyte clones through the TCR or Thy-1. J Immunol 1995; 154:4363-4370.
206. Weiss A. T-cell antigen receptor signal transduction—A tale of tails and cytoplasmic protein-tyrosine kinases. Cell 1993; 73:209-212.
207. Shaw AS, Dustin ML. Making the T cell receptor go the distance: A topological view of T cell activation. Immunity 1997; 6:361-369.

While this review was in press several reports have strongly implicated the sphingolipid-rich membrane microdomains in signaling via the CD3/TCR complex. Following TCR engagement the CD3 z chain is recruited to the microdomains,[1,2] presumably through the mediation of the microdomain-associated Lck and Fyn PTKs. The transmembrane adaptor molecule LAT (Linker for T-cell Activation) is targeted to the microdomains by dual acylation, becomes rapidly tyrosine phosphorylated upon T-cell activation and recruits several signaling molecules including PLCg1 and Vav to the microdomains via protein modular binding domains.[3] Disrupting the membrane microdomain architecture by cholesterol depletion or gangliosides decreased tyrosine phosphorylation of CD3 z and PLCg1, and abolished the intracellular calcium flux.[2] Further evidence suggest that association of CD3 z with actin cytoskeleton upon activation is also dependent on intact microdomains.[4] Finally, under conditions mimicking the interaction of antigen-presenting cells and T lymphocytes, signaling via the costimulatory molecule CD28 appears to be necessary for coupling the microdomain signaling machinery to the CD3/TCR complex.[5] Interestingly, accumulation of polyunsaturated fatty acids in T-lymphocyte membranes decreases the amount of Lck but not CD59 associated with microdomains, suggesting that the microdomain cytoplasmic leaflet may be selectively perturbed in its interactions with PTKs.[6]

1. Montixi C, Langlet C, Bernard AM, et al. Engagement of T-cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. Embo J 1998; 17(18):5334-48.
2. Xavier R, Brennan T, Li Q, McCormack C, Seed B. Membrane compartmentation is required for efficient T-cell activation. Immunity 1998; 8(6):723-732.
3. Zhang W, Trible RP, Samelson LE. LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T-cell activation. Immunity 1998; 9(2):239-246.
4. Moran M, Miceli MC. Engagement of GPI-linked CD48 contributes to TCR signals and cytoskeletal reorganization: a role for lipid rafts in T cell activation. Immunity 1998; 9(6):787-796.
5. Viola A, Schroeder S, Sakakibara Y, Lanzavecchia A. T Lymphocyte Costimulation Mediated by Reorganization of Membrane Microdomains. Science 1999; 283(5402):680-682.
6. Stulnig TM, Berger M, Sigmund T, Raederstorff D, Stockinger H, Waldhausl W. Polyunsaturated fatty acids inhibit T-cell signal transduction by modification of detergent-insoluble membrane domains. J Cell Biol 1998; 143(3):637-644.

CHAPTER 5

GPI-Anchored Proteins of Leucocytes: Inventory, Structural Properties and Functions

Vaclàv Horejsí, Petr Dráber and Hannes Stockinger

In this chapter we describe the structural properties and known functions of GPI-anchored proteins expressed on leucocytes. Basic characteristics of the leucocyte surface GPI-anchored proteins are summarized in Table 5.1 and are not repeated in the separate entries on individual molecules. We will not mention here the numerous observations on the capacity of these molecules to transduce signals into cells upon antibody crosslinking and on their association with membrane microdomains rich in glycosphingolipids and protein tyrosine kinases, as these topics are discussed in chapter 4.

CD14

The polypeptide chain of CD14 contains 10 repeats with partial similarity to the leucine-rich glycoprotein.[1] In addition to its typical expression on myeloid cells (Table 5.1), very low levels of functional CD14 are also expressed by B cells.[2] Significant amounts of CD14 are stored together with other GPI-anchored proteins (CD16b, alkaline phosphatase) and with complement receptor type 3 (CR3; CD11b/CD18) in neutrophil secretory vesicles which fuse upon cell stimulation with plasma membrane, resulting in markedly increased expression of these molecules.[3] Soluble forms of CD14 are present in relatively high concentrations (around 2-3 μg/ml) in normal serum and in urine of nephrotic patients.[4,5] One of the soluble forms (48 kDa) is produced by proteolytic shedding of the cell surface form.[6] A second soluble form is secreted by human monocytes and corresponds to a full length polypeptide with the C-terminal peptide normally removed during GPI anchor addition.[7] It should be noted that the total amount of soluble CD14 in blood exceeds the amount of membrane-bound CD14 by several hundred-fold.

CD14 is a receptor for lipopolysaccharide (LPS; endotoxin) released from Gram-negative bacteria.[8] The binding of LPS to CD14 is facilitated by LPS-binding protein (LBP).[9,10] Myeloid cell surface CD14 with bound LPS appears to interact with another so far poorly characterized receptor(s) responsible for the LPS-induced signaling[11,12] which ultimately leads to secretion of TNF, IL-6 and IL-1. One of the transmembrane receptors capable of reversible interaction with the CD14-LPS complex may be the complement receptor type 3 (CR3; the leucocyte integrin CD11b/CD18).[13] Some reports indicate that the myeloid cell activation by LPS via CD14 is dependent on protein tyrosine kinase activation,[14,15] while others deny it.[16,17] Interestingly, the LPS-triggered signaling capacity of the transmembrane version of CD14 is similar to that of the natural GPI-anchored one.[11]

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

Table 5.1 Leucocyte surface GPI-anchored proteins

Protein	Synonyms	MW (kda)	Amino acids (mature protein)	N-glyco-sylation sites	Chromosome localization	Structural family	Cellular expression	Key refs. reviews
CD14	LPS receptor	55	333	4	5q31	Leucine rich gp?	M, N	208-210
CD16b	Fcγ receptor type III	48-60	186	6	1q23	Ig SF	N	31,211, 212
CD24	BA-1; HSA; J11d (mouse)	42	33	2	6q21		B, N, EPC	47
CD48	BLAST-1; OX-45 (rat); BCM1 or sgp-60 (mouse)	41	194	6	1q21-q23	Ig SF	Leukocytes	64,66-68
CD52	Campath-1; B7(2) (mouse)	21-28	12	1			Leukocytes	75,76,79
CD55	DAF	60-70	319	1	1q32	CR-SF	Most cell types	213-215
CD58	CFA-3	40-65	180	6	1p13.1	Ig SF	Many cell types	85-87
CD59	Protectin, HRF20, MIRL	19-25	77	1	11q13	Ly-6 SF	Most cell types	214,216, 217
CD66b	CD67, NCA95	95-100	286	11	19q13.1-2	Ig SF	N	99,218
CD66c	NCA50	90-95	286	12	19q13.1-2	Ig SF	N	99,218
CD73	Ecto-5' nucleo-tidase VAP-2	69	523	4	6q14-q21		B sub., T sub., EC	110,114, 219
CD87	uPA-R	45-55	283	5	19q13	Ly-6 SF	M, N, EC, tumor cells, fibroblasts, smooth muscle	122,123, 127
CD90	Thy-1	25-35	111	3	11q23.3	Ig SF	BM sub., HEV, neurons, Thy, T (mouse)	220-222
CDw108	JMH blood group antigen	75	Unknown	Unknown	Unknown	Unknown	HPB-ALL (T-cell line) T act, E	162,163
CD109	Gov$^{a/b}$ alloantigen	170	Unknown	Probably	Unknown	Unknown	EC, T act, P	165,166
CD157	BST-1 Mo5	42-50	272	4		NAD-glyco -hydratase family	M, N BM stroma	167,168
RT6		24-35	227	1 or 0 (allelic forms)	Rat	Arg-ribosyl transferase family	T	183,223

Table 5.1 Leucocyte surface GPI-anchored proteins (con't)

Protein	Synonyms	MW (kda)	Amino acids (mature protein)	N-glyco-sylation sites	Chromosome localization	Structural family	Cellular expression	Key refs. reviews
Melano-trans ferrin	gp97	80-97	692	3		Transferrin family	Melanoma, HPB-ALL (T)	188,189
Ly-6A/E (mouse)	Sca-1, TAP	15-18	79	0	15 band E (mouse)	Ly-6 SF	Lymphocytes T sub.	190,191 225
Ly-6C (mouse)		14	76	0	15 band E (mouse)	Ly-6 SF	BM, N, M, T sub.	190,191 225
Ly-6G (mouse)		?	79	0	15 band E (mouse)	Ly-6 SF	BM	190,191, 226
ThB (mouse)		15		0	15 (mouse)	Ly-6 SF	Thymus B, skin	190,191, 227
TSA-1 (mouse)	Sca-2	15-17	82	1	15 (mouse)	Ly-6	BM, thymus, B, T act.	190,191 228,229

Abbreviations: act., activated; B, B cells; BM, bone marrow; CR, complement receptor; DAF, decay accelerating factor; EC, endothelial cells; EPC, epithelial cells; E, erythrocytes; gp, glycoprotein; HEV, high endothelial venules; HRF, homologous restriction factor; HSA, heat stable antigen; HSC, hematopoietic stem cells; Ig, immunoglobulin; LFA, lymphocyte function associated antigen; LPS, lipopolysaccharide; MIRL, membrane inhibitor of reactive lysis; M, monocytes; N, neutrophils; NCA, nonspecific crossreactive antigen; P, platelets; Sca, stem cell antigen; SF, superfamily; sub., subpopulation; T, T-cells; T act, activated T-cells; TAP, T-cell activating protein; Thy, thymus shared antigen; uPA-R, urokinase plasminogen activator receptor; VAP, vascular adhesion protein.

The complex of soluble CD14 (sCD14) with LPS binds to an unidentified receptor on endothelial and epithelial cells and activates these cells.[18,19] Soluble CD14-LPS complexes may also stimulate macrophages, neutrophils[20] and dendritic cells.[21] Soluble CD14 also plays an important role in neutralization and clearance of LPS, which is transferred from the sCD14-LBP-LPS complexes to serum high density lipoprotein particles.[22,23]

In addition to binding of LPS released from bacteria, monocyte cell surface CD14 may also interact with the bacterial surface LPS and induce phagocytosis.[24] The binding ability of CD14 appears to be much broader than originally thought: In addition to LPS it binds and initiates response to other bacterial envelope components from Gram-negative and Gram-positive microorganisms as well as mycobacteria,[25] such as streptococcal polysaccharides,[26] an unidentified minor staphylococcal compound,[27] peptidoglycans,[28] uronic acid polymers[29] or lipoteichoic acids.[30] Thus, CD14 appears to be a versatile "pattern receptor" used by myeloid cells for recognition for a variety of microorganisms.

CD16b

CD16 exists in two distinct forms encoded by two closely linked genes. One of them is a transmembrane protein (CD16a) expressed on NK cells and macrophages, the other one (CD16b) is GPI-anchored and present on neutrophils.[31] The extracellular parts of CD16a and CD16b differ in 6 amino acid residues. Two allelic forms of CD16b are called NA1 and NA2.[32] Interestingly, only the transmembrane form of CD16 exists in the mouse and is expressed on both NK cells and neutrophils.[33] The polypeptide chain of CD16b consists of two Ig-like domains and is richly and variably *N*-glycosylated, producing a heterogeneous range of molecular forms. CD16b serves as a neutrophil low affinity IgG receptor which

plays an important role (in cooperation with other neutrophil Fc receptors and complement receptor type 3) in phagocytosis of antigenic particles opsonized with IgG and in generation of signals leading to respiratory burst.[34-40] Some patients with paroxysmal nocturnal hemoglobulinuria lacking GPI-anchored proteins on a fraction of their neutrophils therefore have increased levels of circulating immune complexes and suffer from increased susceptibility to bacterial infections.[41] CD16b is noncovalently associated with complement receptor type 3 (CR3; the CD11b/CD18 integrin) in the neutrophil membrane; this interaction is based on the lectin-carbohydrate interaction between the carbohydrate-binding site in the CR3 molecule and CD16b oligosaccharide.[42,43] CD16b is released by proteolytic shedding from neutrophil surfaces;[44] and rapid loss of surface CD16b is an early sign of neutrophil apoptotic changes.[45] Carbohydrates of serum CD16b may bind CR3 and CR4 via carbohydrate-lectin interaction. CR3 ligation by soluble CD16b may induce IL-6 and IL-8 production by monocytes.[40] CD16b seems to also collaborate functionally with the bacterial chemotactic formyl-peptide receptors.[46]

CD24

The unusually short polypeptide chain of CD24 is richly *N*- and *O*-glycosylated.[47] The true Mr of the mature glycoprotein is not known, as the apparent value is probably largely overestimated due to anomalously slow migration of heavily glycosylated proteins under the conditions of SDS PAGE. This glycoprotein is characteristic for human granulocytes and B lymphocytes, but its expression decreases after B-cell activation and it is absent on plasma cells.[48] It is also present on approximately 2% of thymocytes and on normal epithelium. CD24 is expressed at high levels on small cell lung carcinoma.[49] A likely mouse homologue molecule is called J11d or HSA (heat stable antigen) and is present on all stages of B cells. HSA is expressed on cortical ($CD4^+CD8^+$) thymocytes but absent from single-positive medullary thymocytes and mature T cells.[50] Mouse erythrocytes, myeloid cells and epidermal dendritic cells are also HSA-positive.[51] The relatively low degree of sequence identity (33%) between mature mouse HSA and human CD24 raises the possibility that these molecules are not true homologues. The data on expression of CD24 and HSA should be interpreted with caution, as most mAbs are directed to carbohydrate epitopes which may be present on other molecules as well.

Mouse CD24 (HSA) may function (in addition to PSGL-1 (CD162)) as a ligand of P-selectin, an important adhesion molecule of thrombocytes and activated endothelia.[52,53] This interaction may play a role in extravasation of murine myeloid cells and perhaps also in the binding of human metastatic tumor cells to thrombi.[54] It may be speculated that interactions between B-cell surface CD24 and an unknown lectin play a regulatory role in B-cell development. In this respect, it is relevant that crosslinking of HSA by antibodies induces apoptosis in B-cell precursors and suppresses CD40-mediated proliferation of mature B lymphocytes,[55] indicating that HSA is possibly a negative regulator of B-cell development and activation.[56] Some of these effects may be indirect and due to modifications of expression or affinity of other adhesion molecules such as the β_1 integrin VLA-4 induced upon CD24 ligation.[57] HSA may also play a negative regulatory role in thymocyte development.[58] HSA expressed on the surface of antigen-presenting cells appears to act as a ligand of an unidentified receptor which delivers important costimulatory signals to T cells.[59-62] It was suggested that this costimulatory activity is due to a homotypic interaction between HSA molecules on the APC and T cells[60] (though mature T cells express very little HSA).

Finally, a single report indicates that HSA may contribute to protection of cells from lysis by homologous complement.[63]

CD48

The polypeptide chain of CD48 consists of two N-glycosylated Ig-like domains. Among other members of the Ig superfamily, the degree of amino acid sequence similarity is highest for CD2, CD58, Ly-9, 2B4 CD150 (SLAM).[64] Human CD48 is widely expressed on leucocytes, except granulocytes and platelets.[65] The expression increases upon activation; actually, one of the first described B-cell activation antigens, Blast-1, is identical to CD48.[66-68] The rat CD48 homologue (OX-45) is detectable also on endothelia, connective tissue and erythrocytes.[69] The mouse homologue is also called BCM1 or sgp60.[70,71] Human CD48 is a low affinity ligand for the adhesion/signaling T-cell receptor CD2.[72] However, the affinity of the CD2-CD48 interaction is more than two orders of magnitude lower than that of the CD2-CD58 interaction.[64] It may be speculated that an alternative CD48 receptor (other than CD2) exists and binds it with a higher affinity. On the other hand, CD48 is the major, medium-affinity CD2 ligand in rat[73] as well as mouse.[74]

CD52

This molecule, called also Campath-1, is generally similar to CD24: It has also a short (only 12 amino acid residues) polypeptide chain[75] carrying a large sialylated, polylactosamine-containing, core-fucosylated, tetra-antennary oligosaccharide.[76] The Mr as determined by SDS PAGE is largely overestimated due to anomalous SDS binding; true Mr is approximately 8 kDa.[76] Two forms of CD52 were described which differ in the structure of the GPI anchor.[76] CD52 is strongly expressed on most leucocytes except plasma cells and platelets and characteristically is strongly positive on eosinophils as compared to neutrophils.[77] The mouse homologue of CD52 was named, somewhat unfortunately, B7(2) (not to be confused with similar "pre-CD" names of CD80 and CD86!).[78] Nothing is known about possible ligands or receptors of CD52, nor its biological function. Some mAbs to CD52 are very efficient in complement-mediated lysis of lymphocytes and therefore are used as therapeutic lymphocyte-depleting immunosuppressants in treatments of transplantation rejections, autoimmune diseases and in autologous bone marrow transplantation in malignant lymphoma patients.[79,80]

CD55

This protein (decay accelerating factor, DAF) is described in the chapter on complement protecting molecules (see chapter 7). Here we will just mention that CD55 interacts with CD97;[81] the physiological role of this adhesion interaction is so far unclear. CD55 may serve as a receptor for several Echoviruses[82,83] and coxsackie B virus.[84]

CD58 (LFA-3)

The structure of CD58 is very similar to that of CD48, i.e., the polypeptide chain consists of two *N*-glycosylated Ig-like domains.[85,86] However, in addition to the GPI-anchored form, an alternative transmembrane form exists which is a product of alternative splicing.[87] CD58 is broadly expressed on various cell types; memory T cells and dendritic cells have particularly high levels of expression.[88,89] CD58 is the major ligand of the adhesive/signaling receptor of T and NK cells, CD2.[90] Ligation of CD2 by CD58 provides regulatory signals to T and NK cells.[91] The homologue of CD58, expressed at high density on sheep erythrocytes, is responsible for the well known "rosetting" of human T cells with sheep erythrocytes.[92] A soluble form of CD58 is found in human body fluids.[93]

CD59

This protein is described in chapter 7. Here we will just mention that CD59 may be an alternative low-affinity ligand of the T-cell adhesion/signaling receptor CD2.[94,95] However, this issue is controversial.[96]

CD66

On human leucocytes, two CD66 glycoproteins are expressed (CD66b and CD66c), which are anchored to the cell surface via a GPI moiety. They belong to the carcinoembryonic antigen (CEA) family, which forms a subgroup within the Ig superfamily. CD66b, (previously called CD67) called also CGM6, W272 or NCA-95, contains 3 Ig-like, heavily glycosylated domains. It is present at low levels on resting mature granulocytes and its expression is rapidly increased following activation with inflammatory agonists.[97-99] CD66c, called also NCA, NCA-50/90 or TEX contains also 3 heavily glycosylated Ig domains. It is expressed mainly in granulocytes and epithelial cells. It seems that CD66c has a signaling role and regulates the adhesion activity of CD11/CD18 in neutrophils.[97-99] Members of the CD66 subgroup have also been found in mice and rats, but none of them is anchored to the cell plasma membrane via a GPI moiety. This finding led to the suggestion that GPI-anchored members of the CD66 family represent part of a gene family in transitional evolution.[100]

All GPI-anchored members of the CD66 family are able to mediate intercellular adhesion under in vitro conditions. CD66b exhibits heterotypic adhesion with CD66c and CD66e, while CD66c and CD66e exhibit both homotypic and heterotypic adhesion.[101-103] The binding sites for these interactions lie within their N-terminal domains. CD66c and CD66e glycoproteins can also bind *E. coli* and *Salmonella* strains, suggesting that these proteins can be involved in recognition of bacteria.[104] Interestingly, CD66 glycoproteins have been found to serve as receptors for gonococcal opacity proteins, and it has been suggested that CD66e and some other members of the CD66 family are involved at early stages of colonization of *Neisseria gonorrhoea* in urogenital tissues.[105-107] It should be also noted that the presence of CD15 (Le^x) on CD66 glycoproteins may modulate adhesive functions, and that CD66c may be able to present CD15s (sialylated Le^x) to E-selectin.[108,109]

CD73

This protein displays 5'-nucleotidase activity and catalyzes the 5'-dephosphorylation of extracellular ribo- and deoxyribonucleoside monophosphates to nucleosides (mainly AMP to adenosine) which can be then transported inside the cell.[110] It is expressed on approximately 75% of peripheral blood B cells, 10% of $CD4^+$ and 50% of $CD8^+$ peripheral T cells.[111] CD73 expression increases during the development of both B and T cells.[110,112] In addition, it is present also on epithelial, endothelial and dendritic cells.[111] In addition to the ectoenzyme activity, CD73 appears to be an adhesion molecule playing a role in lymphocyte-endothelial cell interaction: the endothelial cell surface molecule originally called VAP-2 (vascular adhesion protein 2) was found to be identical to CD73.[113,114] The receptor or ligand for endothelial CD73 on the lymphocyte surface has not been identified as yet. On the other hand, lymphocyte surface CD73 seems to be important in lymphocyte binding to vascular endothelium in inflamed human skin.[115] The interaction between CD73 on the surface of follicular dendritic cells and an unknown ligand on the B cell surface may control the contact between these two cell types.[116] In this context, it may be relevant that chicken gizzard 5'-nucleotidase was reported to be a receptor for the extracellular matrix component fibronectin.[117] Several studies describe induction of T-cell activation by antibody-mediated crosslinking of CD73.[118-120] It is not clear whether this is just an in vitro artefact or a valid model of activation due to CD73 ligation by the putative natural ligand.

CD87

CD87, alias urokinase plasminogen activator receptor (uPA-R), is a polypeptide of 31 kDa which matures into a heavily N-glycosylated, cell surface glycoprotein of 50-60 kDa. The extracellular region consists of three Ly-6 domains which are separated by hinge-like sequences.[121-124] Ly-6 domains are stretches of 70-80 amino acids containing 10 conserved cysteine residues which were first recognized in Ly-6 antigens (see below and refs. 125, 126).

CD87 is a multifunctional component expressed on monocytes, granulocytes, activated T cells and endothelial cells that are capable of migration through the extracellular matrix. It is also expressed on tumor cells, where high level of expression correlates with high risk of recurrence and metastasis.[127]

On the one hand, CD87 localizes and controls the fibrinolytic system at the cell surface. Upon binding to the first (N-terminal) Ly-6 domain of CD87, its ligand, pro-uPA, is proteolytically converted into uPA, which can enzymatically cleave plasminogen into the serine protease plasmin. Plasmin can directly degrade matrix proteins and activates a variety of biologically potent substances.[128,129] Internalization of CD87 involving CD91 (α_2-macroglobulin receptor), ligand clearing and reexpression are crucial devices controlling uPA activity, and therefore the fibrinolytic system.[130,131]

Furthermore, CD87 is directly (via interaction with vitronectin) and indirectly (via interaction with integrins) involved in regulation of cell adhesion during migration. Within domains 2 and 3 of CD87, there is a binding site for the matrix protein vitronectin,[132-134] and this interaction is suggested to mediate cell adhesion to extracellular matrix and other cells coated by vitronectin. Recently, it has been shown that this binding site is also involved in binding of high molecular weight kininogen, but the significance of this binding remains doubtful. However, one could envisage a function in the regulation of cell adhesion involving vitronectin, but also in the formation of uPA, since kininogen can bind prekallikrein which may, upon activation, proteolytically activate pro-uPA.[135]

Several lines of evidence link CD87 and integrins to adhesion regulation and signal transduction. Presence of CD87 at focal adhesion sites and the leading edges of migrating cells where integrins accumulate was found earlier.[136,137] Recently, CD87 was coprecipitated with the β2 integrins CD11a/CD18 (LFA-1) and CD11b/CD18 (CR3, Mac-1) together with Src protein tyrosine kinases from lysates of human monocytes. This complex represents a relatively large, detergent-resistant plasma membrane domain and contains several other so far unidentified molecules.[138] Incubation of monocytes with antibodies to CR3 impaired CD87-dependent adhesion to vitronectin, and engagement of CD87 by uPA inhibited CR3-dependent fibrinogen degradation, while this function (probably important in wound healing) was promoted when CD87 was ligated with vitronectin.[139] Downregulation of CD87 in monocytes by antisense oligonucleotides reduced the adhesiveness of CR3.[140] Association of CD87 and β2 integrins has also been found in various myeloid cell lines including U937 and THP-1 (Godar S and Stockinger H, unpublished data), and in resting neutrophils.[141] Interestingly, neutrophils from leucocyte-adhesion-deficient patients (lacking β2 integrins) showed impaired Ca^{2+} signaling. Moreover, COS transfectants mobilized Ca^{2+} only upon uPA incubation when both CD87 and CR3 were expressed,[142] suggesting that CR3 may act as a transmembrane adaptor for CD87. During neutrophil polarization, CD87 and CR3 dissociate;[143] CR3 moves to the rear, whereas CD87 concentrates at lamellipodia of migrating cells, where it associates in an oscillatory manner with the β2 integrin CD11c/CD18, alias CR4.[144] Together, these studies indicate a physical and functional link of CD87 and β2 integrins on the surface of myeloid cells.

In nonhematopoietic cells which do not express β2 integrins, cooperation of CD87 with β1 and β3 integrins has been observed. In a fibrosarcoma cell line, CD87 associated with members of the β1 and β3 integrin family when the cells were allowed to adhere to

substrates of the respective integrins.[145] Expression of CD87 in a kidney embryonic cell line resulted in complex formation with β1 integrin and inhibition of the adhesive function of the integrin,[146] suggesting that CD87 can act as a disintegrin. On the other hand, integrin-dependent adhesion was reported to be facilitated by CD87,[140,147] suggesting the CD87 may act as an integrin amplifier. In spite of this controversy, CD87 seems to regulate the function of different integrins in various cells.

Besides the link to integrins and adhesion regulation, CD87 is also molecularly connected to cytokine systems. CD87 was found to complex with CD130 (the signal-transducing subunit of receptors for IL-6, IL-11 and several other cytokines) in a tumor cell line and to activate the CD130-associated signaling components JAK1 and STAT1.[148] Whether the CD87/CD130 association is involved in modulating cytokine signals or in the known mitogenic capacity of uPA for various tumor cells[149] remains to be determined. Furthermore, CD87 was found to directly interact with the mannose-6-phosphate/insulin-like growth factor type II receptor, which can simultaneously bind the latent form of transforming growth factor-β1 (TGF-β1); there is evidence that this complex formation is important for conversion of latent TGF-β1 into its active form.[150] Thus, CD87 is not only involved in the regulation of the fibrinolytic system and integrins, but also appears to regulate signaling through cytokine receptors.

CD90

This glycoprotein, also called Thy-1, is similar in structure to a single, highly *N*-glycosylated variable region domain of the immunoglobulin superfamily of cell surface and secreted molecules.[151] In the mouse, Thy-1 occurs in two allelic forms, Thy-1.1 and Thy-1.2, which differ by a single amino acid substitution at residue 89 (arginine in Thy-1.1 and glutamine in Thy-1.2).

CD90 is expressed by hematopoietic stem cells and neurons and in connective tissues in all species studied. In mice it is also expressed in thymocytes and peripheral T cells, whereas in rats it is absent from peripheral T cells. In man, its expression is even more restricted to a small fraction of fetal thymocytes. Interestingly, large amounts of CD90 are found on the surface of rat but not mouse or human mast-cells.[152] Immunohistochemical analysis revealed that CD90 is also found in some other human tissues such as kidney tubules, blood vessel endothelium and skin fibroblasts.[153] Importantly, the composition of CD90 carbohydrates varies between tissues of the same animal and within the same tissue at different stages of differentiation.[151]

The function of CD90 remains vexingly unknown. It has been proposed that Thy-1 is functionally related to the putative Ig superfamily primordial domain and involved directly in cell-cell interactions. CD90 expression on a neural cell line selectively inhibited neurite outgrowth of mature astrocytes in vitro.[154] Although Thy-1 is a dominant surface glycoprotein in brain and thymus cells, mice lacking the CD90 gene exhibited a regional impairment of long term potentiation in the hippocampal dentate gyrus.[155] Thy-$1^{-/-}$ cells also showed altered cell-cell contacts and hyperresponsiveness to TCR triggering, suggesting that CD90 negatively regulates TCR signaling.[156] This might be related to previous data indicating that a substantial fraction of Thy-1 and also TCR are physically associated with CD45, a transmembrane tyrosine phosphatase.[157] Recently it has been shown that rodent CD90 is a high affinity receptor for the channel-forming toxin aerolysin, secreted by virulent *Aeromonas* sp.[158] This could explain the increased sensitivity of rodent T cells to this toxin. Antibody-mediated crosslinking of Thy-1 on T cells is strongly activating, an effect which is probably related to inclusion of Thy-1 (as well as other GPI-anchored proteins) in the protein-tyrosine kinase-rich membrane microdomains (see chapter 4). Similarly, aggregation of surface Thy-1 in rat mast-cells or rat basophilic leukemia cells

induces cell activation and release of mediators independently of the high-affinity IgE receptor.[152,159] On the other hand, it has been shown that immobilized antibodies directed at a defined Thy-1 epitope region can promote apoptosis in cultured CD4$^+$CD8$^+$ double-positive thymocytes. This cell death pathway is developmentally regulated and occurs through a *Bcl-2*-resistant mechanism.[160] It is not clear whether these signaling effects represent in vitro antibody artefacts or whether they reflect a physiological response to Thy-1 ligation with its hypothetical ligand.

CDw108

The primary structure of this protein is so far unknown. Apparent Mr of the glycoprotein is approximately 80 kDa, of which about 28% is *N*-linked oligosaccharide.[161] It is expressed on erythrocytes, activated lymphocytes and some T-cell lines such as HPB-ALL.[162] The CDw108 glycoprotein carries the determinants of the erythrocyte JMH antigen,[163] which is identical to the H105 antigen.[161,164] Nothing is known about CDw108 function.

CD109

CD109 is a monomeric glycoprotein of so far unknown primary structure, bearing two *N*-linked glycan chains[165] and expressed on activated platelets and activated lymphocytes as well as on endothelial and epithelial cells. The molecule carries the Gov$^{a/b}$ thrombocyte alloantigenic determinants.[166] Nothing is known about its function.

CD157 (Mo5, BST-1)

This protein has the ADP-ribosyl cyclase and cyclic-ADP-ribose hydrolase ectoenzyme activities, i.e., it converts NAD into nicotinamide and cyclic ADP-ribose (cADPR) and further hydrolyzes cyclic ADP-ribose into ADP-ribose.[167,168] Intracellular cADPR acts as a second messenger regulating cytoplasmic Ca^{2+} concentrations. The role of extracellular cADPR, and whether the enzyme is transported inside the cells, are not known. CD157 shares limited sequence homology (33%) with a transmembrane leucocyte surface protein, CD38,[167] which also has similar enzymatic activities. CD157 is expressed on myeloid cells, follicular dendritic cells and endothelia; expression in the lymphoid lineage appears to correlate with developmental stages of early progenitors.[169,170] It is not yet established whether antibody-induced costimulatory signaling via CD157[171,172] is an in vitro artefact or reflects a receptor role of CD157 for an unidentified ligand. High levels of soluble CD157 are detectable in sera of patients with severe rheumatoid arthritis.[173]

RT6

This bifunctional T-cell ectoenzyme acts as an NAD-dependent arginine ADP-ribosyltransferase that covalently modifies arginine residues with ADP-ribose.[174] In addition, it is also a potent NAD glycohydrolase (hydrolyzes NAD into nicotinamide and ADP-ribose).[175] The protein substrates of the ADP-ribosylation are not well known, but one of them is RT6 itself[176] and another may be an unidentified 40 kDa protein regulating the kinase activity of Lck.[177] the RT6 molecule was studied mainly in rat and mouse; the human gene is transcriptionally inactive.[178] A similar protein is present in the membrane of human skeletal muscle cells.[179] A GPI-anchored cell surface ADP-ribosyltransferase appears to negatively regulate the function of the adhesion receptor LFA-1 in human T cells;[180] this ectoenzyme is specifically released from cytotoxic T cells upon activation.[181] It may be a product of the recently cloned RT6-related gene.[182] RT6 is a specific marker of rat peripheral T lymphocytes, expressed only after emigration from the thymus.[183] Particularly high levels of RT6 expression were reported for intestinal epithelial T cells.[184] Low expression of RT6

may be related to enhanced T-cell autoreactivity,[185,186] suggesting an immunosuppressive role for this ectoenzyme.

Melanotransferrin

This protein is predominantly expressed on nonhematopoietic cells (melanomas, fetal tissues, liver, epithelia, placenta)[187] but it is also present on some leukemic cell lines such as HPB-ALL. The polypeptide chain is composed of two homologous domains bearing sequence similarity to transferrin.[188,189] Melanotransferrin binds iron ions and plays a role in an alternative route of iron transport into some cell types.

Ly-6

Several members of the Ly-6 family have been characterized in the mouse: Ly-6A/E (Ly-6E, originally considered as a separate gene product, was found to be an allelic form of Ly-6A), Ly-6C, Ly-6F, Ly-6G, ThB and TSA-1 (Sca-2) (for review see refs. 190, 191). All of them are small (mature polypeptide of about 80 amino acids), mostly nonglycosylated GPI-anchored proteins of very different tissue distribution (see Table 5.1) encoded by genes residing in a gene cluster on mouse chromosome 15.[192] The structure of the Ly-6 domains is covalently stabilized by 5 cysteine bridges. The expression of those Ly-6 proteins present on lymphoid cells is markedly regulated by interferons.[193] Recently, three likely human Ly-6 homologues have been described; one of them, E48, appears to be a ThB homologue,[194] another is a TSA-1/Sca-2 homologue[195] and the last one (RIG-E) may be a Ly-6C homologue.[196] The Ly-6 proteins are prototypic members of a structural family which includes also CD59, CD87, squid brain protein Sgp-2, snake venom toxins such as α-bungarotoxin and snake plasma inhibitor or phospholipase-A2 (for a review see ref. 190).

The functions of the Ly-6 proteins are not clearly defined, although tight regulation of their expression suggests functional importance. So far, no Ly-6 knockout mice have been produced. Transgenic mice were prepared with Ly-6A expression under the control of a CD2 promoter[197] and thus not turned off at the early stage of thymocyte development, as is the case for the endogenous Ly-6. This resulted in a block of thymocyte development at the very early stage ($CD4^-CD8^-$). The transgenic thymocytes spontaneously aggregated (and formed also aggregates with normal thymocytes); the aggregation could be blocked by antibodies to Ly-6A.[198] This indicates an adhesion interaction of Ly6A with an unidentified cell surface ligand potentially important in thymocyte development. Ly-6C may play an adhesive role during the interaction between cytotoxic T cells and their targets.[199] Also, E48, the putative human homologue of ThB, appears to be an adhesion molecule (or an adhesion regulator) in keratinocytes.[194]

Crosslinking of Ly-6A/E or Ly-6C by suitable antibodies produces marked cellular responses in T cells similar to those observed upon crosslinking of Thy-1 (CD90) or other GPI-anchored proteins. These effects are probably due to inclusion of the Ly-6 molecules in the membrane microdomains rich in signaling molecules (see chapter 4); antibody crosslinking may perturb these membrane complexes in such a way that signaling cascades are initiated. It is not clear to what extent these antibody-induced effects mimic the effect of Ly-6 ligation with their presumed natural ligands. At least some signaling properties of the Ly-6A/E molecules are not exclusively due to their GPI-anchorage: crosslinking of a transmembrane version of Ly-6A/E was able to inhibit T-cell stimulation triggered by CD3 crosslinking.[200] Surprisingly, antisense-mediated downregulation of Ly-6A/E expression in a T-cell line led to impairment of the Fyn protein tyrosine kinase activity, downregulation of TCR β chain synthesis and loss of surface TCR expression and function.[201,202] This would therefore suggest an important role for Ly-6A in regulation of TCR expression and signaling.

Other Leucocyte GPI-Anchored Proteins

A broadly expressed murine nonclassical (class Ib) MHC glycoprotein, Qa-2, is anchored via a GPI moiety; it binds antigenic peptides and appears to serve as one of the antigen-presenting MHC molecules (for review see refs. 203, 204). At least one isoenzyme of alkaline phosphatase (different from the well known placental isoenzyme) is expressed as a GPI-anchored ectoenzyme on the surface of various cell types including granulocytes[205] and activated murine and rat B cells;[206] it is identical to the mouse Ly-31 alloantigen,[207] but the biological function of this ectoenzyme is still unknown.

References

1. Ferrero E, Hsieh CL, Francke U et al. CD14 is a member of the family of leucine-rich proteins and is encoded by a gene syntenic with multiple receptor genes. J Immunol 1990; 145:331-6.
2. Ziegler-Heitbrock HW, Pechumer H, Petersmann I et al. CD14 is expressed and functional in human B cells. Eur J Immunol 1994; 24:1937-40.
3. Detmers PA, Zhou D, Powell D et al. Endotoxin receptors (CD14) are found with CD16 (Fc gamma RIII) in an intracellular compartment of neutrophils that contains alkaline phosphatase. J Immunol 1995; 155:2085-95.
4. Bazil V, Baudys M, Hilgert I et al. Structural relationship between the soluble and membrane-bound forms of human monocyte surface glycoprotein CD14. Mol Immunol 1989; 26:657-62.
5. Haziot A, Chen S, Ferrero E et al. The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. J Immunol 1988; 141:547-52.
6. Bazil V, Strominger JL. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J Immunol 1991; 147:1567-74.
7. Bufler P, Stiegler G, Schuchmann M et al. Soluble lipopolysaccharide receptor (CD14) is released via two different mechanisms from human monocytes and CD14 transfectants. Eur J Immunol 1995; 25:604-10.
8. Wright SD, Ramos RA, Tobias PS et al. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein [see comments]. Science 1990; 249(4975):1431-3.
9. Hailman E, Lichenstein HS, Wurfel MM et al. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J Exp Med 1994; 179:269-77.
10. Yu B, Wright SD. Catalytic properties of lipopolysaccharide (LPS) binding protein. Transfer of LPS to soluble CD14. J Biol Chem 1996; 271:4100-5.
11. Lee JD, Kravchenko V, Kirkland TN et al. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. Proc Natl Acad Sci USA 1993; 90(21):9930-4.
12. Vita N, Lefort S, Sozzani P et al. Detection and biochemical characteristics of the receptor for complexes of soluble CD14 and bacterial lipopolysaccharide. J Immunol 1997; 158:3457-62.
13. Zarewych DM, Kindzelskii AL, Todd RF III et al. LPS induces CD14 association with complement receptor type 3, which is reversed by neutrophil adhesion. J Immunol 1996; 156:430-3.
14. Stefanova I, Corcoran ML, Horak EM et al. Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56lyn. J Biol Chem 1993; 268(28):20725-8.
15. Weinstein SL, June CH, DeFranco AL. Lipopolysaccharide-induced protein tyrosine phosphorylation in human macrophages is mediated by CD14. J Immunol 1993; 151:3829-38.
16. Delude RL, Fenton MJ, Savedra R Jr et al. CD14-mediated translocation of nuclear factor-kappa B induced by lipopolysaccharide does not require tyrosine kinase activity. J Biol Chem 1994; 269(35):22253-60.
17. Meng F, Lowell CA. Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. J Exp Med 1997; 185:1661-70.

18. Haziot A, Rong GW, Silver J et al. Recombinant soluble CD14 mediates the activation of endothelial cells by lipopolysaccharide. J Immunol 1993; 151:1500-7.
19. Pugin J, Schurer-Maly CC, Leturcq D et al. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc Natl Acad Sci USA 1993; 90:2744-8.
20. Hailman E, Vasselon T, Kelley M et al. Stimulation of macrophages and neutrophils by complexes of lipopolysaccharide and soluble CD14. J Immunol 1996; 156(11):4384-90.
21. Verhasselt V, Buelens C, Willems F et al. Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: Evidence for a soluble CD14-dependent pathway. J Immunol 1997; 158:2919-25.
22. Wurfel MM, Hailman E, Wright SD. Soluble CD14 acts as a shuttle in the neutralization of lipopolysaccharide (LPS) by LPS-binding protein and reconstituted high density lipoprotein. J Exp Med 1995; 181:1743-54.
23. Gegner JA, Ulevitch RJ, Tobias PS. Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14. J Biol Chem 1995; 270(10):5320-5.
24. Grunwald U, Fan X, Jack RS et al. Monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism. J Immunol 1996; 157:4119-25.
25. Pugin J, Heumann ID, Tomasz A et al. CD14 is a pattern recognition receptor. Immunity 1994; 1:509-16.
26. Soell M, Lett E, Holveck F et al. Activation of human monocytes by streptococcal rhamnose glucose polymers is mediated by CD14 antigen, and mannan binding protein inhibits TNF-alpha release. J Immunol 1995; 154:851-60.
27. Kusunoki T, Hailman E, Juan TS et al. Molecules from *Staphylococcus aureus* that bind CD14 and stimulate innate immune responses. J Exp Med 1995; 182:1673-82.
28. Gupta D, Kirkland TN, Viriyakosol S et al. CD14 is a cell-activating receptor for bacterial peptidoglycan. J Biol Chem 1996; 271(38):23310-6.
29. Espevik T, Otterlei M, Skjak-Braek G et al. The involvement of CD14 in stimulation of cytokine production by uronic acid polymers. Eur J Immunol 1993; 23:255-61.
30. Yu B, Hailman E, Wright SD. Lipopolysaccharide binding protein and soluble CD14 catalyze exchange of phospholipids. J Clin Invest 1997; 99:315-24.
31. Ravetch JV, Perussia B. Alternative membrane forms of Fc gamma RIII(CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. J Exp Med 1989; 170:481-97.
32. Ory PA, Clark MR, Kwoh EE et al. Sequences of complementary DNAs that encode the NA1 and NA2 forms of Fc receptor III on human neutrophils. J Clin Invest 1989; 84:1688-91.
33. Ravetch JV, Kinet JP. Fc receptors. Annu Rev Immunol 1991; 9:457-92.
34. Kimberly RP, Ahlstrom JW, Click ME et al. The glycosyl phosphatidylinositol-linked Fc gamma RIIIPMN mediates transmembrane signaling events distinct from Fc gamma RII. J Exp Med 1990; 171:1239-55.
35. Hundt M, Schmidt RE. The glycosylphosphatidylinositol-linked Fc gamma receptor III represents the dominant receptor structure for immune complex activation of neutrophils. Eur J Immunol 1992; 22:811-6.
36. Rosales C, Brown EJ. Signal transduction by neutrophil immunoglobulin G Fc receptors. Dissociation of intracytoplasmic calcium concentration rise from inositol 1,4,5-trisphosphate. J Biol Chem 1992; 267:5265-71.
37. Naziruddin B, Duffy BF, Tucker J et al. Evidence for cross-regulation of Fc gamma RIIIB (CD16) receptor-mediated signaling by Fc gamma RII (CD32) expressed on polymorphonuclear neutrophils. J Immunol 1992; 149(11):3702-9.
38. Zhou MJ, Brown EJ. CR3 (Mac-1, alpha M beta 2, CD11b/CD18) and Fc gamma RIII cooperate in generation of a neutrophil respiratory burst:requirement for Fc gamma RIII and tyrosine phosphorylation. J Cell Biol 1994; 125:1407-16.

39. Edberg JC, Kimberly RP. Modulation of Fc gamma and complement receptor function by the glycosyl-phosphatidylinositol-anchored form of Fc gamma RIII. J Immunol 1994; 152:5826-5835.
40. Galon J, Gauchat JF, Mazieres N et al. Soluble Fc gamma receptor type III (FcgammaRIII, CD16) triggers cell activation through interaction with complement receptors. J Immunol 1996; 157:1184-92.
41. Selvaraj P, Rosse WF, Silber R et al. The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria. Nature 1988; 333(6173):565-7.
42. Stockl J, Majdic O, Pickl WF et al. Granulocyte activation via a binding site near the C-terminal region of complement receptor type 3 alpha-chain (CD11b) potentially involved in intramembrane complex formation with glycosylphosphatidylinositol-anchored Fc gamma RIIIB (CD16) molecules. J Immunol 1995; 154(10):5452-63.
43. Poo H, Krauss JC, Mayo-Bond L et al. Interaction of Fc gamma receptor type IIIB with complement receptor type 3 in fibroblast transfectants: evidence from lateral diffusion and resonance energy transfer studies. J Mol Biol 1995; 247:597-603.
44. Lanier LL, Phillips JH, Testi R. Membrane anchoring and spontaneous release of CD16 (FcR III) by natural killer cells and granulocytes. Eur J Immunol 1989; 19:775-8.
45. Dransfield I, Buckle AM, Savill JS et al. *Neutrophil apoptosis* is associated with a reduction in CD16 (Fc gamma RIII) expression. J Immunol 1994; 153:1254-63.
46. Kew RR, Grimaldi CM, Furie MB et al. Human neutrophil Fc gamma RIIIB and formyl peptide receptors are functionally linked during formyl-methionyl-leucyl-phenylalanine-induced chemotaxis. J Immunol 1992; 149:989-97.
47. Kay R, Rosten PM, Humphries RK. CD24, a signal transducer modulating B cell activation responses, is a very short peptide with a glycosyl phosphatidylinositol membrane anchor. J Immunol 1991; 147:1412-6.
48. Le Bien T. CD24 Workshop panel report. In: Schlossman SF, Boumsell L, Harlan J et al., eds. Leukocyte Typing V. White Cell Differentiation Antigens. Oxford:Oxford University Press 1995:1472-1473.
49. Jackson D, Waibel R, Weber E et al. CD24, a signal-transducing molecule expressed on human B cells, is a major surface antigen on small cell lung carcinomas. Cancer Res 1992; 52(19):5264-70.
50. Crispe IN, Bevan MJ. Expression and functional significance of the J11d marker on mouse thymocytes. J Immunol 1987; 138:2013-8.
51. Enk AH, Katz SI. Heat-stable antigen is an important costimulatory molecule on epidermal Langerhans' cells. J Immunol 1994; 152:3264-70.
52. Sammar M, Aigner S, Hubbe M et al. Heat-stable antigen (CD24) as ligand for mouse P-selectin. Int Immunol 1994; 6:1027-36.
53. Aigner S, Ruppert M, Hubbe M et al. Heat stable antigen (mouse CD24) supports myeloid cell binding to endothelial and platelet P-selectin. Int Immunol 1995; 7(10):1557-65.
54. Aigner S, Sthoeger ZM, Fogel M et al. CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. Blood 1997; 89:3385-95.
55. Chappel MS, Hough MR, Mittel A et al. Crosslinking the murine heat-stable antigen induces apoptosis in B cell precursors and suppresses the anti-CD40-induced proliferation of mature resting B lymphocytes. J Exp Med 1996; 184:1638-49.
56. Hough MR, Chappel MS, Sauvageau G et al. Reduction of early B lymphocyte precursors in transgenic mice overexpressing the murine heat-stable antigen. J Immunol 1996; 156:479-88.
57. Hahne M, Wenger RH, Vestweber D et al. The heat-stable antigen can alter very late antigen 4-mediated adhesion. J Exp Med 1994; 179:1391-5.
58. Hough MR, Takei F, Humphries RK et al. Defective development of thymocytes overexpressing the costimulatory molecule, heat-stable antigen. J Exp Med 1994; 179:177-84.
59. Liu Y, Jones B, Brady W et al. Costimulation of murine CD4 T-cell growth: cooperation between B7 and heat-stable antigen [published erratum appears in Eur J Immunol 1993; 23:780]. Eur J Immunol 1992; 22(11):2855-9.

60. Hubbe M, Altevogt P. Heat-stable antigen/CD24 on mouse T lymphocytes: Evidence for a costimulatory function. Eur J Immunol 1994; 24:731-7.
61. Wang YC, Zhu L, McHugh R et al. Expression of heat-stable antigen on tumor cells provides costimulation for tumor-specific T-cell proliferation and cytotoxicity in mice. Eur J Immunol 1995; 25:1163-7.
62. Liu Y, Wenger RH, Zhao M et al. Distinct costimulatory molecules are required for the induction of effector and memory cytotoxic T lymphocytes. J Exp Med 1997; 185:251-62.
63. Hitsumoto Y, Ohnishi H, Nakano A et al. Inhibition of homologous complement activation by the heat-stable antigen. Int Immunol 1993; 5:805-8.
64. Davis SJ, van der Merwe PA. The structure and ligand interactions of CD2: implications for T-cell function. Immunol Today 1996; 17:177-189.
65. Klickstein LB, Springer TA. CD48 cluster report. In: Schlossman SF, Boumsell L, Harlan J et al, eds. Leukocyte Typing V. White Cell Differentiation Antigens. Oxford:Oxford University Press 1995:1472-1473.
66. Staunton DE, Fisher RC, LeBeau MM et al. Blast-1 possesses a glycosyl-phosphatidylinositol (GPI) membrane anchor, is related to LFA-3 and OX-45, and maps to chromosome 1q21-23. J Exp Med 1989; 169:1087-99.
67. Korinek V, Stefanova I, Angelisova P et al. The human leucocyte antigen CD48 (MEM-102) is closely related to the activation marker Blast-1. Immunogenetics 1991; 33:108-12.
68. Vaughan HA, Henning MM, Purcell DF et al. The isolation of cDNA clones for CD48. Immunogenetics 1991; 33:113-7.
69. Avrieux J, Jefferies WA, Paterson DJ et al. Monoclonal antibodies against a rat leucocyte antigen block antigen- induced T-cell responses via an effect on accessory cells. Immunology 1986; 58:337-342.
70. Cabrero JG, Freeman GJ, Lane WS et al. Identification, by protein sequencing and gene transfection, of sgp-60 as the murine homologue of CD48. Proc Natl Acad Sci USA 1993; 90:3418-22.
71. Wong YW, Williams AF, Kingsmore SF et al. Structure, expression, and genetic linkage of the mouse BCM1 (OX45 or Blast-1) antigen. Evidence for genetic duplication giving rise to the BCM1 region on mouse chromosome 1 and the CD2/LFA3 region on mouse chromosome 3. J Exp Med 1990; 171:2115-30.
72. Sandrin MS, Mouhtouris E, Vaughan HA et al. CD48 is a low affinity ligand for human CD2. J Immunol 1993; 151:4606-13.
73. van der Merwe PA, Brown MH, Davis SJ et al. Affinity and kinetic analysis of the interaction of the cell adhesion molecules rat CD2 and CD48. EMBO J 1993; 12(13):4945-54.
74. Kato K, Koyanagi M, Okada H et al. CD48 is a counter-receptor for mouse CD2 and is involved in T-cell activation. J Exp Med 1992; 176:1241-9.
75. Xia MQ, Tone M, Packman L et al. Characterization of the CAMPATH-1 (CDw52) antigen: Biochemical analysis and cDNA cloning reveal an unusually small peptide backbone. Eur J Immunol 1991; 21:1677-84.
76. Treumann A, Lifely MR, Schneider P et al. Primary structure of CD52. J Biol Chem 1995; 270(11):6088-99.
77. Valentin H, Gelin C, Coulombel L et al. The distribution of the CDW52 molecule on blood cells and characterization of its involvement in T-cell activation. Transplantation 1992; 54:97-104.
78. Kubota H, Okazaki H, Onuma M et al. Identification and gene cloning of a new phosphatidylinositol-linked antigen expressed on mature lymphocytes. Downregulation by lymphocyte activation. J Immunol 1990; 145(11):3924-31.
79. Hale G, Waldmann H. CAMPATH-1 monoclonal antibodies in bone marrow transplantation. J Hematother 1994; 3:15-31.
80. Hale G, Phillips JM. Clinical trials with CAMPATH-I and other monoclonal antibodies. Biochem Soc Trans 1995; 23:1057-63.
81. Hamann J, Vogel B, van Schijndel GMW et al. The seven-span transmembrane receptor CD97 has a cellular ligand (CD55, DAF). J Exp Med 1996; 184:1185-1189.

82. Bergelson JM, Chan M, Solomon KR et al. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc Natl Acad Sci USA 1994; 91(13):6245-9.
83. Ward T, Pipkin PA, Clarkson NA et al. Decay-accelerating factor CD55 is identified as the receptor for echovirus 7 using CELICS, a rapid immuno-focal cloning method. Embo J 1994; 13(21):5070-4.
84. Bergelson JM, Mohanty JG, Crowell RL et al. Coxsackievirus B3 adapted to growth in RD cells binds to decay-accelerating factor (CD55). J Virol 1995; 69:1903-6.
85. Seed B. An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. Nature 1987; 329(6142):840-2.
86. Wallner BP, Frey AZ, Tizard R et al. Primary structure of lymphocyte function-associated antigen 3 (LFA-3). The ligand of the T lymphocyte CD2 glycoprotein. J Exp Med 1987; 166:923-32.
87. Dustin ML, Selvaraj P, Mattaliano RJ et al. Anchoring mechanisms for LFA-3 cell adhesion glycoprotein at membrane surface. Nature 1987; 329(6142):846-8.
88. Sanders ME, Makgoba MW, Sharrow SO et al. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. J Immunol 1988; 140:1401-7.
89. Freudenthal PS, Steinman RM. The distinct surface of human blood dendritic cells, as observed after an improved isolation method. Proc Natl Acad Sci USA 1990; 87(19):7698-702.
90. Plunkett ML, Sanders ME, Selvaraj P et al. Rosetting of activated human T lymphocytes with autologous erythrocytes. Definition of the receptor and ligand molecules as CD2 and lymphocyte function-associated antigen 3 (LFA-3). J Exp Med 1987; 165:664-76.
91. Holter W, Schwarz M, Cerwenka A et al. The role of CD2 as a regulator of human T-cell cytokine production. Immunol Rev 1996; 153:107-22.
92. Selvaraj P, Dustin ML, Mitnacht R et al. Rosetting of human T lymphocytes with sheep and human erythrocytes. Comparison of human and sheep ligand binding using purified E receptor. J Immunol 1987; 139:2690-5.
93. Hoffmann JC, Dengler TJ, Knolle PA et al. A soluble form of the adhesion receptor CD58 (LFA-3) is present in human body fluids. Eur J Immunol 1993; 23(11):3003-10.
94. Deckert M, Kubar J, Zoccola D et al. CD59 molecule: a second ligand for CD2 in T-cell adhesion. Eur J Immunol 1992; 22(11):2943-7.
95. Hahn WC, Menu E, Bothwell ALM et al. Overlapping but nonidentical binding sites on CD2 for CD58 and a second ligand CD59. Science 1992; 256:1805-1807.
96. van der Merwe PA, Barclay AN, Mason DW et al. Human cell-adhesion molecule CD2 binds CD58 (LFA-3) with a very low affinity and an extremely fast dissociation rate, but does not bind CD48 or CD59. Biochemistry 1994; 33(33):10149-60.
97. Watt SM, Sala-Newby G, Hoang T et al. CD66 identifies a neutrophil-specific epitope within the hematopoietic system that is expressed by members of the carcinoembryonic antigen family of adhesion molecules. Blood 1991; 78:63-74.
98. Watt SM, Fawcett J, Murdoch SJ et al. CD66 identifies the biliary glycoprotein (BGP) adhesion molecule: Cloning, expression, and adhesion functions of the BGPc splice variant. Blood 1994; 84:200-210.
99. Skubitz KM. CD66a, CD66b, CD66c, and CD66d each independently stimulate neutrophils. J Leukoc Biol 1996; 60:1-12.
100. Stanners CP, Rojas M, Zhou H et al. The CEA family: A system in transitional evolution? Int J Biol Markers 1992; 7:137-142.
101. Benchimol S, Fuks A, Jothy S et al. Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. Cell 1989; 57:327-334.
102. Oikawa S, Inuzuka C, Kuroki M et al. Cell adhesion activity of nonspecific cross-reacting antigen (NCA) and carcinoembryonic antigen (CEA) expressed on CHO cell surface: Homophilic and heterophilic adhesion. Biochem Biophys Res Commun 1989; 164:39-45.

103. Oikawa S, Inuzuka C, Kuroki M et al. A specific heterotypic cell adhesion activity between members of carcinoembryonic antigen family, W272 and NCA, is mediated by N-domains. J Biol Chem 1991; 266:7995-8001.
104. Leusch HG, Drzeniek Z, Markos-Pusztai Z et al. Binding of *Escherichia coli* and *salmonella* strains to members of carcinoembryonic antigen family: Differential binding inhibition by aromatic alpha-glycosides of mannose. Infect Immun 1991; 59:2051-2057.
105. Chen T, Gotschlich EC. CGM1a antigen of neutrophils, a receptor of gonococcal opacity proteins. Proc Natl Acad Sci USA 1996; 93:14851-14856.
106. Chen T, Grunert F, Medina Marino A et al. Several carcinoembryonic antigens (CD66) serve as receptors for gonococcal opacity proteins. J Exp Med 1997; 185:1557-1564.
107. Gray Owen SD, Dehio C, Haude A et al. CD66 carcinoembryonic antigens mediate |interactions between Opa-expressing *Neisseria gonorrhoea* and human polymorphonuclear phagocytes. EMBO J 1997; 16:3435-3445.
108. Kuijpers TW, Hoogerwerf M, van der Laan LJ et al. CD66 nonspecific crossreacting antigens are involved in neutrophil adherence to cytokine-activated endothelial cells. J Cell Biol 1992; 118:457-466.
109. Stocks SC, Albrechtsen M, Kerr MA. Expression of the CD15 differentiation antigen (3-fucosyl-N- acetyl-lactosamine, Lex) on putative neutrophil adhesion molecules CR3 and NCA-160. Biochem J 1990; 268:275-280.
110. Shipp MA, Look AT. Hematopoietic differentiation antigens that are membrane-associated enzymes: Cutting is the key! Blood 1993; 82:1052-70.
111. Thompson LF, Ruedi JM, Glass A et al. Production and characterization of monoclonal antibodies to the glycosyl phosphatidylinositol-anchored lymphocyte differentiation antigen ecto-5'-nucleotidase (CD73). Tissue Antigens 1990; 35:9-19.
112. Thompson LF, Ruedi JM, O'Connor RD et al. Ecto-5'-nucleotidase expression during human B cell development. An explanation for the heterogeneity in B lymphocyte ecto-5'-nucleotidase activity in patients with hypogammaglobulinemia. J Immunol 1986; 137:2496-2500.
113. Airas L, Salmi M, Jalkanen S. Lymphocyte-vascular adhesion protein-2 is a novel 70-kDa molecule involved in lymphocyte adhesion to vascular endothelium. J Immunol 1993; 151:4228-38.
114. Airas L, Hellman J, Salmi M et al. CD73 is involved in lymphocyte binding to the endothelium: Characterization of lymphocyte-vascular adhesion protein 2 identifies it as CD73. J Exp Med 1995; 182:1603-8.
115. Arvilommi AM, Salmi M, Airas L et al. CD73 mediates lymphocyte binding to vascular endothelium in inflamed human skin. Eur J Immunol 1997; 27:248-54.
116. Airas L, Jalkanen S. CD73 mediates adhesion of B cells to follicular dendritic cells. Blood 1996; 88:1755-64.
117. Stochaj U, Richter H, Mannherz HG. Chicken gizzard 5'-nucleotidase is a receptor for the extracellular matrix component fibronectin. Eur J Cell Biol 1990; 51:335-8.
118. Thompson LF, Ruedi JM, Glass A et al. Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T-cells to proliferate. J Immunol 1989; 143:1815-21.
119. Massaia M, Perrin L, Bianchi A et al. Human T-cell activation. Synergy between CD73 (ecto-5'-nucleotidase) and signals delivered through CD3 and CD2 molecules. J Immunol 1990; 145:1664-74.
120. Airas L, Niemela J, Salmi M et al. Differential regulation and function of CD73, a glycosyl-phosphatidylinositol-linked 70-kD adhesion molecule, on lymphocytes and endothelial cells. J Cell Biol 1997; 136:421-31.
121. Behrendt N, Ronne E, Ploug M et al. The human receptor for urokinase plasminogen activator. NH_2-terminal amino acid sequence and glycosylation variants. J Biol Chem 1990; 265:6453-6460.
122. Min HY, Semnani R, Mizukami IF et al. cDNA for Mo3, a monocyte activation antigen, encodes the human receptor for urokinase plasminogen activator. J Immunol 1992; 148(11):3636-42.

123. Roldan AL, Cubellis MV, Masucci MT et al. Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis [published erratum appears in EMBO J 1990 May; 9:1674]. EMBO J 1990; 9:467-74.
124. Ploug M, Kjalke M, Ronne E et al. Localization of the disulfide bonds in the NH2-terminal domain of the cellular receptor for human urokinase-type plasminogen activator. A domain structure belonging to a novel superfamily of glycolipid-anchored membrane proteins. J Biol Chem 1993; 268:17539-17546.
125. Shevach EM, Korty PE. Ly-6: A multigene family in search of a function. Immunol Today 1989; 10:195-200.
126. Williams AF, Tse AG, Gagnon J. Squid glycoproteins with structural similarities to Thy-1 and Ly-6 antigens. Immunogenetics 1988; 27:265-272.
127. Fazioli F, Blasi F. Urokinase-type plasminogen activator and its receptor: New targets for anti-metastatic therapy? Trends Pharmacol Sci 1994; 15:25-29.
128. Ellis V, Dano K. The urokinase receptor and the regulation of cell surface plasminogen activation. Fibrinolysis 1992; 6:27-34.
129. Plow EF, Herren T, Redlitz A et al. The cell biology of the plasminogen system. FASEB J 1995; 9:939-945.
130. Nykjaer A, Petersen CM, Moller B et al. Purified α2-macroglobulin receptor/LDL receptor-related protein binds urokinase plasminogen activator inhibitor type-1 complex. Evidence that the α2-macroglobulin receptor mediates cellular degradation of urokinase receptor-bound complexes. J Biol Chem 1992; 267:14543-14546.
131. Conese M, Nykjaer A, Petersen CM et al. α2 Macroglobulin receptor/LDL receptor-related protein(LRP)-dependent internalization of the urokinase receptor. J Cell Biol 1995; 131:1609-1622.
132. Wei Y, Waltz DA, Rao N et al. Identification of the urokinase receptor as an adhesion receptor for vitronectin. J Biol Chem 1994; 269:32380-32388.
133. Deng G, Curriden SA, Wang S et al. Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? J Cell Biol 1996; 134:1563-1571.
134. Kinase SM, Kost C, Wilhelm OG et al. The urokinase receptor is a major vitronectin-binding protein on endothelial cells. Exp Cell Res 1996; 224:344-353.
135. Colman RW, Pixley RA, Najamunnisa S et al. Binding of high molecular weight kininogen to human endothelial cells is mediated via a site within domains 2 and 3 of the urokinase receptor. J Clin Invest 1997; 100:1481-1487.
136. Estreicher A, Muhlhauser J, Carpentier JL et al. The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. J Cell Biol 1990; 111:783-792.
137. Vaheri A, Stephens RW, Salonen EM et al. Plasminogen activation at the cell surface-matrix interface. Cell Differ Dev 1990; 32:255-262.
138. Bohuslav J, Horejsi V, Hansmann C et al. Urokinase plasminogen activator receptor, β2-integrins, and Src-kinases within a single receptor complex of human monocytes. J Exp Med 1995; 181:1381-1390.
139. Simon DI, Rao NK, Xu H et al. Mac-1 (CD11b/CD18) and the urokinase receptor (CD87) form a functional unit on monocytic cells. Blood 1996; 88:3185-3194.
140. Sitrin RG, Todd RF, Albrecht E et al. The urokinase receptor (CD87) facilitates CD11b/CD18-mediated adhesion of human monocytes. J Clin Invest 1996; 97:1942-1951.
141. Xue W, Kindzelskii AL, Todd RF et al. Physical association of complement receptor type 3 and urokinase-type plasminogen activator receptor in neutrophil membranes. J Immunol 1994; 152:4630-4640.
142. Cao D, Mizukami IF, Garni WB et al. Human urokinase-type plasminogen activator primes neutrophils for superoxide anion release. Possible roles of complement receptor type 3 and calcium. J Immunol 1995; 154:1817-1829.

143. Kindzelskii AI, Laska ZO, Todd RF et al. Urokinase-type plasminogen activator receptor reversibly dissociates from complement receptor type 3 (αMβ2, CD11b/CD18) during neutrophil polarization. J Immunol 1996; 156:297-309.
144. Kindzelskii Al, Eszes MM, Todd RF et al. Proximity oscillations of complement type 4 (αXβ2) and urokinase receptors on migrating neutrophils. Biophys J 1997; 73:1777-1784.
145. Xue W, Mizukami I, Todd RF et al. Urokinase type plasminogen activator receptors associate with β1 and β3 integrins of fibrosarcoma cells: Dependence on extracellular matrix components. Cancer Res 1997; 57:1682-1689.
146. Wei Y, Lukashev M, Simon DI et al. Regulation of integrin function by the urokinase receptor. Science 1996; 273:1551-1555.
147. Yebra M, Parry G, Stromblad S et al. Requirement of receptor-bound urokinase-type plasminogen activator for integrin αVβ5-directed cell migration. J Biol Chem 1996; 271:29393-29399.
148. Koshelnick Y, Ehart M, Hufnagl P et al. Urokinase receptor is associated with the components of the JAK1/STAT1 signaling pathway and leads to activation of this pathway upon receptor clustering in the human kidney epithelial tumor cell line TCL-598. J Biol Chem 1997; 272:28563-28567.
149. Kirchheimer JC, Wojta J, Christ G et al. Functional inhibition of endogenously produced urokinase decreases cell proliferation in a human melanoma cell line. Proc Natl Acad Sci USA 1989; 86:5424-5428.
150. Godar S, Horejsi V, Weidle UH et al. M6P/IGFII-receptor complexes uPA-receptor for activation of TGF-β1. submitted.
151. Williams AF. The structure of Thy-1 antigen. In: Reif, AE Schlesinger, M, eds. Cell surface antigen Thy-1: Immunology, neurology, and therapeutic. New York and Basel: Marcel Dekker, Inc. 1989:49-69.
152. Dráberová L. The involvement of Thy-1 antigen in the activation of rat mas T-cells. Eur J Immunol 1989; 19:1715-1720.
153. Gordon JW, Chesa PG, Nishimura H et al. Regulation of Thy-1 gene expression in transgenic mice. Cell 1987; 50:445-452.
154. Tiveron MC, Barboni E, Pliego Rivero FB et al. Selective inhibition of neurite outgrowth on mature astrocytes by Thy-1 glycoprotein. Nature 1992; 355:745-748.
155. Nosten-Bertrand M, Errington ML, Murphy KPSJ et al. Normal spatial learning despite regional inhibition of LTP in mice lacking Thy-1. Nature 1996; 379:826-829.
156. Hueber AO, Bernard AM, Battari CLE et al. Thymocytes in Thy-1-/- mice show augmented TCR signaling and impaired differentiation. Current Biol, 1997; 7:705-708.
157. Volarevic S, Burns CM, Sussman JJ et al. Intimate association of Thy-1 and the T-cell antigen receptor with the CD45 tyrosine phosphatase. Proc Natl Acad Sci USA 1990; 87:7085-7089.
158. Nelson Kl, Raja SM, Buckley JT. The glycosylphosphatidylinositol-anchored surface glycoprotein Thy-1 is a receptor for the channel-forming toxin aerolysin. J Biol Chem 1997; 272:12170-12174.
159. Dráberová L, Dráber P. Thy-1-mediated activation of rat basophilic leukemia cells does not require coexpression of the high-affinity IgE receptor. Eur J Immunol 1995; 25:2428-2432.
160. Hueber AO, Raposo G, Pierres M et al. Thy-1 triggers mouse thymocyte apoptosis through a bcl-2-resistant mechanism. J Exp Med 1994; 179:785-796.
161. Angelisová P, Cerny J, Drbal K et al. Characterization of the human leucocyte GPI-anchored glycoprotein CDw108 and its relation to other similar molecules. Eur J Biochem submitted.
162. Klickstein KB, Springer TA. CDw108 cluster report. In Schlossman, SF, Boumsell, L, Harlan, J et al., eds. Leukocyte Typing V. White Cell Differentiation Antigens. Oxford: Oxford University Press, 1995:1477-1478.
163. Mudad R, Rao N, Angelisova P et al. Evidence that CDw108 membrane protein bears the JMH blood group antigen. Transfusion 1995; 35:566-70.

164. Kubo K. A novel phosphatidylinositol anchored protein expressed on human activated lymphocytes recognized by H105 monoclonal antibody. J Kurume Med Assoc 1991; 54:1051-1060.
165. Sutherland DR, Yeo E, Ryan A et al. Identification of a cell-surface antigen associated with activated T lymphoblasts and activated platelets. Blood 1991; 77:84-93.
166. Smith JW, Hayward CP, Horsewood P et al. Characterization and localization of the Gova/b alloantigens to the glycosylphosphatidylinositol-anchored protein CDw109 on human platelets. Blood 1995; 86:2807-14.
167. Itoh M, Ishihara K, Tomizawa H et al. Molecular cloning of murine BST-1 having homology with CD38 and aplysia ADP-ribosyl cyclase. Biochem Biophys Res Commun 1994; 203:1309-17.
168. Hirata Y, Kimura N, Sato K et al. ADP ribosyl cyclase activity of a novel bone marrow stromal cell surface molecule, BST-1. FEBS Lett 1994; 356(2-3):244-8.
169. Todd RF III, Roach JA, Arnaout MA. The modulated expression of Mo5, a human myelomonocytic plasma membrane antigen. Blood 1985; 65:964-973.
170. Ishihara K, Kobune Y, Okuyama Y et al. Stage-specific expression of mouse BST-1/BP-3 on the early B and T-cell progenitors prior to gene rearrangement of antigen receptor. Int Immunol 1996; 8:1395-404.
171. Okuyama Y, Ishihara K, Kimura N et al. Human BST-1 expressed on myeloid cells functions as a receptor molecule. Biochem Biophys Res Commun 1996; 228:838-45.
172. Vicari AP, Bean AG, Zlotnik A. A role for BP-3/BST-1 antigen in early T-cell development. Int Immunol 1996; 8:183-91.
173. Lee BO, Ishihara K, Denno K et al. Elevated levels of the soluble form of bone marrow stromal cell antigen 1 in the sera of patients with severe rheumatoid arthritis. Arthritis Rheum 1996; 39:629-37.
174. Haag F, Andresen V, Karsten S et al. Both allelic forms of the rat T-cell differentiation marker RT6 display nicotinamide adenine dinucleotide (NAD)-glycohydrolase activity, yet only RT6.2 is capable of automodification upon incubation with NAD. Eur J Immunol 1995; 25:2355-61.
175. Maehama T, Nishina H, Hoshino S et al. NAD(+)-dependent ADP-ribosylation of T lymphocyte alloantigen RT6.1 reversibly proceeding in intact rat lymphocytes. J Biol Chem 1995; 270(39):22747-51.
176. Rigby MR, Bortell R, Stevens LA et al. Rat RT6.2 and mouse Rt6 locus 1 are NAD+:arginine ADP ribosyltransferases with auto-ADP ribosylation activity. J Immunol 1996; 156(11):4259-65.
177. Wang J, Nemoto E, Dennert G. Regulation of CTL by ecto-nictinamide adenine dinucleotide (NAD) involves ADP-ribosylation of a p56lck-associated protein. J Immunol 1996; 156:2819-27.
178. Haag F, Koch-Nolte F, Kuhl M et al. Premature stop codons inactivate the RT6 genes of the human and chimpanzee species. J Mol Biol 1994; 243:537-46.
179. Okazaki IJ, Zolkiewska A, Nightingale MS et al. Immunological and structural conservation of mammalian skeletal muscle glycosylphosphatidylinositol-linked ADP-ribosyltransferases. Biochemistry 1994; 33(43):12828-36.
180. Nemoto E, Yu Y, Dennert G. Cell surface ADP-ribosyltransferase regulates lymphocyte function-associated molecule-1 (LFA-1) function in T-cells. J Immunol 1996; 157:3341-9.
181. Nemoto E, Stohlman S, Dennert G. Release of a glycosylphosphatidylinositol-anchored ADP-ribosyltransferase from cytotoxic T-cells upon activation. J Immunol 1996; 156:85-92.
182. Koch-Nolte F, Haag F, Braren R et al. Two novel human members of an emerging mammalian gene family related to mono-ADP-ribosylating bacterial toxins. Genomics 1997; 39:370-6.
183. Koch F, Haag F, Kashan A et al. Primary structure of rat RT6.2, a nonglycosylated phosphatidylinositol-linked surface marker of postthymic T-cells. Proc Natl Acad Sci USA 1990; 87:964-7.

184. Fangmann J, Schwinzer R, Wonigeit K. Unusual phenotype of intestinal intraepithelial lymphocytes in the rat: Predominance of T-cell receptor alpha/beta+/CD2- cells and high expression of the RT6 alloantigen. Eur J Immunol 1991; 21:753-60.
185. Greiner DL, Mordes JP, Handler ES et al. Depletion of RT6.1+ T lymphocytes induces diabetes in resistant biobreeding/Worcester (BB/W) rats. J Exp Med 1987; 166:461-75.
186. Koch-Nolte F, Klein J, Hollmann C et al. Defects in the structure and expression of the genes for the T-cell marker Rt6 in NZW and (NZB x NZW)F1 mice. Int Immunol 1995; 7:883-90.
187. Brown JP, Hewick RM, Hellstrom I et al. Human melanoma-associated antigen p97 is structurally and functionally related to transferrin. Nature 1982; 296:171-173.
188. Kennard ML, Richardson DR, Gabathuler R et al. A novel iron uptake mechanism mediated by GPI-anchored human p97. EMBO J 1995; 14(17):4178-86.
189. Rose TM, Plowman GD, Teplow DB et al. Primary structure of the human melanoma-associated antigen p97 (melanotransferrin) deduced from the mRNA sequence. Proc Natl Acad Sci USA 1986; 83:1261-5.
190. Palfree RG. Ly-6-domain proteins—new insights and new members: A C-terminal Ly-6 domain in sperm acrosomal protein SP-10. Tissue Antigens 1996; 48:71-9.
191. Gumley TP, McKenzie IF, Sandrin MS. Tissue expression, structure and function of the murine Ly-6 family of molecules. Immunol Cell Biol 1995; 73:277-96.
192. LeClair KP, Rabin M, Nesbitt MN et al. Murine Ly-6 multigene family is located on chromosome 15. Proc Natl Acad Sci USA 1987; 84:1638-43.
193. LeClair KP, Bridgett MM, Dumont FJ et al. Kinetic analysis of Ly-6 gene induction in a T lymphoma by interferons and interleukin 1, and demonstration of Ly-6 inducibility in diverse cell types. Eur J Immunol 1989; 19:1233-9.
194. Brakenhoff RH, Gerretsen M, Knippels EM et al. The human E48 antigen, highly homologous to the murine Ly-6 antigen ThB, is a GPI-anchored molecule apparently involved in keratinocyte cell-cell adhesion. J Cell Biol 1995; 129:1677-89.
195. Capone MC, Gorman DM, Ching EP et al. Identification through bioinformatics of cDNAs encoding human thymic shared Ag-1/stem cell Ag-2. A new member of the human Ly-6 family. J Immunol 1996; 157:969-73.
196. Mao M, Yu M, Tong JH et al. RIG-E, a human homolog of the murine Ly-6 family, is induced by retinoic acid during the differentiation of acute promyelocytic leukemia cell. Proc Natl Acad Sci USA 1996; 93(12):5910-4.
197. Bamezai A, Palliser D, Berezovskaya A et al. Regulated expression of Ly-6A.2 is important for T-cell development. J Immunol 1995; 154:4233-9.
198. Bamezai A, Rock KL. Overexpressed Ly-6A.2 mediates cell-cell adhesion by binding a ligand expressed on lymphoid cells. Proc Natl Acad Sci U S A 1995; 92(10):4294-8.
199. Johnson R, Lancki DW, Fitch FW. Accessory molecules involved in antigen-mediated cytolysis and lymphokine production by cytotoxic T lymphocyte subsets. I. Identification of functions for the T-cell surface molecules Ly-6C and Thy-1. J Immunol 1993; 151:2986-99.
200. Fleming TJ, Malek TR. Multiple glycosylphosphatidylinositol-anchored Ly-6 molecules and transmembrane Ly-6E mediate inhibition of IL-2 production. J Immunol 1994; 153:1955-62.
201. Lee SK, Su B, Maher SE et al. Ly-6A is required for T-cell receptor expression and protein tyrosine kinase fyn activity. EMBO J 1994; 13:2167-76.
202. Flood PM, Dougherty JP, Ron Y. Inhibition of Ly-6A antigen expression prevents T-cell activation. J Exp Med 1990; 172:115-20.
203. Stroynowski I. Tissue-specific, peptide-binding transplantation antigens: Lessons from the Qa-2 system. Immunol Rev 1995(147):91-108.
204. Stroynowski I, Tabaczewski P. Multiple products of class Ib Qa-2 genes: Which ones are functional? Res Immunol 1996; 147:290-301.
205. Cain TJ, Robinson JM. Rapid purification of the PI-linked alkaline phosphatase from intact human neutrophils. J Cell Biol 1991; 115:471a.

206. Feldbush TL, Lafrenz D. Alkaline phosphatase on activated B cells. Characterization of the expression of alkaline phosphatase on activated B cells: Kinetics and membrane anchor. J Immunol 1991; 147:3690-3695.
207. Dairiki K, Nakamura S, Ikegami S et al. Mouse Lx-31.1 is an alloantigenic determinant of alkaline phosphatase predominantly expressed in the kidney and bone. Immunogenetics 1989; 29:235-240.
208. Goyert SM, Ferrero E, Rettig WJ et al. The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors. Science 1988; 239(4839):497-500.
209. Kielian TL, Blecha F. CD14 and other recognition molecules for lipopolysaccharide: A review. Immunopharmacology 1995; 29:187-205.
210. Ulevitch RJ, Tobias PS. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol 1995; 13:437-57.
211. Ravetch JV. Fc receptors. Curr Opin Immunol 1997; 9:121-5.
212. Deo YM, Graziano RF, Repp R et al. Clinical significance of IgG Fc receptors and Fc gamma R-directed immunotherapies. Immunol Today 1997; 18:127-35.
213. Caras IW, Davitz MA, Rhee L et al. Cloning of decay-accelerating factor suggests novel use of splicing to generate two proteins. Nature 1987; 325(6104):545-9.
214. Liszewski MK, Farries TC, Lublin DM et al. Control of the complement system. Adv Immunol 1996; 61:201-83.
215. Nicholson-Weller A, Wang CE. Structure and function of decay accelerating factor CD55. J Lab Clin Med 1994; 123:485-91.
216. Meri S. Protectin (CD59). Complement lysis inhibitor and prototype domain in a new protein superfamily. Immunologist 1994; 2:149-155.
217. Davies A, Simmons DL, Hale G et al. CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. J Exp Med 1989; 170:637-54.
218. Thompson JA, Grunert F, Zimmermann W. Carcinoembryonic antigen gene family: Molecular biology and clinical perspectives. J Clin Lab Anal 1991; 5:344-66.
219. Zimmermann H. 5'-Nucleotidase:molecular structure and functional aspects. Biochem J 1992; 285(Pt 2):345-65.
220. Seki T, Spurr N, Obata T et al. The human Thy-1 gene: Structure and chromosomal location. Proc Natl Acad Sci USA 1985; 82:6657-661.
221. Seki T, Chang HC, Moriuchi T et al. A hydrophobic transmembrane segment at the carboxy terminus of Thy-1. Science 1985; 227:649-651.
222. Reif AF, Schlesinger M, eds. Cell surface Thy-1. New York: Marcel Dekker Inc. 1989.
223. Koch-Nolte F, Haag F, Kastelein R et al. Uncovered: The family relationship of a T-cell membrane protein and bacterial toxins. Immunol Today 1996; 17:402-405.
224. Palfree RG, LeClair KP, Bothwell A et al. cDna characterization of an Ly-6.2 gene expressed in Bw5147 tumor cells. Immunogenetics 1987; 26:389-91.
225. Palfree RG, Sirlin S, Dumont FJ et al. N-terminal and cDNA characterization of murine lymphocyte antigen Ly-6C.2. J Immunol 1988; 140:305-10.
226. Fleming TJ, O'hUigin C, Malek TR. Characterization of two novel Ly-6 genes. Protein sequence and potential structural similarity to alpha-bungarotoxin and other neurotoxins. J Immunol 1993; 150(12):5379-90.
227. Gumley TP, McKenzie IF, Kozak CA et al. Isolation and characterization of cDNA clones for the mouse thymocyte B cell antigen (ThB). J Immunol 1992; 149:2615-8.
228. MacNeil I, Kennedy J, Godfrey DI et al. Isolation of a cDNA encoding thymic shared antigen-1. A new member of the Ly6 family with a possible role in T-cell development. J Immunol 1993; 151(12):6913-23.
229. Classon BJ, Coverdale L. Mouse stem cell antigen Sca-2 is a member of the Ly-6 family of cell surface proteins. Proc Natl Acad Sci USA 1994; 91(12):5296-300.

Chapter 6

GPI-Anchored Proteins in the Nervous System

David A. Harris

Of all the cells in the body, those of the nervous and immune systems probably contain the richest variety of proteins with glycosyl-phosphatidylinositol (GPI) anchors. This fact may reflect the complex cell-cell interactions and transmembrane signaling phenomena required for development and plasticity in the nervous system and for response to foreign antigens by the immune system. In the nervous system, GPI-anchored proteins play roles in a wide range of processes, including growth factor signaling, axon pathfinding and fasciculation, synapse formation and modification, and neurodegenerative diseases. Although a great deal is often known about the biology of these proteins, in only a few cases is the functional significance of the GPI anchor itself known with any certainty.

In this chapter, I will review the main classes of GPI-anchored proteins in the nervous system, address the issue of whether caveolae exist in neurons, and discuss the properties and possible functions of detergent-resistant rafts. In addition, I will present recent work from my own laboratory concerning the prion protein, which is involved in a group of unusual neurodegenerative diseases, as a way of illustrating how the GPI anchor plays an important role in the biology of one medically relevant protein.

Growth Factor Receptors

Signal transduction by several important neuronal growth factors has been found to involve a receptor component that is GPI-anchored (Fig. 6.1; see also chapter 4). One group includes glial-derived neurotrophic factor (GDNF),[1] neurturin (NTN)[2] and the recently identified protein persephin (PSP).[3] These proteins, which are distantly related members of the TGF-β family, promote the survival of specific subsets of neurons from both the central and peripheral nervous system, and also serve as inductive factors in embryonic development of the kidney. Each of these factors utilizes a signal transduction system that consists of a GPI-linked receptor (designated GFRα) and a transmembrane tyrosine kinase called Ret.[4,5] The growth factor binds to the GFRα component, which then associates with Ret to form a ternary complex, resulting in activation of the tyrosine kinase activity of Ret. Activated Ret is then thought to autophosphorylate as a result of growth factor-induced dimerization, initiating a signaling cascade that includes components of the MAP kinase pathway.[6] The importance of GFRα in this process is clearly demonstrated by the fact that treatment of cells with PIPLC, a bacterial phospholipase that cleaves the GPI anchor, abolishes their responsiveness to growth factor.[7] There are now three different GFRα variants identified.[8] GFRα-1 appears to be relatively specific for GDNF and GFRα-2 for NTN; the binding specificity of GFRα-3 is uncertain. PSP does not appear to interact with

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

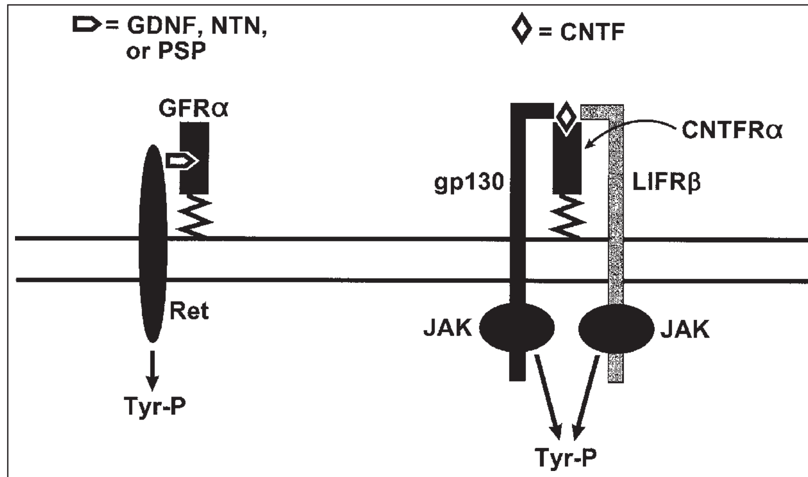

Fig 6.1. Receptor complexes for two classes of neuronal growth factors contain a GPI-anchored component. GFRα and CNTFRα are GPI-anchored proteins that bind GDNF/NTN/PSP and CNTF, respectively. There are several isoforms of GFRα with different binding specificities. Ret and JAK are tyrosine kinases.

either any of the three GFR variants, which suggests that other binding components remain to be discovered.[3]

Another growth factor that utilizes a GPI-anchored binding component is ciliary neurotrophic factor (CNTF), which sustains survival of parasympathetic, motor, and sensory neurons.[9] Binding of CNTF to the GPI-anchored CNTFRα receptor induces association with two transmembrane signal-transducing components, gp130 and LIFRβ.[10] These latter two proteins are not themselves kinases, but their cytoplasmic domains are associated with kinases of the JAK family that phosphorylate substrates responsible for propagating the growth signal to the nucleus.[11] The same gp130 and LIFRβ proteins are also components of the signal transduction mechanism engaged by several other structurally related cytokines active in the hematopoetic system (leukemia inhibitory factor, interleukin 6, and oncostatin M), although GPI-anchored subunits are not involved.[12]

In each of these systems, a ligand-binding α subunit cooperates with one or more transmembrane signaling subunits. The function of the α subunit is presumed to confer ligand specificity, but it may also serve to concentrate the ligand, and so intensify the signal. Another role might be to induce a conformational change in the ligand that increases its affinity for the transmembrane signaling subunits of the complex.[13]

The functional significance of having an α subunit with a GPI anchor is unclear. One intriguing fact is that ligand bound to soluble forms of GRFα and CNTFRα released by treatment with PIPLC are capable of stimulating a response in cells that lack these subunits.[7] This raises the possibility that release of the GPI-anchored components by endogenous phospholipases might subserve a physiological function. One such function may be mediating the response to nerve injury. In support of this idea, it has been found that denervation dramatically increases release of soluble CNTFRα from skeletal muscle, and in a complementary fashion nerve damage stimulates release of CNTF from Schwann cells

Table 6.1 GPI-anchored molecules of the immunoglobulin superfamily involved in axonal and synaptic adhesion

Protein	Number of Ig domains
F3 (F11/Contactin)	6
TAG-1 (Axonin-1)	6
BIG-1	6
BIG-2	6
NCAM*	5
ApCAM*	5
Fasciclin II*	5
OBCAM	3
Neurotrimin	3
CEPU-1	3
LAMP	3
Thy-1	1

For original citations, see ref. 16.
*These molecules exist in both GPI-linked and transmembrane isoforms.

that ensheathe the axon.[14] Thus nerve injury elicits a coordinated release of a GPI-anchored receptor and its cognate ligand.

Molecules Involved in Axonal and Synaptic Adhesion

A number of GPI-anchored proteins in the nervous system are involved in axon outgrowth and fasciculation, as well as the formation and plasticity of synapses (Table 6.1). These proteins are thought to mediate adhesion between the axonal or synaptic membranes of adjacent neurons, or between the axonal membrane of a neuron and its substrate. Many of these proteins are members of the immunoglobulin superfamily, and contain multiple immunoglobulin and fibronectin domains. I will not attempt to review here the extensive literature on the function of these proteins (see refs. 15-17), but only consider several issues relevant to the role of the GPI anchor.

The proteins listed in Table 6.1 are a small subset of a much larger group of neuronal and glial adhesion proteins, the rest of which have a transmembrane rather than a GPI anchor.[16] Thus, the GPI anchor is not a general feature of molecules mediating adhesion in the nervous system, and the presence of this modification on some molecules may endow them with special properties. Several possibilities have been suggested. First, GPI-anchored adhesion proteins, like other glycolipidated proteins, are found in soluble as well as membrane-bound forms, and it has been hypothesized that regulated release of these proteins from the cell surface may play a role in neurite outgrowth.[18,19] Such soluble forms could diffuse over long distances and establish adhesive gradients during development. The soluble forms may also possess different biological activities than the membrane-attached forms.[20]

Second, some adhesion molecules such as NCAM, ApCAM, and fasciclins exist in both GPI-linked and transmembrane isoforms, and it is possible that differential expression, degradation, or localization of these forms may have functional significance. For example, it is hypothesized that the transmembrane form of ApCAM promotes fasciculation of axons, while the GPI-linked form stabilizes synaptic contacts. Neuronal activity is thought to

induce selective endocytosis and degradation of transmembrane ApCAM, which would then allow axonal sprouting and formation of new synaptic sites without loss of existing synapses.[21]

Third, it is now well known that adhesion molecules such as integrins can transduce signals to the cell interior, and GPI-anchored adhesion molecules may be especially suited to this role by virtue of their incorporation into cholesterol-rich raft domains which contain various kinds of signaling components (see below). Signal transduction is likely to be important in processes such as axonal chemoattraction and repulsion, growth cone collapse, and target-induced differentiation. Interactions between neighboring cells may be bidirectional, since several of the molecules in Table 6.1 can function as both receptor and ligand.[17]

Finally, it is possible that the presence of the GPI anchor may play a role in determining where on the surface of the neuron the adhesion molecule is displayed, and therefore the targets with which it will interact. In at least some situations, the GPI anchor may serve as an axonal targeting signal (discussed below), and so may be present on molecules that are meant to be excluded from the soma and dendrites. In addition, the anchor may play a role in determining whether the protein is localized at the terminals of the axon, or along its shaft.[22-24]

Caveolae and Detergent-Resistant Rafts

Current evidence indicates that caveolae are not present in peripheral or central neurons. This conclusion is based on both morphological and biochemical data. First, caveolae have not been observed in cultured neurons or neuronal cell lines by either thin-section or deep-etch electron microscopy (ref. 25 and J. Heuser, personal communication). The latter technique allows unequivocal identification of the striated caveolar coat, which is easily distinguishable from the lattice-like clathrin coat (Fig. 6.2). Second, cultured neurons, neuronal cell lines, and brain tissue express low or undetectable levels of the known caveolin isoforms and their mRNAs.[25-33] Small amounts of caveolin mRNA that have sometimes been detected in brain are likely to be derived from astrocytes, which contain abundant caveolae based on morphological and biochemical evidence.[34] Of course, it is possible that only certain anatomically or developmentally defined subsets of neurons possess caveolae, or that neurons contain caveolin isoforms which are not detectable with current nucleic acid and antibody reagents. However, it seems more likely that whatever function caveolae perform in other cells is dispensable, or is performed by other structures, in nerve cells.

Despite the apparent absence of caveolae in neurons, there is abundant evidence that these cells contain detergent-resistant membrane domains (rafts) that are similar in physical properties, composition, and appearance to the domains isolated from cells such as fibroblasts and smooth muscle cells that contain caveolae. The fact that rafts can be isolated from neurons clearly demonstrates that these domains are not identical to caveolae, although it is likely that rafts are associated with caveolae in cell types that possess these invaginations. The detergent-resistant domains prepared from cultured nerve cells and brain by lysis in cold Triton buffer are large by gel filtration chromatography ($>2 \times 10^7$ daltons),[35] they have a low buoyant density on sucrose gradients,[29,35,36] and in the electron microscope they have the appearance of membranous vesicles of heterogeneous size and shape.[29,35,36] They are enriched in sphingolipid and cholesterol,[29,35] as well as in GPI-anchored proteins, including the prion protein[35,37,38] (Fig. 6.3) and the adhesion molecules F3,[29,39,40] Thy-1,[29] NCAM-120,[29] and axonin-1.[41] They contain a variety of signaling components, including the Src-family kinases Fyn[29,39,40] and Yes,[35] and the heterotrimeric G-protein subunits $G_{0\alpha}$, $G_{i\alpha}$ and $G_{\beta 1}$.[35] A light membrane fraction containing similar kinds of molecules has also been isolated from brain using a procedure that does not involve the use of detergent.[42]

Interestingly, this fraction contains, in addition to cytoplasmic signaling proteins, a number of transmembrane receptors, including insulin receptor, the neurotrophin receptors trkB and p75, and a member of the Eph family of receptors, which are thought to be involved in axon guidance and pathfinding.

Possible Functions of Rafts in the Nervous System

There is considerable debate (see chapters 3 and 4) about whether the detergent-resistant complexes that have been characterized biochemically correspond to membrane domains that exist in intact cells, or whether they are artifacts of detergent extraction. If rafts do exist in vivo, however, they might subserve several possible functions in neurons and glia. Given the wide variety of signal-transducing elements they contain, one plausible hypothesis is that rafts are involved in transmembrane signaling. Several pieces of evidence support this idea. First, the kinases in rafts prepared from brain and neuroblastoma cells are catalytically active, as demonstrated by incorporation of $^{32}PO_4$ and formation of phosphotyrosine upon incubation at 37°C.[35] In addition, binding of ligands to GPI-anchored proteins on intact cells alters the activity or distribution of cytoplasmic kinases. For example, antibody-induced crosslinking of F3 on neurons or transfected cells results in increased association with Fyn, and enhanced tyrosine phosphorylation.[39,40] Less artificial stimuli, including F3-mediated cell aggregation,[40] or binding to F3 of its natural ligand tenascin-R,[39] also activate or redistribute Fyn kinase. Fasciculation of dorsal root ganglion axons induces clustering of axonin-1 with the transmembrane adhesion molecule Ng-CAM, with a concomitant reduction in Fyn kinase activity.[41] These results with neuronal cells are quite similar to those that have been obtained with lymphocytes, in which antibody crosslinking of GPI-anchored surface proteins induces changes in tyrosine phosphorylation. The transmission of a signal from a GPI-anchored protein on the external leaflet of the lipid bilayer to cytoplasmic kinases is presumed to require mediation of a transmembrane linker protein, but the identity of such molecules in the nervous or immune systems has remained a mystery.

Another possible function for detergent-resistant membrane domains in neurons is in polarized sorting of proteins, analogous to the function that has been suggested for them in epithelial cells. In polarized MDCK epithelial cells, most GPI-anchored proteins are delivered preferentially to the apical surface after synthesis, a phenomenon that has been proposed to depend on their incorporation into raft domains in the trans-Golgi network.[43] In neurons, it has been suggested that the axonal membrane is analogous to the apical surface of epithelial cells, and the somatodendritic membrane to the basolateral surface. In support of this notion, GPI-anchored Thy-1, as well as influenza virus hemagglutinin (a transmembrane protein that is apically localized in MDCK cells) are both preferentially localized on the axonal membrane in cultured hippocampal neurons.[44,45]

Once deposited in the axonal membrane, GPI-anchored proteins may be prevented from diffusing back into the soma and dendrites by the presence of a barrier at the axon hillock that prevents mixing of lipids from the two domains.[46] However, the existence of this barrier has recently been challenged, raising the possibility that selective removal as well as delivery might play a role in restricting GPI-anchored proteins to the axonal membrane.[47] It is also possible that the stringency of the diffusion barrier may vary among neurons, explaining why certain GPI-anchored proteins are found on both the axonal and somatodendritic surfaces of some neurons, but are restricted to the axonal surface of other neurons.[24]

A third possible function for rafts, one that would be particular to the nervous system, is in assembly of myelin by oligodendrocytes. Myelin, the multilammelar sheath that insulates large caliber axons, is enriched in glycosphingolipids and cholesterol, and so represents a

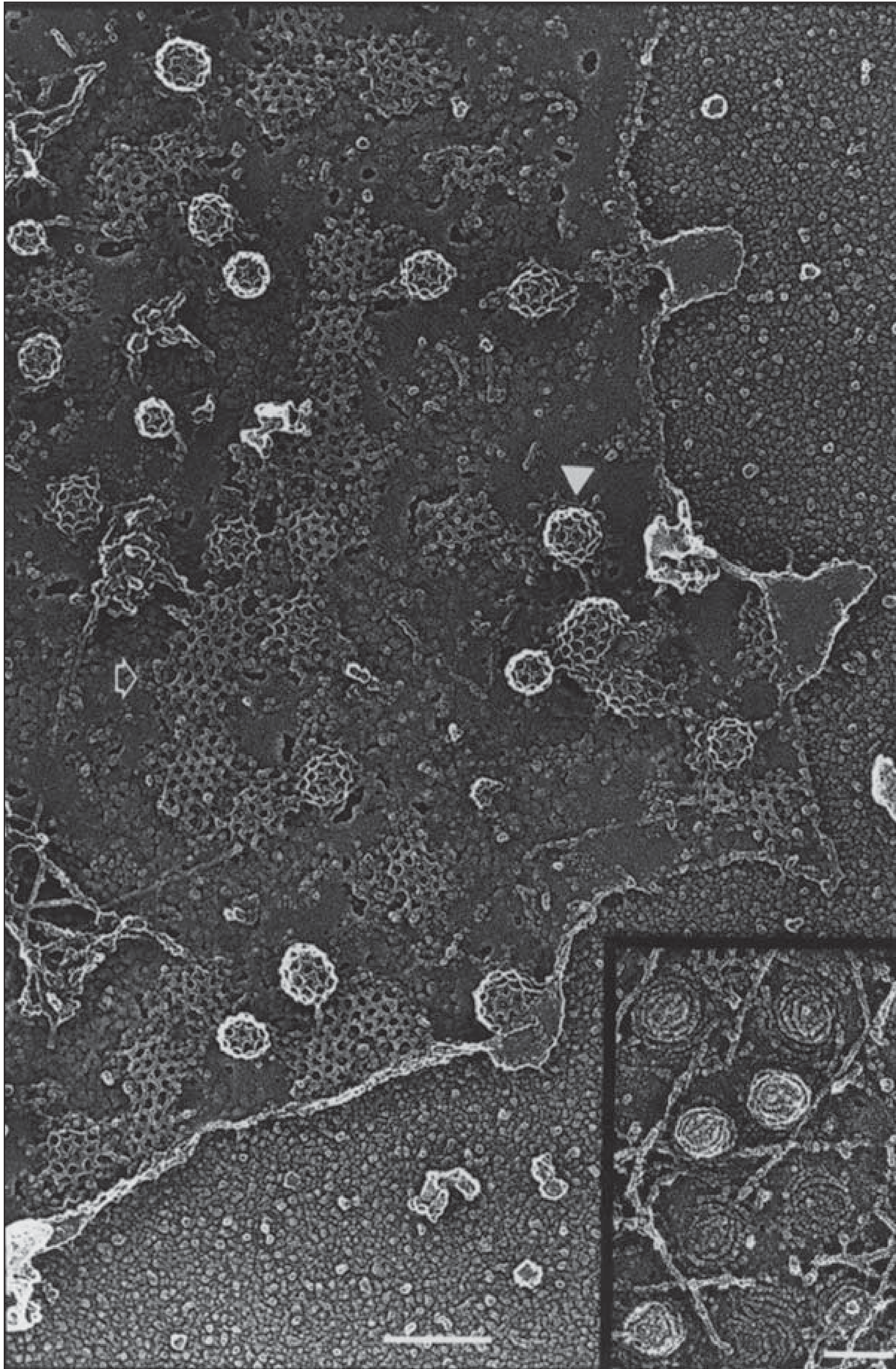

Fig. 6.2. (opposite page) Cultured neuroblastoma cells do not contain caveolae. The electron micrograph in the main panel shows the inner surface of the plasma membrane of an N2a neuroblastoma cell that has been sheared open, quick-frozen and deep-etched. Numerous clathrin lattices are seen; some are relatively flat (open arrowhead), while others curve around a budding vesicle (filled triangle). These polygonal lattices are easily distinguished from the striped coats of caveolae, which are seen in the inset taken from a 3T3-L1 adipocyte. Despite extensive examination, no structures that display a caveolar coat could be found in N2a cells. In addition, N2a cells do not express caveolin protein or mRNA.[25] Scale bars in both panels are 0.2 μm. Reprinted with permission from Shyng SL, Heuser JE, Harris DA. J Cell Biol 1994; 125:1239-1250. ©1994 The Rockefeller University Press.

natural membrane domain that is similar in lipid composition to the rafts produced by detergent extraction. Interestingly, oligodendrocytes do not assemble their GPI-anchored proteins into a detergent-resistant form until after the cells reach a mature stage of differentiation, concomitant with their ability to synthesize glycosphingolipids and cholesterol.[48] This observation suggests that rafts might serve as functional units that are required for assembly of the lipid and protein components of myelin. However, factors other than incorporation into rafts must also influence delivery of molecules to the myelin sheath, since the hemagglutinin (HA) protein of influenza virus is Triton-insoluble in oligodendrocytes but is not found in myelin; conversely the G protein of vesicular stomatitis virus, which is Triton-soluble, is incorporated into myelin.[49]

Prions

For many proteins, it has proven difficult to assign a functional significance to the presence of a GPI anchor. The prion protein (PrP), however, is a good candidate for investigating the role of the anchor, since this modification features in several important ways in the biology of the protein.

Prion Diseases

This group of fatal neurodegenerative disorders includes Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler syndrome (GSS), fatal familial insomnia (FFI) and kuru in human beings, and scrapie and bovine spongiform encephalopathy ("mad cow disease") in animals. These diseases, which are characterized by dementia, motor dysfunction, and cerebral amyloidosis, now pose potentially grave dangers to public health because of the likelihood that bovine spongiform encephalopathy has already been transmitted to human beings by consumption of contaminated beef.[50] Prion diseases are also of enormous scientific interest because infectious, inherited, and sporadic cases are all thought to result from changes in the conformation of a single, highly unusual membrane glycoprotein called PrP (for a review see ref. 51). This molecular transition converts a normal version of the protein (PrP^{C}) into a pathogenic form (PrP^{Sc}) that constitutes the major component of an unprecedented type of infectious particle (prion) devoid of nucleic acid.

The GPI Anchor of PrP

Both PrP^{C} and PrP^{Sc} contain a GPI anchor, based on the sensitivity of the proteins to PIPLC (although this sensitivity is manifested only after denaturation in the case of PrP^{Sc}), the demonstration by chemical analysis or metabolic labeling of anchor components such as fatty acids and ethanolamine, and the presence of a C-terminal hydrophobic domain that is cleaved upon anchor addition.[52-54] A complete structure has been determined for the anchor on PrP^{Sc} from hamster brain.[55] This anchor contains a core structure common to other glycolipidated proteins, including an ethanolamine residue amide-bonded to the

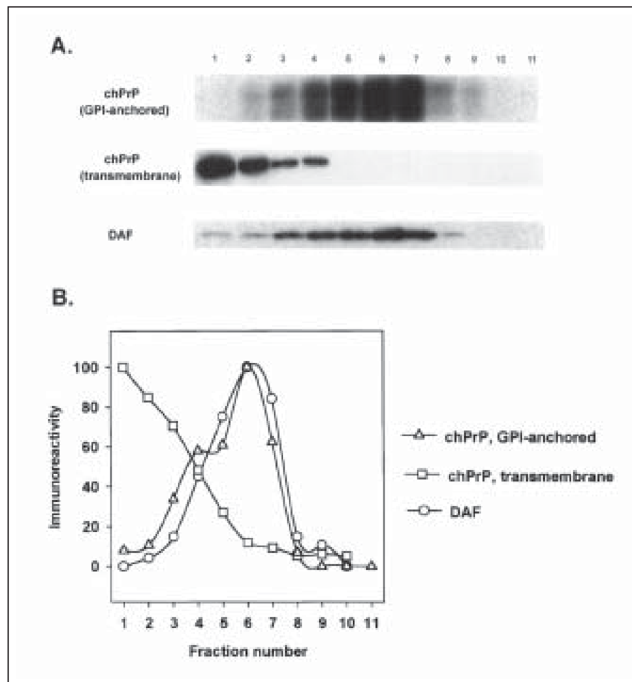

Fig. 6.3. GPI-anchored forms of PrP^C and DAF, but not a transmembrane form of PrP^C, are present in low-density rafts after detergent extraction of neuroblastoma cells. Transfected N2a cells expressing either GPI-anchored chicken prion protein PrP^C (chPrP), transmembrane chPrP (chPrP residues 1-241 fused to the transmembrane and cytoplasmic domains of the cation-independent mannose-6-phosphate receptor), or DAF (decay-accelerating factor) were homogenized at 4°C in a buffer containing 1% Triton X-100. The homogenate was brought to 40% sucrose, overlaid with a continuous gradient of 30% to 5% sucrose, and centrifuged to equilibrium (170,000 x g for 16 h). Gradient fractions were immunoblotted to detect the expressed proteins (A), and the signals quantitated (B). The two GPI-anchored proteins float at 15-20% sucrose (fractions 5-7), while the transmembrane form of chPrP is found near the bottom of the gradient (fractions 1-4) with the bulk of the total cellular protein. Reprinted with permission from Gorodinsky A, Harris DA. J Cell Biol 1995; 129:619-627. ©1995 The Rockefeller University Press.

C-terminal amino acid, three mannose residues, an unacetylated glucosamine residue, and a phosphatidylinositol molecule. This core is modified to a variable extent by additional ethanolamine and mannose residues, as well as by GalNAc, Gal, and sialic acid moieties, the last of which are not found on other GPI anchors described to date.

Endocytosis of PrP^C

Experiments using transfected cell lines indicate that PrP^C constitutively cycles between the plasma membrane and an early endocytic compartment.[56-58] This conclusion is based on several lines of evidence. First, it is possible to directly measure internalization and recycling of surface PrP^C molecules that have been labeled with membrane-impermeant iodination or biotinylation reagents. Kinetic analysis of these data indicate that PrP^C molecules cycle through cultured neuroblastoma cells with a transit time of ~60 minutes. Second, endocytosis of PrP^C can be visualized by using immunofluorescence microscopy to

track internalization of antibodies that have been bound to PrP^{C} on the cell surface. Third, PrP^{C} is subject to a proteolytic cleavage in its mid-region that is inhibited by lysosomotropic amines and leupeptin, suggesting that it occurs within an endosomal compartment that is acidic and protease-containing. This endocytic recycling pathway is of interest because it may be the route along which certain steps in the conversion of PrP^{C} to PrP^{Sc} take place. In addition, this pathway suggests that one physiological function of PrP^{C} might be to facilitate uptake of an as yet unidentified extracellular ligand, by analogy with receptors responsible for uptake of transferrin and low density lipoprotein.

Clathrin-Coated Pits

We have found that clathrin-coated pits and vesicles are the morphological structures responsible for endocytic uptake of PrP^{C}.[25] This conclusion is based on immunogold localization of PrP^{C} in these organelles by electron microscopy, inhibition of PrP^{C} internalization by incubation of cells in hypertonic sucrose which disrupts clathrin lattices, and detection of PrP^{C} in purified preparations of coated vesicles from brain. We hypothesize that PrP^{C} molecules enter clathrin-coated pits after exiting detergent-resistant raft domains on the plasma membrane.[35]

The involvement of clathrin-coated pits in endocytosis of PrP^{C} is surprising, since GPI-anchored proteins like PrP^{C} lack a cytoplasmic domain that could interact directly with the intracellular components of coated pits such as clathrin and adapter proteins. Indeed, it has been speculated that other GPI-anchored proteins are excluded from coated pits, and are internalized via caveolae.[59] To explain this paradox, we have postulated the existence of a "PrP^{C} receptor", a transmembrane protein that has a coated-pit localization signal in its cytoplasmic domain, and whose extracellular domain binds the N-terminal portion of PrP^{C} (Fig. 6.4).[58] This model is consistent with our observation that deletions within the N-terminal domain of PrP^{C} diminish internalization of the protein measured biochemically, and reduce its concentration in coated pits as determined morphometrically.[60] We presume that these deletions reduce the affinity of PrP^{C} for the endocytic receptor. Identification of this receptor is now of great importance, as it is likely to provide clues to the normal function of PrP^{C}, and may allow design of therapeutic strategies for blocking endocytic uptake of PrP^{C}, thereby inhibiting prion replication. Such a receptor might also be involved in the conversion of PrP^{C} into PrP^{Sc}, or in the initial uptake of PrP^{Sc}-containing prion particles into cells.

Membrane Topology of PrP^{Sc} and the Cleavability of its GPI Anchor

Although both isoforms contain a GPI anchor, PrP^{Sc}, in contrast to PrP^{C}, cannot be released by PIPLC from membranes of scrapie-infected cells and brain.[53,61] The same is true for PrP molecules carrying mutations linked to familial CJD, GSS and FFI, which acquire the biochemical attributes of PrP^{Sc} when expressed in transfected CHO cells[54,61] (Fig. 6.5) and in the brains of transgenic mice (Chiesa and Harris, unpublished data). The explanation for this unusual property has been uncertain, although it is likely to reflect an important feature of the PrP^{Sc} molecule and could play a role in the disease process.

Several hypotheses can be envisioned to explain lack of PIPLC release (Fig. 6.6). First, it is possible that the PrP^{Sc} molecule has a second mechanism of membrane attachment in addition to the GPI anchor, such as integration of the PrP polypeptide chain into the lipid bilayer or tight binding to another membrane protein. In favor of this explanation, we find that ^{3}H-fatty acid label incorporated into mutant PrP is partially removed upon treatment of intact cells with PIPLC, even though virtually all of the protein remains tightly associated with the cell membrane.[54] Alternatively, the GPI anchor of PrP^{Sc} could be physically shielded from the phospholipase, either by aggregation of the protein or by intrinsic conformational

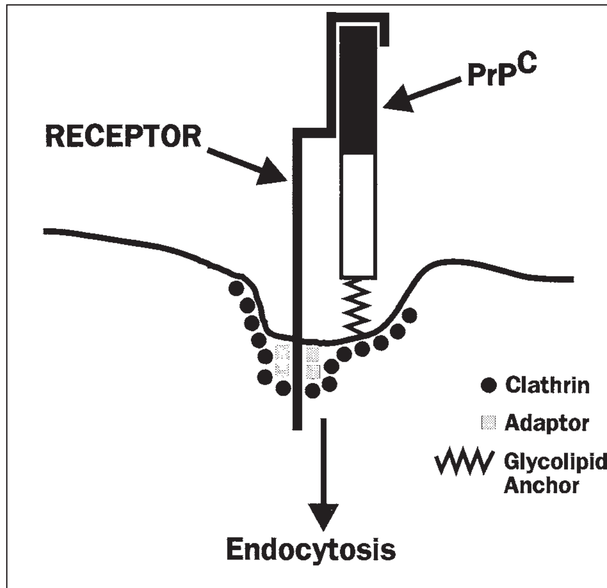

Fig. 6.4. Model for the function of a putative PrP^{C} receptor. Reprinted with permission from Harris DA, Gorodinsky A, Lehmann S et al. Curr Top Microbiol Immunol 1996; 207:77-93. ©1996 Springer-Verlag.

features of the polypeptide chain. This mechanism may also be operative, based on our observation that some mutant PrP molecules remain resistant to PIPLC cleavage after extraction into Triton lysis buffer, but not after boiling in SDS, which would unfold the polypeptide chain and disrupt any intermolecular aggregates (Narwa and Harris, unpublished data). Clearly, further work will be needed to determine the relative contributions of these different models.

Whatever the underlying mechanism, PIPLC resistance is a property that develops very early in the biosynthesis of mutant PrPs.[62] The mutant proteins acquire this property within a 5-10 minute pulse-labeling period, as assayed by partitioning in Triton X-114, or binding to phenyl-Sepharose. This suggests that PIPLC resistance marks a very early step in the conversion of mutant PrPs to a PrP^{Sc} state, one that is likely to take place in the endoplasmic reticulum (ER). The involvement of the ER in the formation of PrP^{Sc} makes sense theoretically, since this is the compartment where proteins fold, usually in association with resident chaperone proteins. It is attractive to think that acquisition of PIPLC resistance reflects the fundamental conformational transition of mutant PrP to the PrP^{Sc} state, and that ER chaperones play an important role in this conversion.

Role of Rafts in the Formation of PrP^{Sc}

There are several pieces of evidence suggesting that detergent-resistant raft domains play a role in at least some steps in PrP^{Sc} generation. First, PrP^{Sc} as well as PrP^{C} are present in Triton-insoluble complexes prepared from scrapie-infected cultured cells and brain.[38] Second, depletion of cellular cholesterol, which disrupts rafts, inhibits formation of PrP^{Sc}.[63] Third, engineered forms of PrP that contain a transmembrane segment instead of a GPI anchor, and which are presumably excluded from rafts, are poor substrates for conversion to PrP^{Sc}.[64]

Our own results with mutant PrPs shed further light on the role of rafts in PrP^{Sc} formation. Insolubility in nondenaturing detergents and partial resistance to protease digestion are two operational characteristics which are commonly used to recognize PrP^{Sc}.

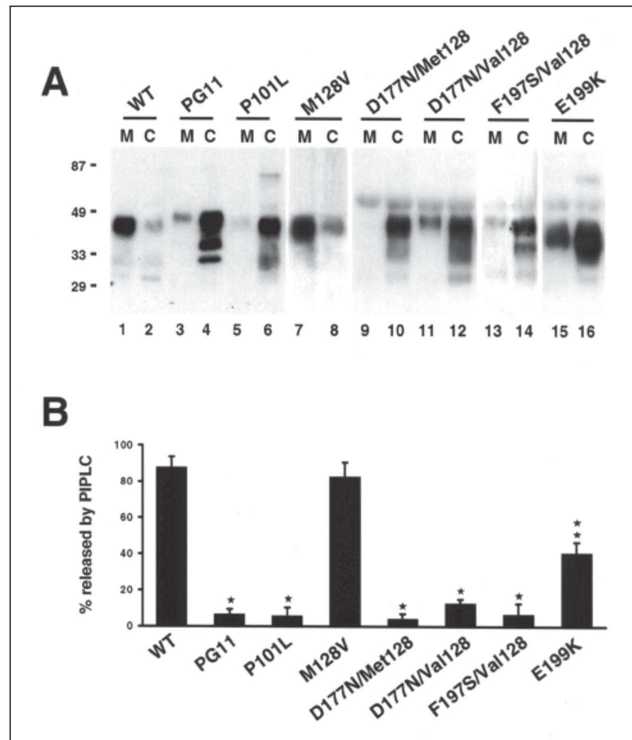

Fig. 6.5. PrP molecules carrying disease-related mutations are not released from the cell surface by PIPLC. (A) CHO cells were stably transfected to express wild type (WT) mouse PrP, or mutant mouse PrPs whose human homologues are associated with familial CJD, GSS or FFI. Cells were biotinylated with the membrane-impermeant reagent sulfo-biotin-X-NHS, and were then incubated with PIPLC prior to lysis. PrP in the PIPLC incubation media (M lanes) and cell lysates (C lanes) was immunoprecipitated, separated by SDS-PAGE, and visualized by developing blots of the gels with HRP-streptavidin and enhanced chemiluminescence. M128V is the mouse homologue of a non-pathogenic polymorphism in human PrP. (B) The amount of PrP released by PIPLC was plotted as a percentage of the total amount of PrP (medium + cell lysate). Each bar represents the mean ± SD. Values that are significantly different from wild type PrP by t-test are indicated by single ($p<0.001$) and double ($p<0.01$) asterisks. Reprinted with permission from Harris DA, Lehmann S. In: Iqbal K, Winblad B, Nishimura T et al., eds. Alzheimer's Disease: Biology, Diagnosis and Therapeutics. 1997:631-643. ©John Wiley and Sons Ltd.

We find that PIPLC treatment of intact cells inhibits the acquisition of detergent insolubility by mutant PrPs, presumably because at least some of the molecules have had their anchors cleaved, and although they are still present on the cell surface, they are no longer present in raft domains (Daude and Harris, unpublished data). Similarly, chimeric forms of mutant PrP that contain a transmembrane domain without a GPI anchor never become detergent insoluble or protease resistant. Taken together, these results argue that detergent insolubility and protease resistance are attributes acquired in raft domains on the plasma membrane.

This interpretation accords with the results of kinetic experiments which demonstrate that detergent insolubility and protease resistance are acquired relatively late (>30 minutes)

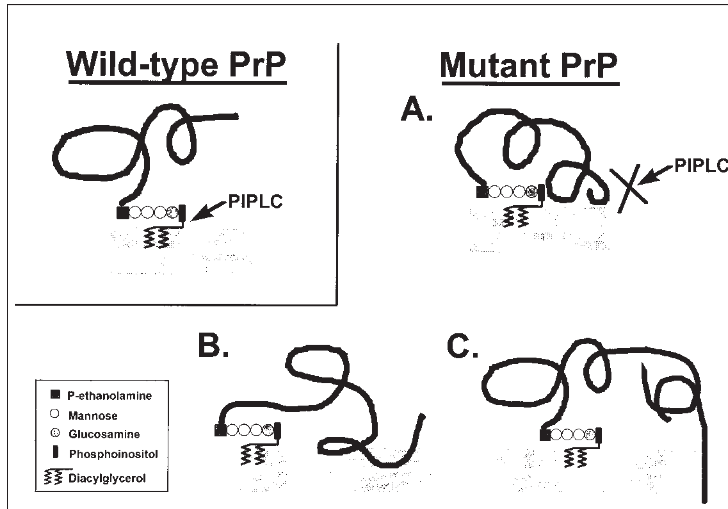

Fig. 6.6. Models to explain why PIPLC releases wild-type but not mutant PrP from cell membranes. Wild type PrP is anchored to the membrane exclusively by its GPI anchor, the core structure of which is illustrated, along with the site cleaved by PIPLC. The polypeptide chain of mutant PrP may adopt a conformation that physically blocks access of PIPLC to the anchor (A). Alternatively, the polypeptide chain of the mutant protein may be integrated into the lipid bilayer (B), or bind tightly to another membrane-associated molecule (C); in these last two cases, the PrP molecule would be retained on the membrane even after the anchor is cleaved.

after synthesis of mutant PrP molecules.[62,65] By this time, the proteins would have already reached the cell surface or been delivered to endocytic compartments. In contrast, acquisition of PIPLC resistance is a much earlier step in PrP^{Sc} formation, one that takes place in the ER and which therefore does not involve rafts (which are thought to be assembled in the trans-Golgi network). It is attractive to speculate that rafts may facilitate formation of a detergent-insoluble and protease-resistant form of PrP^{Sc} by forcing the protein molecules together in a circumscribed area of the membrane, thereby favoring aggregation. Figure 6.7 summarizes how mutant PrPs are converted to a PrP^{Sc} state in a stepwise manner as they traverse different cellular compartments.

Summary

GPI-anchored proteins are numerous and abundant in the nervous system, playing roles in a variety of physiological phenomena including growth factor signaling, axonal outgrowth and fasciculation, synaptic development and plasticity, polarized sorting, and possibly myelination. In addition, at least one important class of neurodegenerative disorders is caused by alterations in the conformation of a highly unusual GPI-anchored protein, PrP. The GPI anchor of PrP is an important factor in its mechanism of endocytic trafficking and its localization in detergent-insoluble membrane domains where some steps in formation of PrP^{Sc} take place. In addition, resistance of the anchor to enzymatic cleavage turns out to be a very early marker of conversion to the PrP^{Sc} state. Although broad generalizations about the role of the GPI-anchored proteins in the nervous system are not possible, additional investigations will undoubtedly uncover new ways in which the anchor figures in the biological function of particular proteins.

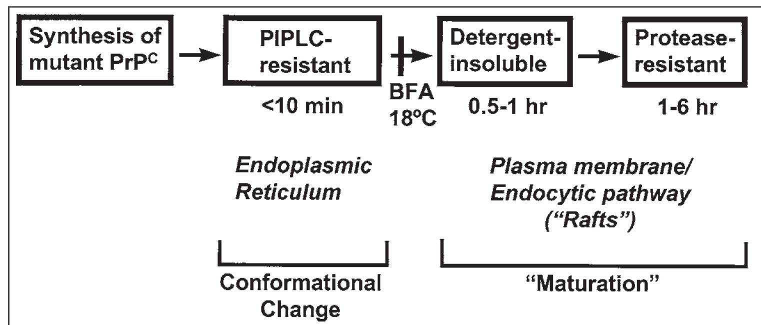

Fig. 6.7. A scheme for transformation of mutant PrPs to a PrP^{Sc} state. Mutant PrPs are initially synthesized in the PrP^{C} state, and acquire PrP^{Sc} properties in a stepwise fashion as they pass through the endoplasmic reticulum and arrive at the plasma membrane. PIPLC resistance reflects folding of the polypeptide chain into the PrP^{Sc} conformation, while detergent insolubility and protease resistance result from subsequent intermolecular aggregation ("maturation"). The times given underneath the boxes indicate when after pulse-labeling the corresponding property is detected. Addition of Brefeldin A (BFA) to cells or incubation at 18°C, treatments which block movement of proteins beyond the Golgi apparatus, inhibit acquisition of detergent insolubility and protease resistance but not PIPLC resistance. Adapted from Daude N, Lehmann S, Harris DA. J Biol Chem 1997; 272:11604-11612. ©1997 The American Society for Biochemistry and Molecular Biology, Inc.

Acknowledgments

Work in the author's laboratory is supported by grants from the National Institutes of Health (AG12925 and NS35496).

References

1. Lin L-F, Doherty DH, Lile JD et al. GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 1993; 269:1130-1132.
2. Kotzbauer PT, Lampe PA, Heuckeroth RO et al. Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature 1996; 384:467-470.
3. Milbrandt J, de Sauvage J, Fahrner JJ et al. Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron 1998; 20:245-253.
4. Treanor JJS, Goodman L, Desauvage F et al. Characterization of a multicomponent receptor for GDNF. Nature 1996; 382:80-83.
5. Klein RD, Sherman D, Ho WH et al. A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature 1997; 387:717-721.
6. Creedon DJ, Tansey MG, Baloh RH et al. Neurturin shares receptors and signal transduction pathways with glial cell line-derived neurotrophic factor in sympathetic neurons. Proc Natl Acad Sci USA 1997; 94:7018-7023.
7. Jing SQ, Wen DZ, Yu YB et al. GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF. Cell 1996; 85:1113-1124.
8. Jing SQ, Yu YB, Fang M et al. GFRα-2 and GFRα-3 are two new receptors for ligands of the GDNF family. J Biol Chem 1997; 272:33111-33117.
9. Lin LF, Mismer D, Lile JD et al. Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). Science 1989; 246:1023-1025.
10. Ip NY, Nye SH, Boulton TG et al. CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. Cell 1992; 69:1121-1132.

11. Taniguchi T. Cytokine signaling through nonreceptor protein tyrosine kinases. Science 1995; 268:251-255.
12. Stahl N, Yancopoulos GD. The alphas, betas, and kinases of cytokine receptor complexes. Cell 1993; 74:587-590.
13. Massagué J. Crossing receptor boundaries. Nature 1996; 382:29-30.
14. Davis S, Aldrich TH, Ip NY et al. Released form of CNTF receptor α component as a soluble mediator of CNTF responses. Science 1993; 259:1736-1739.
15. Walsh FS, Doherty P. Glycosylphosphatidylinositol anchored recognition molecules that function in axonal fasciculation, growth and guidance in the nervous system. Cell Biol Intl Rep 1991; 15:1151-1166.
16. Brümmendorf T, Rathjen FG. Structure/function relationships of axon-associated adhesion receptors of the immunoglobulin superfamily. Curr Op Neurobiol 1996; 6:584-593.
17. Faivre-Sarrailh C, Rougon G. Axonal molecules of the immunoglobulin superfamily bearing a GPI anchor: Their role in controlling neurite outgrowth. Mol Cell Neurosci 1997; 9:109-115.
18. Durbec P, Gennarini G, Goridis C et al. A soluble form of the F3 neuronal adhesion molecule promotes neurite outgrowth. J Cell Biol 1992; 117:877-887.
19. Lierheimer R, Kunz B, Vogt L et al. The neuronal cell-adhesion molecule axonin-1 is specifically released by an endogenous glycosylphosphatidylinositol-specific phospholipase. Eur J Biochem 1997; 243:502-510.
20. Buttiglione M, Revest JM, Rougon G et al. F3 neuronal adhesion molecule controls outgrowth and fasciculation of cerebellar granule cell neurites: A cell-type-specific effect mediated by the Ig-like domains. Mol Cell Neurosci 1996; 8:53-69.
21. Martin KC, Kandel ER. Cell adhesion molecules, CREB, and the formation of new synaptic connections. Neuron 1996; 17:567-570.
22. Vogt L, Giger RJ, Ziegler U et al. Continuous renewal of the axonal pathway sensor apparatus by insertion of new sensor molecules into the growth cone membrane. Curr Biol 1996; 6:1153-1158.
23. Harel R, Futerman AH. A newly-synthesized GPI-anchored protein, TAG-1/axonin-1, is inserted into axonal membranes along the entire length of the axon and not exclusively at the growth cone. Brain Res 1996; 712:345-348.
24. Faivre-Sarrailh C, Gennarini G, Goridis C et al. F3/F11 cell surface molecule expression in the developing mouse cerebellum is polarized at synaptic sites and within granule cells. J Neurosci 1992; 12:257-267.
25. Shyng SL, Heuser JE, Harris DA. A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits. J Cell Biol 1994; 125:1239-1250.
26. Glenney JR. Tyrosine phosphorylation of a 22-kDa protein is correlated with transformation by Rous sarcoma virus. J Biol Chem 1989; 264:20163-20166.
27. Glenney JR. The sequence of human caveolin reveals identity with VIP21, a component of transport vesicles. FEBS Lett 1992; 314:45-48.
28. Lisanti MP, Scherer PE, Vidugiriene J et al. Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. J Cell Biol 1994; 126:111-126.
29. Olive S, Dubois C, Schachner M et al. The F3 neuronal glycosylphosphatidylinositol-linked molecule is localized to glycolipid-enriched membrane subdomains and interacts with L1 and fyn kinase in cerebellum. J Neurochem 1995; 65:2307-2317.
30. Scherer PE, Okamoto T, Chun MY et al. Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. Proc Natl Acad Sci USA 1996; 93:131-135.
31. Tang ZL, Scherer PE, Okamoto T et al. Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. J Biol Chem 1996; 271:2255-2261.
32. Song KS, Scherer PE, Tang ZL et al. Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells: Caveolin-3 is a component of the sarcolemma and cofractionates with dystrophin and dystrophin-associated glycoproteins. J Biol Chem 1996; 271:15160-15165.

33. Scherer PE, Lewis RY, Volonte D et al. Cell-type and tissue-specific expression of caveolin-2: Caveolins 1 and 2 colocalize and form a stable hetero-oligomeric complex in vivo. J Biol Chem 1997; 272:29337-29346.
34. Cameron PL, Ruffin JW, Bollag R et al. Identification of caveolin and caveolin-related proteins in the brain. J Neurosci 1997; 17:9520-9535.
35. Gorodinsky A, Harris DA. Glycolipid-anchored proteins in neuroblastoma cells form detergent-resistant complexes without caveolin. J Cell Biol 1995; 129:619-627.
36. Bouillot C, Prochiantz A, Rougon G et al. Axonal amyloid precursor protein expressed by neurons in vitro is present in a membrane fraction with caveolae-like properties. J Biol Chem 1996; 271:7640-7644.
37. Naslavsky N, Stein R, Yanai A et al. Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform. J Biol Chem 1996; 272:6324-6331.
38. Vey M, Pilkuhn S, Wille H et al. Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. Proc Natl Acad Sci USA 1996; 93:14945-14949.
39. Zisch AH, D'Alessandri L, Amrein K et al. The glypiated neuronal cell adhesion molecule contactin/F11 complexes with src-family protein tyrosine kinase Fyn. Mol Cell Neurosci 1995; 6:263-279.
40. Cervello M, Matranga V, Durbec P et al. The GPI-anchored adhesion molecule F3 induces tyrosine phosphorylation: Involvement of the FNIII repeats. J Cell Sci 1996; 109:699-704.
41. Kunz S, Ziegler U, Kunz B et al. Intracellular signaling is changed after clustering of the neural cell adhesion molecules Axonin-1 and NgCAM during neurite fasciculation. J Cell Biol 1996; 135:253-267.
42. Wu C, Butz S, Ying Y-S et al. Tyrosine kinase receptors concentrated in caveolae-like domains from neuronal plasma membrane. J Biol Chem 1997; 272:3554-3559.
43. Simons K, Ikonen E. Functional rafts in cell membranes. Nature 1997; 387:569-572.
44. Dotti CG, Simons K. Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. Cell 1990; 62:63-72.
45. Dotti CG, Parton RG, Simons S. Polarized sorting of glypiated proteins in hippocampal neurons. Nature 1991; 349:158-161.
46. Kobayashi T, Storrie B, Simons K et al. A functional barrier to movement of lipids in polarized neurons. Nature 1992; 359:647-650.
47. Winckler B, Poo M-M. No diffusion barrier at axon hillock. Nature 1996; 379:213.
48. Krämer E-M, Koch T, Niehaus A et al. Oligodendrocytes direct glycosyl phosphatidylinositol-anchored proteins to the myelin sheath in glycosphingolipid-rich complexes. J Biol Chem 1997; 272:8937-8945.
49. de Vries H, Schrage C, Hoekstra D. An apical-type trafficking pathway is present in cultured oligodendrocytes but the sphingolipid-enriched myelin membrane is the target of a basolateral-type pathway. Mol Biol Cell 1998; 9:599-609.
50. Will RG, Ironside JW, Zeidler M et al. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 1996; 347:921-925.
51. Prusiner SB. Prions. In: Fields BN, Knipe DM, Howley PM, eds. Virology. Philadelphia: Lippincott-Raven Press., 1996:2901-2950.
52. Stahl N, Borchelt DR, Hsiao K et al. Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell 1987; 51:229-249.
53. Stahl N, Borchelt DR, Prusiner SB. Differential release of cellular and scrapie prion proteins from cellular membranes by phosphatidylinositol-specific phospholipase C. Biochemistry 1990; 29:5405-5412.
54. Lehmann S, Harris DA. A mutant prion protein displays an aberrant membrane association when expressed in cultured cells. J Biol Chem 1995; 270:24589-24597.
55. Stahl N, Baldwin MA, Hecker R et al. Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. Biochemistry 1992; 31:5043-5053.

56. Shyng SL, Huber MT, Harris DA. A prion protein cycles between the cell surface and an endocytic compartment in cultured neuroblastoma cells. J Biol Chem 1993; 268:15922-15928.
57. Harris DA, Huber MT, van Dijken P et al. Processing of a cellular prion protein: Identification of N- and C-terminal cleavage sites. Biochemistry 1993; 32:1009-1016.
58. Harris DA, Gorodinsky A, Lehmann S et al. Cell biology of the prion protein. Curr Top Microbiol Immunol 1996; 207:77-93.
59. Anderson RGW. Plasmalemmal caveolae and GPI-anchored membrane proteins. Curr Op Cell Biol 1993; 5:647-652.
60. Shyng SL, Moulder KL, Lesko A et al. The N-terminal domain of a glycolipid-anchored prion protein is essential for its endocytosis via clathrin-coated pits. J Biol Chem 1995; 270:14793-14800.
61. Lehmann S, Harris DA. Mutant and infectious prion proteins display common biochemical properties in cultured cells. J Biol Chem 1996; 271:1633-1637.
62. Daude N, Lehmann S, Harris DA. Identification of intermediate steps in the conversion of a mutant prion protein to a scrapie-like form in cultured cells. J Biol Chem 1997; 272:11604-11612.
63. Taraboulos A, Scott M, Semenov A et al. Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. J Cell Biol 1995; 129:121-132.
64. Kaneko K, Vey M, Scott M et al. COOH-terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform. Proc Natl Acad Sci USA 1997; 94:2333-2338.
65. Lehmann S, Harris DA. Two mutant prion proteins expressed in cultured cells acquire biochemical properties reminiscent of the scrapie isoform. Proc Natl Acad Sci USA 1996; 93:5610-5614.

CHAPTER 7

GPI-Anchored Complement Regulatory Molecules

Carmen W. van den Berg and B. Paul Morgan

The Complement System

The complement (C) system is a major component of the humoral defense system and plays an important role in inflammation. Its primary roles are opsonization of bacteria, production of chemotactic and anaphylactic peptides (all aiding the killing of bacteria by phagocytes) and direct cell killing through formation of a transmembrane pore. The C system consists of approximately 20 plasma proteins and can be activated through 3 different pathways: the classical pathway, the lectin pathway and the alternative pathway, all resulting in a cascade of enzymatic activities (Fig. 7.1). The classical pathway (CP) is activated through binding of C1q to antibodies. Subsequent activation of C1r and C1s leads to activation of C4, C2 and C3. The lectin pathway (LP) is activated after binding of mannan binding lectin (MBL) to mannose-containing proteins on bacteria. This results in the activation of MASP1 and MASP2 followed by C4, C2 and C3. The alternative pathway (AP) is initiated when spontaneously activated C3 binds to the surface of a pathogen. Factor B becomes bound to this bound C3 and is then activated by factor D. This pathway also functions as an amplification loop of the classical pathway. All pathways result in formation of the C3/C5 convertases, which then activate the terminal pathway. Activation of the terminal pathway by cleavage of C5 is the last enzymatic reaction and results in assembly of a multimeric membrane-perturbing complex consisting of C5b, C6, C7, C8 and up to 18 molecules of C9, the membrane attack complex (MAC). Activation of the C system leads to release of protein fragments with anaphylactic (C3a, C4a, C5a) and chemotactic (C5a) properties, thereby attracting phagocytes. Deposition of C3b fragments leads to opsonization and ingestion by phagocytes, and MAC formation leads to membrane perturbations which can cause lysis. Because of the powerful amplification of the cascade and the potentially hazardous side effects, C activation has to be kept under tight control. This regulation is accomplished by a number of fluid phase and membrane bound regulators which act at different stages of activation. The first evidence for the existence of membrane bound regulators of C came from studies using extracts of human erythrocytes.[1] These extracts protected sheep erythrocytes against lysis by guinea pig complement. Several membrane bound inhibitors have now been identified, which act either on the C3/C5 convertase or on the MAC (Fig. 7.1). These inhibitors are of particular importance to protect cells against damage by host complement.

The membrane bound inhibitors acting on the C3/C5 convertase are decay accelerating factor (DAF/CD55), membrane cofactor protein (MCP/CD46) and complement receptor 1

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

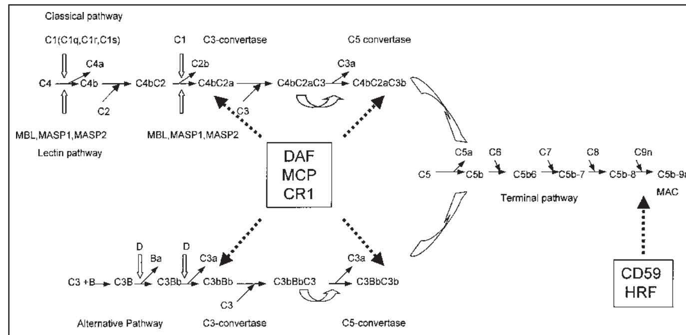

Fig. 7.1. The complement system, showing the division into four interacting pathways. Enzymatic cleavages are represented by open arrows, stages of inhibition are represented by hatched arrows.

(CR1/CD35). These proteins inhibit amplification in the activation pathways and initiation of the terminal pathway either by enhancing dissociation of the convertase (DAF, CR1) or by acting as a cofactor (MCP, CR1) for the serum protease factor I in the cleavage of C3. CD59 and homologous restriction factor (HRF) both act as regulators of the MAC by binding to C8 and C9 and so preventing pore formation. DAF, CD59 and HRF are all anchored to the membrane by a glycosylphosphatidylinositol (GPI) anchor and hence are the focus of this chapter, while MCP and CR1 are transmembrane-anchored proteins (Fig. 7.2). The GPI-anchors of DAF and CD59 are considered to give these proteins more lateral mobility and enhance the ability of these molecules to protect the cells against damage by C. For the purposes of this book, we will limit further discussion to the GPI-anchored C-regulatory proteins DAF, CD59 and HRF. It must, however, be understood that these proteins work in concert with the transmembrane C-regulatory proteins to protect the cells.

Decay Accelerating Factor (DAF, CD55)

Function

Regulation of C activation by DAF occurs at the stage of the C3/C5 convertase (Fig. 7.1). Inhibition of the C3 convertase by DAF prevents amplification of the C-cascade and inhibits release of anaphylatoxins and chemotaxins generated by C3 and C5 activation and activation of the terminal lytic pathway.

DAF functions as a regulator of C-activation by enhancing the dissociation of C2a and Bb from the C4b2a and C3bBb C3-convertase complexes[2] (Fig. 7.3). DAF does not prevent binding of C2 and factor B to the target. Nephritic factors, which are antibodies in serum of patients with rheumatoid arthritis with specificity for the C3-convertase, are able to stabilize these complexes and prevent decay by DAF.[3] DAF, unlike the other membrane-bound C3-convertase regulators CR1 and MCP, has no cofactor activity for the cleavage of C3b or C4b by factor I.[4] The active sites of DAF reside within SCR-2 and SCR-3 for the CP C3-convertase, while AP C3-convertase regulatory function resides within SCR-2, -3, and -4.[5] DAF can reincorporate into cells through its GPI anchor and in standard assays using sheep erythrocytes as target, the activity of DAF is due to reincorporated protein.

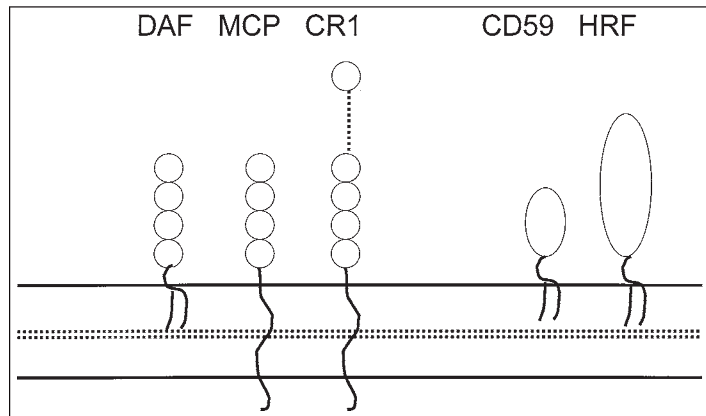

Fig. 7.2. Membrane-bound regulators of complement with different modes of anchorage. GPI-anchored: DAF, CD59 and HRF, and transmembrane anchored: MCP and CR1.

Structure

The membrane-bound regulators of the C3-convertase are structurally related. DAF, MCP and CR1 and also the membrane-bound complement receptor 2 (CR2) and the fluid phase inhibitors factor H and C4-binding protein all originate from a common ancestral gene. These proteins form a family termed the regulators of complement activation (RCA) and are all built up of homologous domains, the short consensus repeats (SCR). Each SCR unit consists of approximately 60 amino acids characterized by four conserved Cys residues and several other highly conserved residues which include Pro, Trp, Tyr, Phe and Gly. From sequence analyses of enzymatic digests of DAF, the disulfide bonds in the 4 SCR units of DAF were found to be between the first and third and between the second and fourth half-cystines within each SCR unit.[6] The RCA proteins are built of between 4 (DAF, MCP) and 30 SCRs. The genes for all these proteins are closely linked and localized to the long arm of chromosome 1, band q32.[7,8]

The DAF gene was originally cloned from cDNA libraries from Hela and HL-60 cells.[9,10] In the human genome there is only one copy of the DAF gene,[11] which consists of 11 exons. Two or three major DAF mRNA species have been identified from Northern blot analysis.[9,10] The first exon encodes the 5' signal peptide, containing a 34 amino acid leader sequence, which is removed upon translocation in the endoplasmic reticulum. Exons 2-6 encode the first 250 amino acids of the mature protein containing the 4 SCRs; exons 7-9 encode amino acid 253-322, the Ser/Thr rich region; and exon 11 encodes the COOH terminus of the spliced and unspliced transcript.[12] Exon 10 is spliced out of the predominant GPI-anchored form of DAF. The last 28 amino acids of the translated DAF are removed upon addition of the GPI anchor at Ser319.[13]

The ability of purified DAF to bind and insert into sheep erythrocytes and protect against C-damage indicated that DAF was anchored to the membrane by a GPI anchor.[14] Further proof was provided by the demonstration that DAF could be cleaved from the membrane by phosphatidylinositol-specific phospholipase C.[15,16] While DAF on most cells has a PIPLC-sensitive GPI anchor, on erythrocytes the anchor contains an additional ester-linked fatty acid on inositol, which makes it resistant to PIPLC.[16]

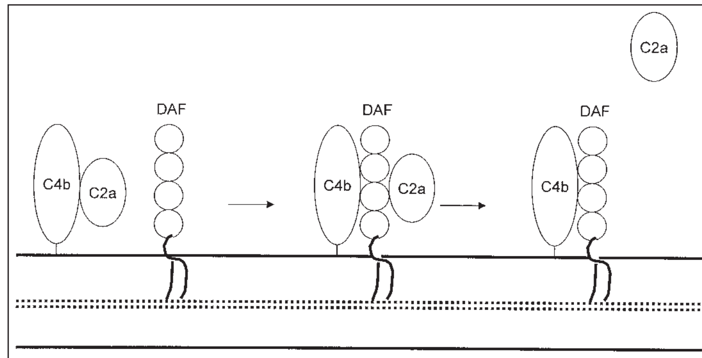

Fig. 7.3. Mechanism of action of DAF. DAF binds C4bC2a and dissociates this complex, thereby inactivating the C3 cleaving enzyme; DAF dissociates the C3bBb complex in a similar manner.

DAF is expressed on cells as a single chain protein with a Mr of 70 kDa, but the calculated Mr of the native protein after removal of the N- and C-terminal signal sequences is about 46 kDa. Endo F decreases the Mr of DAF by about 3 kDa, suggesting that the one *N*-terminal glycosylation site at Asn61 is occupied. The Mr of DAF is decreased by 18 kDa after neuraminidase treatment, indicating that it is heavily sialylated, and by a further 8 kDa after endo-α-N-acetylgalactosylaminidase treatment. Most of the sialic acid is on the *O*-linked side chains.[17] Biosynthetic studies showed that the earliest DAF precursor had a Mr of 43 kDa, and was rapidly converted into a 46 kDa molecule.[17] The *O*-linked carbohydrate side chains are not necessary for DAF function but make DAF resistant to proteolytic degradation.[18]

Alternative forms of DAF have been reported. On erythrocytes, 55 kDa and 63 kDa forms were found which are probably the consequence of proteolytic degradation,[19] while 100 and 140 kDa erythrocyte forms were probably dimers of native DAF.[20,21] A DAF species with a Mr of 44-54 kDa has been found on sperm.[22] Carbohydrate analysis of sperm DAF indicated that it contains non-sialylated *N*- and *O*-linked sugars. The absence of mature oligosaccharides on this protein appears to account for the difference in molecular mass between sperm DAF and the 70 kDa DAF expressed on other human tissues.[23]

Distribution

DAF is very broadly distributed; in the circulation it is present on erythrocytes, granulocytes, monocytes and lymphocytes.[24,25] A subpopulation of lymphocytes, including NK cells, are DAF negative.[25] DAF expressed on leukocytes has a higher Mr than erythrocyte DAF, due to a difference in glycosylation.[17,25] DAF is also expressed in a wide variety of tissues including vascular endothelium, endocardium, uterus, synovium and urinary and gastrointestinal tracts and exocrine glands. On polarized cells, DAF, like other GPI-anchored molecules, is targeted to the apical cell surface.[26] Secreted forms of DAF have been detected in plasma, tears, saliva, synovial fluid, cerebrospinal fluid, urine and seminal fluid.[27] DAF from urine is not able to incorporate into membranes, which suggests that it has lost its anchor.[27]

Regulation of Expression

At sites of inflammation protection against C-mediated damage is essential. Upregulation of expression of C-regulatory molecules would give cells enhanced protection against C-activation products generated at such sites. Agents produced during inflammation, including products of C-activation, have been investigated for their ability to influence the level of expression of DAF. In the synovial lining cell layer and on vascular endothelial cells of synovial tissue, a high expression of DAF has been observed both in rheumatoid arthritis and in osteoarthritis. A significant correlation was found between the expression of DAF and of HLA-DR in the lining layer, suggesting that DAF may be induced during a local inflammatory response.[28] An upregulation of DAF was also observed in diseased cartilage from arthritic joints and in IL-1-treated cartilage compared to normal cartilage.[29] A direct effect of C activation on DAF expression was observed in mesangial cells. Immune complex-induced C-activation increased DAF expression on these cells; this was a consequence of MAC formation on the cells, as no upregulation was observed in the absence of C5 or C8.[30] Upregulation of DAF at sites of inflammation is not always observed. In patients with cutaneous immune complex vasculitis, the endothelial cells of upper dermal vessels in vasculitic lesions were almost completely devoid of DAF, while abundant expression was observed in healthy skin of the same patients. The mechanism of this downregulation or loss is not known, but removal by shedding of membrane including DAF or enzymatic release of DAF may account for low level of expression in the lesion.[31]

Activation of endothelial cells with various concentrations of TNF-α, IL-1β, and especially IL-4 increased expression of DAF.[32] In other studies, stimulation of HUVEC with various cytokines including TNF, IL-1, and IFN-γ did not alter DAF levels, but wheat germ agglutinin and the lectins ConA and PHA increased DAF levels two- to five-fold when incubated with HUVEC for 12 to 24 h.[33] TNF-α and IL-4 also induced a marked increase in DAF mRNA and protein expression in HT-29, T84 and Caco-2 cells. But IL-1β induced only a weak upregulation, and IL-6, IL-8, IL-10, and IFN-γ had no effect.[34,35] Phorbol esters (PMA) induced an increase in expression of DAF in human endothelial cells and the HOG cell line,[36,37] no change in cell surface expression on the erythroblast cell line K562 was observed.[38] These data show that regulation of DAF expression may differ between cells. While in most instances induction of DAF expression required de novo synthesis and thus took hours to occur, an upregulation of DAF cell surface expression was observed within minutes after stimulation of neutrophils. This upregulation of cell surface expression was shown to be due to release from intracellular stores.[39]

Little is known about the regulation of induction of DAF. Nerve growth factor (NGF) treatment of adult dorsal root ganglion (DRG) neurons induced DAF expression, and this was dependent on sequences between -206 and -77 relative to the DAF transcriptional start site.[40] Analysis of the promoter region of DAF revealed a series of transcription start sites in a 10 nucleotide region located 86 nucleotides upstream from the ATG (the first of these start sites is numbered +1). Major enhancer activity was demonstrated between -206 and -77, and between -77 and -54 (containing cAMP responsive element and AP-1 binding site), and between -54 and -34 (containing an Sp1 binding site). The identification of transcription start sites and enhancer regions in the DAF gene are important for studies of the mechanisms whereby cytokines and other factors may modulate DAF expression.[41]

Deficiencies

Deficiency of DAF has been identified in patients with antibodies recognizing the Cromer-related antigens. These antibodies recognized a 70 kDa protein in normal erythrocytes, which was identified as DAF.[42,43] Patients lacking the Cromer antigens were designated as having the Inab phenotype. Patients do not suffer from hemolysis as might

have been predicted, but in in vitro assays significantly more C3 was deposited on cells after C-activation.[43] The Inab phenotype is caused by a point mutation in the DAF gene resulting in introduction of a stop codon.[44,45] Inab erythrocytes are not more sensitive to lysis by acidified serum in the Hamm test.[43,46]

In paroxysmal nocturnal hemoglobinuria (PNH) an autosomal mutation in the bone marrow gives rise to a clonal deficiency of all GPI-anchored molecules, including DAF and CD59, on erythrocytes, monocytes, granulocytes, platelets and sometimes lymphocytes.[47] The deficiency is caused by a mutation in the PIG-A gene, which causes a deficiency of a component of glycosylphosphatidylinositol biosynthesis with the consequence that no GPI anchor is attached to proteins with the correct signal sequence.[48] The absence of both DAF and CD59 makes the cells extremely sensitive to C-mediated lysis, including by acidified serum in the Hamm test.[49] Patients suffering from PNH have recurring episodes of intravascular complement-mediated hemolysis and venous thrombosis.

DAF in Other Mammalian Species

DAF was first isolated from guinea pig erythrocytes.[50] Guinea pig DAF was characterized as a single polypeptide chain with a Mr of 60 kDa. Isolation of guinea pig DAF cDNA clones from a spleen library identified six different classes. All encoded the same four SCR domains, with 58% amino acid sequence identity to human DAF. Alternative splicing of two exons generated transmembrane, GPI-anchored, and secreted forms of guinea pig DAF, and differential usage of splice sites generated variable Ser/Thr-rich regions.[51] Mouse DAF purified from mouse erythrocytes had a Mr of 60 kDa and was susceptible to PIPLC treatment.[52] Cloning of mouse DAF identified two classes of cDNA clones. Rather than representing alternately spliced mRNAs, these were derived from two separate but closely linked genes. While one cDNA encoded a GPI-anchored form, the other encoded a trans-membrane-anchored form.[53] Chromosome localization studies mapped the mouse DAF genes to chromosome 1, where they segregated with the C4-binding protein gene.[53] A GPI-anchored 66 kDa molecule with decay accelerating activity has also been isolated from rabbit erythrocytes. No sequence of this rabbit DAF has been published.[54]

Parasites

An important strategy used by parasites to evade the C system is the expression of C-inhibitory molecules. For example, a molecule exhibiting DAF-like activity was found in trypomastigotes, the C-resistant stage of *Trypanosma cruzi*, but could not be detected in the C-sensitive epimastigotes.[55] The purified molecule (T-DAF) had a Mr of about 90 kDa,[56] but isolated T-DNA showed only limited homology with human DAF and no SCRs could be identified.[57] Comparison of the partial T-DAF sequence with recently submitted sequences in Genbank databases suggest that T-DAF may be a trans-sialidase and confer its protective effect by transfer of sialic acid, a potent inhibitor of C, to the trypomastigotes. *T. cruzi*-derived trans-sialidases have been been shown to confer resistance on desialylated sheep and human erythrocytes by transfer of sialic acid from serum onto the target cell.[58] Desialylated *T.cruzi* replenish sialic acid from serum and so regain C-resistance.[59] Another molecule, which restricts C-activation by binding C3b and inhibiting C3 convertase formation, has been purified from *T.cruzi*. This molecule, with a Mr of 160 kDa, was cloned and hybridized with human DAF, suggesting sequence similarity.[60] Comparison of the full length coding sequence identified this protein as a member of the FL-160 gene family, a group of trypanosome surface molecules of unknown function.[61] However, again a high degree of homology with trans-sialidases was found, suggesting that this molecule exerts its inhibitory effects in a manner similar to that of T-DAF.

Another way for parasites to evade the immune system is by capturing GPI-linked C-regulatory molecules from the host. Anti-human DAF immunoprecipitates of *Schistosoma mansoni* isolated from primates revealed a 70 kDa molecule which appeared to be DAF acquired from the host.[62] In an in vitro experiment where schistosomula were incubated with normal human erythrocytes, parasites became resistant to C by acquisition of a 70 kDa surface protein which could be immunoprecipitated by anti-DAF antibodies; DAF-deficient erythrocytes did not confer resistance.[63]

Viruses and Virus Infected Cells

Virus infection of cells often changes the expression of cell surface antigens, and viruses, when budding from the cell, often capture part of the host cell membrane. Increasing the level of C-regulatory molecules on the cell surface and capture of C-regulatory molecules by the budding virus is a potential strategy to aid the survival of the virus against lysis by C. Indeed, DAF has been found on primary isolates of HIV virions and blocking of DAF with antibodies made the virions susceptible to C.[64-66] Human cytomegalovirus infection induced an eight-fold increase in DAF expression on fibroblasts and glioblastoma, but no change in DAF expression occurred after infection with HSV-1 or adenovirus.[67] As a consequence of this, CMV virions were protected by DAF captured from the host cell.[68]

Signaling

Like most GPI-anchored molecules, DAF induces cell activation upon crosslinking with antibodies (see also chapter 4). The importance of the GPI anchor in this signaling capacity was demonstrated by replacing the GPI anchor with the TM anchor of MCP. This abrogated the ability of DAF to induce tyrosine phosphorylation and IL-2 production.[69] When anti-DAF immunoprecipitates were prepared from the transfected cells, only the GPI-anchored form of DAF coprecipitated kinase activity. Both $p56^{lck}$ and $p59^{fyn}$ were associated with DAF in DAF-transfected EL-4 cells. The putative transmembrane signal transducing molecule has not yet been identified.

Other Ligands

It has recently been reported that DAF functions as a ligand for the seven-span transmembrane molecule CD97.[70] CD97 is an activation-induced antigen homologous to the secretin receptor superfamily. An anti-DAF mAb recognizing SCR-2 blocked the adhesion between CD97 transfectants and red cells. Erythrocytes from PNH patients or from individuals with the Inab phenotype did not adhere to CD97 transfectants, confirming that DAF was the ligand. The significance of the role of DAF as a ligand for CD97 is not known.

DAF as a Receptor for Microorganisms

Cell surface molecules are often used by bacteria and viruses as receptors to which to adhere, or to enter and infect the host. DAF has been shown to function as a receptor for several viruses and bacteria.

Viruses

Echoviruses are human pathogens belonging to the picornavirus family and have been shown to use DAF to attach to and possibly enter the cell. Anti-DAF monoclonal antibodies or PIPLC treatment prevents attachment of Echoviruses of at least six serotypes to susceptible cells and protects cells from infection. Transfection with DAF of cells that are not normally susceptible to echovirus facilitated binding of the echovirus to the cells.[71] Similar studies showed that DAF also functioned as a major cell attachment receptor for coxsackieviruses B1, B3, and B5.[72] However, expression of human DAF on the surface of nonpermissive murine

fibroblasts led only to virus attachment without subsequent replication, and it was shown that an unidentified 50 kDa cell surface coreceptor is required to facilitate cell entry and subsequent replication.[73]

Enterovirus 70 (EV70) is a recently emerged human pathogen belonging to the family Picornaviridae, it has been demonstrated that this virus also uses DAF as a receptor for cell entrance.[74]

Bacteria

Bacterial adhesins are important virulence factors that allow colonization of the human urogenital tract by *Escherichia coli*. Adhesins of the Dr family have been found to be more frequently expressed in strains associated with symptomatic urinary tract infections. The ligand of the Dr adhesin has been shown to be DAF and binding of Dr occurs to SCR-3 and -4.[75,76] Change of expression of DAF as observed, e.g., on the endometrium during the menstrual cycle may explain the differences in susceptibility to infections observed.[77]

CD59

Of the two known membrane-bound inhibitors of the terminal pathway, CD59 is the best characterized. An inhibitor of the final stage of C-cascade was isolated independently by several different groups in 1988-89.[78] The protein was given several different names including P-18, MACIF,[79] MIRL (membrane inhibitor of reactive lysis),[80] 1F5 antigen and HRF-20 (homologous restriction factor with Mr 20 kDa).[81] Two groups identified the protein, independently of its role in C-regulation, as a widely distributed cell surface antigen recognized by a specific monoclonal antibody (MEM-43 antigen[82]) or as a molecule involved in adhesion of T cells to erythrocytes (H19[83]). Only upon submitting the various monoclonal antibodies to the 4th Leukocyte Workshop was it realized that all these antibodies recognized the same antigen, which was placed in the cluster designated CD59.[78] CD59 is now the generally used name for this inhibitor of C.

Function

CD59 is a powerful inhibitor of the membrane attack complex (MAC) and acts by restricting the binding of C9 to the C5b-8 complex and so preventing pore formation (Fig. 7.4).[84,85] CD59 has binding sites both for C8α and the ‘b’ domain of C9[86] and can, through its GPI anchor, reinsert into membranes and confer resistance to C-mediated lysis upon these cells.[78,87] The first indication that CD59 was GPI-anchored to the membrane was provided by the observation that if cells were washed after incubation with purified protein or erythrocyte extract, the cells were still protected against C.[78,79] Like other GPI-anchored molecules on erythrocytes, CD59 is refractory to PIPLC release due to an extra lipid anchor on inositol. On nucleated cells, about 70% of the CD59 can be removed from the cell surface by PIPLC treatment.

The ability of CD59 to prevent lysis by autologous serum led to the hypothesis that CD59 was responsible for the phenomenon of ‘homologous restriction’. Homologous restriction is the finding that cells are resistant to lysis by complement of the same species. However we have shown in an extensive study that human CD59 is able to inhibit lysis by C8 and C9 from a variety of species and is thus not a ‘homologous restriction factor’.[88] In the same series of studies we showed that pig CD59 is an efficient inhibitor of human C.[89] In these studies the ability to confer resistance by incorporation of human CD59 was shown to be partially dependent on the target cell used. While human CD59 efficiently protected guinea pig and chicken erythrocytes against lysis by human C, sheep erythrocytes were refractory to protection by human CD59 due to the presence of high levels of endogenous CD59.[88] However, some incompatibility between CD59 and C8/C9 from different species

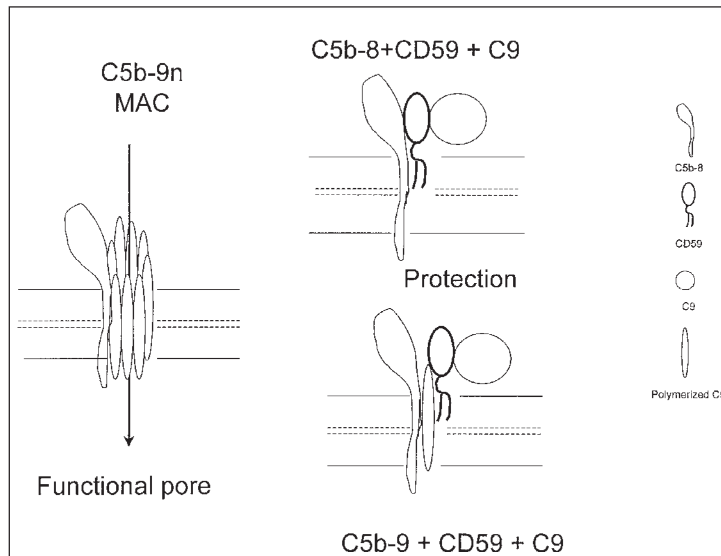

Fig. 7.4. Mechanism of action of CD59. CD59 binds to C5b-8 or C5b-9 and prevents binding and polymerization of C9, thereby inhibiting functional MAC formation.

exists. Based on this species specificity, the binding site for CD59 in C9 has been localized between Cys359 and Cys384 of C9, with an additional contribution by residues C-terminal to this segment,[90] while the binding site in C8 was assigned to the region between Cys345 and Cys369 in the C8α-chain, a region homologous to C9.[91]

To inhibit C, CD59 has to be in close proximity to the membrane, and when CD59 is spaced away from the membrane by attaching to the amino-terminus of DAF it is unable to prevent MAC formation.[92] Soluble CD59, either purified from urine or generated by deletion of the GPI anchor attachment site, is at least 100 times less efficient than membrane-incorporated CD59 in preventing MAC formation.[93]

Structure

The primary sequence of CD59 has been deduced from cDNA clones.[78,94] CD59 belongs to a superfamily of proteins that include Ly-6 and the urokinase plasminogen-activator receptor (CD87). Because of the homology found with members of the Ly-6 family (27%), including the conservation of the 10 cysteines, it had been suggested that Ly-6 was the mouse analogue of human CD59. However, lack of C-inhibitory activity, different chromosomal localization and the recent cloning of mouse CD59 has shown that this is not the case.[95]

The CD59 gene is about 27 kb and consists of one 5'-untranslated exon and three coding exons.[96,97] The second exon encodes the hydrophobic leader sequence of the protein, and the third exon encodes the amino-terminal portion of the mature protein. The fourth exon encodes the remainder of the mature protein, including the hydrophobic sequence necessary for GPI anchor attachment. The gene structure is similar to that of mouse Ly-6 except for the larger size of CD59 introns. Northern blot analysis showed that more than four different CD59 mRNA molecules were generated by alternative polyadenylation.

Karyotypic analysis showed that the gene for CD59 was localized in the region p13 of the short arm of chromosome 11.[98,99]

Translation of the 370 bp CD59 mRNA coding sequence generates a 128 aa protein. N-terminal amino acid sequencing has determined that the first 25 amino acids of the translated protein are cleaved off, and lysine is the N-terminal amino acid of the mature protein.[82] Tryptic digestion of CD59 has identified the GPI-anchoring site at asparagine 77 of the mature protein.[100] This study also assigned the following disulfide-linked cysteine pairs: Cys3-Cys26, Cys6-Cys13, Cys19-Cys39, Cys45-Cys63 (or 64) and Cys63 (or 64)-Cys69, generating 5 different loops.

CD59 runs on gels as an 18-20 kDa protein, a Mr much larger than expected based on its amino acid contents. Deglycosylation with Endo-F resulted in a reduction of 6 kDa, while treatment with neuraminidase had only a marginal effect.[78,82] Removal of the GPI anchor with PIPLC even increased the Mr by 1 kDa.[78] CD59 has 2 potential *N*-glycosylation sites at position 8 and 18, but sequencing showed that only position 18 is occupied; position 8 is unusable because residue 9 is a proline.[78] Detailed analysis of the carbohydrate structure revealed that CD59 may also have some *O*-linked carbohydrate, although the site of attachment could not be determined.[101] Enzymatic removal of the *N*-linked carbohydrate of CD59 abolished its functional activity,[102] but eliminating the *N*-glycosylation site by deleting N at position 18 had no effect on[103,104] or even enhanced[105] the complement inhibitory activity of CD59.

Detailed mutational analysis by three different groups has assigned the C-inhibiting site of CD59 to a region between amino acids 40 and 61, indicating the involvement of the third and fourth loops.[104-106] Nuclear magnetic resonance has resolved the three dimensional structure of urine and erythrocyte-derived CD59 and confirmed the localization of the *N*-glycosylation site and disulfide bonds.[107,108]

On Western blots, CD59 of nucleated cells often shows a laddered pattern. This is most likely due to differences in glycosylation, and Mr up to 25 kDa can be found. Sometimes 40 and 80 kDa proteins with anti-CD59 reactivity can be observed. These are most likely aggregates that often arise during purification, (ref. 78 and our unpublished observations). In cells overexpressing CD59, a 14 kDa protein can be found which probably reflects newly synthesized but not yet glycosylated CD59.[38]

Distribution

CD59 has a very wide tissue distribution and has been found on all cells of hemopoietic origin. It is also widely expressed on tissues including endothelial cells, cells of the peripheral and central nervous system and on spermatozoa. Soluble forms of CD59 have been found in urine,[78] seminal plasma,[109] breast milk[110] and in supernatants of cultured cells.[38] CD59 found in urine has lost its GPI anchor and cannot reinsert in a membrane.[78]

Regulation of Expression

Upregulation of expression of C-inhibitors at sites of inflammation will protect the cells from harmful side effects of C-activation. Many cell activation events induced by C are mediated through the MAC.[111] Protection against MAC by upregulation of CD59 would be an important strategy to prevent tissue damage. Inflammatory agents, including C, have been studied for their effects on the level of CD59 expression.

C is thought to be involved in damaging the colonic mucosa in ulcerative colitis (UC). While in normal colonic epithelia, GPI-anchored molecules are targeted to the apical surface, enhanced expression of DAF and CD59 on epithelial cells of UC also results in redistribution to the basolateral surfaces.[112] This altered cell distribution was also observed in colonic mucosa in other inflammatory bowel diseases, such as ischemic colitis. In

rheumatoid arthritis, CD59 expression was increased in the diseased cartilage of joints compared to normal cartilage. IL-1 treatment elicited the same effect in normal cartilage.[29]

Although sublethal C-attack caused increased resistance to subsequent C-attack, it did not result in an increase in expression of CD59 or other C-regulatory molecules on K562 cells.[113] The same study showed that expression of CD59 and C-resistance were inversely correlated; confluent cells were low CD59 and C-resistant, whereas cells growing in log phase were high CD59 and C-susceptible, showing that expression of C-regulators is not the only factor involved in resistance to C-lysis.

The expression of CD59 and several other GPI-anchored molecules, including DAF, were decreased in non-lesional psoriatic skin and virtually abolished in lesional psoriatic skin. The reduction of all GPI-anchored molecules, but not of the TM molecules, suggested a shedding or enzymatic cleavage specific for the GPI-anchored molecules.[114]

Tumor cells often have a high level of expression of C-regulatory molecules. Therapeutic downregulation of expression would augment C-mediated immune surveillance. Levamisole was found to downregulate CD59 expression on the human colorectal cell lines HT29 and Caco-2 which may partially explain the effects of Levamisole in the reduction of incidence of colon cancer relapse following surgical resection.[115]

PMA has been shown to induce an up to 15-fold increase in CD59 expression on a variety of cells including the EA.hy 926 endothelial cell line,[116] K562 erythroblasts[110,117] and the HOG oligodendrocyte cell line.[37] This increased expression was due to de novo mRNA and protein synthesis. Analysis of the promoter region of CD59 showed that the first 70 nucleotides immediately 5' of the transcriptional start site of the CD59 gene were essential for both constitutive and PMA-responsive transcription; however, responsiveness to PMA was cell line-specific and could not be induced in all cell lines.[118] Exposure of K562 erythroleukemia cells to a variety of stimulants (dexamethasone, calcium ionophore, lipopolysaccharide, IL-1, TNF-α, hemin, and cyclic AMP) had no effect on CD59 expression.[117] Endothelial cells (EC) slightly upregulated CD59 upon incubation with TNF-α and downregulated when incubated with IL-1β.[119] Activation of neutrophils by stimulation with fMLP or calcium ionophore A23187 induced an immediate upregulation of CD59 as a consequence of release from intracellular stores. Upregulation was dependent on release of calcium from intracellular stores.[112]

Deficiencies

PNH is caused by an autosomal mutation in the PIG-A gene, which causes a deficiency of a component of GPI biosynthesis with the consequence that no GPI anchor is attached to proteins with the correct signal sequence.[48] Patients suffer from recurrent hemolysis. The importance of deficiency of CD59 in PNH was demonstrated in one patient with severe PNH who was shown to be deficient only in CD59. All other GPI-anchored molecules, including DAF, were found to be normally expressed.[121,122] Other circulating cells and tissues of this patient were also found to be completely devoid of CD59. The molecular basis of this deficiency was a base deletion which caused a codon frame shift resulting in failure to produce intact CD59.[123] The parents and cousins of the patient had decreased CD59, suggesting that the deficiency is hereditary and that complete deletion was brought about by a homozygous abnormality in the CD59 gene.

Other Species

CD59 analogues have been purified and/or cloned from a variety of mammalian species, including monkeys (baboon, owl monkey, African green monkey, marmoset),[124] rat,[125,126] mouse,[95] sheep,[127] pig,[89,128] goat and rabbit (our unpublished results). Overall identity at the protein level between human and baboon, rat and mouse was 82%, 44% and

34% respectively, while rat and mouse were 60% identical. In all cases the cysteines and the *N*-glycosylation site were conserved. The highest degree of homology observed was between sheep and goat (90% of first 20 amino acids), and this was the only combination where antibodies showed cross-species reactivity (our unpublished results).

The CD59 analogues purified and characterized all had a similar Mr to CD59 and showed C-inhibitory activity toward human C8 and C9. All of them, however, showed some species selectivity. CD59 on rat, sheep and pig erythrocyte were all sensitive to PIPLC cleavage, demonstrating that these species expressed the simple two lipid GPI anchor on their erythrocytes.[125,127]

The gene encoding the mouse analogue of human CD59 has been localized to the E2-E4 region of mouse chromosome 2, a region that is syntenous with the location of the human CD59 gene on chromosome 11p13.[95] No transmembrane species of any CD59 analogue has yet been found and is not known if any of the analogues is encoded by more than one gene. The distribution of the rat and sheep CD59 species is different from that of human CD59. Rat CD59 was not detected on platelets and lymphocytes[125] and newborn rats did not express CD59 on oligodendrocytes.[129] Sheep CD59 was not detected on sheep platelets.[127]

Parasites

Expression of inhibitors of the terminal pathway by parasites or acquisition of GPI-anchored inhibitors from the host would enhance parasite survival in the blood. Some stages of the parasite *Trypanosoma cruzi* are very resistant to lysis by C. While the amastigote and epimastigote activate C via the AP to similar degrees, only the epimastigotes are destroyed.[130] Restriction at the final stage of MAC assembly was demonstrated by showing a reduced binding of C9 to amastigotes. Reactivity of a polyclonal anti-human CD59 antibody with cell lysates of amastigotes and trypomastigotes but not epimastigotes, and the isolation of MAC-inhibitory activity from cell supernatants of cultured amastigotes and trypomastigotes, suggests that *T. cruzi* expresses a molecule with functional and antigenic similarity to CD59 (Tambourgi DV, personal communication).

Crossreactivity of a polyclonal anti-human CD59 antibody with a 94 kDa GPI-anchored protein on *Schistosoma mansoni* has been demonstrated. The same antibody enhanced the susceptibility of schistosomula to C-mediated lysis, suggesting that this protein had functional and antigenic similarity to CD59. No sequence is known for this protein and it is not clear whether it is host derived or parasite encoded.[131]

Viruses and Virus-Infected Cells

Mimicking, enhancing expression of and/or capturing human C-regulators would aid the survival of viruses and virus-infected cells in the host. The only virus so far identified to express an analogue of CD59 is Herpesvirus saimiri (HVS). This virus is a T lymphotropic tumor virus that causes lymphomas and leukemias in various New World primates other than its natural host, the squirrel monkey (*Saimiri sciureus*). An open reading frame of 363 nucleotides in the viral genome encoded a 121 amino acid protein which showed 48% homology to human CD59.[132] BALB/3T3 cells stably expressing HVS-CD59 or HuCD59 were equally well protected from C-mediated lysis by human serum.[133] Other mechanisms used by viruses to avoid C-mediated lysis include increasing expression of C-regulators on the surface of the host cell and capturing C-regulators on the budding virion. HIV-1 virions, grown in CHO cells expressing either human CD46, CD55 or CD59, were shown to incorporate GPI-anchored CD55 and CD59 as well as transmembrane CD46.[134] Further evidence that HIV captures CD59 from the host cell is provided by the observation that HIV virus grown in GPI anchor-deficient cells was more sensitive to C-mediated lysis than virus grown in normal cells.[135] Plasma HIV-1 virus appeared more sensitive to C than

primary isolates. While primary isolated virions contained CD46, CD55, and CD59, only CD59 was detected on plasma virus.[65] Both CD59 and CD55 were associated with the external membrane of HTLV-I derived from MT2 cells, and antisera to CD55 and CD59 induced C-mediated lysis of HTLV-I virions. Purified HCMV produced in human foreskin fibroblasts also contained both CD55 and CD59.[136] Infection of fibroblasts or glioblastoma cells with human cytomegalovirus did not alter the expression of CD59 on these cells.[67]

Signaling

Like other GPI-anchored molecules (see chapter 4) crosslinking of CD59 induces a wide range of signaling events (rise in intracellular calcium, tyrosine phosphorylation, inositol phosphate production, IL-2 production, cell proliferation) in a variety of nucleated cells.[137-139] Incorporation of purified CD59 into a CD59 negative cell line showed that association of CD59 with Src-kinases in cholesterol-rich patches is an essential requirement for the signaling capacity of CD59.[140] In T cells, presence of the T-cell receptor is a requirement for cell activation through CD59, but on other cells no similar association with a receptor-signaling complex has yet been found.

Ligand

One of the groups which first discovered CD59 identified it as a cell surface molecule involved in the CD2-dependent rosetting of T cells with erythrocytes.[83] In an in vitro binding assay with purified CD58 and CD59, $CD2^+$ cells bound to immobilized CD58 and also to CD59. It was demonstrated that the binding sites on CD2 for CD58 and CD59 were overlapping but nonidentical. These observations suggest that direct interactions between CD2 and both CD58 and CD59 contribute to T-cell activation and adhesion.[141] Binding of CD59 could be inhibited by some but not all anti-CD59 antibodies.[142] However, other groups, using the highly sensitive surface plasmon resonance technique, could not find any interaction of CD59 with CD2.[143]

Homologous Restriction Factor

The first inhibitor of the membrane attack complex discovered was identified and purified by its ability to bind C8 and C9 and is known by the names homologous restriction factor (HRF),[144] C8-binding protein[145] and MAC inhibiting protein (MIP).[146] It was first isolated from human erythrocytes as a 38 kDa protein by affinity chromatography using a C9-Sepharose column.[144] Because of its ability to protect against lysis by complement of the same species, it was named homologous restriction factor (HRF). Antibodies raised against this protein reacted mainly with a 65 kDa molecule, suggesting that the 38 kDa protein constituted a fragment of membrane HRF. A soluble form of HRF with Mr of 65 kDa (HRF-U) was isolated from normal human urine.[147] HRF purified from erythrocyte membranes by virtue of its binding to C8 had a Mr of 65 kDa.[145] Erythrocyte-derived HRF had a Mr of 55 kDa under nonreducing conditions and 65 kDa under reducing conditions.[146] HRF was also found in plasma, urine, saliva and cerebrospinal fluid.

HRF had a similar function to CD59 in that it restricted the binding of C9 to C5b-8 and so limited cell damage. The capacity to incorporate into chicken erythrocytes, the absence on erythrocytes of PNH patients[148,149] and susceptibility to release by PIPLC[150] showed that HRF was also a GPI-anchored C-regulator. Very little is known about this protein; there is no amino-terminal amino acid or DNA sequence known and no analogues in other animals have been described. The only relation with disease is the absence of the protein on erythrocytes of PNH patients.[148,149]

Why Are So Many C-Regulatory Molecules Anchored through a GPI Anchor?

Of the five known C-regulatory molecules, three are anchored to the membrane through a GPI anchor. Originally it was thought that the GPI anchors would give the molecules more lateral mobility, enabling them to reach their target more quickly and so exert their function more efficiently. However, TM forms of DAF and CD59, generated by replacing the GPI anchor sequence by the TM and cytoplasmic tail of MCP, showed similar ability to decay the C3-convertase[151] or prevent MAC formation[152] (our unpublished results). One possibility is that the selection of GPI anchoring for C-regulatory proteins relates to the ability of GPI-anchored molecules to induce cell activation (see chapter 4). The precise link is still poorly understood, but further study may reveal a role in protection against C for the GPI anchor on these molecules. Another possible reason for GPI anchoring relates to the ability of these molecules to transfer to other cells (see chapter 10). Transfer of GPI-anchored C-regulatory molecules is an efficient way of conferring C-resistance on cells that do not express endogenous C-regulatory molecules. This phenomenon is best illustrated by the observation that mice and pigs, expressing human DAF and CD59 under control of the globin promotor, (resulting in exclusive expression of these molecules on erythrocytes), were found also to have DAF and CD59 on the endothelial cells lining the vascular system in sufficient quantities to protect against C.[153,154] The high content of DAF and CD59 in prostasomes in seminal fluid may, by a similar transfer process, confer protection on the sperm in the female genital tract.[155]

References

1. Hoffmann EM. Inhibition of complement by a substance isolated from human erythrocytes. II. Studies on the site and mechanism of action. Immunochem 1969; 6:405-419.
2. Fujita T, Inoue T, Ogawa K et al. The mechanism of action of decay-accelerating factor (DAF). DAF inhibits the assembly of C-3 convertases by dissociating C2a and Bb. J Exp Med 1987; 166:1221-1228.
3. Ito S, Tamura N, Fujita T. Effect of decay-accelerating factor on the assembly of the classical and alternative pathway C3-convertases in the presence of C-4 or C-3 nephritic factor. Immunol 1989; 68:449-452.
4. Pangburn MK, Schreiber RD, Muller-Eberhard HJ. Deficiency of an erythrocyte membrane protein with complement regulatory activity in paroxysmal nocturnal hemoglobinuria. Proc Nat Acad Sci USA 1983; 80:5430-5434.
5. Brodbeck WG, Liu DC, Sperry J et al. Localization of classical and alternative pathway regulatory activity within the decay-accelerating factor. J Immunol 1996; 156:2528-2533.
6. Nakano Y, Sumida K, Kikuta N et al. Complete determination of disulfide bonds localized within the short consensus repeat units of decay accelerating factor (CD55 antigen). Biochim Biophys Acta 1992; 1116:235-240.
7. Rey Campos J, Rubinstein P, De Cordoba SR. Decay-accelerating factor—genetic-polymorphism and linkage to the RCA (regulator of complement activation) gene-cluster in humans. J Exp Med 1987; 166:246-252.
8. Lublin DM, Krsekstaples J, Pangburn MK et al. Biosynthesis and glycosylation of the human-complement regulatory protein decay-accelerating factor. J Immunol 1986; 137:1629-1635.
9. Caras IW, Davitz MA, Rhee L et al. Cloning of decay-accelerating factor suggests novel use of splicing to generate 2 proteins. Nature 1987; 325:545-549.
10. Medof ME, Lublin DM, Holers VM et al. Cloning and characterization of cDNAs encoding the complete sequence of decay-accelerating factor of human-complement. Proc Natl Acad Sci USA 1987; 84:2007-2011.

11. Stafford HA, Tykocinski ML, Lublin DM et al. Normal polymorphic variations and transcription of the decay accelerating factor gene in paroxysmal nocturnal hemoglobinuria cells. Proc Natl Acad Sci USA 1988; 85:880-884.
12. Post TW, Arce MA, Liszewski MK et al. Structure of the gene for human-complement protein decay accelerating factor. J Immunol 1990; 144:740-744.
13. Moran P, Raab H, Kohr WJ et al. Glycophospholipid membrane anchor attachment—molecular analysis of the cleavage attachment site. J Biol Chem 1991; 266:1250-1257.
14. Medof ME, Kinoshita T, Nussenzweig V. Inhibition of complement activation on the surface of cells after incorporation of decay-accelerating factor (DAF) into their membranes. J Exp Med 1984; 160:1558-1578.
15. Davitz MA, Low MG, Nussenzweig V. Release of decay-accelerating factor (DAF) from the cell-membrane by phosphatidylinositol-specific phospholipase-C (PIPLC)—selective modification of a complement regulatory protein. J Exp Med 1986; 163:1150-1161.
16. Medof ME, Walter EI, Roberts WL et al. Decay accelerating factor of complement is anchored to cells by a C-terminal glycolipid. Biochemistry 1986; 25:6740-6747.
17. Lublin DM, Krsek-Staples J, Pangburn MK et al. Biosynthesis and glycosylation of the human complement regulatory protein decay-accelerating factor. J Immunol 1986; 137:1629-1635.
18. Reddy P, Caras I, Krieger M. Effects of *O*-linked glycosylation on the cell-surface expression and stability of decay-accelerating factor, a glycophospholipid-anchored membrane-protein. J Biol Chem 1989; 264:17329-17336.
19. Seya T, Atkinson JP. Purification and characterization of additional forms of decay-accelerating factor (DAF). Fed Proc 1987; 46:773-773.
20. Kinoshita T, Rosenfeld SI, Nussenzweig V. A high Mw form of decay-accelerating factor (DAF-2) exhibits size abnormalities in paroxysmal nocturnal hemoglobinuria erythrocytes. J Immunol 1987; 138:2994-2998.
21. Lass JH, Walter EI, Burris TE et al. Expression of 2 molecular-forms of the complement decay-accelerating factor in the eye and lacrimal gland. Invest Ophthal Visual Sci 1990; 31:1136-1148.
22. Rooney IA, Davies A, Morgan BP. Membrane attack complex (MAC)-mediated damage to spermatozoa—protection of the cells by the presence on their membranes of MAC inhibitory proteins. Immunology 1992; 75:499-506.
23. Cervoni F, Oglesby TJ, Fenichel P et al. Expression of decay-accelerating factor (CD55) of the complement system on human spermatozoa. J Immunol 1993; 151:939-948.
24. Kinoshita T, Medof ME, Silber R et al. Distribution of decay-accelerating factor in the peripheral-blood of normal individuals and patients with paroxysmal-nocturnal hemoglobinuria. J Exp Med 1985; 162:75-92.
25. Nicholson-Weller A, March JP, Rosen CE et al. Surface membrane expression by human blood leukocytes and platelets of decay-accelerating factor, a regulatory protein of the complement system. Blood 1985; 65:1237-1244.
26. Lisanti MP, Caras IW, Davitz MA et al. A glycophospholipid membrane anchor acts as an apical targeting signal in polarized epithelial cells. J Cell Biol 1989; 109:2145-2156.
27. Medof ME, Walter EI, Rutgers JL et al. Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. J Exp Med 1987; 165:848-864.
28. Tarkowski A, Trollmo C, Seifert PS et al. Expression of decay-accelerating factor on synovial lining cells in inflammatory and degenerative arthritides. Rheumatol Internat 1992; 12:201-205.
29. Davies ME, Horner A, Loveland BE et al. Upregulation of complement regulators MCP (CD46), DAF (CD55) and protectin (CD59) in arthritic joint disease. Scand J Rheumatol 1994; 23:316-321.
30. Shibata T, Cosio FG, Birmingham DJ. Complement activation induces the expression of decay-accelerating factor on human mesangial cells. J Immunol 1991; 147:3901-3908.

31. Boom BW, Mommaas AM, Daha MR et al. Decreased expression of decay-accelerating factor on endothelial cells of immune complex-mediated vasculitic skin lesions. J Dermatol Sci 1991; 2:308-315.
32. Moutabarrik A, Nakanishi I, Namiki M et al. Cytokine-mediated regulation of the surface expression of complement regulatory proteins, CD46(MCP), CD55(DAF), and CD59 on human vascular endothelial cells. Lymphokine Cytokine Res 1993; 12:167-172.
33. Bryant RW, Granzow CA, Siegel MI et al. Wheat germ agglutinin and other selected lectins increase synthesis of decay-accelerating factor in human endothelial cells. J Immunol 1991; 147:1856-1862.
34. Andoh A, Fujiyama Y, Sumiyoshi K et al. Interleukin-4 acts as an inducer of decay-accelerating factor gene-expression in human intestinal epithelial-cells. Gastroenterology 1996; 111:911-918.
35. Andoh A, Fujiyama Y, Sumiyoshi K et al. Tumour necrosis factor-α up-regulates decay-accelerating factor gene expression in human intestinal epithelial cells. Immunology 1997; 90:358-363.
36. Bryant RW, Granzow CA, Siegel MI et al. Phorbol esters increase synthesis of decay-accelerating factor, a phosphatidylinositol-anchored surface protein, in human-endothelial cells. J Immunol 1990; 144:593-598.
37. Gasque P, Morgan BP. Complement regulatory protein expression by a human oligodendrocyte cell-line—cytokine regulation and comparison with astrocytes. Immunology 1996; 89:338-347.
38. Marchbank KJ, Morgan BP, Van den Berg CW. Regulation of CD59 expression on K562 cells: Effects of phorbol myristate acetate, crosslinking antibody and nonlethal complement attack. Immunol 1995; 85:146-152.
39. Berger M, Medof ME. Increased expression of complement decay-accelerating factor during activation of human neutrophils. J Clin Invest 1987; 79:214-220.
40. Kendall G, Crankson H, Ensor E et al. Activation of the gene encoding decay accelerating factor following nerve growth factor treatment of sensory neurons is mediated by promoter sequences within 206 bases of the transcriptional start site. J Neurosci Res 1996; 45:96-103.
41. Thomas DJ, Lublin DM. Identification of 5'-flanking regions affecting the expression of the human decay accelerating factor gene and their role in tissue-specific expression. J Immunol 1993; 150:151-160.
42. Spring FA, Judson PA, Daniels GL et al. A human cell-surface glycoprotein that carries cromer-related blood-group antigens on erythrocytes and is also expressed on leukocytes and platelets. Immunology 1987; 62:307-313.
43. Telen MJ, Green AM. The Inab phenotype: Characterization of the membrane protein and complement regulatory defect. Blood 1989; 74:437-441.
44. Reid ME, Mallinson G, Sim RB et al. Biochemical studies on red blood cells from a patient with the Inab phenotype (decay-accelerating factor deficiency). Blood 1991; 78:3291-3297.
45. Lublin DM, Mallinson G, Reid ME et al. Molecular-basis of reduced or absent expression of decay accelerating factor in Dr(a-) and Inab phenotypes of Cromer blood-group. Transfusion 1992; 32:S 47.
46. Merry AH, Rawlinson VI, Uchikawa M et al. Studies on the sensitivity to complement-mediated lysis of erythrocytes (Inab phenotype) with a deficiency of DAF (decay accelerating factor). Br J Haematol 1989; 73:248-253.
47. Schubert J, Alvarado M, Uciechowski P et al. Diagnosis of paroxysmal-nocturnal hemoglobinuria using immunophenotyping of peripheral-blood cells. Br J Haematol 1991; 79:487-492.
48. Miyata T, Yamada N, Iida Y et al. Abnormalities of pig-a transcripts in granulocytes from patients with paroxysmal-nocturnal hemoglobinuria. New England J Med 1994; 330:249-255.
49. Nicholson-Weller A, March JP, Rosenfeld SI et al. Affected erythrocytes of patients with paroxysmal-nocturnal hemoglobinuria are deficient in the complement regulatory protein, decay accelerating factor. Proc Natl Acad Sci USA 1983; 80:5066-5070.

50. Nicholson-Weller A, Burge J, Austen KF. Purification from guinea-pig erythrocyte stroma of a decay-accelerating factor for the classical C-3 convertase, C4b,2a. J Immunol 1981; 127:2035-2039.
51. Nonaka M, Miwa T, Okada N et al. Multiple isoforms of guinea-pig decay-accelerating factor (DAF) generated by alternative splicing. J Immunol 1995; 155:3037-3048.
52. Kameyoshi Y, Matsushita M, Okada H. Murine membrane inhibitor of complement which accelerates decay of human C3 convertase. Immunology 1989; 68:439-444.
53. Spicer AP, Seldin MF, Gendler SJ. Molecular-cloning and chromosomal localization of the mouse decay-accelerating factor genes—duplicated genes encode glycosylphosphatidylinositol-anchored and transmembrane forms. J Immunol 1995; 155:3079-3091.
54. Sugita Y, Uzawa M, Tomita M. Isolation of decay-accelerating factor (DAF) from rabbit erythrocyte-membranes. J Immunol Meth 1987; 104:123-130.
55. Kipnis TL, Tambourgi DV, Sucupira M et al. Effect of *Trypanosoma-cruzi* membrane-components on the formation of the classical pathway C-3 convertase. Brazilian J Med Biol Res1986; 19:271-278.
56. Joiner KA, Da Silva WD, Rimoldi MT et al. Biochemical-characterization of a factor produced by trypomastigotes of *Trypanosoma cruzi* that accelerates the decay of complement C-3 convertases. J Biol Chem 1988; 263:11327-11335.
57. Tambourgi DV, Kipnis TL, Da Silva WD et al. A partial cDNA clone of trypomastigote decay-accelerating factor (T-DAF), a developmentally regulated complement inhibitor of *Trypanosoma cruzi*, has genetic and functional similarities to the human complement inhibitor DAF. Infect Immun 1993; 61:3656-3663.
58. Tomlinson S, Decarvalho LP, Vandekerckhove F et al. Resialylation of sialidase-treated sheep and human erythrocytes by *Trypanosoma cruzi* trans-sialidase—restoration of complement resistance of desialylated sheep erythrocytes. Glycobiology 1992; 2:549-551.
59. Tomlinson S, Decarvalho LCP, Vandekerckhove F et al. Role of sialic-acid in the resistance of *Trypanosoma cruzi* trypomastigotes to complement. J Immunol 1994; 153:3141-3147.
60. Norris KA, Bradt B, Cooper NR et al. Characterization of a *Trypanosoma cruzi* C3-binding-protein with functional and genetic similarities to the human-complement regulatory protein, decay-accelerating factor. J Immunol 1991; 147:2240-2247.
61. Norris KA, Schrimpf JE, Szabo MJ. Identification of the gene family encoding the 160-kilodalton *Trypanosoma cruzi* complement regulatory protein. Infect Immun 1997; 65:349-357.
62. Pearce EJ, Hall BF, Sher A. Host-specific evasion of the alternative complement pathway by schistosomes correlates with the presence of a phospholipase C- sensitive surface-molecule resembling human decay accelerating factor. J Immunol 1990; 144:2751-2756.
63. Fatima M, Horta M, Ramalho Pinto FJ. Role of human decay-accelerating factor in the evasion of *Schistosoma-mansoni* from the complement-mediated killing in vitro. J Exp Med 1991; 174:1399-1406.
64. Marschang P, Sodroski J, Wurzner R et al. Decay-accelerating factor (CD55) protects human-immunodeficiency virus type-1 from inactivation by human-complement. Eur J Immunol 1995; 25:285-290.
65. Sullivan BL, Knopoff EJ, Saifuddin M et al. Susceptibility of HIV-1 plasma virus to complement-mediated lysis—evidence for a role in clearance of virus in-vivo. J Immunol 1996; 157:1791-1798.
66. Stoiber H, Pinter C, Siccardi AG et al. Efficient destruction of human-immunodeficiency-virus in human serum by inhibiting the protective action of complement factor-H and decay accelerating factor (DAF, CD55). J Exp Med 1996; 183:307-310.
67. Spiller OB, Morgan BP, Tufaro F et al. Altered expression of host-encoded complement regulators on human cytomegalovirus-infected cells. Eur J Immunol 1996; 26:1532-1538.
68. Spiller OB, Hanna SM, Devine DV et al. Neutralization of cytomegalovirus virions: The role of complement. J Infect Dis 1997; 176:339-347.
69. Shenoyscaria AM, Kwong J, Fujita T et al. Signal transduction through decay-accelerating factor—interaction of glycosyl-phophatidylinositol anchor and protein tyrosine kinases p56(Lck) and p59(Fyn-1). J Immunol 1992; 149:3535-3541.

70. Hamann J, Vogel B, Van Schijndel GMW et al. The seven-span transmembrane receptor CD97 has a cellular ligand (CD55, DAF). J Exp Med 1996; 184:1185-1189.
71. Bergelson JM, Chan M, Solomon KR et al. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc Natl Acad Sci, USA 1994; 91:6245-6248.
72. Shafren DR, Bates RC, Agrez MV et al. Coxsackieviruses B1, B3, and B5 use decay-accelerating factor as a receptor for cell attachment. J Virol 1995; 69:3873-3877.
73. Modlin JF, Bergelson JM, Wielandalter W et al. Group-b coxsackieviruses (cbv) use both decay-accelerating factor and a 50 kd protein as receptors. Pediatric Res 1996; 39:1064-1064.
74. Karnauchow TM, Tolson DL, Harrison BA et al. The Hela-cell receptor for enterovirus-70 is decay-accelerating factor (CD55). J Virol 1996; 70:5143-5152.
75. Nowicki B, Hart A, Coyne KE et al. Short consensus repeat-3 domain of recombinant decay-accelerating factor is recognized by *Escherichia coli* recombinant Dr adhesion in a model of a cell-cell interaction. J Exp Med 1993; 178:2115-2121.
76. Pham T, Kaul A, Hart A et al. Dra-related X adhesions of gestational pyelonephritis-associated *Escherichia coli* recognize SCR-3 and SCR-4 domains of recombinant decay-accelerating factor. Infect Immun 1995; 63:1663-1668.
77. Kaul AK, Kumar D, Nagamani M et al. Rapid cyclic changes in density and accessibility of endometrial ligands for *Escherichia coli* Dr fimbriae. Infect Immun 1996; 64:611-615.
78. Davies A, Simmons DL, Hale G et al. CD59, an ly-6-like protein expressed in human lymphoid-cells, regulates the action of the complement membrane attack complex on homologous cells. J Exp Med 1989; 170:637-654.
79. Sugita Y, Tobe T, Tomita M et al. Molecular cloning and characterization of MACIF, an inhibitor of membrane channel formation of complement. J Biochem 1989; 106:555-557.
80. Holguin MH, Wilcox LA, Bernshaw NJ et al. Relationship between the membrane inhibitor of reactive lysis and the erythrocyte phenotypes of paroxysmal nocturnal hemoglobinuria. J Clin Invest 1989; 84:1387-1394.
81. Okada N, Harada R, Fujita T et al. Monoclonal antibodies capable of causing hemolysis of neuraminidase-treated human erythrocytes by homologous complement. J Immunol 1989; 143:2262-2266.
82. Stefanova I, Hilgert I, Kristofova H et al. Characterization of a broadly expressed human leucocyte surface antigen MEM-43 anchored in membrane through phosphatidylinositol. Mol Immunol 1989; 26:153-161.
83. Groux H, Huet S, Aubrit F et al. A 19-kDa human-erythrocyte molecule H19 is involved in rosettes, present on nucleated cells, and required for T-cell activation—comparison of the roles of H19 and LFA-3 molecules in T-cell activation. J Immunol 1989; 142:3013-3020.
84. Rollins SA, Sims PJ. The complement-inhibitory activity of CD59 resides in its capacity to block incorporation of C9 into membrane C5b-9. J Immunol 1990; 144:3478-3483.
85. Meri S, Morgan BP, Davies A et al. Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. Immunology 1990; 71:1-9.
86. Ninomiya H, Sims PJ. The human complement regulatory protein CD59 binds to the alpha-chain of C8 and to the 'b' domain of. J Biol Chem 1992; 267:13675-13680.
87. Sugita Y, Nakano Y, Tomita M. Isolation from human erythrocytes of a new membrane protein which inhibits the formation of complement transmembrane channels. J Biochem 1988; 104:633-637.
88. van den Berg CW, Morgan BP. Complement-inhibiting activities of human CD59 and analogs from rat, sheep, and pig are not homologously restricted. J Immunol 1994; 152:4095-4101.
89. van den Berg CW, Harrison RA, Morgan BP. A rapid method for the isolation of analogs of human CD59 by preparative SDS-page—application to pig CD59. J Immunol Meth 1995; 179:223-231.
90. Husler T, Lockert DH, Sims PJ. Role of a disulfide-bonded peptide loop within human complement C9 in the species-selectivity of complement inhibitor CD59. Biochemistry 1996; 35:3263-3269.

91. Lockert DH, Kaufman KM, Chang CP et al. Identity of the segment of human-complement C8 recognized by complement regulatory protein CD59. J Biol Chem 1995; 270:19723-19728.
92. Fodor WL, Rollins SA, Guilmette ER et al. A novel bifunctional chimeric complement inhibitor that regulates C3 convertase and formation of the membrane attack complex. J Immunol 1995; 155:4135-4138.
93. Sugita Y, Ito K, Shiozuka K et al. Recombinant soluble CD59 inhibits reactive haemolysis with complement. Immunology 1994; 82:34-41.
94. Sawada R, Ohashi K, Anaguchi H et al. Isolation and expression of the full-length cDNA encoding CD59 antigen of human lymphocytes. DNA & Cell Biol 1990; 9:213-220.
95. Powell MB, Marchbank KJ, Rushmere NK et al. Molecular cloning, chromosomal localization, expression, and functional characterization of the mouse analogue of human CD59. J Immunol 1997; 158:1692-1702.
96. Tone M, Walsh LA, Waldmann H. Gene structure of human CD59 and demonstration that discrete mRNAs are generated by alternative polyadenylation. J Mol Biol 1992; 227:971-976.
97. Petranka JG, Fleenor DE, Sykes K et al. Structure of the CD59-encoding gene: Further evidence of a relationship to murine lymphocyte antigen Ly-6 protein. Proc Natl Acad Sci USA 1992; 89:7876-7879.
98. Forsberg UH, Bazil V, Stefanova I et al. Gene for human CD59 (likely Ly-6 homologue) is located on the short arm of chromosome 11. Immunogen 1989; 30:188-193.
99. Bickmore WA, Longbottom D, Oghene K et al. Colocalization of the human CD59 gene to 11p13 with the MIC11 cell surface antigen. Genomics 1993; 17:129-135.
100. Sugita Y, Nakano Y, Oda E et al. Determination of carboxyl-terminal residue and disulfide bonds of MACIF (CD59), a glycosyl-phosphatidylinositol-anchored membrane protein. J Biochem 1993; 114:473-477.
101. Rudd PM, Morgan BP, Wormald MR et al. The glycosylation of the complement regulatory protein, human erythrocyte CD59. J Biol Chem 1997; 272:7229-7244.
102. Ninomiya H, Stewart BH, Rollins SA et al. Contribution of the *N*-linked carbohydrate of erythrocyte antigen CD59 to its complement-inhibitory activity. J Biol Chem 1992; 267:8404-8410.
103. Rother RP, Zhao J, Zhou QS et al. Elimination of potential sites of glycosylation fails to abrogate complement regulatory function of cell-surface CD59. J Biol Chem 1996; 271:23842-23845.
104. Bodian DL, Davis SJ, Morgan BP et al. Mutational analysis of the active site and antibody epitopes of the complement-inhibitory glycoprotein, CD59. J Exp Med 1997; 185:507-516.
105. Yu J, Dong S, Rushmere NK et al. Mapping the regions of the complement inhibitor CD59 responsible for its species selective activity. Biochemistry 1997; 36:9423-9428.
106. Petranka J, Zhao J, Norris J et al. Structure-function relationships of the complement regulatory protein, CD59. Blood Cells Mol Dis 1996; 22:281-296.
107. Fletcher CM, Harrison RA, Lachmann PJ et al. Sequence-specific 1H-NMR assignments and folding topology of human CD59. Protein Sci 1993; 2:2015-2027.
108. Kieffer B, Driscoll PC, Campbell ID et al. Three-dimensional solution structure of the extracellular region of the complement regulatory protein CD59, a new cell-surface protein domain related to snake venom neurotoxins. Biochemistry 1994; 33:4471-4482.
109. Rooney IA, Davies A, Morgan BP. Membrane attack complex (MAC)-mediated damage to spermatozoa: Protection of the cells by the presence on their membranes of MAC inhibitory proteins. Immunology 1992; 75:499-506.
110. Bjorge L, Jensen TS, Kristoffersen EK et al. Identification of the complement regulatory protein CD59 in human colostrum and milk. Am J Reprod Immunol 1996; 35:43-50.
111. Morgan BP. Complement membrane attack on nucleated cells—resistance, recovery and nonlethal effects. Biochem J 1989; 264:1-14.
112. Uesu T, Mizuno M, Inoue H et al. Enhanced expression of decay-accelerating factor and CD59 homologous restriction factor-20 on the colonic epithelium of ulcerative-colitis. Lab Invest 1995; 72:587-591.

113. Marchbank KJ, van den Berg CW, Morgan BP. Mechanisms of complement resistance induced by nonlethal complement attack and by growth arrest. Immunology 1997; 90:647-653.
114. Venneker GT, Das PK, Meinardi MMHM et al. Glycosylphosphatidylinositol (GPI)-anchored membrane proteins are constitutively down-regulated in psoriatic skin. J Pathol 1994; 172:189-197.
115. Bjorge L, Matre R. Downregulation of CD59 (protectin) expression on human colorectal adenocarcinoma cell lines by levamisole. Scand J Immunol 1995; 42:512-516.
116. Meri S, Mattila P, Renkonen R. Regulation of CD59 expression on the human endothelial cell line EA.hy 926. Eur J Immunol 1993; 23:2511-2516.
117. Holguin MH, Martin CB, Weis JH et al. Enhanced expression of the complement regulatory protein, membrane inhibitor of reactive lysis (CD59), is regulated at the level of transcription. Blood 1993; 82:968-977.
118. Holguin MH, Martin CB, Eggett T et al. Analysis of the gene that encodes the complement regulatory protein, membrane inhibitor of reactive lysis (CD59)—identification of an alternatively spliced exon and characterization of the transcriptional regulatory regions of the promoter. J Immunol 1996; 157:1659-1668.
119. Moutabarrik A, Nakanishi I, Namiki M et al. Cytokine-mediated regulation of the surface expression of complement regulatory proteins, CD46(MCP) CD55(DAF), and CD59 on human vascular endothelial-cells. Lymphokine Cytokine Res 1993; 12:167-172.
120. Gordon DL, Papazaharoudakis H, Sadlon TA et al. Upregulation of human neutrophil CD59, a regulator of the membrane attack complex of complement, following cell activation. Immunol & Cell Biol 1994; 72:222-229.
121. Yamashina M, Ueda E, Kinoshita T et al. Inherited complete deficiency of 20-kilodalton homologous restriction factor-(CD59) as a cause of paroxysmal-nocturnal hemoglobinuria. New Eng J Med 1990; 323:1184-1189.
122. Taguchi R, Funahashi Y, Ikezawa H et al. Analysis of PI (phosphatidylinositol)-anchoring antigens in a patient of paroxysmal nocturnal hemoglobinuria (PNH) reveals deficiency of 1F5 antigen (CD59), a new complement-regulatory factor. FEBS Letters 1990; 261:142-146.
123. Motoyama N, Okada N, Yamashina M et al. Paroxysmal nocturnal hemoglobinuria due to hereditary nucleotide deletion in the HRF20 (CD59) gene. Eur J Immunol 1992; 22:2669-2673.
124. Fodor WF, Rollins SA, Bianco-Caron S et al. Primate terminal complement inhibitor homologues of human CD59. Immunogenetics 1995; 41:51.
125. Hughes TR, Piddlesden SJ, Williams JD et al. Isolation and characterization of a membrane protein from rat erythrocytes which inhibits lysis by the membrane attack complex of rat complement. Biochem J 1992; 284:169-176.
126. Rushmere NK, Harrison RA, van den Berg CW et al. Molecular-cloning of the rat analog of human CD59—structural comparison with human CD59 and identification of a putative active-site. Biochem J 1994; 304:595-601.
127. van den Berg CW, Harrison RA, Morgan BP. The sheep analog of human CD59—purification and characterization of its complement inhibitory activity. Immunology 1993; 78:349-357.
128. Hinchliffe SJ, Rushmere NK, Hanna SM et al. Molecular cloning and functional characterization of the pig analogue of CD59: Relevance to xenotransplantation. J Immunol 1998;160:3924-3932.
129. Piddlesden SJ, Morgan BP. Killing of rat glial-cells by complement—deficiency of the rat analog of CD59 is the cause of oligodendrocyte susceptibility to lysis. J Neuroimmunol 1993; 48:169-176.
130. Iida K, Whitlow MB, Nussenzweig V. Amastigotes of Trypanosoma cruzi escape destruction by the terminal complement components. J Exp Med 1989; 169:881-891.
131. Parizade M, Arnon R, Lachmann PJ et al. Functional and antigenic similarities between a 94-kD protein of Schistosoma mansoni (SCIP-1) and human CD59. J Exp Med 1994; 179:1625-1636.

132. Albrecht J-C, Nicholas J, Cameron K et al. Herpesvirus saimiri has a gene specifying a homologue of the cellular membrane glycoprotein CD59. Virol 1992; 190:527-530.
133. Rother RP, Rollins SA, Fodor WL et al. Inhibition of complement-mediated cytolysis by the terminal complement inhibitor of herpesvirus saimiri. J Virol 1994; 68:730-737.
134. Saifuddin M, Hedayati T, Atkinson JP et al. Human immunodeficiency virus type 1 incorporates both glycosyl phosphatidylinositol-anchored CD55 and CD59 and integral membrane CD46 at levels that protect from complement-mediated destruction. J Gen Virol 1997; 78:1907-1911.
135. Saifuddin M, Ghassemi M, Patki C et al. Host-cell components affect the sensitivity of HIV type-1 to complement-mediated virolysis. Aids Res Human Retroviruses 1994; 10:829-837.
136. Spear GT, Lurain NS, Parker CJ et al. Host cell-derived complement control proteins CD55 and CD59 are incorporated into the virions of 2 unrelated enveloped viruses—human T-cell leukemia/lymphoma virus type-I (HTLV-I) and human cytomegalovirus (HCMV). J Immunol 1995; 155:4376-4381.
137. Korty PE, Brando C, Shevach EM. CD59 functions as a signal-transducing molecule for human T cell activation. J Immunol 1991; 146:4092-4098.
138. Morgan BP, van den Berg CW, Davies EV et al. Crosslinking of CD59 and of other glycosyl phosphatidylinositol- anchored molecules on neutrophils triggers cell activation via tyrosine kinase. Eur J Immunol 1993; 23:2841-2850.
139. Lund Johansen F, Olweus J, Symington FW et al. Activation of human monocytes and granulocytes by monoclonal- antibodies to glycosylphosphatidylinositol-anchored antigens. Eur J Immunol 1993; 23:2782-2791.
140. van den Berg CW, Cinek T, Hallett MB et al. Exogenous glycosyl phosphatidylinositol-anchored CD59 associates with kinases in membrane clusters on U937 cells and becomes Ca2+-signaling competent. J Cell Biol 1995; 131:669-677.
141. Hahn WC, Menu E, Bothwell ALM et al. Overlapping but nonidentical binding sites on CD2 for CD58 and a second ligand CD59. Science 1805; 256:1805-1807.
142. Deckert M, Kubar J, Zoccola D et al. CD59 molecule: A second ligand for CD2 in T cell adhesion. Eur J Immunol 1992; 22:2943-2947.
143. Van der Merwe PA, Barclay AN, Mason DW et al. Human cell-adhesion molecule CD2 binds CD58 (LFA-3) with a very low affinity and an extremely fast dissociation rate but does not bind CD48 or CD59. Biochemistry 1994; 33:10149-10160.
144. Zalman LS, Wood LM, Muller-Eberhard HJ. Isolation of a human erythrocyte membrane protein capable of inhibiting expression of homologous complement transmembrane channels. Proc Natl Acad Sci USA 1986; 83:6975-6979.
145. Schonermark S, Rauterberg EW, Shin ML et al. Homologous species restriction in lysis of human erythrocytes: A membrane-derived protein with C8-binding capacity functions as an inhibitor. J Immunol 1986; 136:1772-1776.
146. Watts MJ, Dankert JR, Morgan BP. Isolation and characterization of a membrane-attack-complex-inhibiting protein present in human serum and other biological fluids. Biochem J 1990; 265:471-477.
147. Zalman LS, Brothers MA, Muller-Eberhard HJ. Isolation of homologous restriction factor from human urine. Immunochemical properties and biologic activity. J Immunol 1986; 143:1943-1947.
148. Hansch GM, Schonermark S, Roelcke D. Paroxysmal nocturnal hemoglobinuria type III. Lack of an erythrocyte membrane protein restricting the lysis by C5b-9. J Clin Invest 1987; 80:7-12.
149. Zalman LS, Wood LM, Frank MM et al. Deficiency of the homologous restriction factor in paroxysmal nocturnal hemoglobinuria. J Exp Med 1987; 165:572-577.
150. Hansch GM, Weller PF, Nicholson-Weller A. Release of C8 binding protein (C8bp) from the cell membrane by phosphatidylinositol-specific phospholipase C. Blood 1988; 72:1089-1092.

151. Lublin DM, Coyne KE. Phospholipid-anchored and transmembrane versions of either decay-accelerating factor or membrane cofactor protein show equal efficiency in protection from complement-mediated cell-damage. J Exp Med 1991; 174:35-44.
152. Rother RP, Rollins SA, Mennone J et al. Expression of recombinant transmembrane CD59 in paroxysmal-nocturnal hemoglobinuria B-cells confers resistance to human-complement. Blood 1994; 84:2604-2611.
153. Kooyman DL, Byrne GW, McClellan S et al. In vivo transfer of GPI-linked complement restriction factors from erythrocytes to the endothelium. Science 1995; 269:89-92.
154. McCurry KR, Kooyman DL, Alvarado CG et al. Human-complement regulatory proteins protect swine-to-primate cardiac xenografts from humoral injury. Nature Medicine 1995; 1:423-427.
155. Rooney IA, Heuser JE, Atkinson JP. GPI-anchored complement regulatory proteins in seminal plasma—an analysis of their physical condition and the mechanisms of their binding to exogenous cells. J Clin Invest 1996; 97:1675-1686.

CHAPTER 8

GPI-Containing Molecules of Pathogenic Mycobacteria and Protozoa

Pascal Schneider and Daniel C. Hoessli

GPI-anchored proteins and glycolipids are widely distributed throughout eukaryotes. They are extremely abundant in protozoan parasites and often represent major cell surface constituents of these organisms which are exposed to variable and mostly unfavorable environments throughout their life cycles. It is likely that these surface molecules have evolved to accommodate these harsh conditions, and the use of GPI moieties rather than transmembrane segments to anchor surface antigens may be advantageous in allowing very high levels of protein packing and in reducing the interactions of extracellular proteins with the interior of the cell. The GPI-linked glycolipids, coat proteins and enzymes of parasites are also important players in the interactions with arthropod or mammalian hosts and in the avoidance of destruction by the host's immune system. More recently, several GPI structures of parasite origin have been shown to induce or modulate host cell functions to the parasite's benefit, a finding which could partially explain the outcome of certain diseases. These effects are mediated by changes in the physical properties and/or signaling pathways of host cell membranes, and the understanding of the molecular mechanisms underlying the signaling interferences caused by parasite products is of considerable interest in explaining both parasite virulence and transmembrane signaling in mammalian cells.

Mycobacterial GPI-Linked Molecules

The causative agents of tuberculosis and leprosy belong to the species of mycobacteria. Successful tissue reactions to mycobacteria consist of granuloma wherein macrophages phagocytose and kill those microorganisms. Neither *M. tuberculosis* nor *M. leprae* secrete any toxin and they owe their virulence properties to the nature and constituents of their envelopes. The mycobacterial envelopes are thick and impermeable barriers consisting of a framework of peptidoglycans, arabinogalactans and mycolic acids. The GPI-linked lipomannans (LM) and lipoarabinomannans (LAMs) are inserted within this framework and can amount to 5 mg/g of bacterial weight.[1] They are readily released in blood and infected tissues.[2,3] Part of these LMs and LAMs are integrated in the underlying mycobacterial plasma membrane by means of their GPI anchor. LM and LAM polymers originate from phosphatidylinositolmannosides (PIMs) through biosynthetic pathways that are still incompletely defined.[4] The mycobacterial GPIs only contain mannoses in the glycan that links the phosphatidylinositol to the mannan or arabinan polymers[5] and so differ from the canonical Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol GPI glycan core.[6,7] The

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

arabinan termini of the large LAM polymers may either be unsubstituted (Ara-LAMs) or capped with mannose (Man-LAMs). Whether distinct biological properties (virulence, speed of growth) characterize Mycobacteria that elaborate the different LAMs is not yet fully understood.

Biological Effects of Mycobacterial GPI-Linked Molecules

LAMs play a key role in the recognition and phagocytosis of Mycobacteria and serve as a ligand for the mannose receptor of macrophages.[8-10] In addition, released LAMs exert widespread effects on monocytes and macrophages in vitro.[12,13] In particular, LAMs are able to deactivate the phagocytic functions of macrophages and diminish their responsiveness to T lymphocyte-derived interferon-γ,[11-13] while at the same time stimulating the macrophage production of several proinflammatory cytokines.[14] Man-LAMs appear less potent than Ara-LAMs in inducing TNF-α, IL-1, IL-6 and IL-10, but retain the capacity to induce expression of the "deactivating" TGF-β cytokine.[15] The effects of LAMs on cytokine production by macrophages[5,16] and T lymphocytes[14,17] are all abolished by deacylation of the molecule. This points to the importance of the membrane-seeking, lipophilic portion of the GPI anchor in promoting the biological effects of LAMs. Recently, the polar PIM structure on the mycobacterial surface was shown to serve as an adhesin for nonphagocytic cells in a mannan-inhibitable manner.[18] The neutralization of cytotoxic free oxygen radicals by LAMs also diminishes the macrophage bactericidal capacity,[19] but paradoxically stimulates the production of nitric oxide.[20] In any event, the main block in the bactericidal pathway of macrophages towards live Mycobacteria resides in the early endosomes,[21-23] which become unable to fuse with lysosomes and to accumulate the membrane proton ATPase complexes responsible for vesicle acidification.[24] LAMs have been identified histochemically in vesicles free of Mycobacteria and therefore must become redistributed among intracellular membranes of the infected macrophage,[25] possibly through transfer to host cell membranes.[26] Whether LAMs and related molecules have any disruptive role to play in the vesicular traffic of Mycobacteria-infected macrophages remains an important question to be addressed.

The T lymphocyte proliferative response is inhibited by LAMs,[14,16,17,27] and downregulation of IL-2, IL-5 and GM-CSF gene expression in response to LAMs has been observed in human T cells.[28] However, detailed investigations of the direct effects of LAMs on T lymphocytes are still lacking.

LAMs are therefore nontoxic molecules capable of inducing contrasting responses in phagocytes and T cells. On the one hand, they enhance cytokine production, and on the other considerably impair phagocytosis and bactericidal activity. LAMs have been reported to stimulate tyrosine phosphatases in macrophage membranes and could indeed deactivate transmembrane signaling in this manner.[29] It is therefore expected that LAMs should profoundly perturb cellular interactions leading to protective responses against mycobacterial pathogens.

Interactions of Mycobacterial GPI-Linked Molecules with Host Cell Surface

PIMs, LMs and LAMs released by mycobacteria can integrate mammalian plasma membranes, a capacity which is dependent on the presence of the acyl chains as shown by deacylation and competition experiments.[30] Integration of LAMs in target cells occurs preferentially in plasma membrane domains already enriched in endogenous GPI-linked surface glycoproteins and glycosphingolipids.[30] In addition, LAMs and their derivatives, either as released molecules or as part of mycobacterial envelopes, also interact with defined receptors on the surface of specialized cells.

Mannose Receptors

The interaction of the mannosyl chains of LAMs with mannose receptors on phagocytes is more efficient with high virulence mycobacterial strains (Erdman and H37Rv) than low virulence (H37Ra) ones,[10] and free LAMs inhibit the nonopsonic binding of Mycobacteria to macrophages.[8] The terminal di-mannosyl unit present in both high and low virulence strains is necessary for interaction with the mannose receptor, but other undefined structural features of the LAM molecule are required to account for the difference in virulence. LAM molecules with arabinan groups at their termini (Ara-LAMs) no longer bind to mannose receptors.[9,10]

CD14

LAMs and several other lipoglycans[31] as well as peptidoglycans[32] bind to the GPI-linked lipopolysaccharide (LPS) receptor CD14 (see chapters 4 and 5). This receptor has been proposed to bind LAMs and transmit activation signals to phagocytic cells involved in the innate defense mechanisms.[31] GPI-anchored CD14 may be confined to specialized domains of the plasma membrane and could therefore determine the kind of interactions undergone by LPS or LAMs with cell membranes. Soluble CD14 and LAM-binding proteins could also cooperate in transferring LAMs to phospholipid bilayers, utilizing a mechanism similar to that suggested for LPS.[32]

CD1

LAMs also interact with the CD1 family of antigen-presenting molecules.[15,34] Unlike the molecules encoded in the major histocompatibility complex, CD1b is a nonpolymorphic antigen-presenting surface molecule characterized by an unusual hydrophobic groove which accommodates the lipid part of mycobacterial lipoglycans and may also present their carbohydrate portion to CD1-restricted T lymphocytes.[35,36] The association of LAMs with CD1 is thought to take place in late endosome/lysosome vesicles, where MHC class II molecules also acquire their antigenic peptides.[37] LAM and related lipoglycans elicit strong immune responses in infected individuals.[3,4] It is possible that the anti-carbohydrate antibody response observed in patients is initiated by the presentation of LAM antigens to CD1-restricted T lymphocytes[34,37] and that CD1-restricted, cytotoxic T cells may likewise contribute to killing mycobacteria-infected macrophages.[38] However, the nonprotective humoral response appears to prevail over the protective cellular one in naive human populations.[39]

GPI in Protozoa

Several methods are used to suggest or demonstrate the presence of a GPI anchor in protozoan antigens, e.g., metabolic labeling experiments using specific precursors of GPIs, direct chemical characterization of the anchor, determination of cDNA sequences which encode characteristic hydrophobic carboxy-termini,[40] and solubilization of the antigen after exposure to phosphatidylinositiol-specific phospholipase C (PI-PLC). GPI anchors in which the inositol is acylated are, however, resistant to PI-PLC treatment. The hallmark of GPI-anchored proteins is the remarkably conserved linear polysaccharide structure linking the carboxy-terminal amino acid of the protein to the lipid. The lipid moiety itself and the side chains of the conserved backbone are, however, very heterogeneous.[6] Protozoan GPI anchors for which structural information is available are depicted in Figure 8.1. It is noteworthy that a given protein may be anchored by different types of lipids. The structure of some constituents of GPI and GPI-related glycolipids is shown in Figure 8.2.

Besides GPI-anchored proteins, free glycolipids called glycoinositol phospholipids (GIPLs), which contain the conserved structure Manα1-4GlcNα1-6*myo*-inositol-1-PO_4, are

Protein
EtN-PO$_4$–6
Manα1-2 Manα1-6 Manα1-4 GlcNα1-6 *myo*-Ino-1 PO$_4$
R1 (2), R2 (3), R2' (4), R3 (6), R4 (2)
Glycerolipid or ceramide

Protein (species)	R1	R2 / R2'	R3	R4	Lipid
VSG (*T. brucei* MITat 1.4)	OH	±Galα1-2Galα1-6Galα1-3 ±Galα1-2	OH	OH	Glycerolipid: 1-*O*-acyl (C14:0) / 2-*O*-acyl (C14:0)
VSG (*T. congolense*)	OH	Galβ1-6GlcNAcβ1-4	OH	OH	Glycerolipid: 1-*O*-acyl (C14:0) / 2-*O*-acyl (C14:0)
Procyclin/PARP (*T. brucei*)	OH	$[NANA]_5[\beta Gal]_9[\beta GlcNAc]_9$ [a]	OH	Acyl various	Glycerolipid: **1-*O*-acyl (C18:0)**, 1-*O*-alkyl (C18:0) / 2-*O*-*lyso*
35/50 mucin [b] epimastigote (*T. cruzi*)	Manα1-2	OH	2-aminoethyl-phosphonate	OH	Glycerolipid [c]: 1-*O*-alkyl (C16:0) / 2-*O*-acyl (C16:0)
35/50 mucin metacyclic trypomastigote (*T. cruzi*)	Manα1-2	OH	2-aminoethyl-phosphonate	OH	Ceramide [c]: Sphinganine / *N*-acyl **(C16:0)** (C24:0)
gp90/1G7 (*T. cruzi*)	Manα1-2	OH	2-aminoethyl-phosphonate	OH	Glycerolipid: 1-*O*-alkyl (C16:0) / 2-*O*-acyl (C16:0)
Tc-85 (*T. cruzi*)	OH	OH	?	OH [d]	Glycerolipid: 1-*O*-alkyl (C16:0) / ?
Ssp-4 (*T. cruzi*)	?	?	?	?	Ceramide: Sphinganine / *N*-acyl **(C16:0)** (C18:0)
Gp63 (*L. major* *L. mexicana*)	OH	OH	OH	OH	Glycerolipid: 1-*O*-alkyl (C24:0) / 2-*O*-acyl
MSP-1 / MSP-2 (*P. falciparum*)	Xxx-Manα1-2 [e]	OH	OH	Acyl	Glycerolipid: 1-*O*-acyl / 2-*O*-acyl
P30 / gp23 (*T. gondii*)	OH	±Glcα1-4GalNAcβ1-3	OH	Acyl	Glycerolipid: 1-*O*-acyl / 2-*O*-acyl

Fig. 8.1. Structural features of GPI anchors found in protozoan proteins. The core structure depicted at the top of the Figure is conserved in all GPI anchors. This core structure may carry additional substituents at various positions (R1 to R4), which are listed in the included table. The lipid moiety is either a glycerolipid or a ceramide. In glycerolipids, the hydroxyl group at position 1 of the glycerol is either esterified with a fatty acid (acyl) or etherified with a fatty alcohol (alkyl). When known, the length of the carbon chain and the number of double bonds are indicated. If various types of lipids exist on the same protein, the major species is indicated in boldface. Microheterogeneity is present in most lipids, except in those of the VSGs that consist exclusively of myristic acid. For the detailed structure of the lipids, see Figure 8.2. a) The side chain of procyclin/PARP is attached at position R2 and contains an undefined sialylated polylactosamine; b) A portion of the 35/50 kDa mucin of *T. cruzi* is linked to the GPI via AEP instead of the conserved ethanolamine phosphate; c) The ceramide lipid is enriched in the metacyclic trypomastigote stage;[131] d) The shed form of the protein is acylated on the inositol;[118] e) Xxx is a nitrous acid-sensitive, uncharacterized substituent. References: *T. brucei* VSG,[46] *T. congolense* VSG,[292] procyclin,[68,69,293] 35/50 kDa mucin epimastigote,[130,131] 35/50 kDa mucin trypo-mastigote;[131] gp90/1G7;[132,133] Tc-85;[117] Ssp-4;[138] Gp63;[182,183] MSP-1/MSP-2;[272,277] P30[294] and gp23.[288]

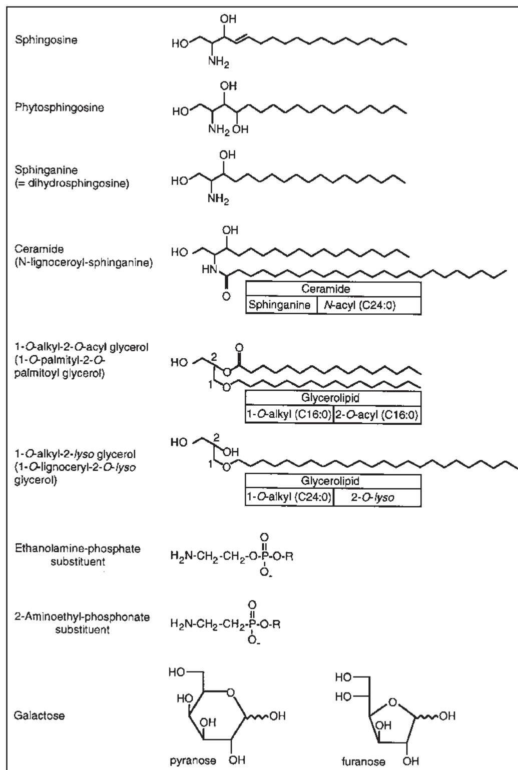

Fig. 8.2. Structures occurring in GPIs, GIPLs and LPG. Sphingosine, sphinganine and phytosphingosine are long base constituents of ceramides which are found in some GPI-anchored proteins and in several GIPLs. Alkyl-acyl glycerol is a common lipid component of both glycoinositol phospholipids (GIPLs) and protein GPIs. *Lyso*-alkyl glycerol is found in the LPG of *Leishmania* and in some GIPLs. Lipid structures are summarized in boxes showing the long base chain and fatty acid components for ceramides and the major acyl and/or alkyl chains for glycerolipids. Aminoethyl-phosphonate is a common substituent in *T. cruzi*, Herpetomonas and Leptomonas proteins and GIPLs. Galactose in the furanose ring configuration occurs in *T. cruzi O-* and *N*-linked oligosaccharides, in the lipophosphoglycan of *Leishmania*, in the lipoarabinogalactan of *Crithidia* and in GIPLs of *T. cruzi*, *Leishmania*, Endotrypanum and Leptomonas.

often major constituents of parasite surfaces (Fig. 8.3). GIPLs are characterized by a wide variety of carbohydrate and lipid structures, some of which provide the membrane anchor for bigger hydrophilic polymers such as in *Crithidia* lipoarabinogalactan[41] and in *Leishmania* lipophosphoglycan (Fig. 8.4).

African Trypanosomes

Introduction and Life Cycle

Trypanosomes are the causative agents of animal trypanosomiases (due to *T. vivax*, *T. congolense*, *T. evansi*, *T. equiperdum* and *T. brucei*) and of sleeping sickness in man (due to *T. brucei rhodiense* and *T. brucei gambiense*). These diseases are characterized by cyclic waves of fever that correlate with spikes of trypanosomes in the blood. The African *T. vivax*, *T. congolense* and *T. brucei* parasites are generally transmitted by the *Glossina* species of tsetse fly. In the mammalian bloodstream, the trypanosomes consist of a pleomorphic population of long, slender dividing forms, intermediate forms, and nondividing short stumpy forms, all of which are surface-coated with variant surface glycoprotein (VSG). Metabolic changes in the stumpy forms facilitate their developmental adaptation after ingestion by the fly, whereupon they differentiate and multiply in the tsetse midgut as noninfective procyclic trypomastigote forms. Procyclic forms migrate to the anterior portion of the gut (*T. vivax*, *T. congolense*) or colonize the salivary glands (*T. brucei*) where they differentiate and multiply as epimastigotes. Procyclic trypomastigotes and epimastigotes no longer express VSG, but switch to a different surface protein (procyclin/PARP in *T. brucei*;

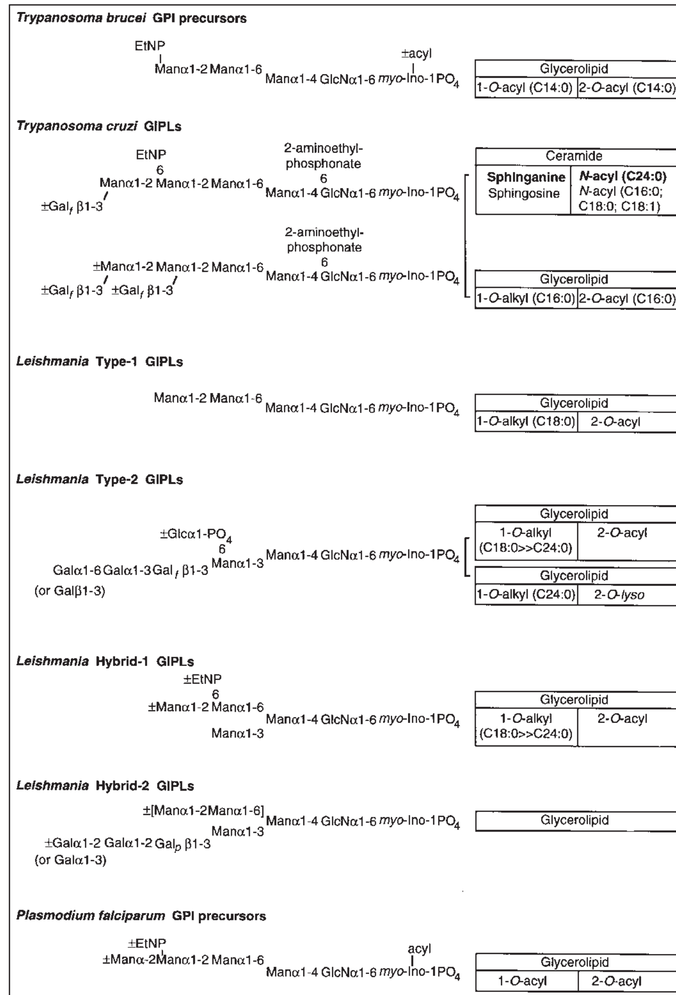

Fig. 8.3. Structure of *Trypanosoma*, *Leishmania* and *Plasmodium* GIPLs and GPI precursors. Only representative GIPL species are shown. In most cases shorter end products or intermediates have also been characterized. When known, the nature and composition of the lipid component is shown in a box (see Fig. 8.2 for details). References: *T. brucei*,[50,295,296] *T. cruzi*,[154-156] *Leishmania* Type-1,[209] Type-2,[210,213,297] Hybrid-1,[183,208,298] Hybrid-2 (McConville MJ, personal communication), *P. falciparum*.[278]

GARP in *T. congolense*). After division, epimastigotes resume synthesis of the VSG coat and mature to nondividing, free-swimming, infective metacyclic trypomastigotes (for a review, see refs. 42,43).

Variant Surface Glycoprotein (VSG)

The trypanosome uses the large, serially expressed repertoire of antigenically distinct VSGs, for which over a thousand genes are present in its genome. The VSG has the dual function of preventing complement-activated lysis by the alternative pathway and allowing

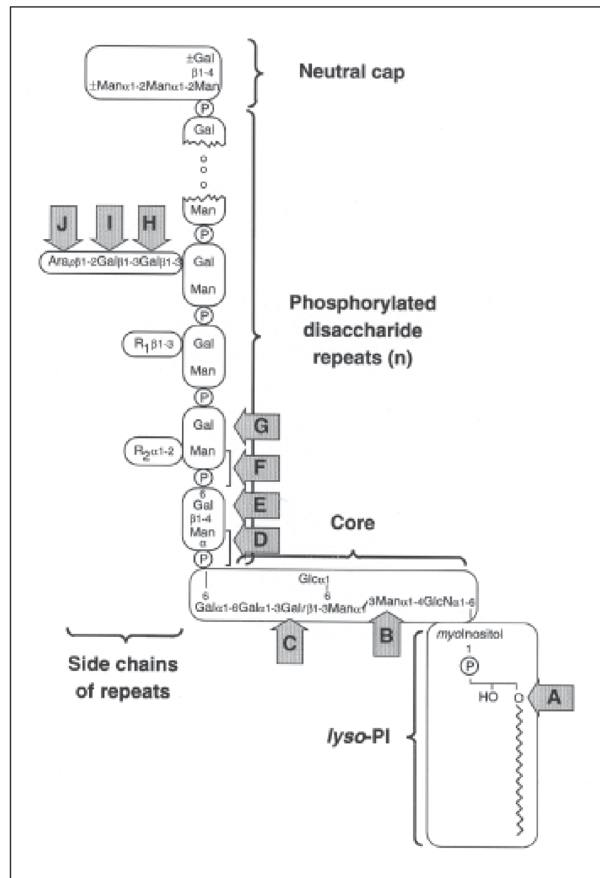

Fig. 8.4. Schematic structure and biosynthesis of *Leishmania* lipophosphoglycan (LPG). Schematic representation of LPG showing phosphatidylinositol (PI), core, phosphorylated disaccharide repeats and cap structures. The *lyso*-PI contains mainly C24:0 and C26:0 long alkyl chains. The phosphorylated disaccharide repeats can be substituted either on the Gal (R_1) or on the Man (R_2) residues. Residues for which activated precursors or biosynthetic enzymes have been characterized are indicated with arrowheads containing a capital letter: A. NADH-dependent alkyl-glycerol cleavage enzyme.[299] Biosynthesis occurs in glycosomes;[300] B. Transferred by a Dol-P-Man: GlcN-PIα1-4-mannosyltransferase;[301] C. Defective addition in *L. donovani* R2D2 mutant ($lpg1^-$).[302,303] LPG1 is a Golgi enzyme;[304] D. Defective addition in *L. donovani* C3PO mutant ($lpg2^-$).[305] LPG2 is a Golgi transporter allowing GDP-Man uptake into the Golgi.[306] The initiating mannosyl-phosphate is transferred by a Mn^{2+}-dependent GDP-Man: Galα1-6Gal-R α-mannosylphosphate transferase whose activity is downregulated in the amastigote stage;[307] E. Defective addition in OB1 mutant ($lpg3^-$). LPG3 is a chaperone which allows the first repeating unit to have the Gal residue attached (S. J. Turco and S. Beverley, unpublished observations); F. Transferred from GDP-Man[308] by a GDP-Man: Galβ1-4Manα-1-PO4-R α-mannosylphosphate transferase.[309] Defective addition in JEDI mutant ($lpg4^-$). LPG4 might be the elongating mannosylphosphate transferase (S. J. Turco and S. Beverley, unpublished observations); G. Transferred from UDP-Gal;[308] H. Defective addition in *L. major* Spock mutant.[229] Transferred on phosphorylated disaccharide repeats from UDP-Gal by a Mn^{2+}-dependent β1-3 galactosyl transferase;[310,311] I.- Transferred from UDP-Gal by a β1-3 galactosyl transferase activity distinct from the previous one (see point G);[310] J. Transferred from GDP-α-D-Ara*p*.[312,313]

the parasite to escape immune recognition by periodic switching to a new antigen type.[42] The surface-exposed N-terminal domains of VSGs are highly variable and yet display similar tertiary structures,[44] whereas the C-terminal domains are conserved. The GPI moiety of VSGs contain a typical dimyristoyl-glycerol lipid moiety and a side chain of branched and space-filling Gal residues.[45,46] The specific use of myristic acid in the GPI of VSG may be required to achieve very dense surface packing of this protein (10^7 VSG molecules per cell, representing 10% of the total protein content of the cell).

Although the GPI backbone of trypanosome and mammalian proteins is identical, the enzymes involved in its biosynthesis are markedly different in their substrate specificities and inhibitor sensitivities, and therefore represent potential targets for anti-protozoan chemotherapy.[47-49] In addition, the trypanosome-specific fatty acid exchange and remodeling reactions occurring in GPI precursors and mature VSG[50,51] are essential for trypanosome survival, and myristic acid analogs are toxic to the parasite.[52]

A GPI-specific phospholipase C, which is associated with the cytosolic face of intracellular membranes, is expressed in bloodstream trypanosomes.[53,54] PI-PLC-mediated release of VSG occurs upon disruption of the parasite,[55] under stress conditions[56] or during normal protein turnover.[57] However, the rapid release of VSG during differentiation to the procyclic form is mediated by a protease which also cleaves stage-specific, transmembrane invariant proteins.[58,59]

GPI anchors isolated from VSG or its precursors induce macrophages to secrete IL-1α and TNF-α. Similar effects have been obtained with crude parasite extracts. This activation depends on protein tyrosine kinase activation, and can be inhibited by a monoclonal antibody raised against the GPI moiety of *Plasmodium* antigens (see below). This mechanism could explain the cachexia frequently observed during trypanosome infection.[60] Interestingly, recombinant human IL-1β specifically binds the Man-6-phosphate of the VSG anchor.[61] This could result in the sequestration of IL-1β and thus decrease its biological efficacy.

Procyclin

During differentiation of the bloodstream to the procyclic form, VSG is released from the cell surface[62,63] and is replaced by a new invariant protein coat consisting of procyclin (also called procyclic acidic repetitive protein, PARP).[62,64-66] There are two types of procyclins which are named according to their glutamic acid- and proline-rich repetitive sequences, i.e., EP- and GEEPT-procyclins. Both types can be expressed simultaneously in varying ratio at the cell surface.[67,68] Procyclin possesses a complex, sialylated GPI anchor (Fig. 8.1),[69] which in GEEPT-procyclin accounts for over two-thirds of the apparent molecular weight.[67] As trypanosomes are unable to synthesize sialic acid, they transfer it from host proteins to the GPI anchor of PARP by means of a stage-specific, membrane-bound *trans*-sialidase.[70-72]

In *T. congolense*, the functional homologue of procyclin is a glutamic acid/alanine-rich protein (GARP).[73,74] The procyclin and GARP coats may protect parasites from attack by digestive enzymes in the insect gut, or may serve as a means of direct attachment to epithelial membranes, and influence tropism and development in the fly (for a review, see ref. 75). Knockout experiments have revealed that all of the six to seven EP-procyclin genes could be deleted, but that at least one of the two GPEET-procyclin genes was required, for parasite viability.[76] The EP-procyclin knockouts were, however, limited in their ability to establish a heavy infection in the insect midgut. The two forms of procyclin thus appear to play different roles: GPEET-procyclin is required for cell growth, and EP-procyclin enhances survival in the fly.[76]

Transferrin Receptor

Trypanosomes are dependent upon host transferrin for growth.[77] The transferrin receptor of trypanosomes is a heterodimeric complex of low abundance (about 3000 molecules per cell) encoded by two related genes, *ESAG6* and *ESAG7* that are associated with the VSG expression site. A functional transferrin receptor is only obtained when *ESAG6* and *ESAG7* proteins are coexpressed.[77-83] *ESAG6*, but not *ESAG7*, encodes a GPI-addition signal; the two genes are otherwise very similar.[78] The *ESAG6* product is indeed a glycoprotein of 50-60 kDa modified by a GPI anchor, whereas the 42 kDa *ESAG7* product has an unmodified COOH-terminus.[80]

Transferring binding activity has been observed in the flagellar pocket, a membrane invagination specialized in secretion and endocytosis.[84,85] Interestingly, both transferrin and the transferrin receptor predominantly localize to the lumen of the flagellar pocket, whereas a few of these proteins are located on the membrane of the flagellar pocket and in intracellular vesicles.[80] These results suggest that the transferrin receptor may shuttle between the membrane and the lumen of the flagellar pocket, where it may bind its ligand more efficiently. After transferrin binding, the ternary complex is endocytosed to an acidic endosomal compartment wherein iron is released from transferrin, and transferrin from its receptor.[86] Apo-transferrin is then degraded in lysosomes, and only the receptor is recycled. This mechanism is distinct from the mammalian one where both the receptor and apo-transferrin are recycled (for a review, see ref. 87).

Trypanosoma Cruzi

Introduction and Life Cycle

Trypanosoma cruzi causes Chagas disease, which is endemic in South and Central America. Unlike its African relative *T. brucei*, *T. cruzi* does not undergo antigenic variation, but avoids the immune response by invading and multiplying within host cells. Infection is characterized by an acute phase with high parasitemia, followed by an asymptomatic phase which may last for more than 10 years and lead to a chronic disease, with characteristic megasyndromes of the heart and digestive tract. In the course of a complex life cycle involving mammals and insect vectors (reduviid bugs), several life stages of *T. cruzi* are recognized: the dividing epimastigotes which colonize the midgut of the vector; the nondividing, infective metacyclic trypomastigotes which are excreted within feces of the feeding bug and enter the mammalian host via the micro-wound generated by the biting vector; the amastigotes, which proliferate in the cytoplasm of a variety of host cells before differentiation into nondividing trypomastigotes; and trypomastigotes which are released in the bloodstream upon host cell lysis and provoke a transient parasitemia before invading new host cells.

T. cruzi expresses numerous GPI-anchored surface antigens, which can be either stage-specific or common to different life stages.[88] Establishing the link between a gene and a mature surface antigen is not easy in *T. cruzi*: first, the genes encoding surface antigens often belong to large families, and protein products of closely related genes can be expressed simultaneously.[89] Second, carbohydrate substituents of these antigens carry strain- or stage-specific immunogenic epitopes[90-92] that do not always correlate with actual protein expression.[93,94] This latter point can generate some confusion when monoclonal antibodies against these epitopes are used for antigen identification.[88,95] The GPI-anchored antigens discussed below are expressed on various life stages of *T. cruzi*, as summarized in Table 8.1.

***Table 8.1 GPI-anchored antigens expressed on various life cycle stages of* T. cruzi.**

Life Cycle Stages	GPI-Anchored Molecules
Epimastigotes	GIPLs; 35/5.0 kDa mucins; Cruzain
Metacyclic trypomastigotes	GIPLs; 35/50 kDa mucins; Cruzain; *trans*-sialidases: gp82, gp90/IG7
Amastigotes	Cruzain; Ssp-4; *trans*-sialidases: ASP-1, ASP-2; SA85-1
Cellular and bloodstream trypomastigotes	GIPLs; Cruzain; *trans*-sialidase: SAPA; SA85-1; F2/F3 mucins; Tc-85

Sialidase/*Trans*-Sialidase Family

The genes encoding *trans*-sialidase and related proteins devoid of enzymatic activity belong to a large family of several hundred members characterized by:

1. GPI-addition signals; and
2. Sequences encoding a conserved amino acid motif of 11 residues (VTVxNVxLYNR).

These genes are classified into four subfamilies according to sequence criteria.[96,97] Group I contains the active *trans*-sialidase, the inactive shed acute phase antigen (SAPA) and related sequences sharing homologies with bacterial and viral sialidases. Group II comprises genes coding for the so-called gp85 surface glycoprotein family, such as the metacyclic trypomastigote-specific gp82[98] and gp90/1G7, the amastigote-specific ASP-1[99] and ASP-2,[100,101] the trypomastigote-specific *TSA-1*[102] and Tt34l, and the SA85-1 family which is expressed in both amastigotes and trypomastigotes. Group III contains proteins of about 160 kDa, including a flagellar protein, Fl-160[103] and group IV consists of the more distantly related Tc13 family (refs. 96, 97 and references therein).

T. cruzi parasites circumvent their inability to synthesize sialic acid precursors[104] by expressing a unique *trans*-sialidase, which transfers α2-3-linked sialic acids from host glycolipids and glycoproteins to β-Gal-containing mucins present on the parasite surface. The 120-220 kDa sialidase/*trans*-sialidase is an oligomeric GPI-anchored protein mostly expressed in infective trypomastigotes; its heterogeneity results from variations in the presence and length of a noncatalytic, but very immunogenic, carboxy-terminal repetitive domain.[105] Its activity is dependent on a tyrosine residue (Tyr342) as shown by natural or experimental point mutations.[106-108] The enzyme is absent in amastigotes, whereas epimastigotes express a distinct monomeric *trans*-sialidase which is devoid of COOH-terminal repeats and is not anchored to the membrane via a GPI anchor.[97,109,110] Sialylation of the F2/F3 mucin complex of trypomastigotes (see below) only occurs on parasites exposed to extracellular environments and leads to the formation of a novel, stage-specific surface epitope, Ssp-3.[88,111] Antibodies directed against Ssp-3 block attachment to host cells,[111,112] and populations of trypomastigotes not expressing *trans*-sialidase are attenuated, the latter phenotype being fully reversed by addition of exogenous *trans*-sialidase.[113] Surface sialylation has also been implicated in trypomastigote resistance to complement-mediated lysis (for a review, see ref. 97) and in the exit of intracellular parasites from parasitophorous vacuoles into the cytoplasm.[114] In this case, PI-PLC released sialidase activity appears to desialylate lysosomal glycoproteins, rendering the membrane more susceptible to Tc-TOX, a parasite hemolysin related to the mammalian C9 lytic component of the complement.[115] Finally, *trans*-sialidase is readily shed from parasites and

may favor the establishment of an infection. Indeed, mice primed with small doses of *trans*-sialidase before parasite injection display greatly enhanced parasitemia and mortality rates, an effect which is dependent on the *trans*-sialidase activity.[116]

Tc-85 Antigens

Trypomastigote-specific, GPI-anchored Tc-85 antigens[117] are possible products of the *TSA-1* and *Tt34l* genes and contain both acylated and nonacylated inositol. Unexpectedly, the acylated form is shed from the parasite surface, whereas the nonacylated one remains cell-associated.[118] Monoclonal antibodies to Tc-85 reduced invasion of cultured mammalian cells by trypomastigotes,[119] and an acidic component of the Tc-85 family was shown to bind the host's extracellular matrix protein laminin,[120] pointing to the involvement of Tc-85 proteins in the attachment and cell invasion processes.

SA85-1 Antigens

The SA85-1 protein family expressed in amastigotes and trypomastigotes is encoded by a large number of genes,[89] resulting in the simultaneous expression of related proteins. This polymorphism may play a role in immune evasion in a way distinct from the antigenic variation of African trypanosomes. In *T. cruzi*, protein heterogeneity may limit (or dilute) the amount of epitopes required to fully activate T lymphocytes, and would therefore not favor an efficient antiparasitic Th1 response.[121]

Gp82 and Gp90/1G7 Antigens

Metacyclic trypomastigotes express a specific set of GPI-anchored surface proteins,[95] including gp90/1G7 and gp82, which are involved in infectivity and host cell invasion.[122-124] This indicates that the molecular mechanism of host cell invasion by metacyclic and bloodstream form trypomastigotes are at least partially distinct.

Surface Mucins (35/50 kDa Mucins)

These antigens identified in epimastigotes more than 20 years ago[125] are GPI-anchored, *O*-glycosylated mucins[92,104] also expressed on metacyclic trypomastigotes.[126,127] The "LPG-like antigen" described in epimastigotes most probably represents the same molecule.[128] A complex gene family encodes these antigens, which differ in the number and sequences of characteristic threonine- and proline-rich repeats[94,129] carrying a series of branched, *O*-linked oligosaccharides harboring terminal β-Gal residues which act as sialic acid acceptors.[92,130,131] These carbohydrate structures are unusual in that they are linked to threonine residues via GlcNAc and contain terminal and internal Gal_f residues,[92,131] unlike mammalian *O*-linked oligosaccharides.

The only structural variation of the 35/50 kDa mucins detected during transition from noninfective epimastigote to infective metacyclic trypomastigote forms occurs in the GPI lipid, which changes in 70% of the cases from an alkylacyl-glycerol to a ceramide (Fig. 8.1).[131] This exchange probably occurs after the addition of the GPI to the protein, as GPI precursors in *T. cruzi* do not contain ceramide.[132] This developmentally regulated lipid exchange might correlate with the ability of the parasite to selectively shed the ceramide-containing mucins upon invasion of the host cell,[127] but not the alkylacyl-PI-anchored 1G7 antigen.[131,132] Interestingly, ceramides of similar structure originating from *T. cruzi* GIPLs have a potential to induce biological responses in the host's immune system (see below).[134]

F2/F3 Mucins

In the mammalian trypomastigote stage, the F2/F3 mucin complex is a group of GPI-anchored molecules sharing the stage-specific epitope Ssp-3,[88] which is dependent on parasite sialylation.[111] The *O*-linked oligosaccharides of the F2/F3 mucins are linked to threonine residues via GlcNAc rather than by GalNAc, and contain terminal α-Gal residues[90] which are also present in the *N*-linked oligosaccharides of Tc-85.[135] These α-Gal residues are the targets of lytic antibodies found in patients with chronic Chagas disease and which lyse parasites by intense agglutination in a complement-independent fashion. Although anti-Gal antibodies exist in normal human serum, these are not lytic to *T. cruzi* and are very poor binders of the F2/F3 mucins compared to Chagas anti-Gal antibodies.[90]

Ssp-4 Antigen

The amastigote-specific Ssp-4 surface protein(s) is named after its characteristic epitope Ssp-4, which is detected both in vitro and in vivo.[88] This PI-PLC sensitive, GPI-anchored antigen is progressively shed during transformation of amastigote to trypomastigote, or epimastigote forms.[136,137] The lipid moiety of Ssp-4 is a ceramide, and newly synthesized, free ceramides of similar composition have been specifically detected in freshly differentiated amastigotes.[138] Ssp-4 can be released from the parasite together with membrane material from the flagellar pocket of the amastigote.[139] The gene(s) encoding Ssp-4 are not known, although the amastin gene family represents a possible candidate.[140,141] The amastin genes encode surface proteins of about 170 amino acids with a possible GPI-addition signal and several putative *O*-glycosylation sites reminiscent of mucins.

Membrane-Bound Cruzain

T. cruzi expresses a glycosylated lysosomal cysteine protease of 51/57 kDa, called cruzain or cruzipain, which is present in the three main stages of the parasite. Cruzain is encoded by a large number a tandemly arranged genes, clustered on two to four chromosomes.[142-145] Most of these genes are apparently identical and encode a predicted soluble form of lysosomal cruzain. However, the genes at the 3' extremities of the arrays are different and encode GPI-addition signals.[145] In common with other cysteine proteinases of *Trypanosomatid* origin, the soluble cruzain contains a noncatalytic, posttranslationally modified and immunodominant COOH-terminal domain.[146] Cruzain inhibitors block both intracellular amastigote replication and differentiation into trypomastigotes and decrease the ability of trypomastigotes to invade host cells.[147] These inhibitors are taken up by intracellular amastigotes in which they accumulate in vesicles, but they do not reach lysosomes of the mammalian host.[148] The existence of a GPI-anchored form of cruzain is suggested by the fact that a GPI-anchored surface antigen of 50/55 kDa identified in the epimastigote, trypomastigote and amastigote stages[149,150] and displaying cysteinyl protease activity[151] can be detected with anti-cruzain antibodies in freeze-fractured amastigote membranes.[137] Whether cruzain inhibitors prevent amastigote replication by inhibiting soluble cruzain, or whether inhibition of GPI-anchored cruzain or of other proteases also contribute to this phenotype is not entirely resolved. Interestingly, *T. cruzi* stably expressing the PI-PLC of *T. brucei* showed impaired surface expression of several GPI-anchored proteins, including cruzain. These parasites were still infective, although with reduced efficiency, but were arrested at the amastigote stage by failure to duplicate their nucleus.[152,153]

GIPLs

Epimastigotes synthesize large amounts of GIPLs[125,154] (also known as LPPG in the literature) which resemble a protein GPI anchor in the core structure and are characterized by the presence of additional galactofuranose residues, an aminoethylphosphonate

substituent and a ceramide lipid[154-156] (Fig. 8.3). *T. cruzi* GIPLs either contain an ethanolamine phosphate substituent on the third Man residue (as in protein GPIs), or additional Gal_f residues.[156] These GIPLs downregulate activation of both $CD4^+$ and $CD8^+$ T cells in vitro and in vivo, and this effect was mapped to their ceramide moiety,[134] suggesting that either a bulky substituent does not affect ceramide activity or that target cells contain the necessary enzymes to release active ceramide from their GIPL precursors. The effect was also obtained with palmitoyl(C16:0)-sphingosine and lignoceroyl(C24:0)-sphinganine (which is the major ceramide found in GIPLs), but not with palmitoyl-sphinganine, indicating an effect of the 4,5 double bond (Fig. 8.2). These results also indicate that beside the 4,5 double bond, the nature of the fatty acid chain can also modify the biological activity of ceramides.[134,135] The effect of exogenously added ceramide on cells has been well studied and can cause cell cycle arrest or apoptosis.[157,158] The immunomodulatory action of GIPLs would favor Th2 type responses, since $CD4^+$ T cells exposed to GIPLs display a decreased production of IL-2, but not IL-4.[134] This phenomenon could contribute to chronic infections by generating a predominantly useless Th2 instead of a Th1-type response. Trypomastigotes express significant amounts of GIPLs (about 10% of the epimastigote content),[159] together with other ceramide-containing molecules.[131,132] A drift towards ceramide anchors rather than alkyl-acyl glycerol has been reported during epimastigote transition from log to stationary phase,[160] or epimastigote differentiation to metacyclic trypomastigotes,[131] and free ceramide is also present in *T. cruzi*.[161] These molecules are a potential source of active ceramides and may transfer from parasites to host cells, similarly to ceramide-containing mucins during cell invasion.[127]

The *T. cruzi* GIPLs are potent activators of immunoglobulin secretion by B cells and act synergistically with cytokines. This activity was mapped to the inositolphosphoglycan moiety, which was even more active than the native molecule.[162] In another study, GIPL headgroups were able to reduce ACTH action in calf adrenocortical cells, and this inhibition was markedly reinforced after removal of the Gal_f residues.[163] These effects could be due to solubilized GIPLs that interfere with a putative signaling pathway of the host responding to soluble, extracellular inositolphosphoglycans.

Leishmania

Introduction and Life Cycle

Leishmania is a protozoan parasite with a digenetic life cycle occurring in a mammalian host and a sandfly vector. Three major stages of the parasite can be recognized in its life cycle. The amastigote form is an obligate intracellular parasite of mammalian macrophages, where it lives and proliferates as round, nonmotile cells with a cryptic flagellum. The procyclic promastigote form infests the digestive tract of sandfly vectors of the *Phlebotominae* or *Lutzomia* genus and is characterized by the presence of an anterior flagellum. Finally, the free-swimming metacyclic promastigotes present in the mouth parts of the sandfly have an elongated and flagellated morphology, a high motility and have undergone the biochemical preadaptations for successful infection of the mammalian host. All three stages of the parasite express surface macromolecules GPI-anchored to the plasma membrane. GIPLs are abundantly expressed in all three stages of the parasite, while GPI-anchored proteins (gp63) and lipophosphoglycans (LPG) are predominantly expressed at the surface of procyclic and metacyclic promastigotes.

Surface Protease Gp63

This abundant surface glycoprotein is a zinc endopeptidase with broad substrate specificity[164-167] that has been identified in promastigotes of virtually all *Leishmania* species

investigated.[168-170] Gp63 genes organized in tandem arrays located on one or more chromosomes are present in 5 to more than 50 copies, according to the species, and some of them can be expressed simultaneously.[171-176] Gp63 genes can be either constitutively expressed or specifically transcribed in procyclic or metacyclic promastigote stages.[173,177] Specific 3'-untranslated regions are essential for stage-specific expression in metacyclic promastigotes.[178,179] Most gp63 cDNAs predict the addition of the GPI anchor, which is found in the mature protease[180,181] and consists of the minimally required structure to anchor a protein[182,183] (Fig. 8.1). Mutation of the GPI attachment site results in the release of a soluble protease.[184] In metacyclic promastigotes of *L. chagasi*, gp63 expression is increased by ten-fold and a distinct protease of 59 kDa, encoded by one of the metacyclic-specific genes, is expressed and correlates with infectivity of the parasite.[185] It is noteworthy that expression levels of gp63 do not correlate with infectivity, as exemplified by the avirulent, LPG-deficient strain of *L. major* which overexpresses gp63.[171]

Gp63, like other surface proteins, is not accessible to antibodies[186] or to the protease inhibitor α_2-macroglobulin,[187] probably because it is buried under the thick glycocalyx of metacyclic stages.[188,189] Complement activation occurs at the promastigote surface independently of gp63, but may be regulated by gp63. In gp63-deficient promastigotes, or in promastigotes expressing proteolytically inactive gp63, fixation of terminal complement components occurs, leading to parasite lysis. The same parasites transfected with active gp63 display accelerated conversion of C3b to an inactive iC3b-like molecule, thus preventing formation of the membrane attack complex and leading to enhanced resistance to serum-mediated lysis.[190] In addition, iC3b provides the ligand for Mac-1-mediated recognition of promastigotes by macrophages,[190,191] without activating the microbicidal defenses of the latter. The surface protease might contribute to parasite protection within macrophage phagolysosomes[164,192] and might also prevent presentation of parasite-derived peptides by class I molecules of the major histocompatibility complex. In a particular case, presentation of an ovalbumin peptide derived from parasites transfected with ovalbumin was inhibited by the endopeptidase activity of gp63.[193] It remains to be investigated whether this effect was fortuitous or could be of more general significance. In this respect, it is noteworthy that live promastigotes and amastigotes can degrade extracellular peptides with peptidase activities distinct from that of gp63.[194]

Occurrence of gp63 in the amastigote stage seems to depend on the parasite species. It is virtually absent in *L. major*,[195] but it is quite abundant in *L. mexicana* where it is expressed as a soluble protein apparently confined to the lysosomal compartment of the parasite,[196,197] a remarkably unusual location for a metalloprotease. Amastigote gp63 is the probable product of the C1 family of gp63 genes encoding transmembrane proteases lacking the GPI addition signal.[173,177] The production of soluble gp63 may be explained either by a post-translational proteolytic event or by the use of a gene directly encoding a soluble protein, as has been reported in *L. guyanensis*.[176]

Gp46/M2 (PSA-2)

The 46 kDa surface antigen (gp46/M2) first described in *L. amazonensis*[198] and a group of proteins of 94, 90 and 80 kDa (promastigote surface antigen-2 complex; PSA-2) detected in *L. major*[199] are products of a single gene family comprising more than 14 members. These proteins contain a short N-terminal motif, repeated 3 to 7 times depending on the species[179,200,201] followed by serine-, threonine- and proline-rich sequences reminiscent of mucins and a conserved C-terminal domain carrying the GPI anchor.[199,202] mRNA levels of gp46/PSA-2 are developmentally regulated and increase by more than 30-fold when promastigotes of *L. chagasi* progress to the infectious metacyclic stage.[179] The protein is also expressed at the surface of *L. major* amastigotes as a PI-PLC-resistant 50 kDa molecule,[203]

but has not yet been assigned enzymatic or other functions. Its conformation or post-translational modifications are probably unusual, as suggested by the observation that protective immunity in mice was obtained by vaccination with the recombinant protein produced in *L. mexicana*, but not by a bacterial recombinant protein.[204] Gp46/PSA-2 genes are present in a number of *Leishmania* species except in the *L. braziliensis* complex (*L. braziliensis*, *L. guyanensis* and *L. panamensis*) where all copies have been deleted.[205-207]

Glycoinositol Phospholipids (GIPLs)

GIPLs are free GPI molecules which are very abundant in all developmental stages of the parasite. They are classified (Fig. 8.3) according to structural criteria: Type-1 GIPLs are related to protein GPI anchors (Fig. 8.1) and Type-2 GIPLs resemble the GPI core of LPG (Fig. 8.4). Glycolipids displaying features of both Type-1 and Type-2 GIPLs are referred to as Hybrid-1 GIPLs whereas Hybrid-2 GIPLs contain distinct branched oligosaccharides. Only the minor fraction of these GIPLs containing very long alkyl chains on the *sn*-1 position of the glycerol (C24:0 or C26:0) are biosynthetic intermediates of GPI-anchored proteins and LPG,[183] whereas the bulk of GIPLs, containing shorter alkyl chains (C18:0), are metabolic end products incorporated into the glycocalyx architecture of the parasite surface. In promastigotes, GIPLs are buried under a thick LPG coat and may not be accessible to host macromolecules. The amastigote glycocalyx is thinner,[188] containing no other GPI-linked molecules, and probably displays surface-exposed GIPLs along with host-derived glycosphingolipids.[6] Depending on the subset of exposed GIPLs, the surface may be rich in terminal Man (Type-1 GIPLs of *L. donovani* and Hybrid-1 GIPLs of *L. mexicana*),[208,209] or Gal residues (Type-2 GIPLs of *L. major*).[210] These carbohydrate epitopes may be recognized by macrophages either directly (mannose-fucose receptor)[211] or after opsonization with -mannose-binding protein of the serum[212] or specific anti-Gal antibodies,[213] and therefore facilitate the entry of amastigotes into a new host macrophage.

Interaction of GIPLs with the Host

Parasites certainly modulate to their advantage some of the host cell functions involved in macrophage activation and cytokine production. GIPLs, or GIPL degradation products, have been proposed to be directly implicated in these phenomena by virtue of their structural homology with host signaling molecules. The carbohydrate headgroup of iM4, a Hybrid-1 GIPL of *L. mexicana* containing the evolutionarily conserved core glycan of protein GPI anchors, could induce activation of the protein tyrosine kinase $p59^{hck}$ in macrophages, but was unable to activate PKCε.[214] On the contrary, both Type-2[215] and Hybrid-1 GIPLs[216] have a tendency to downregulate this response and could inhibit production of nitric oxide by the macrophage in response to IFN-γ, concomitantly reducing their leishmanicidal activity. This effect was mapped to the alkyl-*acyl* glycerol lipid moiety of GIPLs.[215] A similar effect on inducible nitric oxide synthase was obtained with LPG but with distinct structural requirements[217] (see below). GIPLs and LPG may thus participate in *Leishmania*l immune evasion. Indeed, clinical and experimental evidence indicates that the control of cutaneous *Leishmania*sis requires IL-12 production by activated macrophages which drive Th1 cells to differentiate and proliferate. Th1 cells produce IFN-γ that activates macrophages to produce NO and kill intracellular parasites (for a review, see ref. 218). The inhibition of inducible nitric oxide synthase expression by LPG might be therefore important for intracellular survival of the parasite.

Lipophosphoglycan (LPG)

The LPG molecule is predominantly expressed in promastigotes. It is characterized by several unusual structural features and consists of a backbone of phosphorylated disaccharide

repeats which is capped with neutral oligosaccharides (Fig. 8.4). The repeats can be substituted with various branching oligosaccharides and the molecule is anchored in the membrane via a GPI anchor related to that of Type-2 GIPLs and containing a *lyso*-ether lipid (mainly 1-*O*-lignoceryl-2-*O*-*lyso*-glycerol). Three classes of LPG are recognized according to the presence and structure of substituents on the phosphorylated disaccharide repeats. Type-1 LPG (e.g., in East African strains of *L. donovani*) is unsubstituted.[219,220] Type-2 LPG (e.g., *L. mexicana*, *L. major* and *L. tropica*) are substituted on the C-3 position of the Gal residues present in the disaccharide repeats with side chains of varying complexity depending on species and strains.[221-224] Type-3 LPG (of which L. *aethiopica* is the only known member) contain side chains on both the C-3 position of the Gal residue and the C-2 position of the Man residue in the disaccharide repeat, the latter substitution being likely to affect the extended helical conformation of LPG.[224,225]

The biosynthesis of LPG requires a number of steps, some of which have been characterized by functional complementation of LPG-deficient *Leishmania* mutants (Fig. 8.4). The GPI anchor is synthesized first and serves as a support for initiation and elongation of the phosphorylated disaccharide repeats. The half life of surface LPG is considerably shorter than that of GIPLs because it is readily shed from the parasite surface.[223,226]

Interaction of LPG with the Vector

Parasites ingested during a blood meal infest the digestive tract of the vector, and successful infection correlates with the ability of parasites to bind to the midgut epithelium. In a later stage, differentiation to the infective metacyclic promastigotes is accompanied by detachment of the parasite and migration to the mouth parts of the insect. The LPG appears to be required in the initial stages of infection, as LPG-deficient mutants proliferate less than wild type parasites in the insect's digestive tract.[227] Proliferation alone, however, is not sufficient to establish a long-term infection if attachment to the midgut epithelium does not taken place.[227] This interaction occurs in specific pairs of vectors and parasites and depends on the complementarity of the insect's lectins and the parasite's carbohydrate epitopes.[227-229] Subsequent detachment of *L. major* metacyclic promastigotes depends on developmental modifications of the LPG, leading to its elongation and to the masking of the lectin-interacting terminal β-Gal residues by β-Ara residues.[221-228] The metacyclic form of LPG is first detected 5 days after infection of the fly and is found in 100% of the parasites egested by the fly 10 days later.[230] In *L. donovani*, the metacyclic LPG is altered in its packing or conformation and no longer interacts with gut lectins.[189] The notion of a developmentally-controlled, conformational change in the LPG molecule is supported by the fact that human C-reactive protein specifically binds to metacyclic LPG but not to procyclic LPG of *L. donovani*.[231] Thus, midgut adhesion appears to be a sufficiently critical component of vectorial competence to provide the evolutionary drive for LPG structure polymorphisms.

Interaction of LPG with the Host

Between the time of inoculation and infection of macrophages, promastigotes are exposed to the potential lytic effects of normal serum. The developmental modifications of *L. major* and *L. donovani* LPG are similar, particularly with respect to the increase in the number of repeat units, resulting in a doubling of the glycocalyx thickness in metacyclic promastigotes.[189,232] This modification prevents binding of the membrane attack complex to *L. major* metacyclic promastigotes.[233,234] *L. donovani* metacyclic promastigotes also display higher resistance to complement,[235,236] probably for the same reason. Complement activation and C3 deposition on the parasite surface could be important in targeting the parasite to macrophages and promoting their phagocytosis and intracellular survival.[237,238]

Alternative pathways implicate mannose-fucose receptors,[211,239] direct binding of LPG to macrophage receptors,[240,241] and opsonization by either serum mannan-binding protein that recognizes the oligosaccharide caps of LPG[212] or C-reactive protein recognizing phosphorylated disaccharide units of metacyclic *L. donovani* LPG.[231]

LPG is undetectable in *L. donovani* and *L. mexicana* amastigotes,[209,242] whereas amastigotes of *L. major* express low levels of an antigenically distinct LPG[243-244] recognized by macrophage receptors.[245] In *L. mexicana* amastigotes, however, there is no intrinsic surface ligand to enable high affinity interaction with macrophages.[246] For both *L. major* and *L. mexicana*, opsonization with specific host immunoglobulins may be the key factor in vivo because amastigotes carry detectable surface immunoglobulins and because progression of the disease is slower in mice lacking immunoglobulins.[246,247] Possible targets for the immunoglobulins may be the abundant surface GIPLs of the amastigote stage.[208-210]LPG mediates downregulation of a variety of biological functions such as production of proinflamatory cytokines by macrophages,[248,249] expression of inducible nitric oxide synthase,[217] induction of c-Fos transcription factor,[250] oxidative burst[251-254] and monocyte chemotaxis.[251,252] These effects are similar to those of *Leishmania* GIPLs in that they downregulate macrophage functions and activation, but contrast with those observed in response to malarial GPIs, suggesting that the different structural features of these glycolipids can lead to distinct effects on host cells. A number of the cellular functions downregulated by LPG depend on the activation of protein kinase C (PKC), and it is interesting in this context that *trans*-bilayer inhibition of PKC by LPG has been observed.[255,256] This does not appear to be the result of direct inhibition of PKC by LPG, although this activity has been reported,[257] but rather of specific alterations of membrane properties by LPG.[258] Indeed, incorporation of full length LPG into lipid bilayers results in decreased fusogenic capacity of the membranes by stabilizing them against the formation of an inverted hexagonal structure.[258,259] LPG also modulate the bending rigidity and the spontaneous curvature of the membrane, making the destabilization and rearrangement of the underlying lipid bilayer more difficult. This affects the activity of several membrane-interacting proteins, including PKC,[258,260] and could provide a clue as to how LPG modulates PKC-dependent cellular functions. The anti-fusogenic properties of LPG have been shown to be essential for successful infection of host macrophages by *Leishmania* promastigotes.[261] In this study, it was observed that phagosomes containing wild type promastigotes do not fuse with endosomes, whereas the reverse was true for LPG-deficient mutants. This effect was attributed to LPG, as passive transfer of LPG or functional genetic complementation of the mutant fully restored its capacity to inhibit phagosome-endosome fusion.[261] LPG is transferred within minutes from the promastigote surface to the macrophage membrane at the immediate area of internalization[262] and thus prevents destruction of the parasite after fusion of the endosome with the parasitophorous vacuole. Transformation of promastigotes into amastigotes occurs concomitantly with the release of phagosome-endosome fusion inhibition. Amastigotes then proliferate inside acidic, hydrolase-rich vacuoles,[263-265] and the fact that they produce little or no LPG suggests that the role of LPG may be restricted to the establishment of infection during promastigote to amastigote conversion. These results confirm numerous reports showing that a variety of mutants partially or completely deficient in LPG biosynthesis display partial or total loss of virulence,[266-268] a phenotype which could be rescued by the addition of exogenous LPG[269,270] or reversion to normal levels of LPG expression.[271]

Plasmodium

Among the 4 *Plasmodium* species that infect man, *P. falciparum* is the most virulent. *P. falciparum* undergoes a complex life cycle involving an anopheles insect vector and

human host. Clinical symptoms of malaria are entirely caused by the asexual, proliferative intraerythrocytic stage of the parasite. Infected erythrocytes in the peripheral blood only contain ring and early trophozoite stage parasites, whereas erythrocytes containing mature trophozoites and schizonts may be sequestered from the peripheral circulation by adhesion to the endothelial cells of capillaries in several organs. In the small venous capillaries of the brain, the attached infected erythrocytes can actually interrupt the blood flow, leading to local anoxia and tissue necrosis. This pathology is known as "cerebral malaria" characterized by numerous dysfunctions, coma and often death.

GPI-Anchored Proteins

GPI-anchored proteins are present in several proteins of the erythrocytic stage of *P. falciparum*, including a 195 kDa merozoite surface protein 1 (MSP-1) and its C-terminal proteolytic fragments,[272,273] a 45 kDa merozoite surface protein-2 (MSP-2),[272,274] a 102 kDa transferrin receptor found at the erythrocyte surface,[275] and a 76 kDa serine-protease.[276] In *P. falciparum*, GPI appears to be the major carbohydrate modification of intraerythrocytic parasite proteins.[277] Precursors of GPI anchors have been structurally characterized.[278]

Interaction of Plasmodial GPIs with the Host

Schofield and collaborators have studied the biological effects of GPIs isolated from *P. falciparum* on a variety of host cells and attempted to define the molecular mechanism of malaria GPI signaling in macrophages.[60,214,216,279-281] At least two macrophage enzymes appear to be implicated in early events of signal transduction: $p59^{hck}$, a protein tyrosine kinase of the Src family, and PKCε, one of the six isoforms of protein kinase C present in macrophages. These two protein kinases synergize to activate transcription factors of the c-Rel/NF-κB family which then turn on transcription of a specific set of genes, including those of cytokines, inducible nitric oxide synthase and adhesion molecules. The structural features of GPI required to activate $p59^{hck}$ and PKCε was investigated using GPIs of various origins, which in some cases were further modified by enzymatic treatment. The minimal GPI structure tested that was able to activate $p59^{hck}$ consisted of a soluble Manα1-2Manα1-6Manα1-4GlcN-inositol core glycan. Removal of one Man residue at the nonreducing end, or replacement of GlcN-inositol by anhydromannitol abolished this activation. In contrast, various substituents, including ethanolamine phosphate, single Man residues and acyl group on the inositol had no effect. For PKCε activation, the diacyl glycerol moiety attached to the GPI was required and substitution of diacyl glycerol for either alkylacyl glycerol or 1-*O-acyl*-2-*lyso*-glycerol resulted in loss of activity. Both $p59^{hck}$ and PKCε activation by GPI was inhibited by a monoclonal antibody recognizing the GPI moiety of *P. falciparum* glycoproteins. Identical results were obtained using crude parasite extracts, suggesting that GPI are major signaling-competent molecules of the parasite.

These results suggest the existence of a lectin-like receptor for carbohydrate epitopes present in the GPI, regardless of whether these are linked to lipids or proteins. This binding results in $p59^{hck}$ activation, whereas the signaling via PKCε requires a membrane bound GPI with specific (i.e., diacyl glycerol) lipid component. Whether hydrolysis of the GPI will occur during this process is not known.

The downstream effects of cellular activation by *P. falciparum* GPI, i.e., cytokine release and adhesion molecule expression, could contribute to the pathology of cerebral malaria. TNF-α levels correlate with the severity of malaria[282] and upregulate the expression of adhesion molecules in the vascular endothelium, favoring the sequestration of the parasite-infected erythrocytes in the postcapillary veinules of the brain.[283,284]

Signaling via second messengers derived from GPI structures has been proposed to occur for several mammalian receptors (see chapter 4), but the molecular mechanism of

these signaling pathways and the structural characterization of the second messengers still remain unknown (for a review, see ref. 285). The use of parasite GPIs, which offer a large structural diversity, will probably prove useful in addressing these questions. GIPLs have been described at the surface of mammalian cells and could serve as candidate precursors of second messengers for these pathways.[286] Interestingly, these glycolipids, which resemble GPI precursors, contain diacylglycerol, in contrast to mature GPI-anchored proteins of the same cell which contain alkylacyl glycerol. A comprehensive evaluation of the effects of malarial GPI precursors and those derived from MSP-1 by proteolysis[60,214,216,279-281] should therefore await further studies to define the transmembrane signaling pathways involved.

Toxoplasma

Toxoplasma gondii is a protozoan parasite infecting nearly all warm-blooded animals and is the causative agent of toxoplasmosis in man. The parasite only undergoes sexual replication in cats and other felines. Development and multiplication of *Toxoplasma* tachyzoites is obligatorily intracellular, and the parasite has the ability to invade almost any nucleated cell in a complex process.[287] Tachyzoites express several GPI-anchored proteins (P22, gp23, P30, P35 and P45)[288] among which P30 which has been implicated in host cell invasion.[289] As an immune response is established, a second stage of the parasite is observed, the bradyzoite, which expresses a different set of surface proteins. Bradyzoites divide less rapidly and persist within large cysts found in tissues not readily accessible to the immune system such as the brain and skeletal muscle. Toxoplasmosis is a benign infection except if it occurs during pregnancy, or if dormant brain cysts are reactivated in an immunocompromised individual, leading to *Toxoplasma* encephalitis.

The GPI structures of P30 and gp23 surface proteins as well as immunogenic surface GIPLs have been determined.[288,290,291] These molecules are characterized by a unique and highly immunogenic Glcα1-4GalNAc side chain on the GPI core which appears to be widely expressed in various clinical isolates of *T. gondii* and might prove useful for diagnosis purposes.[291] As in *Plasmodium*, modifications of the conserved GPI backbone appear to take place in the ER before transfer to the protein, which is in contrast with the situation observed in trypanosomes, yeast and mammals.

Summary

Free GPIs and GPI-anchored molecules are major components of mycobacterial envelopes and surface membranes of *Trypanosoma*, *Leishmania*, *Plasmodia* and *Toxoplasma* parasites. GPI-containing molecules critically influence the outcomes of host-parasite and vector-parasite relationships in various contexts. In particular, it has become evident that the outcome of infection depends not only on the immune recognition of microbial antigens but also on microbial products, especially GPI-containing ones, which act as major modulators of host cell function. GPI-containing molecules modify host cell behavior by interfering with both transmembrane signaling pathways and membrane fusion processes of the cells involved in immune recognition, microbe phagocytosis and cytokine production. Several target molecules involved in host cell signaling pathways have already been identified. In the future, the availability of synthetic GPI structures will help define the effects of microbial molecules on host cell function.

Acknowledgments

We thank S.J. Turco, S. Beverley and M.J. McConville for sharing unpublished data.

References

1. Brennan PJ, Nikaido H. The envelope of mycobacteria. Annu Rev Biochem 1995; 64:29-63.
2. Gelber RH, Li F, Cho SN et al. Serum antibodies to defined carbohydrate antigens during the course of treated leprosy. Int J Lepr Other Mycobact Dis 1989; 57:744-751.
3. Sada E, Aguilar D, Torres M et al. Detection of lipoarabinomannan as a diagnostic test for tuberculosis. J Clin Microbiol 1992; 30:2415-2418.
4. Besra GS, Morehouse CB, Rittner CM et al. Biosynthesis of mycobacterial lipoarabinomannan. J Biol Chem 1997; 272:18460-18466.
5. Chatterjee, D, Hunter SW, McNeil M et al. Lipoarabinomannan. Multiglycosylated form of the mycobacterial mannosylphosphatidylinositols. J Biol Chem 1992; 267:6228-33.
6. McConville, MJ, Ferguson MAJ. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem J 1993; 294:305-324.
7. Ferguson MAJ. What can GPI do for you. Parasitol Today 1994; 10:48-52.
8. Stokes RW, Speert DP. Lipoarabinomannan inhibits nonopsonic binding of *Mycobacterium tuberculosis* to murine macrophages. J Immunol 1995; 155:1361-1369.
9. Schlesinger LS, Hull SR, Kaufman TM. Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of *Mycobacterium tuberculosis* to human macrophages. J Immunol 1994; 152:4070-4079.
10. Schlesinger LS, Kaufman TM, Iyer S et al. Differences in mannose receptor-mediated uptake of lipoarabinomannan from virulent and attenuated strains of *Mycobacterium tuberculosis* by human macrophages. J Immunol 1996; 157:4568-4575.
11. Chan J, Fujiwara T, Brennan PJ et al. Microbial glycolipids: Possible virulence factors that scavenge oxygen radicals. Proc Natl Acad Sci USA 1989; 86:2453-2457.
12. Sibley LD, Hunter SW, Brennan PJ et al. Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. Infect Immun 1988; 56:1232-1236.
13. Sibley LD, Adams LB, Krahenbuhl JL. Inhibition of interferon-γ-mediated activation in mouse macrophages treated with lipoarabinomannan. Clin Exp Immunol 1990; 80:141-148.
14. Barnes PF, Chatterjee D, Abrams JS et al. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan. Relationship to chemical structure. J Immunol 1992; 149:541-547.
15. Dahl KE, Shiratsuchi H, Hamilton BD et al. Selective induction of transforming growth factor β in human monocytes by lipoarabinomannan of *Mycobacterium tuberculosis*. Infect Immun 1996; 64:399-405.
16. Moreno C, Mehlert A, Lamb J. The inhibitory effects of mycobacterial lipoarabinomannan and polysaccharides upon polyclonal and monoclonal human T cell proliferation. Clin Exp Immunol 1988; 74:206-210.
17. Molloy A, Gaudernack G, Levis WR et al. Suppression of T-cell proliferation by *Mycobacterium leprae* and its products: The role of lipopolysaccharide. Proc Natl Acad Sci USA 1990; 87:973-977.
18. Hoppe HC, de Wet BJ, Cywes C et al. Identification of phosphatidylinositol mannoside as a mycobacterial adhesin mediating both direct and opsonic binding to nonphagocytic mammalian cells. Infect Immun 1997; 65:3896-3905.
19. Chan J, Fan XD, Hunter SW et al. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. Infect Immun 1991; 59:1755-1761.
20. Schuller-Levis GB, Levis WR, Ammazzalorso M et al. Mycobacterial lipoarabinomannan induces nitric oxide and tumor necrosis factor α production in a macrophage cell line: Down regulation by taurine chloramine. Infect Immun 1994; 62:4671-4674.
21. Russell DG. Mycobaterium and *Leishmania*: Stowaways in the endosomal network. Trends Cell Biol 1995; 5:125-128.
22. Clemens DL, Horwitz MA. The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. J Exp Med 1996; 184:1349-1355.

23. Clemens DL, Horwitz MA. Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. J Exp Med 1995; 181:257-270.
24. Sturgill-Koszycki S, Schaible UE, Russell DG. *Mycobacterium*-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. EMBO J 1996; 15:6960-6968.
25. Xu S, Cooper A, Sturgill-Koszycki S et al. Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. J Immunol 1994; 153:2568-2578.
26. Ilangumaran S, Robinson PJ, Hoessli DC. Transfer of exogenous glycosylphosphatidylinositol (GPI)-linked molecules to plasma membranes. Trends Cell Biol 1996; 6:163-167.
27. Kaplan G, Gandhi RR, Weinstein DE et al. *Mycobacterium leprae* antigen-induced suppression of T cell proliferation in vitro. J Immunol 1987; 138:3028-3034.
28. Chujor CS, Kuhn B, Schwerer B et al. Specific inhibition of mRNA accumulation for lymphokines in human T cell line Jurkat by mycobacterial lipoarabinomannan antigen. Clin Exp Immunol 1992; 87:398-403.
29. Knutson KL, Hmama Z, Herrera-Velit P et al. Lipoarabinomannan of *Mycobacterium tuberculosis* promotes protein tyrosine dephosphorylation and inhibition of mitogen-activated protein kinase in human mononuclear phagocytes. Role of the Src homology 2 containing tyrosine phosphatase 1. J Biol Chem 1998; 273:645-652.
30. Ilangumaran S, Arni S, Poincelet M et al. Integration of mycobacterial lipoarabinomannans into glycosylphosphatidylinositol-rich domains of lymphomonocytic cell plasma membranes. J Immunol 1995; 155:1334-1342.
31. Pugin J, Heumann ID, Tomasz A et al. CD14 is a pattern recognition receptor. Immunity 1994; 1:509-516.
32. Gupta D, Kirkland TN, Viriyakosol S et al. CD14 is a cell-activating receptor for bacterial peptidoglycan. J Biol Chem 1996; 271:23310-23316.
33. Wurfel MM, Wright SD. Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers: Preferential interaction with particular classes of lipid. J Immunol 1997; 158:3925-3394.
34. Sieling PA, Chatterjee D, Porcelli SA et al. CD1-restricted T cell recognition of microbial lipoglycan antigens. Science 1995; 269:227-230.
35. Moody DB, Sugita M, Peters PJ et al. The CD1-restricted T-cell response to mycobacteria. Res Immunol 1996; 147:550-9
36. Zeng ZH, Castano AR, Segelke BW et al. Crystal structure of mouse CD1: An MHC-like fold with a large hydrophobic binding groove. Science 1997; 277:339-345.
37. Prigozy, T.I., Sieling PA, Clemens D et al. The mannose receptor delivers lipoglycan antigens to endosomes for presentation to T cells by CD1b molecules. Immunity 1997; 6:187-197.
38. Stenger S, Mazzaccaro RJ, Uyemura K et al. Differential effects of cytolytic T cell subsets on intracellular infection. Science 1997; 276:1684-1687.
39. Sousa AO, Salem JI, Lee FK et al. An epidemic of tuberculosis with a high rate of tuberculin anergy among a population previously unexposed to tuberculosis, the Yanomami Indians of the Brazilian Amazon. Proc Natl Acad Sci USA 1997; 94:13227-13232.
40. Udenfriend S, Kodukula K. Prediction of ω site in nascent precursor of glycosylphosphatidylinositol protein. Methods Enzymol 1995; 250:571-582.
41. Schneider P, Treumann A, Milne KG et al. Structural studies on a lipoarabinogalactan of *Crithidia fasciculata*. Biochem J 1996; 313:963-971.
42. Cross GAM. Cellular and genetic aspects of antigenic variation in trypanosomes. Annu Rev Immunol 1990; 8:83-110.
43. Cross GAM. Antigenic variation in trypanosomes: Secrets surface slowly. Bioessays 1996; 18:283-291.
44. Blum ML, Down JA, Gurnett AM et al. A structural motif in the variant surface glycoproteins of *Trypanosoma brucei*. Nature 1993; 362:603-609.

45. Homans SW, Edge CJ, Ferguson MAJ et al. Solution structure of the glycosylphosphatidylinositol membrane anchor glycan of *Trypanosoma brucei* variant surface glycoprotein. Biochemistry 1989; 28:881-7
46. Ferguson MAJ, Homans SW, Dwek RA et al. Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. Science 1988; 239:753-759.
47. Smith TK, Sharma DK, Crossman A et al. Parasite and mammalian GPI biosynthetic pathways can be distinguished using synthetic substrate analogues. EMBO J 1997; 167667-6675.
48. Sütterlin C, Horvath A, Gerold P et al. Identification of a species-specific inhibitor of glycosylphosphatidylinositol synthesis. EMBO J 1997; 16:6374-6383.
49. Guther ML, Masterson WJ, Ferguson MAJ. The effects of phenylmethylsulfonyl fluoride on inositol-acylation and fatty acid remodeling in African trypanosomes. J Biol Chem 1994; 269:18694-18701.
50. Masterson WJ, Raper J, Doering TL et al. Fatty acid remodeling: A novel reaction sequence in the biosynthesis of trypanosome glycosyl phosphatidylinositol membrane anchors. Cell 1990; 62:73-80.
51. Buxbaum LU, Milne KG, Werbovetz KA et al. Myristate exchange on the *Trypanosoma brucei* variant surface glycoprotein. Proc Natl Acad Sci USA 1996; 93:1178-1183.
52. Doering TL, Raper J, Buxbaum LU et al. An analog of myristic acid with selective toxicity for African trypanosomes. Science 1991; 252:1851-1854.
53. Bülow R, Griffiths G, Webster P et al. Intracellular localization of the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*. J Cell Sci 1989; 93:233-240.
54. Carrington M, Bulow R, Reinke H et al. Sequence and expression of the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*. Mol Biochem Parasitol. 1989; 33:289-296.
55. Cross GAM. Release and purification of *Trypanosoma brucei* variant surface glycoprotein. J Cell Biochem 1984; 24:79-90.
56. Rolin S, Hanocq-Quertier J, Paturiaux-Hanocq F et al. Simultaneous but independent activation of adenylate cyclase and glycosylphosphatidylinositol-phospholipase C under stress conditions in *Trypanosoma brucei*. J Biol Chem 1996; 271:10844-10852.
57. Bulow R, Nonnengasser C, Overath . Release of the variant surface glycoprotein during differentiation of bloodstream to procyclic forms of *Trypanosoma brucei*. Mol Biochem Parasitol 1989; 32:85-92
58. Overath P, Chaudhri M, Sterverding D et al. Invariant surface proteins in bloodstream forms of *Trypanosoma brucei*. Parasitol Today 1994; 10:53-58.
59. Ziegelbauer K, Stahl B, Karas M et al. Proteolytic release of cell surface proteins during differentiation of *Trypanosoma brucei*. Biochemistry 1993; 32:3737-3742.
60. Tachado SD, Schofield L. Glycosylphosphatidylinositol toxin of *Trypanosoma brucei* regulates IL-1 alpha and TNF-α expression in macrophages by protein tyrosine kinase mediated signal transduction. Biochem Biophys Res Comm 1994; 205:984-991.
61. Fukushima K, Hara-Kuge S, Ohkura T et al. Lectin-like characteristics of recombinant human interleukin-1β recognizing glycans of the glycosylphosphatidylinositol anchor. J Biol Chem 1997; 272: 10579-10584.
62. Roditi I,Schwarz H, Pearson TW et al. Procyclin gene expression and loss of the variant surface glycoprotein during differentiation of *Trypanosoma brucei*. J Cell Biol 1989; 108:737-746.
63. Matthews KR, Gull K. Evidence for an interplay between cell cycle progression and the initiation of differentiation between life cycle forms of African trypanosomes. J Cell Biol 1994; 125:1147-1156.
64. Mowatt MR, Clayton CE. Developmental regulation of a novel repetitive protein of *Trypanosoma brucei*. Mol Cell Biol 1987; 7:2838-2844.

65. Richardson JP, Beecroft RP, Tolson DL et al. Procyclin: An unusual immunodominant glycoprotein surface antigen from the procyclic stage of African trypanosomes. Mol Biochem Parasitol 1988; 31:203-216.
66. Roditi I, Carrington M. Turner M. Expression of a polypeptide containing a dipeptide repeat is confined to the insect stage of *Trypanosoma brucei*. Nature 1987; 325:272-274.
67. Butikofer P, Ruepp S, Boschung M et al. 'GPEET' procyclin is the major surface protein of procyclic culture forms of *Trypanosoma brucei* brucei strain 427. Biochem J 1997; 326:415-423.
68. Treumann A, Zitzmann N, Hulsmeier A et al. Structural characterization of two forms of procyclic acidic repetitive protein expressed by procyclic forms of *Trypanosoma brucei*. J Mol Biol 1997; 269:529-547.
69. Ferguson MAJ, Murray P, Rutherford H et al. A simple purification of procyclic acidic repetitive protein and demonstration of a sialylated glycosyl-phosphatidylinositol membrane anchor. Biochem J 1993; 291:51-55.
70. Pontes de Carvalho LC, Tomlinson S, Vandekerckhove F et al. Characterization of a novel *trans*-sialidase of *Trypanosoma brucei* procyclic trypomastigotes and identification of procyclin as the main sialic acid acceptor. J Exp Med 1993; 177:465-474.
71. Engstler M, Reuter G, Schauer R. Purification and characterization of a novel sialidase found in procyclic culture forms of *Trypanosoma brucei*. Mol Biochem Parasitol 1992; 54:21-30.
72. Engstler M, Reuter G, Schauer R. The developmentally regulated *trans*-sialidase from *Trypanosoma brucei* sialylates the procyclic acidic repetitive protein. Mol Biochem Parasitol 1993; 61:1-13.
73. Bayne RA, Kilbride EA, Lainson FA et al. A major surface antigen of procyclic stage *Trypanosoma congolense*. Mol Biochem Parasitol 1993; 61:295-310.
74. Beecroftt RP, Roditi I, Pearson TW. Identification and characterization of an acidic major surface glycoprotein from procyclic stage *Trypanosoma congolense*. Mol Biochem Parasitol 1993; 61:285-294.
75. Miller, N, Lehane MJ. Peritrophic membranes, cell surface molecules and parasite tropism within arthropod vectors. Parasitol Today 1993; 9:45-50.
76. Ruepp S, Furger A, Kurath U et al. Survival of *Trypanosoma brucei* in the tsetse fly is enhanced by the expression of specific forms of procyclin. J Cell Biol 1997; 137:1369-1379.
77. Schell D, Borowy NK, Overath P. Transferrin is a growth factor for the bloodstream form of *Trypanosoma brucei*. Parasitol Res 1991; 77:558-560.
78. Pays E, Tebabi P, Pays A et al. The genes and transcripts of an antigen gene expression site from *T. brucei*. Cell 1989; 57:835-845.
79. Salmon D, Geuskens M, Hanocq F et al. A novel heterodimeric transferrin receptor encoded by a pair of VSG expression site-associated genes in *T. brucei*. Cell 1994; 78:75-86.
80. Steverding D, Stierhof YD, Chaudhri M et al. ESAG 6 and 7 products of *Trypanosoma brucei* form a transferrin binding protein complex. Eur J Cell Biol 1994; 64:78-87.
81. Steverding D, Stierhof YD, Fuchs H et al. Transferrin-binding protein complex is the receptor for transferrin uptake in *Trypanosoma brucei*. J Cell Biol 1995; 131:1173-1182.
82. Chaudhri M, Steverding D, Kittelberger D et al. Expression of a glycosylphosphatidylinositol-anchored *Trypanosoma brucei* transferrin-binding protein complex in insect cells. Proc Natl Acad Sci USA 1994; 91:6443-6447.
83. Ligtenberg MJ, Bitter W, Kieft R et al. Reconstitution of a surface transferrin binding complex in insect form *Trypanosoma brucei*. EMBO J 1994; 13:2565-2573.
84. Overath P, Stierhof YD, Wiese M. Endocytosis and secretion in trypanosomatid parasites—tumultuous traffic in a pocket. Trends Cell Biol 1997; 7:27-33
85. Webster P, Russell DG. The flagellar pocket of trypanosomatids. Parasitol Today 1993; 9:210-206.
86. Maier A, Steverding D. Low affinity of *Trypanosoma brucei* transferrin receptor to apotransferrin at pH 5 explains the fate of the ligand during endocytosis. FEBS Lett 1996; 396:87-89.

87. Jefferies WA, Gabathuler R, Rothenberger S et al. Pumping iron in the '90s. Trends Cell Biol 1996; 6:223-228.
88. Andrews NW, Hong KS, Robbins ES et al. Stage-specific surface antigens expressed during the morphogenesis of vertebrate forms of *Trypanosoma cruzi*. Exp Parasitol 1987; 64:474-484.
89. Kahn S, Van Voorhis WC, Eisen H. The major 85-kD surface antigen of the mammalian form of *Trypanosoma cruzi* is encoded by a large heterogeneous family of simultaneously expressed genes. J Exp Med 1990; 172:589-597.
90. Almeida IC, Ferguson MAJ, Schenkman S et al. Lytic anti- α-galactosyl antibodies from patients with chronic Chagas' disease recognize novel *O*-linked oligosaccharides on mucin-like glycosyl-phosphatidylinositol-anchored glycoproteins of *Trypanosoma cruzi*. Biochem J 1994; 304: 793-802.
91. Haynes PA, Ferguson MAJ, Cross GAM. Structural characterization of novel oligosaccharides of cell-surface glycoproteins of *Trypanosoma cruzi*. Glycobiology 1996; 6:869-878.
92. Previato JO, Jones C, Goncalves LP et al. *O*-glycosidically linked N-acetylglucosamine-bound oligosaccharides from glycoproteins of *Trypanosoma cruzi*. Biochem J 1994; 301:151-159.
93. Haynes PA, Russell DG, Cross GAM. Subcellular localization of *Trypanosoma cruzi* glycoprotein Gp72. J Cell Sci 1996; 109:2979-2988.
94. Di Noia JM, Pollevick GD, Xavier MT et al. High diversity in mucin genes and mucin molecules in *Trypanosoma cruzi*. J Biol Chem 1996; 271:32078-32083.
95. Teixeira MM, Yoshida N. Stage-specific surface antigens of metacyclic trypomastigotes of *Trypanosoma cruzi* identified by monoclonal antibodies. Mol Biochem Parasitol 1986; 18:271-282.
96. Cross GAM, Takle GB. The surface *trans*-sialidase family of *Trypanosoma cruzi*. Annu Rev Microbiol 1993; 47:385-411.
97. Schenkman S, Eichinger D, Pereira ME et al. Structural and functional properties of *Trypanosoma trans*-sialidase. Annu Rev Microbiol 1994; 48:499-523.
98. Araya JE, Cano MI, Yoshida N et al. Cloning and characterization of a gene for the stage-specific 82-kDa surface antigen of metacyclic trypomastigotes of *Trypanosoma cruzi*. Mol Biochem Parasitol 1994; 65:161-169.
99. Santos MA, Garg N, Tarleton RL. The identification and molecular characterization of *Trypanosoma cruzi* amastigote surface protein-1, a member of the *trans*-sialidase gene super-family. Mol Biochem Parasitol 1997; 86:1-11.
100. Low HP, Tarleton RP. Molecular cloning of the gene encoding the 83 kDa amastigote surface protein and its identification as a member of the *Trypanosoma cruzi* sialidase superfamily. Mol Biochem Parasitol 1997; 88:137-149.
101. Pan AA, McMahon-Pratt D. Amastigote and epimastigote stage-specific components of *Trypanosoma cruzi* characterized by using monoclonal antibodies. Purification and molecular characterization of an 83-kilodalton amastigote protein. J Immunol 1989; 143:1001-1008.
102. Ruef BJ, Dawson BD, Tewari D et al. Expression and evolution of members of the *Trypanosoma cruzi* trypomastigote surface antigen multigene family. Mol Biochem Parasitol 1994; 63:109-120.
103. Van Voorhis WC., Pham VT, De Vos T. The FL-160 genes of *Trypanosoma cruzi* are dispersed on multiple chromosomes. Exp Parasitol 1995; 80:578-582.
104. Previato JO, Andrade AF, Pessolani MC et al. Incorporation of sialic acid into *Trypanosoma cruzi* macromolecules. A proposal for a new metabolic route. Mol Biochem Parasitol 1985; 16:85-96.
105. Schenkman S, Chaves LB, Pontes de Carvalho LC et al. A proteolytic fragment of *Trypanosoma cruzi trans*-sialidase lacking the carboxyl-terminal domain is active, monomeric, and generates antibodies that inhibit enzymatic activity. J Biol Chem 1994; 269:7970-7975.
106. Schenkman S, Pontes de Carvalho LC, Nussenzweig V. *Trypanosoma cruzi trans*-sialidase and neuraminidase activities can be mediated by the same enzymes. J Exp Med 1992; 175:567-575.

107. Cremona ML, Sanchez DO, Frasch AC et al. A single tyrosine differentiates active and inactive *Trypanosoma cruzi trans*-sialidases. Gene 1995; 160:123-128.
108. Cremona ML, Pollevick GD, Frasch AC et al. Effect of primary structure modifications in *Trypanosoma cruzi* neuraminidase/*trans*-sialidase activities. Cell Mol. Biol 1996; 42:697-702.
109. Briones MR, Egima CM, Schenkman S. *Trypanosoma cruzi trans*-sialidase gene lacking C-terminal repeats and expressed in epimastigote forms. Mol Biochem Parasitol 1995; 70:9-17.
110. Briones MR, C.M. Egima, D. Eichinger, S. Schenkman. *Trans*-sialidase genes expressed in mammalian forms of *Trypanosoma cruzi* evolved from ancestor genes expressed in insect forms of the parasite. J Mol Evolution 1995; 41:120-131.
111. Schenkman S, Jiang MS, Hart GW et al. A novel cell surface *trans*-sialidase of *Trypanosoma cruzi* generates a stage-specific epitope required for invasion of mammalian cells. Cell 1991; 65:1117-1125.
112. Schenkman S, Diaz C, Nussenzweig V. Attachment of *Trypanosoma cruzi* trypomastigotes to receptors at restricted cell surface domains. Exp Parasitol 1991; 72:76-86.
113. Pereira ME, Zhang K, Gong Y et al. Invasive phenotype of *Trypanosoma cruzi* restricted to a population expressing *trans*-sialidase. Infect Immun 1996; 64:3884-3892.
114. Hall BF, Webster P,Ma AK et al. Desialylation of lysosomal membrane glycoproteins by *Trypanosoma cruzi*: A role for the surface neuraminidase in facilitating parasite entry into the host cell cytoplasm. J Exp Med 1992; 176:313-325.
115. Andrews NW, Abrams CK, Slatin SL et al. A *T. cruzi*-secreted protein immunologically related to the complement component C9: Evidence for membrane pore-forming activity at low pH. Cell 1990; 61:1277-1287.
116. Chuenkova M, Pereira ME. *Trypanosoma cruzi trans*-sialidase: Enhancement of virulence in a murine model of Chagas' disease. J Exp Med 1995; 181:1693-1703.
117. Couto AS, De Lederkremer RM, Colli W et al. The glycosylphosphatidylinositol anchor of the trypomastigote-specific Tc-85 glycoprotein from *Trypanosoma cruzi*. Metabolic-labeling and structural studies. Eur J Biochem 1993; 217:597-602.
118. Abuin G, Couto AS, de Lederkremer RM et al. *Trypanosoma cruzi*: The Tc-85 surface glycoprotein shed by trypomastigotes bears a modified glycosylphosphatidylinositol anchor. Exp Parasitol 1996; 82:290-297.
119. Alves MJ, Abuin G, Kuwajima VY et al. Partial inhibition of trypomastigote entry into cultured mammalian cells by monoclonal antibodies against a surface glycoprotein of *Trypanosoma cruzi*. Mol Biochem Parasitol 1986; 21:75-82.
120. Giordano R, Chammas R, Veiga SS et al. 1994. An acidic component of the heterogeneous Tc-85 protein family from the surface of *Trypanosoma cruzi* is a laminin binding glycoprotein. Mol Biochem Parasitol 1994; 65:85-94.
121. Kahn SJ, Wleklinski M. The surface glycoproteins of *Trypanosoma cruzi* encode a superfamily of variant T cell epitopes. J Immunol 1997; 159:4444-4451.
122. Yoshida N, Blanco SA, Araguth MF et al. The stage-specific 90-kilodalton surface antigen of metacyclic trypomastigotes of *Trypanosoma cruzi*. Mol Biochem Parasitol 1990; 39:39-46.
123. Ramirez MI, Ruiz RC, Araya JE et al. Involvement of the stage-specific 82-kilodalton adhesion molecule of *Trypanosoma cruzi* metacyclic trypomastigotes in host cell invasion. Infect Immun 1993; 61:3636-3641.
124. Santori FR, Dorta ML, Juliano L et al. Identification of a domain of *Trypanosoma cruzi* metacyclic trypomastigote surface molecule gp82 required for attachment and invasion of mammalian cells. Mol Biochem Parasitol 1996; 78:209-216.
125. Alves MJ, Colli W. Glycoproteins from *Trypanosoma cruzi*: Partial purification by gel chromatography. FEBS Lett 1975; 52:188-190.
126. Yoshida N, Mortara RA, Araguth MF et al. Metacyclic neutralizing effect of monoclonal antibody 10D8 directed to the 35- and 50-kilodalton surface glycoconjugates of *Trypanosoma cruzi*. Infect Immun 1989; 57:1663-1667.

127. Schenkman S, Ferguson MAJ, Heise N et al. Mucin-like glycoproteins linked to the membrane by glycosylphosphatidylinositol anchor are the major acceptors of sialic acid in a reaction catalyzed by *trans*-sialidase in metacyclic forms of *Trypanosoma cruzi*. Mol Biochem Parasitol 1993, 59:293-303.
128. Singh BN, Lucas JJ, Beach DH et al. Expression of a novel cell surface lipophosphoglycan-like glycoconjugate in *Trypanosoma cruzi* epimastigotes. J Biol Chem 1994; 269: 21972-21982.
129. Di Noia JM, Sanchez DO, Frasch AC. The protozoan *Trypanosoma cruzi* has a family of genes resembling the mucin genes of mammalian cells. J Biol Chem 1995; 270: 24146-24149.
130. Previato JO, Jones C, Xavier MT et al. Structural characterization of the major glycosylphosphatidylinositol membrane-anchored glycoprotein from epimastigote forms of *Trypanosoma cruzi* Y-strain. J Biol Chem 1995; 270:7241-7250.
131. Serrano AA, Schenkman S, Yoshida N et al. The lipid structure of the glycosylphosphatidylinositol-anchored mucin-like sialic acid acceptors of *Trypanosoma cruzi* changes during parasite differentiation from epimastigotes to infective metacyclic trypomastigote forms. J Biol Chem 1995; 270:27244-27253.
132. Heise N, de Almeida ML, Ferguson MAJ. Characterization of the lipid moiety of the glycosylphosphatidylinositol anchor of *Trypanosoma cruzi* 1G7-antigen. Mol Biochem Parasitol 1995; 70:71-84.
133. Guther ML, de Almeida ML, Yoshida N et al. Structural studies on the glycosylphosphatidylinositol membrane anchor of *Trypanosoma cruzi* 1G7-antigen. The structure of the glycan core. J Biol Chem 1992; 267:6820-6828.
134. Gomes NA, Previato JO, Zingales B et al. Downregulation of T lymphocyte activation in vitro and in vivo induced by glycoinositolphospholipids from *Trypanosoma cruzi*. Assignment of the T cell-suppressive determinant to the ceramide domain. J Immunol 1996; 156:628-635.
135. Couto AS, Goncalves MF, Colli W et al. The N-linked carbohydrate chain of the 85-kilodalton glycoprotein from *Trypanosoma cruzi* trypomastigotes contains sialyl, fucosyl and galactosyl (α1-3)galactose units. Mol Biochem Parasitol 1990; 39:101-107.
136. Andrews NW, Robbins ES, Ley V et al. Developmentally regulated, phospholipase C-mediated release of the major surface glycoprotein of amastigotes of *Trypanosoma cruzi*. J Exp Med 1988; 167:300-314.
137. Nascimento AE, de Souza W. High resolution localization of cruzipain and Ssp4 in *Trypanosoma cruzi* by replica staining label fracture. Biology of the Cell 1996; 86:53-58.
138. Bertello LE, Andrews NW, de Lederkremer RM. Developmentally regulated expression of ceramide in *Trypanosoma cruzi*. Mol Biochem Parasitol 1996; 79:143-151.
139. Barros HC, Da Silva S, Verbisck NV et al. Release of membrane-bound trails by *Trypanosoma cruzi* amastigotes onto modified surfaces and mammalian cells. J Euk Microbiol 1996; 43:275-285.
140. Teixeira SM, Russell DG, Kirchhoff LV et al. A differentially expressed gene family encoding "amastin", a surface protein of *Trypanosoma cruzi* amastigotes. J Biol Chem 1994; 269:20509-20516.
141. Teixeira SM, Kirchhoff LV, Donelson JE. Posttranscriptional elements regulating expression of mRNAs from the amastin/tuzin gene cluster of *Trypanosoma cruzi*. J Biol Chem 1995; 270:22586-22594.
142. Campetella O, Martinez J, Cazzulo JJ. A major cysteine proteinase is developmentally regulated in *Trypanosoma cruzi*. FEMS Microbiol Lett 1990; 55:145-149.
143. Campetella O, Henriksson J, Aslund L et al. The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is encoded by multiple polymorphic tandemly organized genes located on different chromosomes. Mol Biochem Parasitol 1992; 50:225-234.
144. Eakin AE, Mills AA, Harth G et al. The sequence, organization, and expression of the major cysteine protease (cruzain) from *Trypanosoma cruzi*. J Biol Chem 1992; 267:7411-7420.
145. Tomas AM, Kelly JM. Stage-regulated expression of cruzipain, the major cysteine protease of *Trypanosoma cruzi* is independent of the level of RNA1. Mol Biochem Parasitol 1996; 76:91-103.

146. Parodi AJ, Labriola C, Cazzulo JJ. The presence of complex-type oligosaccharides at the C-terminal domain glycosylation site of some molecules of cruzipain. Mol Biochem Parasitol 1995; 69:247-255.
147. Meirelles MN, Juliano L, Carmona E et al. Inhibitors of the major cysteinyl proteinase (GP57/51) impair host cell invasion and arrest the intracellular development of *Trypanosoma cruzi* in vitro. Mol Biochem Parasitol 1992; 52:175-184.
148. McGrath ME, Eakin AE, Engel JC et al. The crystal structure of cruzain: A therapeutic target for Chagas' disease. J Mol Biol 1995; 247:251-259.
149. Hernandez-Munain C, Fernandez MA, Alcina A et al. Characterization of a glycosyl-phosphatidylinositol-anchored membrane protein from *Trypanosoma cruzi*. Infect Immun 1991; 59:1409-1416.
150. Souto-Padron T, Campetella OE, Cazzulo JJ. Cysteine proteinase in *Trypanosoma cruzi*: Immunocytochemical localization and involvement in parasite-host cell interaction. J Cell Sci 1990; 96:485-490.
151. Fresno M, Hernandez-Munain C, de-Diego J et al. *Trypanosoma cruzi*: Identification of a membrane cysteine proteinase linked through a GPI anchor. Braz J Med Biol Res 1994; 27:431-437.
152. Garg N, Postan M, Mensa-Wilmot K et al. Glycosylphosphatidylinositols are required for the development of *Trypanosoma cruzi* amastigotes. Infect Immun 1997; 65:4055-4060.
153. Garg N, Tarleton RL, Mensa-Wilmot K. Proteins with glycosylphosphatidylinositol (GPI) signal sequences have divergent fates during a GPI deficiency. GPIs are essential for nuclear division in *Trypanosoma cruzi*. J Biol Chem 1997; 272:12482-12491.
154. de Lederkremer RM, Lima C, Ramirez MI et al. Complete structure of the glycan of lipopeptidophosphoglycan from *Trypanosoma cruzi* epimastigotes. J Biol Chem 1991; 266:23670-23675.
155. Previato JO, Gorin PA, Mazurek M et al. Primary structure of the oligosaccharide chain of lipopeptidophosphoglycan of epimastigote forms of *Trypanosoma cruzi*. J Biol Chem 1990; 265:2518-2526.
156. Carreira JC, Jones C, Wait R et al. Structural variation in the glycoinositolphospholipids of different strains of *Trypanosoma cruzi*. Glycoconjugate J 1996; 13:955-966.
157. Hannun YA, Obeid LM. Ceramide: An intracellular signal for apoptosis. Trends Biochem Sci 1995; 20:73-77.
158. Pushkareva M, Obeid LM, Hannun YA. Ceramide: An endogenous regulator of apoptosis and growth suppression. Immunol Today 1995; 16:294-297.
159. Golgher DB, Colli W, Souto-Padron T et al. Galactofuranose-containing glycoconjugates of epimastigote and trypomastigote forms of *Trypanosoma cruzi*. Mol Biochem Parasitol 1993; 60:249-264.
160. de Lederkremer RM, Lima CE, Ramirez MI et al. Hexadecylpalmitoylglycerol or ceramide is linked to similar glycophosphoinositol anchor-like structures in *Trypanosoma cruzi*. Eur J Biochem 1993; 218:929-936.
161. Bertello LE, Goncalvez MF, Colli W et al. Structural analysis of inositol phospholipids from *Trypanosoma cruzi* epimastigote forms. Biochem J 1995; 310:255-261.
162. Bento CA, Melo MB, Previato JO et al. Glycoinositolphospholipids purified from *Trypanosoma cruzi* stimulate Ig production in vitro. J Immunol 1996; 157:4996-5001.
163. Vila MC, Cozza EN, Lima C et al. An inositol phosphoglycan from *Trypanosoma cruzi* inhibits ACTH action in calf adrenocortical cells. Cellular Signalling 1995; 7:331-339.
164. Chaudhuri G, Chaudhuri M, Pan A et al. Surface acid proteinase (gp63) of *Leishmania mexicana*. A metalloenzyme capable of protecting liposome-encapsulated proteins from phagolysosomal degradation by macrophages. J Biol Chem 1989; 264:7483-7489.
165. Etges R, Bouvier J, Bordier C. The major surface protein of *Leishmania* promastigotes is a protease. J Biol Chem 1986; 261:9098-9101.
166. Bouvier J, Bordier C, Vogel H et al. Characterization of the promastigote surface protease of *Leishmania* as a membrane-bound zinc endopeptidase. Mol Biochem Parasitol 1989; 37:235-45.

167. Bouvier J, Schneider P, Etges R et al. Peptide substrate specificity of the membrane-bound metalloprotease of *Leishmania*. Biochemistry 1990; 29:10113-10119.
168. Tzinia AK, Soteriadou KP. Substrate-dependent pH optima of gp63 purified from seven strains of *Leishmania*. Mol Biochem Parasitol 1991; 47:83-89.
169. Bouvier J, Etges R, Bordier C. Identification of the promastigote surface protease in seven species of *Leishmania*. Mol Biochem Parasitol 1987; 24:73-79.
170. Etges R. Identification of a surface metalloproteinase on 13 species of *Leishmania* isolated from humans, *Crithidia fasciculata*, and *Herpetomonas samuelpessoai*. Acta Tropica 1992; 50:205-217.
171. Murray PJ, Handman E, Glaser TA et al. *Leishmania major*: Expression and gene structure of the glycoprotein 63 molecule in virulent and avirulent clones and strains. Exp Parasitol 1990; 71:294-304.
172. Button LL, Russell DG, Klein HL et al. Genes encoding the major surface glycoprotein in *Leishmania* are tandemly linked at a single chromosomal locus and are constitutively transcribed. Mol Biochem Parasitol 1989; 32:271-283.
173. Medina-Acosta E, Karess RE, Russell DG. Structurally distinct genes for the surface protease of *Leishmania mexicana* are developmentally regulated. Mol Biochem Parasitol 1993; 57:31-45.
174. Webb JR, Button LL, McMaster WR. Heterogeneity of the genes encoding the major surface glycoprotein of *Leishmania donovani*. Mol Biochem Parasitol 1991; 48:173-184.
175. Roberts SC, Swihart KG, Agey MW et al. Sequence diversity and organization of the *msp* gene family encoding gp63 of *Leishmania chagasi*. Mol Biochem Parasitol 1993; 62:157-171.
176. Steinkraus HB, Greer JM, Stephenson DC et al. Sequence heterogeneity and polymorphic gene arrangements of the *Leishmania guyanensis* gp63 genes. Mol Biochem Parasitol 1993; 62:173-185.
177. Ramamoorthy R, Donelson JE, Paetz KE et al. Three distinct RNAs for the surface protease gp63 are differentially expressed during development of *Leishmania donovani chagasi* promastigotes to an infectious form. J Biol Chem 1992; 267:1888-1895.
178. Ramamoorthy R, Swihart KG, McCoy JJ et al. Intergenic regions between tandem gp63 genes influence the differential expression of gp63 RNAs in *Leishmania chagasi* promastigotes. J Biol Chem 1995; 270:12133-9
179. Beetham JK, Myung KS, McCoy JJ et al. Glycoprotein 46 mRNA abundance is post-transcriptionally regulated during development of *Leishmania chagasi* promastigotes to an infectious form. J Biol Chem 1997; 272:17360-17366.
180. Bordier C, Etges RJ, Ward J et al. *Leishmania* and *Trypanosoma* surface glycoproteins have a common glycophospholipid membrane anchor. Proc Natl Acad Sci USA 1986; 83:5988-5991.
181. Etges RJ, Bouvier J, Bordier C. The major surface protein of *Leishmania* promastigotes is anchored in the membrane by a myristic acid-labeled phospholipid. EMBO J 1986; 5:597-601.
182. Schneider P, Ferguson MAJ, McConville MJ et al. Structure of the glycosyl-phosphatidylinositol membrane anchor of the *Leishmania major* promastigote surface protease. J Biol Chem 1990; 265:16955-16964.
183. McConville MJ, Collidge TA, Ferguson MAJ et al. The glycoinositol phospholipids of *Leishmania mexicana* promastigotes. Evidence for the presence of three distinct pathways of glycolipid biosynthesis. J Biol Chem 1993; 268:15595-15604.
184. McGwire BS, Chang KP. Posttranslational regulation of a *Leishmania* HEXXH metalloprotease (gp63). The effects of site-specific mutagenesis of catalytic, zinc binding, N-glycosylation, and glycosyl phosphatidylinositol addition sites on *N*-terminal end cleavage, intracellular stability, and extracellular exit. J Biol Chem 1996; 271:7903-7909.
185. Roberts SC, Wilson ME, Donelson JE. Developmentally regulated expression of a novel 59-kDa product of the major surface protease (Msp or gp63) gene family of *Leishmania chagasi*. J Biol Chem 1995; 270:8884-8892.
186. Karp CL, Turco SJ, Sacks DL. Lipophosphoglycan masks recognition of the *Leishmania donovani* promastigote surface by human kala-azar serum. J Immunol 1991; 147:680-684.

187. Heumann D, Burger D, Vischer T et al. Molecular interactions of *Leishmania* promastigote surface protease with human *a*2-macroglobulin. Mol Biochem Parasitol 1989; 33:67-72.
188. Pimenta PF, Saraiva EM, Sacks DL. The comparative fine structure and surface glycoconjugate expression of three life stages of *Leishmania major*. Exp Parasitol 1991; 72:191-204.
189. Sacks DL, Pimenta PF, McConville MJ et al. Stage-specific binding of *Leishmania donovani* to the sand fly vector midgut is regulated by conformational changes in the abundant surface lipophosphoglycan. J Exp Med 1995; 181:685-697.
190. Brittingham A, Morrison CJ, McMaster WR et al. Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. J Immunol 1995; 155:3102-3111.
191. Mosser DM, Springer TA, Diamond MS. *Leishmania* promastigotes require opsonic complement to bind to the human leukocyte integrin Mac-1 (CD11b/CD18). J Cell Biol 1992; 116:511-520.
192. Seay MB, Heard PL, Chaudhuri G. Surface Zn-proteinase as a molecule for defense of *Leishmania mexicana amazonensis* promastigotes against cytolysis inside macrophage phagolysosomes. Infect Immun 1996; 64:5129-5137.
193. Garcia MR, Graham S, Harris RA et al. Epitope cleavage by *Leishmania* endopeptidase(s) limits the efficiency of the exogenous pathway of major histocompatibility complex class I-associated antigen presentation. Eur J Immunol 1997; 27:1005-1013.
194. Schneider P, Glaser TA. Characterization of two soluble metalloexopeptidases in the protozoan parasite *Leishmania major*. Mol Biochem Parasitol 1993; 62:223-231.
195. Schneider P, Rosat JP, Bouvier J et al. *Leishmania major*: Differential regulation of the surface metalloprotease in amastigote and promastigote stages. Exp Parasitol 1992; 75:196-206.
196. Medina-Acosta E, Karess RE, Schwartz H et al. The promastigote surface protease (gp63) of *Leishmania* is expressed but differentially processed and localized in the amastigote stage. Mol Biochem Parasitol 1989; 37:263-273.
197. Ilg T, Harbecke D, Overath P. The lysosomal gp63-related protein in *Leishmania mexicana* amastigotes is a soluble metalloproteinase with an acidic pH optimum. FEBS Lett 1993; 327:103-107.
198. Kahl LP, McMahon-Pratt D. Structural and antigenic characterization of a species- and promastigote-specific *Leishmania mexicana amazonensis* membrane protein. J Immunol 1987; 138:1587-1595.
199. Murray PJ, Spithill TW, Handman E. The PSA-2 glycoprotein complex of *Leishmania major* is a glycosylphosphatidylinositol-linked promastigote surface antigen. J Immunol 1989; 143:4221-4226.
200. Murray PJ Spithill TW. Variants of a *Leishmania* surface antigen derived from a multigenic family. J Biol Chem 1991; 266:24477-24484.
201. Lohman KL, Langer PJ, McMahon-Pratt D. Molecular cloning and characterization of the immunologically protective surface glycoprotein GP46/M-2 of *Leishmania amazonensis*. Proc Natl Acad Sci USA 1990; 87:8393-8397.
202. Rivas L, Kahl L, Manson K et al. Biochemical characterization of the protective membrane glycoprotein GP46/M-2 of *Leishmania amazonensis*. Mol Biochem Parasitol 1991; 47: 235-243.
203. Handman, E, Osborn AH, Symons F et al. 1995. The *Leishmania* promastigote surface antigen 2 complex is differentially expressed during the parasite life cycle. Mol Biochem Parasitol 74:189-200.
204. Handman E, Symons FM, Baldwin TM et al. 1995. Protective vaccination with promastigote surface antigen 2 from *Leishmania major* is mediated by a TH1 type of immune response. Infect Immun 63:4261-4267.
205. McMahon-Pratt D, Traub-Cseko Y, Lohman KL et al. Loss of the GP46/M-2 surface membrane glycoprotein gene family in the *Leishmania braziliensis* complex. Mol Biochem Parasitol 1992; 50:151-160.

206. Hanekamp T, Langer PJ. Molecular karyotype and chromosomal localization of genes encoding two major surface glycoproteins, gp63 and gp46/M2, hsp70, and β-tubulin in cloned strains of several *Leishmania* species. Mol Biochem Parasitol 1991; 48:27-37.
207. Symons FM, Murray, PJ, Ji H et al. Characterization of a polymorphic family of integral membrane proteins in promastigotes of different *Leishmania* species. Mol Biochem Parasitol 1994; 67:103-113.
208. Winter G, Fuchs M, McConville MJ et al. Surface antigens of *Leishmania mexicana* amastigotes: Characterization of glycoinositol phospholipids and a macrophage-derived glycosphingolipid. J Cell Sci 1994; 107:2471-2482
209. McConville MJ, Blackwell JM. Developmental changes in the glycosylated phosphatidylinositols of *Leishmania donovani*. Characterization of the promastigote and amastigote glycolipids. J Biol Chem 1991; 266:15170-15179.
210. Schneider P, Rosat JP, Ransijn A et al. Characterization of glycoinositol phospholipids in the amastigote stage of the protozoan parasite *Leishmania major*. Biochem J 1993; 295:555-564.
211. Wilson ME, Pearson RD. Evidence that *Leishmania donovani* utilizes a mannose receptor on human mononuclear phagocytes to establish intracellular parasitism. J Immunol 1986; 136:4681-4688.
212. Green PJ, Feizi T, Stoll MS et al. Recognition of the major cell surface glycoconjugates of *Leishmania* parasites by the human serum mannan-binding protein. Mol Biochem Parasitol 1994; 66:319-328.
213. McConville MJ, Homans SW, Thomas-Oates JE et al. Structures of the glyco-inositolphospholipids from *Leishmania major*. A family of novel galactofuranose-containing glycolipids. J Biol Chem 1990; 265:7385-7394.
214. Tachado SD, Gerold P, Schwarz R et al. Signal transduction in macrophages by glycosylphosphatidylinositols of *Plasmodium*, *Trypanosoma*, and *Leishmania*: Activation of protein tyrosine kinases and protein kinase C by inositolglycan and diacylglycerol moieties. Proc Natl Acad Sci USA 1997; 94:4022-4027.
215. Proudfoot L, O'Donnell CA, Liew FY. Glycoinositolphospholipids of *Leishmania major* inhibit nitric oxide synthesis and reduce leishmanicidal activity in murine macrophages. Eur J Immunol 1995; 25:745-750.
216. Tachado SD, Gerold P, McConville MJ et al. Glycosylphosphatidylinositol toxin of *Plasmodium* induces nitric oxide synthase expression in macrophages and vascular endothelial cells by a protein tyrosine kinase-dependent and protein kinase C-dependent signaling pathway. J Immunol 1996; 156:1897-1907.
217. Proudfoot L, Nikolaev AV, Feng GJ et al. Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of *Leishmania* lipophosphoglycan in murine macrophages. Proc Natl Acad Sci USA 1996; 93:10984-10989.
218. Reiner SL, Locksley RM. The regulation of immunity to *Leishmania major*. Annu Rev Immunol 1995; 13:151-177.
219. Thomas JR, McConville MJ, Thomas-Oates JE et al. Refined structure of the lipophosphoglycan of *Leishmania donovani*. J Biol Chem 1992; 267:6829-6833.
220. Turco SJ, Orlandi PA, Homans SW et al. Structure of the phosphosaccharide-inositol core of the *Leishmania donovani* lipophosphoglycan. J Biol Chem 1989; 264:6711-6715.
221. McConville MJ, Turco SJ, Ferguson MAJ et al. Developmental modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. EMBO J 1992; 11:3593-6300.
222. McConville MJ, Thomas-Oates JE, Ferguson MAJ et al. Structure of the lipophosphoglycan from *Leishmania major*. J Biol Chem 1990; 265:19611-19623.
223. Ilg T, Etges R, Overath P et al. Structure of *Leishmania mexicana* lipophosphoglycan. J Biol Chem 1992; 267:6834-6840.
224. McConville MJ, Schnur LF, Jaffe C et al. Structure of *Leishmania* lipophosphoglycan: Inter- and intra-specific polymorphism in Old World species. Biochem J 1995; 310: 807-818.
225. Homans SW, Mehlert A, Turco SJ. Solution structure of the lipophosphoglycan of *Leishmania donovani*. Biochemistry 1992; 31:654-61.

226. Proudfoot L, Schneider P, Ferguson MAJ et al. Biosynthesis of the glycolipid anchor of lipophosphoglycan and the structurally related glycoinositolphospholipids from *Leishmania major*. Biochem J 1995; 308:45-55.
227. Pimenta PF, Saraiva EM, Rowton E et al. Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. Proc Natl Acad Sci USA 1994; 91:9155-9156.
228. Pimenta PF, Turco SJ, McConville, MJ et al. Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. Science 1992; 256:1812-1815.
229. Butcher BA, Turco SJ, Hilty BA et al. Deficiency in β1,3-galactosyltransferase of a *Leishmania major* lipophosphoglycan mutant adversely influences the *Leishmania*-sand fly interaction. J Biol Chem 1996; 271:20573-10579.
230. Saraiva EM, Pimenta PF, Brodin TN et al. Changes in lipophosphoglycan and gene expression associated with the development of *Leishmania major* in *Phlebotomus papatasi*. Parasitology 1995; 111:275-287.
231. Culley FJ, Harris RA, Kaye PM et al. C-reactive protein binds to a novel ligand on *Leishmania donovani* and increases uptake into human macrophages. J Immunol 1996; 156:4691-4696.
232. Pimenta PF, da Silva RP, Sacks DL et al. Cell surface nanoanatomy of *Leishmania major* as revealed by fracture-flip. A surface meshwork of 44 nm fusiform filaments identifies infective developmental stage promastigotes. Eur J Cell Biol 1989; 48:180-190.
233. Puentes SM, Da Silva RP, Sacks DL et al. Serum resistance of metacyclic stage *Leishmania major* promastigotes is due to release of C5b-9. J Immunol 1990; 145:4311-4316.
234. Puentes SM, Sacks DL, da Silva RP. Complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. J Exp Med 1988; 167:887-902.
235. Howard MK, Sayers G, Miles MA. *Leishmania donovani* metacyclic promastigotes: Transformation in vitro, lectin agglutination, complement resistance, and infectivity. Exp Parasitol 1987; 64:147-156.
236. Franke ED, McGreevy PB, Katz SP et al. Growth cycle-dependent generation of complement-resistant *Leishmania* promastigotes. J Immunol 1985; 134:2713-2718.
237. Da Silva RP, Hall BF, Joiner KA et al. CR1, the C3b receptor, mediates binding of infective *Leishmania major* metacyclic promastigotes to human macrophages. J Immunol 1989; 143:617-622.
238. Mosser DM, Edelson PJ. The third component of complement (C3) is responsible for the intracellular survival of *Leishmania major*. Nature 1987; 327:329-331.
239. Wilson ME, Hardin KK. The major concanavalin A-binding surface glycoprotein of *Leishmania donovani chagasi* promastigotes is involved in attachment to human macrophages. J Immunol 1988; 141:265-272.
240. Talamas-Rohana P, Wright, SD, Lennartz MR et al. Lipophosphoglycan from *Leishmania mexicana* promastigotes binds to members of the CR3, p150,95 and LFA-1 family of leukocyte integrins. J Immunol 1990; 144:4817-4824.
241. Kelleher M, Bacic A, Handman E. Identification of a macrophage-binding determinant on lipophosphoglycan from *Leishmania major* promastigotes. Proc Natl Acad Sci USA 1992; 89:6-10.
242. Bahr V, Stierhof YD, Ilg T et al. Expression of lipophosphoglycan, high-molecular weight phosphoglycan and glycoprotein 63 in promastigotes and amastigotes of *Leishmania mexicana*. Mol Biochem Parasitol 1993; 58:107-121.
243. Glaser TA, Moody SF, Handman E et al. An antigenically distinct lipophosphoglycan on amastigotes of *Leishmania major*. Mol Biochem Parasitol 1991; 45:337-344.
244. Moody SF, Handman E, McConville MJ et al. The structure of *Leishmania major* amastigote lipophosphoglycan. J Biol Chem 1993; 268:18457-18466.
245. Kelleher M, Moody SF, Mirabile P et al. Lipophosphoglycan blocks attachment of *Leishmania major* amastigotes to macrophages. Infect Immun 1995; 63: 43-50.
246. Peters C, Aebischer T, Stierhof YD et al. The role of macrophage receptors in adhesion and uptake of *Leishmania mexicana* amastigotes. J Cell Sci 1995; 108:3715-3724.

247. Guy RA, Belosevic M. Comparison of receptors required for entry of *Leishmania major* amastigotes into macrophages. Infect Immun 1993; 61:1553-1558.
248. Ho JL, Kim HK, Sass PM et al. Structure-function analysis of *Leishmania* lipophosphoglycan—distinct domains that mediate binding and inhibition of endothelial cell function. J Immunol 1996; 157: 3013-3020.
249. Hatzigeorgiou DE, Geng JY, Zhu BX et al. Lipophosphoglycan from *Leishmania* suppresses agonist-induced interleukin 1- β gene expression in human monocytes via a unique promoter sequence. Proc Natl Acad Sci USA 1996; 93:14708-14713.
250. Descoteaux A, Turco SJ, Sacks DL et al. *Leishmania donovani* lipophosphoglycan selectively inhibits signal transduction in macrophages. J Immunol 1991; 146:2747-2753.
251. Frankenburg S, Leibovici V, Mansbach N et al. Effect of glycolipids of *Leishmania* parasites on human monocyte activity. Inhibition by lipophosphoglycan. J Immunol 1990; 145:4284-4289.
252. Frankenburg S, Gross A, Leibovici V. *Leishmania major* and *Leishmania donovani*: effect of LPG-containing and LPG-deficient strains on monocyte chemotaxis and chemiluminescence. Exp Parasitol 1992; 75:442-448.
253. Panaro MA, Panunzio M, Jirillo E et al. Parasite escape mechanisms: The role of *Leishmania* lipophosphoglycan on the human phagocyte functions. A review. Immunopharmacol Immunotoxicol 1995; 17:595-605.
254. Brandonisio O, Panaro MA, Marzio R et al. Impairment of the human phagocyte oxidative responses caused by *Leishmania* lipophosphoglycan (LPG): in vitro studies. FEMS Immunol Med Microbiol 1994; 8:57-62.
255. Descoteaux A, Matlashewski G, Turco SJ. Inhibition of macrophage protein kinase C-mediated protein phosphorylation by *Leishmania donovani* lipophosphoglycan. J Immunol 1992; 149:3008-3015.
256. Giorgione JR, Turco SJ, Epand RM. Transbilayer inhibition of protein kinase C by the lipophosphoglycan from *Leishmania donovani*. Proc Natl Acad Sci USA 1996; 93:11634-11639.
257. McNeely TB, Rosen G, Londner MV et al. Inhibitory effects on protein kinase C activity by lipophosphoglycan fragments and glycosylphosphatidylinositol antigens of the protozoan parasite *Leishmania*. Biochem J 1989; 259:601-604.
258. Miao L, Stafford A, Nir S et al. Potent inhibition of viral fusion by the lipophosphoglycan of *Leishmania donovani*. Biochemistry 1995; 34:4676-4683.
259. Easterbrook MD, Levy MH, Gomez AM et al. Inhibition of HIV-1-induced syncytia formation and infectivity by lipophosphoglycan from *Leishmania*. J Acqu Imm Deficiency Syndromes Hum Retrovirol 1995; 10:496-505.
260. Senisterra G, Epand RM. Role of membrane defects in the regulation of the activity of protein kinase C. Arch Biochem Biophys 1993; 300:378-383.
261. Desjardins M, Descoteaux A. Inhibition of phagolysosomal biogenesis by the *Leishmania* lipophosphoglycan. J Exp Med 1997; 185:2061-2068.
262. Tolson DL, Turco SJ, Pearson TW. Expression of a repeating phosphorylated disaccharide lipophosphoglycan epitope on the surface of macrophages infected with *Leishmania donovani*. Infect Immun 1990; 58:3500-3507.
263. Prina E, Antoine JC, Wiederanders B et al. Localization and activity of various lysosomal proteases in *Leishmania amazonensis*-infected macrophages. Infect Immun 1990; 58:1730-1737.
264. Antoine JC, Prina E, Jouanne C et al. Parasitophorous vacuoles of *Leishmania amazonensis*-infected macrophages maintain an acidic pH. Infect Immun 1990; 58:779-787.
265. Russell DG, Xu S, Chakraborty P. Intracellular trafficking and the parasitophorous vacuole of *Leishmania mexicana*-infected macrophages. J Cell Sci 1992; 103:1193-1210.
266. Elhay M, Kelleher M, Bacic A et al. Lipophosphoglycan expression and virulence in ricin-resistant variants of *Leishmania major*. Mol Biochem Parasitol 1990; 40:255-267.
267. Cappai R, Morris L, Aebischer T et al. Ricin-resistant mutants of *Leishmania major* which express modified lipophosphoglycan remain infective for mice. Parasitology 1994; 108:397-405.

268. Opat A, Ng K, Currie G et al. Characterization of lipophosphoglycan from a ricin-resistant mutant of *Leishmania major*. Glycobiology 1996; 6:387-397.
269. Handman E, Schnur LF, Spithill TW et al. Passive transfer of *Leishmania* lipopolysaccharide confers parasite survival in macrophages. J Immunol 1986;137:3608-3613.
270. McNeely TB, Turco SJ. Requirement of lipophosphoglycan for intracellular survival of *Leishmania donovani* within human monocytes. J Immunol 1990; 144:2745-2750.
271. Shankar A, Mitchen TK, Hall LRe et al. Reversion to virulence in *Leishmania major* correlates with expression of surface lipophosphoglycan. Mol Biochem Parasitol 1993; 61:207-216.
272. Gerold P, Schofield L, Blackman MJ et al. 1996. Structural analysis of the glycosyl-phosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of *Plasmodium falciparum*. Mol Biochem Parasitol 75:131-143.
273. Haldar K, Ferguson MAJ, Cross GAM. Acylation of a *Plasmodium falciparum* merozoite surface antigen via *sn*-1,2-diacyl glycerol. J Biol Chem 1985; 260:4969-4974.
274. Smythe JA, Coppel RL, Brown GV et al. Identification of two integral membrane proteins of *Plasmodium falciparum*. Proc Natl Acad Sci USA 1988; 85:5195-5199.
275. Haldar K, Henderson CL, Cross GAM. Identification of the parasite transferrin receptor of *Plasmodium falciparum*-infected erythrocytes and its acylation via 1,2-diacyl-*sn*-glycerol. Proc Natl Acad Sci USA 1986; 83:8565-8569.
276. Braun-Breton C, Rosenberry TL, da Silva LP. Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidyl inositol-specific phospholipase C. Nature 1988; 332:457-459.
277. Gowda DC, Gupta P, Davidson EA. Glycosylphosphatidylinositol anchors represent the major carbohydrate modification in proteins of intraerythrocytic stage *Plasmodium falciparum*. J Biol Chem 1997; 272:6428-6439.
278. Gerold P, Dieckmann-Schuppert A, Schwarz RT. Glycosylphosphatidylinositols synthesized by asexual erythrocytic stages of the malarial parasite, *Plasmodium falciparum*. Candidates for plasmodial glycosylphosphatidylinositol membrane anchor precursors and pathogenicity factors. J Biol Chem 1994; 269:2597-2606.
279. Schofield L, Hackett F. Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. J Exp Med 1993; 177:145-153.
280. Schofield L, Vivas L, Hackett F et al. Neutralizing monoclonal antibodies to glycosylphosphatidylinositol, the dominant TNF-α-inducing toxin of *Plasmodium falciparum*: Prospects for the immunotherapy of severe malaria. Ann Tropical Med Parasitol 1993; 87:617-626.
281. Schofield L, Novakovic S, Gerold P et al. Glycosylphosphatidylinositol toxin of *Plasmodium* upregulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction. J Immunol 1996; 156:1886-1896.
282. Kwiatkowski D, Hill AV, Sambou I et al. TNF concentration in fatal cerebral, nonfatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. Lancet 1990; 336:1201-1204.
283. Berendt AR, Simmons DL, Tansey J et al. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. Nature 1989; 341:57-59.
284. Berendt AR, Turner GDH, Newbold CI. Cerebral malaria: The sequestration hypothesis. Parasitol.Today 1994; 10:412-414.
285. Field MC. Is there evidence for phospho-oligosaccharides as insulin mediators? Glycobiology 1997; 7:161-168.
286. Singh N, Liang LN,. Tykocinski ML et al. A novel class of cell surface glycolipids of mammalian cells. Free glycosyl phosphatidylinositols. J Biol Chem 1996; 271:12879-12884.
287. Joiner KA, Dubremetz JF. *Toxoplasma gondii*: A protozoan for the nineties. Infect Immun 1993; 61:1169-1172.
288. Tomavo S, Dubremetz JF, Schwarz RT. Structural analysis of glycosyl-phosphatidylinositol membrane anchor of the *Toxoplasma gondii* tachyzoite surface glycoprotein gp23. Biology of the Cell 1993; 78:155-162.

289. Kasper LH, Mineo JR. Attachment and invasion of host cells by *Toxoplasma gondii*. Parasitol Today 1994; 10:184-188.
290. Tomavo S, Dubremetz JF, Schwarz RT. A family of glycolipids from *Toxoplasma gondii*. Identification of candidate glycolipid precursor(s) for *Toxoplasma gondii* glycosylphosphatidylinositol membrane anchors. J Biol Chem 1992; 267:11721-11728.
291. Striepen B, Zinecker CF, Damm JB et al. Molecular structure of the "low molecular weight antigen" of *Toxoplasma gondii*: A glucose α 1-4 N-acetylgalactosamine makes free glycosyl-phosphatidylinositols highly immunogenic. J Mol Biol 1997; 266:797-813.
292. Gerold P, Striepen B, Reitter B et al. Glycosyl-phosphatidylinositols of *Trypanosoma congolense*: Two common precursors but a new protein-anchor. J Mol Biol 1996; 261:181-194.
293. Ferguson MAJ. Site of palmitoylation of a phospholipase C-resistant glycosylphosphatidylinositol membrane anchor. Biochem J 1992; 284:297-300.
294. Tomavo S, Dubremetz JF, Schwarz RT. Biosynthesis of glycolipid precursors for glycosylphosphatidylinositol membrane anchors in a *Toxoplasma gondii* cell-free system. J Biol Chem 1992; 267:21446-21458.
295. Guther ML, Treumann A, Ferguson MAJ. Molecular species analysis and quantification of the glycosylphosphatidylinositol intermediate glycolipid C from *Trypanosoma brucei*. Mol Biochem Parasitol 1996; 77:137-145.
296. Masterson WJ, Doering TL, Hart GW et al. A novel pathway for glycan assembly: biosynthesis of the glycosyl-phosphatidylinositol anchor of the trypanosome variant surface glycoprotein. Cell 1989; 56:793-800.
297. Schneider P, Schnur LF, Jaffe CL et al. Glycoinositol-phospholipid profiles of four serotypically distinct Old World *Leishmania* strains. Biochem J 1994; 304:603-609.
298. Previato JO, Jones C, Wait R et al. *Leishmania adleri*, a lizard parasite, expresses structurally similar glycoinositolphospholipids to mammalian *Leishmania*. Glycobiology 1997; 7:687-965.
299. Ma DQ, Russell DG, Beverley SM et al. Golgi GDP-mannose uptake requires *Leishmania* LPG2—a member of a eukaryotic family of putative nucleotide-sugar transporters. J Biol Chem 1997; 272:3799-3805.
300. Heise N, Opperdoes FR. The dihydroxyacetonephosphate pathway for biosynthesis of ether lipids in *Leishmania mexicana* promastigotes. Mol Biochem Parasitol 1997; 89:61-72.
301. Smith TK, Milne FC, Sharma DK et al. Early steps in glycosylphosphatidylinositol biosynthesis in *Leishmania major*. Biochem J 1997; 326:393-400.
302. Huang C, Turco SJ. Defective galactofuranose addition in lipophosphoglycan biosynthesis in a mutant of *Leishmania donovani*. J Biol Chem 1993; 268:240602406-6.
303. Ryan KA, Garraway LA, Descoteaux A et al. Isolation of virulence genes directing surface glycosyl-phosphatidylinositol synthesis by functional complementation of *Leishmania*. Proc Natl Acad Sci USA 1993; 90:8609-8613.
304. Ha DS, Schwarz JK, Turco SJ et al. Use of the green fluorescent protein as a marker in transfected *Leishmania*. Mol Biochem Parasitol 1996; 77:57-64.
305. Descoteaux A, Luo Y, Turco SJ et al. 1995. A specialized pathway affecting virulence glycoconjugates of *Leishmania*. Science 269:1869-72
306. Ma DQ, Beverley SM, Turco SJ. *Leishmania donovani* possess a NADPH-dependent alkylglycerol cleavage enzyme. Biochem Biophys Res Comm 1996; 227:885-889.
307. Mengeling BJ, Zilberstein D, Turco SJ. Biosynthesis of *Leishmania* lipophosphoglycan—solubilization and partial characterization of the initiating mannosylphos-phoryltransferase. Glycobiology 1997; 7:847-853.
308. Carver MA, Turco SJ. Biosynthesis of lipophosphoglycan from *Leishmania donovani*: Characterization of mannosylphosphate transfer in vitro. Arch Biochem Biophys 1992; 295:309-317.
309. Brown GM, Millar AR, Masterson C et al. Synthetic phospho-oligosaccharide fragments of lipophosphoglycan as acceptors for *Leishmania major* α-D-mannosylphosphate transferase. Eur J Biochem 1996; 242:410-416.
310. Ng K, Handman E, Bacic A. Biosynthesis of lipophosphoglycan from *Leishmania major*: Characterization of (β 1-3)-galactosyltransferase(s). Glycobiology 1994; 4:845-853.

311. Ng K, Handman E, Bacic A. Biosynthesis of lipophosphoglycan from *Leishmania major*: Solubilization and characterization of a (β 1-3)-galactosyltransferase. Biochem J 1996; 317:247-255.
312. Schneider P, McConville MJ, Ferguson MAJ. Characterization of GDP-α-D-arabinopyranose, the precursor of D-Arap in *Leishmania major* lipophosphoglycan. J BiolChem 1994; 269:18332-18337.
313. Park S, Pastuszak I,Mengeling BJ et al. Synthesis and utilization of GDP-D-arabinopyranoside. Anal Biochem 1997; 244:321-327.

Chapter 9

Enzymes Cleaving the Phosphodiester Bond in the GPI Anchor

Martin G. Low and Urs Brodbeck

Since the discovery of GPI-anchored proteins, anchor hydrolyzing phospholipases from bacteria have been prominent tools in establishing this particular membrane link. There are two activities which cleave the phosphodiester bond in a GPI anchor: phosphatidylinositol-specific phospholipases C (PI-PLC) and glycosylphosphatidylinositol-specific phospholipases D (GPI-PLD). The products of GPI-PLD hydrolysis are phosphatidic acid and the corresponding hydrophilic inositol glycan (Fig. 9.1). PI-PLC produces diradylglycerol (a collective term for diacyl-, alkyacyl- and alk-1-enylacyl-glycerol) as hydrophobic cleavage product, and the hydrophilic glycan, with a terminal inositol residue containing (in most cases) a 1-2 cyclic phosphodiester. From work on the variant surface glycoprotein (VSG) from trypanosomes, the inositol-cyclic-phosphodiester is known as a key component of the so-called crossreacting determinant (CRD). This antigenic site is common to all GPI-anchored proteins after cleavage by bacterial PI-PLC and (G)PI-PLC from trypanosomes, and its detection by anti-CRD antibodies provides key evidence for GPI anchoring. If a GPI-anchored protein is solubilized by a phospholipase of unknown cleavage specificity, the occurrence of the CRD provides evidence for a C-type cleavage.

GPI Anchor-Cleaving Phospholipases from Bacteria

As detailed in chapter 1, work in the early 60s on a factor from bacteria releasing alkaline phosphatase from the cell surface led to the identification and purification of bacterial phospholipases that later on became indispensable tools in establishing the GPI membrane anchors of proteins. In 1968 Lundin[1] reported that cholinesterase from plaice body muscle was solubilized when a bacterium of the species *Cytophaga* was grown on minced tissue of fish; however, the mechanism of cholinesterase solubilization remained unknown. Subsequently, work on the release of alkaline phosphatase, 5′-nucleotidase, and acetylcholinesterase from cell membranes proposed a link between the activity of bacterial phospholipases and the membrane anchors of these proteins.[2] The phospholipases isolated from *Bacillus cereus*, *Bacillus thuringiensis*, *Staphylococcus aureus* and *Clostridium novyi* were found to cleave phosphatidylinositol (PI) with the specificity of a phospholipase C, but showed virtually no activity towards phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine, and thus were termed PI-specific phospholipase C (PI-PLC). Subsequently, bacterial PI-PLCs were found to also cleave the PI moiety of GPI-anchored proteins and glycolipids. In fact, it was this unique property that led to the initial discovery of GPI-anchored proteins. Ever since, PI-PLCs have been widely used in the detection and analysis of GPI-anchored molecules. Accordingly the *Cytophaga* factor responsible for the release of cholinesterase from fish tissue was later also identified as a PI-PLC.[3]

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

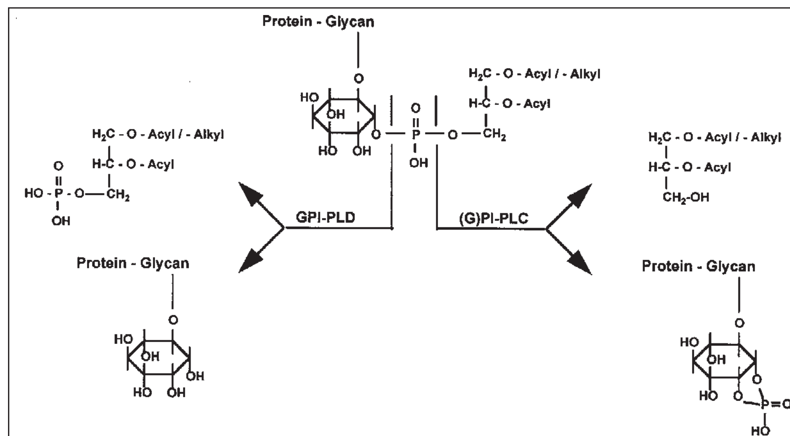

Fig. 9.1: Cleavage specificity of GPI-hydrolyzing phospholipases.

Bacterial PI-PLCs have been purified from several sources, including *B. cereus* and *B. thuringiensis*.[4] The enzymes from these two strains differ in only 8 amino acids and consequently show nearly identical properties with respect to molecular mass, electrophoretic mobility, substrate specificity, sensitivity to inhibitors and interaction with inhibitory antibodies. The properties of bacterial phospholipases have been extensively reviewed (see for example, ref . 5) and their role in bacterial virulence is of special interest.[6] The crystal structure of PI-PLC from *B. cereus* has been solved to 2.5 Å resolution in complex with myo-inositol,[7] and more recently to 2.2 Å in complex with glucosaminyl(α1-6)-D-myo-inositol.[8]

It was shown that the myo-inositol moiety of GlcN(α1-6)Ins occupies the same position in the active site as does free myo-inositol, which provides convincing evidence that the enzyme utilizes the same catalytic mechanism for cleavage of PI and GPI. The myo-inositol moiety is well defined in the crystal structure and undergoes several specific hydrogen bonding interactions with active site residues. In the crystal structure the glucosamine moiety is less well defined, suggesting enhanced conformational flexibility. This is consistent with the view that the glucosamine moiety lies exposed to solvent at the entrance of the active site with minimal specific protein contacts. On the basis of the positioning of GlcN(α1-6)Ins in the active site, it is predicted that the remainder of the GPI glycan moiety makes little or no specific interactions with *B. cereus* PI-PLC. This explains why *B. cereus* PI-PLC is able to cleave GPI anchors having variable glycan structures.[8] By probing the roles of the active site residues in PI-PLC from *B. cereus* by site-directed mutagenesis, the general acid-base catalysis including the four residues directly involved in catalysis could be substantiated.[9] In contrast to other GPI anchor-degrading phospholipases, bacterial PI-PLCs effectively cleave the GPIs in both intact membranes and artificial lipid bilayers. The enzymes were shown to exhibit a 5- to 6-fold "interfacial activation" when its substrate is present in an interface as opposed to existing as a monomer in solution.[10] As with other phosphodiesterases such as RNase, GPI-anchor cleavage by PI-PLC occurs via formation of an inositol-1-2-cyclic-phosphate.[11] Whether the cyclic phosphate is stably formed, thus exposing the CRD, or further hydrolyzed to inositol-phosphomonoester is kinetically controlled and depends on the polarity of the solvent. Organic solvents miscible in water (dimethyl sulfoxide, dimethylformamide, and 2-propanol) increased more than

80-fold the catalytic efficiency (V_{max}/K_m) of cyclic phosphodiesterase by lowering the K_m for myo-inositol 1, 2-cyclic phosphate and substantially increasing V_{max}.[12] The formation of an inositol-cyclic,1-2-phosphate is possible only if the 2-OH of inositol is not acylated. An inositol-acylated GPI anchor was first described for acetylcholinesterase from human erythrocytes, a modification which rendered the GPI anchor insensitive to the hydrolysis by PI-PLC.[13] A possible insensitivity of a membrane protein to PI-PLC thus either means that the protein is not GPI anchored or that it contains a GPI anchor with a fatty acid linked to the 2-OH of inositol.

GPI Anchor-Cleaving Phospholipase C (GPI-PLC) from Trypanosomes

The surface of the bloodstream form of the African trypanosome *Trypanosoma brucei* is covered with about 10^7 molecules of the variant surface glycoprotein (VSG), a protein tethered to the plasma membrane by a GPI anchor. Independent of the work on the bacterial factor solubilizing alkaline-phosphatase, 5′-nucleotidase and acetylcholinesterase from mammalian cells, the characterization of VSG from trypanosomes led to the discovery of a GPI anchor-hydrolyzing phospholipase C in these parasites.[14-17] The initial identification of GPI-anchored proteins in *T. brucei* was complicated by the presence of relatively large amounts of endogenous anchor-degrading phospholipase which removed the anchor during extraction.[18] In living trypanosomes, GPI-PLC does not release VSG from the cell surface and it was only under the peculiar conditions used for extraction (osmotic lysis) that its activity was noticed. Although the enzyme was shown to be active against intracellular GPIs after transfection into *Leishmania* parasites,[19] no clear role in GPI metabolism has been attributed to this enzyme in vivo.[20] To investigate whether the ability to cleave the membrane anchor of the VSG is an essential function of the enzyme in vivo, a GPI-PLC null mutant trypanosome had been generated by targeted gene deletion. The mutant trypanosomes were fully viable; they could go through an entire life cycle and maintain a persistent infection in mice. Thus, the GPI-PLC is not an essential activity and is also not required for antigenic variation in the bloodstream form of trypanosomes. However, mice infected with mutant trypanosomes had a reduced parasitemia and survived longer than those infected with control trypanosomes.[21]

GPI-PLC from *T. brucei* has a relatively low sequence similarity to the bacterial PI-PLCs, and originally the enzyme was thought to be GPI specific. This notion derived from early work in which the enzyme was assayed under conditions that did not reveal the hydrolysis of PI (for a review see ref. 22). More recently, the substrate specificity was reinvestigated and it was shown that PI hydrolysis indeed occurred.[23] In addition, endogenous PI was hydrolyzed during osmotic and detergent lysis of trypanosomes under conditions used to obtain quantitative hydrolysis of VSG.

GPI-PLC activity is downregulated when trypanosomes differentiate from the bloodstream form to the procyclic form found in the tsetse fly vector. The GPI-PLC locus in the trypanosome genome was mapped and the mechanism for this developmental regulation in *T. brucei* examined.[29] Surprisingly, the enzyme does not appear to be involved in the shedding of the VSG coat during differentiation from the bloodstream form to the procyclic form.[30] However, recent data indicates that the GPI-PLC is involved in the release of VSG in response to celç•lar stress induced by low pH or trypsin.[31] GPI-PLC is associated with the cytoplasmic surface of intracellular vesicles, consistent with its lack of a signal sequence.[32] Its mechanism of association with the membrane is unclear, because it has no obvious hydrophobic transmembrane domains.[33] Initially, the cytoplasmic orientation of this enzyme was difficult to understand; however, the subsequent discovery that GPI precursors are synthesized at the cytoplasmic surface of the endoplasmic reticulum suggest that its real

Table 9.1 GPI anchor and PI hydrolysis by phospholipases

	Km-values (μM) for	
Phospholipase	**GPI anchor hydrolysis**	**PI hydrolysis**
Pi-PLC (*B. cereus*)[a]	12.0	2000
PI-PLC (*Cytophaga* sp.)[b]	3.0	2500
(G)PI-PLC (peanut seeds)[c]	2.8	9590
GPI-PLC (*T. brucei*)[d]	0.8	37
GPI-PLD (bovine serum)[e]	0.1	–[f]
GPI-PLD (bovine brain)[e]	0.05	–[f]

a) Mean value from experiments using acetylcholinesterase from bovine erythrocytes and *Torpedo maromorata* as substrates;[24] b-e) Values determined using acetylcholinesterase from bovine erythrocytes as substrate; b) Taken from ref. 3; c) Taken from ref. 25; d) data obtained using the recombinant enzyme[26], values taken from ref. 23; e) Taken from refs. 27 and 28; f) Ny hydrolysis.

function is in the metabolism of the protein-free lipid rather than GPI-anchored proteins (see chapter 2). The recent observation that GPI-PLC also hydrolyzes PI in trypanosomes[23] raises the possibility that PI rather than GPI may be the true intracellular substrate.

PI-PLCs differ with respect to their ability to act on substrates (Table 9.1). PI-PLC from *B. cereus*, *B. thuringiensis*, and *S. aureus* readily cleave PI and GPI anchors in intact cell membranes, while the enzymes from trypanosomes and *Listeria monocytogenes*[34] efficiently cleave GPI anchors only when they are present in a detergent micelle (Table 9.2). In this respect they resemble mammalian GPI-PLD, which has a low membrane activity (see below). (G)PI-PLCs are overall hydrophilic enzymes, and their binding to the membrane interface is a necessary step to make a long chain phospholipid substrate accessible to hydrolysis. A possible reason for the different behavior towards substrates in cell membranes could lie in the different interfacial activation of the phospholipases (see ref. 33 for a review). Allosteric activation of lipolytic enzymes at the water lipid interphase is a necessary prerequisite for catalytic activity on the substrate in the lipid bilayer, and in this context the allosteric activation of PI-PLC[36] and GPI-PLC[37] by several aminoglycoside antibiotics may be of special interest.

GPI Anchor-Cleaving Activity in Plants

A GPI anchor-cleaving enzyme in peanut seeds has been identified and partially purified by a series of column chromatographic steps.[25] It cleaves detergent-solubilized GPI anchors but does not act on membrane-bound GPI nor on inositol-acylated GPI anchors. In addition, PI is also hydrolyzed, while other phospholipids (PC, PE, and PS) are not. The water-soluble products of phosphatidylinositol hydrolysis by peanut phospholipase C were characterized as a mixture of inositol 1,2-cyclic phosphate and inositol phosphate,[24] identifying the activity from peanut seeds as a (G)PI-PLC.[25] However, until recently, potential GPI substrates for this enzyme had not been observed in higher vascular plants. Six proteins on the outer surface of cultured *Nicotiana tabacum* cells with molecular weights of 92, 84, 60.5, 54.5, 39.5 and 37 kDa were found to move from a Triton X-114 detergent-rich phase to an aqueous phase following incubation with PI-PLC. Seven GPI-anchored proteins were

Table 9.2 Substrate specificity of GPI anchor-hydrolyzing phospholipases

Substrate	Bacterial PI-PLCs[a]	*T. brucei* (G)PI-PLC	Peanut (G)PI-PLC	Mammalian GPI-PLD
Detergent solubilized GPI anchor, not inositol-acylated	+	+	+	+
Detergent solubilized, inositol-acylated GPI anchor	–	–	–	+
Membrane-bound GPI, not inositol-acylated	+	–	–	–
Membrane-bound GPI, inositol-acylated	–	–	–	–
PI	+	+	+	–

a) Except for PI-PLC from Listeria monocytogenes[34] which poorly hydrolyzes membrane bound GPI anchors

also detected on the surface of tobacco leaf protoplasts with molecular weights of 67.5, 62, 39, 33.5, 27, 23 and 15.6 kDa.[38]

Mammalian GPI-PLC

The existence of a mammalian GPI-PLC has been described for rat liver[27,39] and mouse brain.[40] The main evidence for a C-type cleavage specificity was based on the release of diacylglycerol from the GPI-anchored VSG, and diradylglycerol from the GPI anchor of bovine red cell acetylcholinesterase. However, later work on rat hepatocyte membranes showed that if the assays were carried out in the presence of NaF and sodium orthovanadate, which are known inhibitors of phosphatidic acid phosphohydrolases, phosphatidic acid was detected in addition to diradylglycerol.[41] In addition, the GPI anchor-converting activity showed properties similar to those of GPI-PLD from serum, i.e., it was Ca^{2+}-sensitive and inhibited by heavy metal chelating agents. These findings strongly suggested that in rat liver the GPI anchor-hydrolyzing activity was due to a phospholipase D and not a phospholipase C.[41]

In conjunction with the mechanism of insulin action, the effect of insulin on the turnover of GPI-anchored structures in cell membranes has been widely studied, and a large body of evidence has accumulated indicating that the action of insulin may, in part, be mediated by the hydrolysis of a phosphatidylinositol-glycan. Part of insulin action is thought to result in the generation of soluble inositol phosphoglycans and diradylglycerol as putative second messengers of this hormone (for recent reviews see refs. 42-46). To date, however, a putative insulin-sensitive GPI-PLC responsible for the hydrolysis of GPI-anchored proteins and lipids has not been isolated and, thus, its existence is only conjectural.[42-46] In several reports, GPI-PLC activities have been postulated to be involved in the release of GPI-anchored ecto-proteins after stimulation of insulin-sensitive cells. Anti-diabetic compounds like sulphonylurea drugs are known to stimulate glucose transport and metabolism in muscle and fat cells in vitro. It was shown that incubation of 3T3 adipocytes with glimepiride, a sulphonylurea compound, caused a time- and concentration-dependent release of the GPI-anchored ecto-proteins 5′- nucleotidase, lipoprotein lipase, and cAMP-binding

protein from the plasma membrane into the culture medium.[47] The conversion of the membrane-anchored amphiphilic proteins into their soluble hydrophilic forms was accompanied by the appearance of the CRD in the released proteins. This, together with the release of inositol phosphate from these proteins after deamination with nitrous acid, suggested that the GPI membrane anchors were hydrolyzed by a GPI-PLC.[47] Similarly, incubation of 3T3-L1 adipocytes with either insulin or glimepiride was found to induce a rapid concentration- and time-dependent release of GPI-anchored membrane dipeptidase from the cell surface.[48] Again, the hydrophilic form of membrane dipeptidase after stimulation of the cells with insulin was recognized by anti-CRD antibodies, suggesting that the GPI anchor was cleaved through the action of a GPI-PLC.[48]

In a further report,[49] stimulation of adipocytes by insulin and glimepiride was found to result in solubilization by lipolytic cleavage of two other GPI-anchored proteins, lipoprotein lipase and cAMP-binding ecto-protein. Inositol-phosphate was retained in the residual protein-linked anchor structure, suggesting cleavage of the GPI anchor by an endogenous GPI-specific insulin- and glimepiride-sensitive GPI-PLC. Interestingly, despite GPI cleavage, the hydrophilic forms of these proteins remained membrane associated and were released only if a competitor, e.g., inositol-(cyclic)monophosphate, was added.[49] Other constituents of the GPI anchor (glucosamine and mannose) were less efficient. This suggests peripheral interaction of lipolytically cleaved lipoprotein lipase and cAMP-binding ecto-protein with the adipocyte cell surface involving the terminal inositol-(cyclic)monophosphate epitope and, presumably, a receptor of the adipocyte plasma membrane.[49]

Signal-induced release of GPI-anchored proteins from the cell surface seems to be a general phenomenon, as it is not restricted to the action of insulin.[50-52] In these cases, however, product identification was less stringent than in the work cited in conjunction with the effect of insulin on the release of GPI-anchored proteins. Altogether, the involvement of GPI-anchored molecules in insulin signaling remains controversial until definite proof of the structure(s) of the putative GPI mediator(s) becomes available and the enzyme producing the phospho-oligosaccharide second messenger has been identified and characterized (see chapter 4).

Mammalian GPI-PLD

Distribution of GPI-PLD

A mammalian GPI-degrading activity was first described during early investigations of the anchoring mechanism of alkaline phosphatase (see chapter 1). It was observed that the standard procedure for extracting alkaline phosphatase from mammalian tissues (incubation of a homogenate or membrane fraction with aqueous butanol) produced a soluble form that was similar in its physical characteristics to that released from membranes with purified bacterial PI-PLC.[53] The butanol treatment had converted this tightly bound membrane enzyme to a dimeric, hydrophilic form that was no longer able to bind liposomes. By contrast, when the extraction was done with cold butanol, or if EDTA was added, an aggregated hydrophobic form was produced which could bind liposomes. The hydrophobic form of alkaline phosphatase could then be converted to the hydrophilic form by incubation with PI-PLC. The simplest interpretation of this phenomenon was that butanol had activated an endogenous enzyme capable of removing the anchor from alkaline phosphatase. The Ca^{2+}-dependent phosphoinositide-specific phospholipase C, found in the cytosolic and membrane fractions of most mammalian tissues, was proposed as the most likely culprit.[53,54] The observation that anchor degradation was strongly pH dependent over the range 5.5-8.0 supported the idea that an endogenous enzyme was responsible.[55-57]

However, more detailed characterization of this activity was not possible until the membrane anchor on alkaline phosphatase was found to be a GPI molecule.

Although phosphatidylinositol hydrolysis and GPI degradation (using alkaline phosphatase and VSG as substrates) exhibited comparable sensitivities to pH and EGTA, it was observed that the GPI-degrading activity was relatively heat stable.[57] Furthermore, the GPI anchor-degrading activity, but not phosphatidylinositol hydrolysis, could be inhibited by 1,10-phenanthroline.[58] The distinction between these two activities was reinforced when it was shown that the GPI-degrading activity released phosphatidic acid but did not expose the CRD epitope on VSG.[57,59] Collectively, this data indicated that the mammalian anchor-degrading activity was a phospholipase D with a preference for GPI substrates.

The anchor-degrading activity was subsequently found to be relatively abundant in plasma and serum from several mammalian species.[58,60,61] In fact, the activity of the GPI-specific phospholipase D (GPI-PLD) in serum is so high that most of the GPI-degrading activity that has been detected in homogenates of unperfused tissues probably results from contamination with plasma.[59,62] However, there is recent evidence indicating that GPI-PLD activity is also tightly associated with liver plasma membranes or located in lysosomes, and it is possible that these are distinct forms of the enzyme.[63-65] Although all mammalian species tested have substantial activity, the amount in human serum and plasma is relatively low (10-30% of that found in other species).[58,66] The GPI-PLD activity has been purified from human and bovine serum and shown to be capable of degrading the GPI anchors on several different proteins but not to hydrolyze any other phospholipid, including phosphatidylinositol.[66-69]

Although the abundance of GPI-PLD in plasma proved useful for purification and molecular characterization, it has severely hampered studies on GPI-PLD located in cells. Using a combination of enzyme assay and immunostaining techniques, GPI-PLD has been detected, often at relatively low levels, in several cell types: neurons, keratinocytes, bone marrow, leukocytes, pancreatic cells and mast cells.[62,70-72] However GPI-PLD is known to be taken up into cells in vitro, and a major concern with the localization studies is the potential for contamination by residual, exogenous GPI-PLD (originating from the serum normally present in culture media).[73] This may explain the substantial decrease in cell-associated GPI-PLD activity often observed following culture in serum-free media.[62,72] The presence of GPI-PLD mRNA has been confirmed in several tissue/cell types by Northern blotting or PCR amplification of cDNA libraries.[71,74-76] However, it has only been detected by in situ hybridization in mast cells and there is no biosynthetic labeling data for GPI-PLD in any cell type.[76]

There is also a great deal of uncertainty regarding cellular sources of the GPI-PLD found in plasma, although the recent demonstration of regulated secretion from pancreatic β cells makes them a good candidate.[77] Constitutive "secretion" has also been reported from myeloid cell lines, raising the possibility that circulating leukocytes are another source of the plasma GPI-PLD.[72] It should be emphasized that the specific activity of GPI-PLD in serum is several orders of magnitude higher than in any tissue/cell type analyzed to date. This suggests that the cells which secrete GPI-PLD must be quite abundant, the GPI-PLD is stored in the secretory cells in a relatively inactive form or the secreted GPI-PLD has a long half life in the bloodstream. In two clinical studies, serum GPI-PLD was reduced in patients with impaired liver function and it was suggested that this organ might be an important source of the enzyme.[78,79] GPI-PLD has also been detected in milk and cerebrospinal fluid, but the concentrations are relatively low, $<1\%$ of that found in plasma.[66] There is relatively little information on the distribution of GPI-PLD in other organisms besides mammals. GPI-PLD is present in chicken plasma and neural tissue, but there are no reports of a similar enzyme in other vertebrates or in nonvertebrates.[58,80]

Structure/Function Studies on GPI-PLD

GPI-PLD has been purified from bovine and human serum and has a molecular mass of 100-120 kDa by SDS-PAGE, with a pI of 5.6.[66-69,81] The predicted molecular mass from the bovine liver DNA sequence (816 residues; Genbank accession # M60804) is 90.2 kDa.[82] Two cDNAs with similar, but not identical, sequences predicting a protein of 817 residues have been isolated from human liver and pancreatic cDNA libraries (Genbank accession #s L11701 and L11702). The C-terminal region of GPI-PLD is highly homologous to the N-terminal domain of integrin α subunits (see below and Figs. 9.2 and 9.3) but there is no apparent homology with any other protein, including the phospholipases D that hydrolyze phosphatidylcholine. The cDNA sequences predict the presence of eight potential N-linked glycosylation sites. Although there is no detailed information on the glycan structure, at least three of these sites are likely to be glycosylated based on sensitivity to N-glycosidase F or glycan staining of tryptic fragments (Li J-Y, Low MG, unpublished work). Four of these sites are clustered around the junction between the two major domains of GPI-PLD (Figs. 9.2, 9.3). GPI-PLD is insensitive to O-glycanase.[66] Presumably the N-glycosylation accounts for the ability of human and bovine GPI-PLD to bind to concanavalin A or wheat germ lectin, as well as the difference between the predicted molecular mass and that determined by SDS-PAGE.[61,66,68,69]

Several gel filtration studies have indicated that purified GPI-PLD is an aggregate with a molecular mass of >400 kDa, which decreases in the presence of Triton X-100 to 200-350 kDa.[66,69,83] Disruption of GPI-PLD aggregates is consistent with the decrease in sedimentation coefficient from >9.5S to approx 6S induced by detergent.[66] However, there is also one report suggesting that purified GPI-PLD has a molecular mass of approximately 100 kDa (by gel filtration) even in the absence of detergent.[68] At present, inherent inaccuracies in the methods used to estimate the size of the monomeric and aggregated forms make it difficult to draw any firm conclusions as to the number of GPI-PLD molecules in the aggregates. Although it seems likely that GPI-PLD is mostly dimeric in the presence of detergent, other possibilities (e.g., an asymmetric monomer, binding to detergent micelles, etc.) cannot be excluded at present. In plasma, GPI-PLD appears to have a molecular mass of approx 500 kDa by gel filtration.[58] However, this is probably due to the association of most of the GPI-PLD in plasma with the apolipoprotein AI fraction of HDL rather than formation of aggregates consisting entirely of GPI-PLD.[66,84] Purified apoAI will disaggregate GPI-PLD, suggesting that this amphipathic protein is able to interact directly with GPI-PLD.[85]

In two independent studies, tryptic degradation of purified GPI-PLD cleaved the protein into two fragments of approximately 40 kDa and one of approximately 30 kDa.[83,86] However, the formation of fragments does not inactivate the GPI-PLD; in fact, under some assay conditions, it produces a substantial increase in activity. A similar stimulation is given by several other proteases.[83] Apart from increased activity, there were no other major changes in its properties. Attempts to isolate a catalytic domain from the tryptic digest were difficult because the three fragments remain associated unless they were previously denatured. After chromatography in 6 M urea followed by renaturation, enzymatic activity was detected in the N-terminal fragment.[86] No activity was present in the other two fragments, either alone or in combination with the N-terminal fragment. Although recovery of activity after this procedure was poor, the work strongly suggests that the catalytic site is located in the N-terminal region of the GPI-PLD molecule (see Fig. 9.2).

The GPI-PLD cDNA sequence predicted the presence of four Ca^{2+}-binding sites in the C-terminal region (see Fig. 9.2).[82] This prediction was consistent with the sensitivity of GPI-PLD to inhibition by EGTA and EDTA, which can be blocked by excess Ca^{2+} ions.[27,28,57,68] However, more detailed studies comparing the ability of different cations to reverse the inhibitory effects of EGTA have indicated that Ca^{2+} can be removed from the GPI-PLD

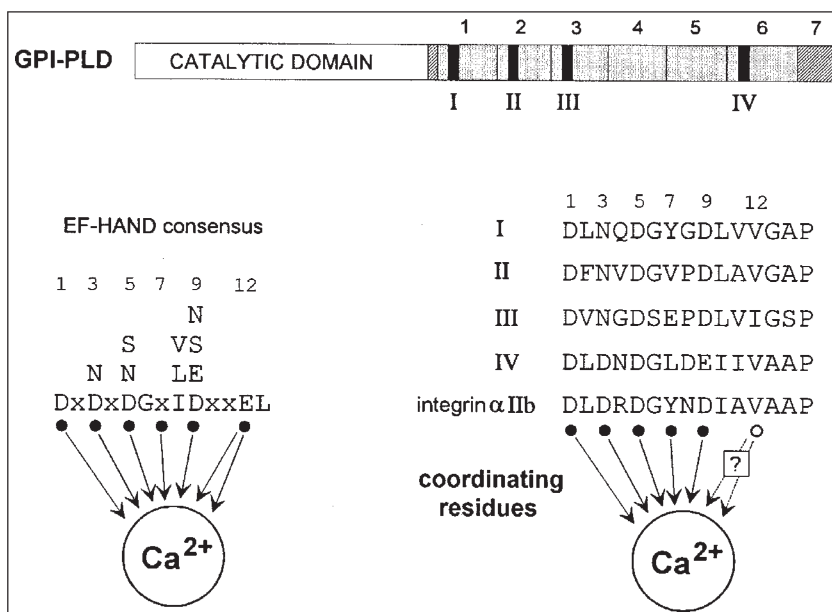

Fig. 9. 2. Predicted Ca^{2+}-binding sites in GPI-PLD. The location of predicted Ca^{2+}-binding sites (solid shading) within GPI-PLD is shown at the top of the figure. The amino acid sequences of these sites are shown at the lower right. For comparison, a typical divalent cation binding site sequence from an integrin α-subunit (sixth repeat of IIb) is shown below the GPI-PLD sequences. The locations of predicted Ca^{2+}-coordinating residues (solid circle) are based on comparisons with the EF-hand sequences.[87] Coordination at position 7 is provided by the main chain carbonyl and at position 9 is via a H-bonded water molecule. The bidentate coordination provided in conventional EF-hands by a glutamic acid at position 12 is absent in integrin EF-hands, where it is usually replaced by an isoleucine or a valine residue. The identity of the "missing" coordinating residue at position 12 is unknown for both GPI-PLD and integrins.

without affecting enzymatic activity.[83] They further suggest that the inhibitory effects of EDTA and EGTA (as well as 1,10-phenanthroline) are probably due to their ability to remove bound zinc from the protein. Although Ca^{2+} does not appear to be necessary for activity, it will bind to GPI-PLD.[83] The binding appears to be relatively specific for Ca^{2+} since it is not blocked by other divalent cations, including zinc. Metal analyses and equilibrium dialysis indicate that approximately 5 Ca^{2+} ions are bound to each GPI-PLD molecule.

The metal analysis also indicated that GPI-PLD contains ten zinc ions.[83] Although this result provides independent support for the inhibitor studies described above, it is not obvious how and where such a large number of zinc ions could be bound by GPI-PLD. It is unlikely that all of these divalent cations could be located in the active center and it is probable that most, if not all, of the zinc ions have a structural rather than a catalytic role. Comparable numbers of structural zinc ions have been found in several zinc finger proteins, but there are no zinc fingers or other well characterized zinc-binding domains in GPI-PLD. At least two closely-spaced cysteines are required to bind a zinc ion, but bovine GPI-PLD is predicted to contain only ten cysteine residues in total, and of these only two are close together. Histidine is a more likely ligand for binding the zinc ions in GPI-PLD

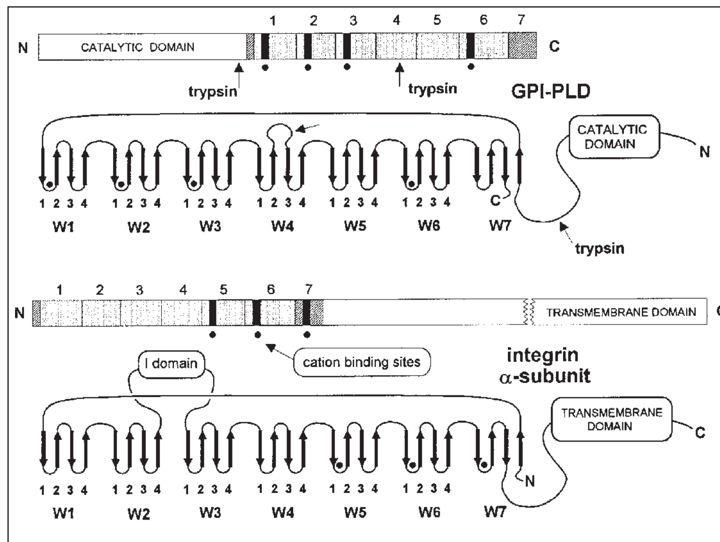

Fig. 9.3. Structural comparison of GPI-PLD and integrin α-subunits. This figure compares the seven-fold repeat structure of GPI-PLD and a representative integrin α-subunit based on modeling studies.[88,89] For each protein the location of the repeats (stippled shading) and the EF-hand-like Ca^{2+}-binding sites (solid shading) is shown schematically in the upper figure. The folding predicted by the β-propeller model[89] is shown in the lower figure. Each repeat (W1-W7) is predicted to fold as a four-stranded antiparallel sheet with the Ca^{2+}-coordinating residues located in a loop between the first and second strands (see Fig. 9.2). Also shown is the location of the I domain, which is inserted between the second and third repeat in some integrins. The location of predicted calcium ion binding sites (filled circles) and tryptic cleavage sites in GPI-PLD (arrows) are also indicated.

because in several different enzymes only two histidine residues, in conjunction with an acidic residue, are required to coordinate one zinc ion.

Initial sequence comparisons indicated that the predicted Ca^{2+}-binding sites have a strong resemblance to EF-hand-like sites found in the N-terminal region of α-subunits of integrins.[82,87] A distinctive feature of the EF-hand-like sites in both integrins and GPI-PLD is that they lack a suitable coordinating residue at position 12 (Fig. 9.2). A subsequent structure modeling study suggested that the similarity between GPI-PLD and integrin α-subunits was even more extensive and encompassed the 400-500 residues contained in the seven FG-GAP repeats common to all integrin α-subunits.[88] In fact the only major differences in this region between integrin α-subunits and GPI-PLD are the location of the FG-GAP repeats relative to the rest of the protein and the distribution of the predicted Ca^{2+}-binding sites amongst the FG-GAP repeats. More recently, all of the FG-GAP repeats in integrin α-subunits and GPI-PLD have been predicted to fold into a single domain: a seven-bladed β-propeller (Fig. 9.3).[89] This model locates all of the Ca^{2+}-binding sites relatively close together on the 'lower' face of the β-propeller, but does not indicate what the function of these sites is, either in integrins or in GPI-PLD. It is of interest to note that the most sensitive tryptic cleavage site on GPI-PLD is predicted to be in an exposed loop on the upper face of the β-propeller (Fig. 9.3).[83,86] The β-propeller model may also explain why relatively small deletions at the C-terminus have such a drastic effect on the enzymatic activity of GPI-PLD when the catalytic site is predicted to be in the N-terminal region of the protein.[90] These

regions of the molecule are in fact adjacent to each other as a consequence of the circular arrangement of the FG-GAP repeats in the β-propeller. However, it should be emphasized that, even though the limited "structural" information available for GPI-PLD is consistent with the β-propeller structure, it does not provide a rigorous test for any of its predictions.

Regulation of Activity on In Vitro Model Systems

GPI-PLD (either purified or in serum) is active on detergent-solubilized substrates but it has very low or undetectable activity towards GPI-anchored proteins located in cell membranes.[60,67,91] The reason why proteins in cell membranes are resistant to GPI-PLD is unknown, but it plays an important role in protecting cells from the high levels of this enzyme in the circulation. It has been calculated that if GPI-PLD were fully active on cells, there would be sufficient activity in the plasma to remove all GPI-anchored proteins from an exposed cell surface in a few minutes.[91] One potential explanation for resistance of GPI-anchored proteins in cells to hydrolysis by GPI-PLD could be their sequestration in the cholesterol and sphingolipid-rich microdomains (see chapter 3). However, there is conflicting evidence as to whether cholesterol-binding agents such as saponin (which would be expected to disrupt the microdomains) can stimulate GPI-PLD-mediated release of GPI-anchored proteins.[67,92] Furthermore, most of the resistance to GPI-PLD exhibited by cell membranes can be restored when the detergent-solubilized substrates are inserted into liposomes containing phosphatidylcholine alone.[67,91]

GPI-PLD is unable to bind to phosphatidylcholine liposomes even if they contain substrate molecules (Low MG, unpublished work). Thus, the relatively low activity towards membranes may be a simple result of the inability of GPI-PLD to bind to the phospholipid bilayer (or cell membranes) and gain the kinetic advantage resulting from processive hydrolysis at the interface. It is not known if the stimulatory effect of detergent on hydrolytic activity is due to disruption of GPI-PLD aggregates (discussed in the previous section) or more effective substrate presentation. In this regard, it is relevant to note that apoAI not only disaggregates GPI-PLD but will also stimulate enzymatic activity.[85] Although this effect is only seen with a large molar excess of apoAI, it is nevertheless significant, because it occurs even in the presence of detergent. It seems likely that activity of GPI-PLD towards substrates dispersed in detergent is a complex interaction between GPI-PLD aggregation state and interfacial properties of the substrate particle, as well as dilution of substrate in the interface. In addition to the nonspecific "inhibition" GPI-PLD activity by the phospholipid bilayer, it has also been found that some lipids have a more specific inhibitory effect which is not reversed by detergents. Thus phosphatidic acid, lysophosphatidic acid and lipid A are all capable of inhibiting GPI-PLD activity in the 0.5-2 μM concentration range. Although the mechanism of this effect is unknown, it is unlikely that it is due to alteration in substrate presentation or to the surface characteristics of the substrate particle, because the effect is seen when the mole fraction of inhibitor:detergent in the assay is <0.01.[93] The observation that phosphatidic acid also interacts with integrin IIb 3 complexes and thereby increases their ability to bind fibrinogen raises the intriguing possibility that the β-propeller domain might contain a binding site for this lipid in both GPI-PLD and integrin α-subunits.[94]

Does GPI-PLD Release GPI-Anchored Proteins from Cells In Vivo?

Low molecular weight, hydrophilic forms of alkaline phosphatase are often found in serum, and it has been suggested that the GPI anchor-degrading enzyme might be responsible for their release from tissues in vivo.[54-57] Although the amount of liver alkaline phosphatase increases in serum of patients with cholestasis and in experimental animals following bile duct ligation, the mechanism of this effect is uncertain. The alkaline phosphatase is present

in the circulation as a complex mixture of both hydrophilic (dimers with anchor removed) and hydrophobic (aggregates and membrane fragments) species. However, the hydrophobic forms appear to be stable and resistant to the action of the GPI-PLD in serum. It has therefore been proposed that GPI-PLD only acts for a brief period of time on alkaline phosphatase aggregates released from the hepatocyte membrane by bile salts leaking into the liver sinusoids.[95] As soon as the alkaline phosphatase aggregates enter the circulation, the bile salts become too dilute to allow further GPI-PLD action. GPI-PLD-mediated anchor degradation would be accelerated in pathological situations (e.g., cholestasis) which increase bile salt concentration in liver sinusoids. Recent studies with glucocorticoid-treated dogs indicate that the increased concentration of bile acids in the liver, resulting from the normal enterohepatic circulation, could also be sufficient to produce a transient activation GPI-PLD.[96,97]

In addition to alkaline phosphatase, several other GPI-anchored proteins have been identified in plasma or other body fluids, or are released from cells into the culture media (summarized in Table 9.3). However, direct evidence that anchor degradation by GPI-PLD is responsible for these observations has proven surprisingly difficult to obtain. In this regard it should be emphasized that for most, if not all of these proteins, there is no compelling physiological reason why they should be converted to a soluble form. In the absence of any well characterized physiological processes that require phospholipase-mediated GPI degradation, studies in this area have so far been confined to identifying the mechanism of protein release from cells, without any idea as to what its ultimate purpose might be (see chapter 1).

Progress in this area has also been hampered by a number of technical problems. First, there are several mechanisms beside GPI degradation which can cause release of GPI-anchored proteins from cells. The ability of GPI-anchored proteins to be enriched in small membrane vesicles or to form soluble, but hydrophobic, oligomeric aggregates can be mistaken for GPI degradation unless the released protein is analyzed (i.e., by Triton X-114 phase separation) to show that it no longer has a hydrophobic anchor.[92,95,98] This analysis is itself problematic because the conditions of the phase separation will activate any contaminating GPI-PLD (particularly in a serum sample) giving a completely erroneous result. Proteolysis adjacent to the GPI anchor or alternative splicing could also cause release of hydrophilic proteins from cells and it is necessary to eliminate them by a positive ^{3}H-ethanolamine labeling result (see below). An interesting example of this type of problem was the proposal that a hydrophilic form of intestinal alkaline phosphatase found in the circulation might have arisen by alternative splicing. Initially, the identification of two distinct cDNAs without discernible C-terminal GPI addition signals supported this possibility. However, it was subsequently shown that this cDNA directed the synthesis of GPI-anchored forms of alkaline phosphatase due to the presence of a cryptic GPI addition signal.[99]

The second problem is that release of GPI-anchored proteins from cells is generally slow (i.e., hours to days) and constitutive, rather than an acutely regulated process. Slow release of a protein increases the likelihood of secondary degradation by a protease, cyclic phosphodiesterase or endoglycosidase which would obscure the location of the original cleavage event. One notable exception is the release of ADP ribosyltransferase from cytotoxic T cells, which can be stimulated acutely (i.e., 30-180 min) by activation of protein kinase C with PMA, crosslinking with anti-CD3 or incubation with IL-2.[100] However, other agents which stimulate release of GPI-anchored proteins from cells perturb membrane structure also.[67,92,95] Conversely, attempts to block release are restricted by the lack of specific GPI-PLD inhibitors. 1,10-phenanthroline is an effective inhibitor of serum GPI-PLD and blocks release of GPI-anchored proteins in several systems.[74,75,80,100] However, this

Table 9.3 GPI-PLD mediated release of proteins from cells and tissues

Protein	Source	Evidence in support of GPI-PLD mediated cleavage	Ref.
Thy-1	human cerebro-spinal fluid	chromoatographic behavior of C-terminal glycopeptide	103
Alkaline phosphatase	canine serum	inositol analysis; no CRD activity, electrophoretic mobility	96
CD59	human urine	structure of C-terminal glycopeptide	104,105
DAF	HeLa cells	^{3}H-ethanolamine labeling; no CRD reactivity; 1,10-phenanthroline inhibition	75
NCAM	murine myoblast cells	^{3}H-inositol labeled product released by nitrous acid deamination	106
Axonin-1	chicken dorsal root ganglion cells	^{3}H-ehtanolamine/^{3}H-inositol labeling; no CRD reactivity; 1,10-phenanthroline inhibition	80
Heparan sulfate proteoglycan	human bone marrow cells	^{3}H-ethanolamine labeling; no CRD reactivity; 1,10-phenanthroline inhibition	74
ADP ribosyl-transferase	murine cytotoxic T-cells	1,10-phenanthroline inhibition	100

transition metal chelator would also be capable of blocking other enzymes that might be responsible for release (e.g., metalloproteases).

The final problem is the poor sensitivity, specificity and the nonquantitative nature of the analytical techniques used to determine the site of GPI anchor cleavage. Labeling cells with ^{3}H-GPI precursors is commonly used to demonstrate that a GPI-anchored protein was not released by proteolysis. ^{3}H-inositol is the most suitable label because it is located immediately distal to the presumed site of cleavage. However, ^{3}H-ethanolamine is often used instead, because incorporation of ^{3}H-inositol into GPI precursors is relatively poor. Prolonged labeling periods increase the likelihood that secondary metabolism of the ^{3}H-labeled precursors will give misleading results. Another technique that has been widely used is immunoblotting with the CRD antibody.[74,75,80,96] The CRD antibody reacts with epitopes present in many GPIs (primarily inositol 1,2 cyclic phosphate) that are exposed by phospholipase C cleavage. However, this is essentially a negative result which only serves to eliminate release mediated by cellular GPI-PLC. By itself, the CRD technique cannot distinguish between release mechanisms as diverse as proteolysis, vesiculation or GPI-PLD-mediated degradation, all of which give a negative result.

In spite of the potential problems indicated above, the data summarized in Table 9.3 provide reasonable evidence that GPI-anchored proteins are released from cells by an endogenous phospholipase D. The subcellular location where GPI cleavage takes place is not known. GPI-anchored proteins are released from cells following transfection with GPI-PLD (native or a GPI-anchored chimera of GPI-PLD), but GPI-PLD secreted from transfected cells was unable to release proteins when added to control cells.[82,101,102] This data suggests that cell-associated GPI-PLD is more effective than soluble GPI-PLD, but there is no evidence to indicate that cell association "activates" GPI-PLD directly. Release could be

due to a concentrating effect resulting from colocalization of GPI-PLD and its substrate in the secretory pathway, in some other intracellular compartment or even at the cell surface. By contrast, several studies of release by endogenous GPI-PLD suggest that GPI degradation does not occur at an intracellular location because it is not blocked by Brefeldin A or monensin.[74,75,100] Furthermore, the ability of a cell impermeant analog of 1,10-phenanthroline (bathophenanthroline disulfonic acid) to inhibit PMA-stimulated ADP ribosyltransferase release suggests that in T lymphocytes GPI anchor degradation does in fact occur on the cell surface.[100]

One criticism of GPI-PLD-mediated anchor degradation as a physiologically relevant mechanism for releasing proteins from cells is that it would be relatively nonspecific. However, there is some data which suggests that release might be selective for particular GPI-anchored proteins. Whereas ADP ribosyltransferase and Thy-1 or several unidentifed ^{3}H-inositol-labeled proteins are all released from cytotoxic T cells by PI-PLC treatment, only ADP ribosyltransferase is released following stimulation with PMA.[100] Axonin-1 is released from cultured chick dorsal root ganglion neurons or from transfected myeloma cells to a much greater extent than the closely related GPI-anchored protein F11. A comparison of the secretion exhibited by chimeric axonin-1/F11 or axonin-1 deletion mutants indicate that the fourth fibronectin type III-like domain of axonin-1 is required for high levels of release.[80] Antibodies specifically directed against the fourth fibronectin type III-like domain also blocked axonin-1 release. This selectivity suggests that the GPI-PLD-mediated release of particular proteins can be regulated independently, although the mechanism remains uncertain.

Summary

A feature common to most GPI-anchored proteins and other biomolecules is their susceptibility to degradation by specific phospholipases that cleave the phosphodiester bond between the lipid and the inositol glycan moieties. Although these enzymes are absolutely specific for the site of cleavage, they are relatively insensitive to the precise structure of the GPI. A phospholipase C (PI-PLC) capable of cleaving phosphatidylinositol as well as GPIs is secreted by several different bacteria and is widely used as a means of identification and experimental manipulation of GPI-anchored biomolecules. However, bacteria do not synthesize GPI and the physiological role of PI-PLC is unknown. Phospholipases C and D capable of degrading GPIs are also produced by eukaryotes, and it has been proposed that these enzymes might play a role in the physiological release of GPI-anchored proteins from cell surfaces.

References

1. Lundin SJ. A bacterial factor capable of solubilizing cholinesterase from plaice body muscle. Acta Chem Scand 1968; 22:2519-2528.
2. Low MG, Finean JB. The action of phosphatidylinositol-specific phospholipases C on membranes. Biochem J 1976; 154:203-208.
3. Jäger K, Stieger S, Brodbeck U. Cholinesterase solubilizing factor from *Cytophaga* sp. is a phosphatidyl-inositol-specific phospholipase C. Biochim Biophys Acta 1991; 1074:45-51.
4. Griffith OH, Volwerk JJ, Kuppe A. Phosphatidylinositol-specific phospholipases C from *Bacillus cereus* and *Bacillus thuringiensis*. Methods Enzymol 1991; 197:493-502.
5. Titball RW. Bacterial phospholipases C. Microbiol Rev 1993; 57:347-66.
6. Songer JG. Bacterial phospholipases and their role in virulence. Trends Microbiol 1997; 5:156-161.
7. Heinz DW, Ryan M, Bullock TL et al. Crystal structure of the phosphatidylinositol-specific phospholipase C from *Bacillus cereus* in complex with myo-inositol. EMBO J 1995; 14:3855-3863.

8. Heinz DW, Ryan M, Smith MP et al. Crystal structure of phosphatidylinositol-specific phospholipase C from *Bacillus cereus* in complex with glucosaminyl(α-1-6)-D-myo-inositol, an essential fragment of GPI anchors. Biochemistry 1996; 35:9496-9504.
9. Gassler CS, Ryan M, Liu T et al. Probing the roles of active site residues in phosphatidylinositol-specific phospholipase C from *Bacillus cereus* by site-directed mutagenesis. Biochemistry 1997; 36:12802-12813.
10. Lewis KA, Garigapati VR, Zhou C et al. Substrate requirements of bacterial phosphatidylinositol-specific phospholipase C. Biochemistry 1993; 32:8836-8841.
11. Volwerk JJ, Shashidhar MS, Kuppe A et al. Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* combines intrinsic phosphotransferase and cyclic phosphodiesterase activities: A 31P NMR study. Biochemistry 1990; 29:8056-8062.
12. Wu Y, Roberts MF. Phosphatidylinositol-specific phospholipase C cyclic phosphodiesterase activity depends on solvent polarity. Biochemistry 1997; 36:8514-8521.
13. Roberts WL, Myher JJ, Kuksis A. et al. Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase. Palmitoylation of inositol results in resistance to phosphatidylinositol-specific phospholipase C. J Biol Chem 1988; 263:18766-18775.
14. Ferguson MA, Low MG, Cross GA. Glycosyl-sn-1,2-dimyristyl-phosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. J Biol Chem 1985; 260:14547-14555.
15. Bulow R, Overath P. Purification and characterization of the membrane-form variant surface glycoprotein hydrolase of *Trypanosoma brucei*. J Biol Chem 1986; 261:11918-11923.
16. Fox JA, Duszenko M, Ferguson MA et al. Purification and characterization of a novel glycan-phosphatidylinositol-specific phospholipase C from *Trypanosoma brucei*. J Biol Chem 1986; 261:15767-15771.
17. Hereld D, Krakow JL, Bangs JD et al. A phospholipase C from *Trypanosoma brucei* which selectively cleaves the glycolipid on the variant surface glycoprotein. J Biol Chem 1986; 261:13813-13819.
18. Cardoso de Almeida ML, Turner MJ. The membrane form of the vatiant surface glycoproteins of *Trypanosoma brucei*. Nature 1983; 302:349-352.
19. Mensa-Wilmot K, LeBowitz JH, Chang KP et al. A glycosyl-phosphatidylinositol (GPI)-negative phenotype produced in *Leishmania* major by GPI phospholipase C from *Trypanosoma brucei*: Topography of two GPI pathways. J Cell Biol 1994; 124:935-47.
20. Webb H, Carnall N, Carrington M. The role of GPI-PLC in *Trypanosoma brucei*. Braz J Med Biol Res 1994; 27:349-356.
21. Webb H, Carnall N, Vanhamme L et al. The GPI-phospholipase C of *Trypanosoma brucei* is nonessential but influences parasitemia in mice. J Cell Biol 1997; 139:103-114.
22. Carrington M, Walters D, Webb H. The biology of the glycosylphosphatidyl-inositol-specific phospholipase C of *Trypanosoma brucei*. Cell Biol Int Rep 1991 15:1101-1114.
23. Butikofer P, Boschung M, Brodbeck U et al. Phosphatidylinositol hydrolysis by *Trypanosoma brucei* glycosylphosphatidylinositol phospholipase C. J Biol Chem 1996; 271:15533-15541.
24. Stieger S, Brodbeck U. Glycosyl-phosphatidylinositol anchored acetyl-cholinesterase as substrate for phosphatidylinositol-specific phospholipase C from *B. cereus*. Biochimie 73; 1991:1179-1186.
25. Butikofer P, Brodbeck U. Partial purification and characterization of a (glycosyl) inositol phospholipid-specific phospholipase C from peanut. J Biol Chem 1993; 268:17794-17802.
26. Mensa-Wilmot K, Englund PT. Glycosyl phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*: Expression in Escherichia coli. Mol Biochem Parasitol 1992; 56:311-321.
27. Stieger S, Diem S, Jakob A et al. Enzymatic properties of phosphatidylinositol-glycan-specific phospholipase C from rat liver and phosphatidylinositol-glycan-specific phospholipase D from rat serum. Eur J Biochem 1991; 197:67-73.
28. Hoener MC, Stieger S, Brodbeck U. Isolation and characterization of a phosphatidylinositol-glycan-anchor-specific phospholipase D from bovine brain. Eur J Biochem. 1990; 190:593-601.

29. Mensa-Wilmot K, Hereld D, Englund PT. Genomic organization, chromosomal localization, and developmentally regulated expression of the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*. Mol Cell Biol 1990; 10:720-726.
30. Bulow R, Nonnengasser C, Overath P. Release of the variant surface glycoprotein during differentiation of bloodstream to procyclic forms of *Trypanosoma brucei*. Mol Biochem Parasitol 1989; 32:85-92.
31. Rolin S, Hanocq-Quertier J, Paturiaux-Hanocq F et al. Simultaneous but independent activation of adenylate cyclase and glycosylphosphatidyl-inositol-phospholipase C under stress condition in *Trypanosoma brucei*. J Biol Chem 1996; 271:10844-10852.
32. Bulow R, Griffith G, Webster P et al. Intracellular localization of the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*. J Cell Sci 1989; 93:233-240.
33. Hereld D, Hart GW, Englund PT. cDNA encoding the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*. Proc Natl Acad Sci USA 1988; 85:8914-8918.
34. Ghandi AJ, Percussia B, Goldfine H. *Listeria monocytogenes* phosphatidylinositol (PI)-specific phopsholipase C has low activity on glycosyl-PI-anchored proteins. J Bacteriol 1993; 175:8014-8017.
35. Gelb MH, Jain MK, Hanel AM et al. Interfacial enzymology of glycerolipid hydrolases: Lessons from secreted phospholipases A2. Annu Rev Biochem 1995; 64:653-88.
36. Morris JC, Ping-Sheng L, Zhai HX et al. Phosphatidylinositol phospholipase C is activated allosterically by the aminoglycoside G418. 2-deoxy-2-fluoro-scyllo-inositol-1-O-dodecylphosphonate and its analogs inhibit glycosylphosphatidylinositol phospholipase C. J Biol Chem 1996; 271:15468-15477.
37. Morris JC, Mensa-Wilmot K. Role of 2,6-dideoxy-2,6-diaminoglucose in activation of a eukaryotic phospholipase C by aminoglycoside antibiotics. J Biol Chem 1997; 272:29554-29559.
38. Takos AM, Dry IB, Soole KL. Detection of glycosyl-phosphatidylinositol-anchored proteins on the surface of *Nicotiana tabacum* protoplasts. FEBS Lett 405 1997; 1-4.
39. Fox JA, Soliz NM, Saltiel AR. Purification of a phosphatidylinositol-glycan-specific phospholipase C from liver plasma membranes: A possible target of insulin action. Proc Natl Acad Sci USA 1987; 84:2663-2667.
40. Fouchier F, Baltz T, Rougon G. Identification of glycosylphosphatidyl-inositol-specific phospholipases C in mouse brain membranes. Biochem J 1990; 269:321-327.
41. Heller M, Bieri S, Brodbeck U. A novel form of glycosylphosphatidyl-inositol-anchor converting activity with a specificity of a phospholipase D in mammalian liver membranes. Biochim Biophys Acta 1992; 1109:109-116.
42. Saltiel A. Structural and functional roles of glycosylphosphoinositides. Subcell Biochem. 1996; 26:165-185.
43. Varela-Nieto I, Leon Y, Caro HN. Cell signalling by inositol phosphoglycans from different species. Comp Biochem Physiol B Biochem Mol Biol 1996; 115:223-241.
44. Muller G, Geisen K. Characterization of the molecular mode of action of the sulfonylurea, glimepiride, at adipocytes. Horm Metab Res 1996; 28:469-487.
45. Stralfors P. Insulin second messengers. Bioessays 1997; 19:327-335.
46. Field MC. Is there evidence for phospho-oligosaccharides as insulin mediators? Glycobiology 1997; 7:161-168.
47. Muller G, Dearey EA, Punter J. The sulphonylurea drug, glimepiride, stimulates release of glycosylphosphatidylinositol-anchored plasma-membrane proteins from 3T3 adipocytes. Biochem J 1993; 289: 509-521.
48. Movahedi S, Hooper NM. Insulin stimulates the release of the glycosyl phosphatidylinositol-anchored membrane dipeptidase from 3T3-L1 adipocytes through the action of a phospholipase C. Biochem J 1997; 326:531-537.
49. Muller G, Dearey EA, Korndorfer A et al. Stimulation of a glycosyl-phosphatidylinositol-specific phospholipase by insulin and the sulfonylurea, glimepiride, in rat adipocytes depends on increased glucose transport. J Cell Biol 1994; 126:1267-76.

50. Nazih-Sanderson F, Pinchon G, Nion S et al. HDL3-signalling in HepG2 cells involves glycosyl-phosphatidylinositol-anchored proteins. Biochim Biophys Acta 1997; 1346:45-60.
51. Roberts JM, Kenton P, Johnson PM. Growth factor-induced release of a glycosyl-phosphatidylinositol (GPI)-linked protein from the HEp-2 human carcinoma cell line. FEBS Lett 1990; 267:213-216.
52. Jacquemin C. Glycosyl phosphatidylinositol in thyroid: Cell signalling or protein anchor? Biochimie 1991; 73: 37-40.
53. Low MG, Zilversmit DB. Role of phosphatidylinositol in attachment of alkaline phosphatase to membranes. Biochemistry 1980; 19:3913-3918.
54. Low MG, Weglicki WB. Release of membrane-bound alkaline phosphatase by activation of cardiac or renal phosphatidylinositol-specific phospholipase C. Fed Proc 1981; 40:826.
55. Kominami T, Miki A, Ikehara Y. Electrophoretic characterization of hepatic alkaline phosphatase released by phosphatidylinositol-specific phospholipase C. Biochem J 1985; 227:183-189.
56. Miki A, Kominami T, Ikehara Y. pH-dependent conversion of liver-membranous alkaline phosphatase to a serum-soluble form by n-butanol extraction. Biochem Biophys Res Commun 1985; 126:89-95.
57. Malik AS, Low MG. Conversion of human placental alkaline phosphatase from a high M_r form to a low M_r during butanol extraction: An investigation of the role of endogenous phosphoinositide-specific phospholipases. Biochem J 1986; 240:519-527.
58. Low MG, Prasad ARS. A phospholipase D specific for the phosphatidylinositol anchor of cell surface proteins is abundant in plasma. Proc Natl Acad Sci USA 1988; 85:980-984.
59. Low MG. Anchoring of membrane proteins by glycosyl-phosphatidylinositol. In: Op den Kamp JAF, ed. NATO ASI Series, Vol. H16 Membrane Biogenesis. Berlin Heidelberg: Springer-Verlag, 1988:219-233.
60. Davitz MA, Hereld D, Shak S et al. A glycan-phosphatidylinositol-specific phospholipase D in human serum. Science 1987; 238:81-84.
61. Cardoso de Almeida ML, Turner MJ, Stambuk BB et al. Identification of an acid-lipase in human serum which is capable of solubilizing glycosylphosphatidylinositol-anchored proteins. Biochem Biophys Res Commun 1988; 150:476-482.
62. Xie M, Sesko AM, Low MG. Glycosylphosphatidylinositol-specific phospholipase D is localized in keratinocytes. Am J Physiol Cell Physiol 1993; 265:C1156-C1168.
63. Heller M, Bieri S, Brodbeck U. A novel form of glycosylphosphatidyl-inositol-anchor converting activity with a specificity of a phospholipase D in mammalian liver membranes. Biochim Biophys Acta Bio-Membr 1992; 1109:109-116.
64. Stinson RA, Hamilton BA. Human liver plasma membranes contain an enzyme activity that removes membrane anchor from alkaline phosphatase and converts it to a plasma-like form. Clin Biochemistry 1994; 27:49-55.
65. Hari T, Kunze H, Bohn E et al. Subcellular distribution at glycosylphosphatidyl-inositol-specific phospholipase D in rat liver. Biochem J 1996; 320:315-319.
66. Hoener MC, Brodbeck U. Phosphatidylinositol-glycan-specific phospholipase D is an amphiphilic glycoprotein that in serum is associated with high-density lipoproteins. Eur J Biochem 1992; 206:747-757.
67. Bergman A-S, Carlsson SR. Saponin-induced release of cell-surface-anchored Thy-1 by serum glycosylphosphatidylinositol-specific phospholipase D. Biochem J 1994; 298:661-668.
68. Davitz MA, Hom J, Schenkman S. Purification of a glycosyl-phosphatidylinositol- specific phospholipase D from human plasma. J Biol Chem 1989; 264:13760-13764.
69. Huang K-S, Li S, Fung W-JC et al. Purification and characterization of glycosyl-phosphatidylinositol-specific phospholipase D. J Biol Chem 1990; 265:17738-17745.
70. Metz CN, Thomas P, Davitz MA. Immunolocalization of a glycosylphosphatidyl-inositol-specific phospholipase D in mast cells found in normal tissue and neurofibromatosis lesions. Am J Pathol 1992; 140:1275-1281.
71. Metz CN, Zhang Y, Guo Y et al. Production of the glycosylphosphatidyl-inositol-specific phospholipase D by the islets of Langerhans. J Biol Chem 1991; 266:17733-17736.

72. Xie M, Low MG. Expression and secretion of glycosylphosphatidylinositol-specific phospholipase D by myeloid cells. Biochem J 1994; 297:547-554.
73. Hari T, Bütikofer P, Wiesmann UN et al. Uptake and intracellular stability of glycosylphosphatidylinositol-specific phospholipase D in neuroblastoma cells. Biochim Biophys Acta Mol Cell Res 1997; 1355:293-302.
74. Brunner G, Metz CN, Nguyen H et al. An endogenous glycosylphosphatidylinositol-specific phospholipase D releases basic fibroblast growth factor-heparan sulfate proteoglycan complexes from human bone marrow cultures. Blood 1994; 83:2115-2125.
75. Metz CN, Brunner G, Choi-Muira NH et al. Release of GPI-anchored membrane proteins by a cell-associated GPI-specific phospholipase D. EMBO J 1994; 13:1741-1751.
76. Stadelmann B, Zurbriggen A, Brodbeck U. Distribution of glycosylphosphatidyl-inositol-specific phospholipase D mRNA in bovine tissue sections. Cell Tissue Res 1993; 274:547-552.
77. Deeg MA, Verchere CB. Regulation of glycosylphosphatidylinositol-specific phospholipase D secretion from TC3 cells. Endocrinology 1997; 138:819-826.
78. Raymond FD, Fortunato G, Moss DW et al. Inositol-specific phospholipase D activity in health and disease. Clin Sci 1994; 86:447-451.
79. Maguire GA, Gossner A. Glycosyl phosphatidylinositol phospholipase D activity in human serum. Ann.Clin.Biochem. 1995; 32:74-78.
80. Lierheimer R, Kunz B, Vogt L et al. The neuronal cell-adhesion molecule axonin-1 is specifically released by an endogenous glycosylphosphatidylinositol-specific phospholipase. Eur J Biochem 1997; 243:502-510.
81. Huang K-S, Li S, Low MG. Glycosylphosphatidylinositol-specific phospholipase D. Meth Enzymol 1991; 197:567-575.
82. Scallon B, Fung W-J, Tsang C et al. Primary structure and functional activity of a phosphatidylinositol-glycan specific phospholipase D. Science 1991; 252:446-448.
83. Li J-Y, Hollfelder K, Huang K-S et al. Structural features of GPI-specific phospholipase D revealed by proteolytic fragmentation and Ca^{2+} binding studies. J Biol Chem 1994; 269:28963-28971.
84. Deeg MA. GPI-specific phospholipase D as an apolipoprotein. Brazilian J Med Biol Res 1994; 27:375-381.
85. Hoener MC, Bolli R, Brodbeck U. Glycosyl-phosphatidylinositol-specific phospholipase D: Interaction with and stimulation by apolipoprotein A-I. FEBS Lett 1993; 327:203-206.
86. Heller M, Bütikofer P, Brodbeck U. Generation by limited proteolysis of a catalytically active 39 kDa protein from the 115-kDa form of phosphatidylinositol-glycan-specific phospholipase D from bovine serum. Eur J Biochem 1994; 224:823-833.
87. Tuckwell DS, Brass A, Humphries MJ. Homology modelling of integrin EF-hands. Evidence for widespread use of a conserved cation-binding site. Biochem J 1992; 285:325-331.
88. Tuckwell DS, Humphries MJ, Brass A. A secondary structure model of the integrin α-subunit N-terminal domain based on analysis of multiple alignments. Cell Adhesion and Communication 1994; 2:385-402.
89. Springer TA. Folding of the N-terminal, ligand-binding region of integrin α-subunits into a β-propeller domain. Proc Natl Acad Sci USA 1997; 94:65-72.
90. Stadelmann B, Bütikofer P, König A et al. The C-terminus of glycosylphosphatidylinositol-specific phospholipase D is essential for biological activity. Biochim Biophys Acta Mol Cell Res 1997; 1355:107-113.
91. Low MG, Huang K-S. Factors affecting the ability of glycosylphosphatidyl-inositol-specific phospholipase D to degrade the membrane anchors of cell surface proteins. Biochem J 1991; 279:483-493.
92. Xie M, Low MG. Streptolysin-O induces release of glycosylphosphatidyl-inositol-anchored alkaline phosphatase from ROS cells by vesiculation independently of phospholipase action. Biochem J 1995; 305:529-537.

93. Low MG, Huang K-S. Phosphatidic acid, lysophosphatidic acid, and lipid A are inhibitors of glycosylphosphatidylinositol-specific phospholipase D. Specific inhibition of a phospholipase by product analogues. J Biol Chem 1993; 268:8480-8490.
94. Smyth S, Hillery CA, Parise LV. Fibrinogen binding to purified platelet glycoprotein IIb-IIIa (Integrin $_{\text{IIb 3}}$) is modulated by lipids. J Biol Chem 1992; 267:15568-15577.
95. Deng JT, Hoylaerts MF, De Broe ME et al. Hydrolysis of membrane-bound liver alkaline phosphatase by GPI-PLD requires bile salts. Am J Physiol Gastrointest Liver Physiol 1996; 271:G655-G663.
96. Solter PF, Hoffmann WE. Canine corticosteroid-induced alkaline phosphatase in serum was solubilized by phospholipase activity in vivo. Am J Physiol Gastrointest Liver Physiol 1995; 269:G278-G286.
97. Solter PF, Hoffmann WE, Chambers MD et al. CCK-8 infusion increases plasma LMW alkaline phosphatase coincident with enterohepatic circulation of bile acids. Am J Physiol Gastrointest Liver Physiol 1997; 273:G381-G388.
98. Küng M, Stadelmann B, Brodbeck U et al. Addition of G418 and other aminoglycoside antibiotics to mammalian cells results in the release of GPI-anchored proteins. FEBS Lett 1997; 409:333-338.
99. Engle MJ, Mahmood A, Alpers DH. Two rat intestinal alkaline phosphatase isoforms with different carboxyl-terminal peptides are both membrane-bound by a glycan phosphatidylinositol linkage. J Biol Chem 1995; 270:11935-11940.
100. Nemoto E, Stohlman S, Dennert G. Release of a glycosylphosphatidylinositol-anchored ADP-ribosyltransferase from cytotoxic T cells upon activation. J Immunol 1996; 156:85-92.
101. Scallon BJ, Kado-Fong H, Nettleton MY et al. Novel strategy for secreting proteins: Use of phosphatidylinositol-glycan-specific phospholipase D to release chimeric phosphatidylinositol-glycan anchored proteins. Biotechnology 1992; 10:550-556.
102. Küng M, Bütikofer P, Brodbeck U et al. Expression of intracellular and GPI-anchored forms of GPI-specific phospholipase D in COS-1 cells. Biochim Biophys Acta Mol Cell Res 1997; 1357:329-338.
103. Almqvist P, Carlsson SR. Characterization of a hydrophilic form of Thy-1 purified from human cerebrospinal fluid. J Biol Chem 1988; 263:12709-12715.
104. Nakano Y, Noda K, Endo T et al. Structural study on the glycosyl-phosphatidylinositol anchor and the asparagine-linked sugar chain of a soluble form of CD59 in human urine. Arch Biochem Biophys 1994; 311:117-126.
105. Meri S, Lehto T, Sutton CW et al. Structural composition and functional characterization of soluble CD59: Heterogeneity of the oligosaccharide and glycophosphoinositol (GPI) anchor revealed by laser-desorption mass spectrometric analysis. Biochem J 1996; 316:923-935.
106. Mukasa R, Umeda M, Endo T et al. Characterization of glycosylphosphatidylinositol (GPI)-anchored NCAM on mouse skeletal muscle cell line C2C12: The structure of the GPI glycan and release during myogenesis. Arch Biochem Biophys 1995; 318:182-190.

Chapter 10

Intercellular Transfer of GPI-Anchored Molecules

Isabelle A. Rooney

The proteins which are attached to cells by a glycosylphosphatidylinositol (GPI) anchor are structurally and functionally diverse.[1-3] Included in this group are cell surface enzymes, adhesion molecules, parasite antigens, complement regulatory proteins, lymphocyte antigens and others. In common, these proteins have at their C terminus a GPI moiety which is attached in the rough endoplasmic reticulum as an early posttranslational event, after cleavage of a C-terminal signal sequence from the nascent protein.[1,4,5] The GPI moiety is inserted into the outer leaflet of the cell's membrane bilayer, while the entire protein component of the molecule is extracellular.

Possible Functions of the GPI Anchor

The biological advantage of the GPI anchor is poorly understood, and the diverse functions of the proteins which utilize it complicate the issue. On epithelial and endothelial cells, most anchored proteins are expressed preferentially at the apical surface of the cell.[6,7] This location suggests a role for the anchor at tissue interfaces. A number of authors have presented evidence that GPI-anchored proteins are associated with tyrosine kinases and that the anchor may be involved in cell signaling following ligand binding of the extracellular protein moiety.[8-11] This issue, although still controversial, continues to be the focus of intense research. Alternatively, the anchor's value may relate to the potential for removal and export of anchored proteins from the cell surface. The anchor can be cleaved by specific phospholipase enzymes (PI-PLC and PI-PLD) of both eukaryotic and prokaryotic origin.[1] Further, vesiculating cells release a higher percentage of GPI-anchored proteins than of other cell surface proteins.[12,13] In some cases the mechanisms which facilitate removal of GPI-anchored proteins from cells confer a clear biologic advantage. Tumor cells and parasitic trypanosomes have been observed to release GPI-anchored proteins,[14-18] and this may be a mechanism by which they evade the immune response.

Export of functional GPI-anchored proteins from cells, either in soluble, anchor-negative form after phospholipase cleavage, or on the surface of vesicles, may also benefit the organism. Complement regulators, exported from vesiculating cells at sites of inflammation, may inhibit complement activation in the fluid phase, thus reducing the level of complement attack on cell surfaces; digestive enzymes, exported from intestinal cells, might increase the amount of food which these cells can process.

In recent years evidence has accumulated which supports the hypothesis that, in some situations, GPI-anchored proteins transfer between cell membranes in vivo either during direct membrane-membrane contact or via a fluid phase, micellar intermediate. The implications of this phenomenon both as a biologic principle, and a tool for experimentation

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

and therapy are considerable. This chapter attempts to summarize current knowledge regarding this issue.

Evidence that GPI-Anchored Proteins can Transfer between Cell Membranes

In 1977, Bouma et al demonstrated transfer of the GPI-anchored enzyme acetylcholinesterase from erythrocytes to liposomes in vitro.[19] It was subsequently demonstrated that the complement inhibitory protein decay accelerating factor (DAF) can be purified from the membranes of detergent-solubilized erythrocytes with the anchor intact, and that this purified DAF can insert into cell membranes and function to protect the acceptor cells from attack by human complement.[20-21] Medof et al later demonstrated that purified DAF could incorporate into erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH, a condition in which erythrocytes fail to express GPI-anchored proteins including complement inhibitors and are therefore extremely sensitive to complement mediated damage) and that this incorporated DAF rendered the PNH cells more resistant to human complement.[22] At this time, however, there was no evidence to indicate that this phenomenon occurred in vivo.

In 1990, Pearce et al[23] reported that schistosomes isolated from host animals possess host DAF on their tegument, although they did not determine whether the parasites acquire this protein as a result of adherence of host cell membrane fragments, or by direct intermembrane transfer of DAF's GPI moiety. Rifkin and Landsberger[24] demonstrated that, during incubation with live trypanosomes, erythrocytes acquired copies of the GPI-linked trypanosome variant surface glycoprotein (VSG) in a time and temperature dependent manner, based on lysis of the erythrocytes in the presence of anti-VSG and complement. Erythrocytes acquired VSG not only during incubation with live trypanosomes, but also during incubation with high speed supernatants of medium in which trypanosomes had been incubated, establishing that cellular acquisition of VSG was independent of direct cell-cell contact. This report did not, however, demonstrate conclusively that the VSG inserted into erythrocytes via the GPI anchor and was not present on small trypanosome membrane fragments or in protein complexes which became adherent to the erythrocyte surface membrane.

Recent evidence suggests that GPI-anchored proteins may transfer between the membranes of mammalian cells in vivo and in vitro. Kooyiman et al,[25] by linking the coding sequences of two human GPI-linked complement regulatory proteins, CD59 and DAF, to the regulatory sequence of the globin gene, created transgenic mice which expressed these human proteins in an erythroid cell specific manner. Using immunoflourescent staining they showed that the endothelium of these animals possessed CD59 and DAF immunoreactivity, and that this immunoreactivity was lost during cell culture. Further, they demonstrated that hearts from these animals, when perfused with human plasma, showed significantly less complement-mediated endothelial damage than did hearts from control animals. These data indicate that the endothelium acquired copies of exogenous, functional CD59 and DAF from circulating erythrocytes, although the results might be explained by adherence of erythrocyte membrane fragments to the endothelial cells, and do not provide conclusive evidence of intercellular GPI transfer.

Dunn et al[26] created a "knockout" mouse negative for the PIG-A gene, which is necessary for GPI anchor synthesis, so that the "knockouts" failed to express all GPI-anchored proteins. Although the mutation was lethal and embryos did not develop beyond an early stage, the authors were able to generate GPI anchor-negative cell lines using this technique. GPI-anchor-negative cells also contained a neomycin resistance gene and a fluorescent marker to facilitate their separation from normal cells after coculture. Cell lines generated from

disaggregated PIG-A negative embryoid bodies did not form the hematopoietic colonies which are typically formed by cells from normal embryoid bodies. When the PIG-A negative cells were cocultured with cells from normal embryos for three to five days, however, they acquired the GPI-anchored protein CD24, and also gained the ability to form hematopoietic colonies, suggesting that acquisition of a GPI-anchored protein allowed these cells to function normally. In these experiments, PIG-A negative cells could not acquire GPI-anchored proteins from culture supernatant of normal cells, indicating that, in this system, direct cell-cell contact was necessary for protein acquisition by the acceptor cells. The mechanism by which PIG-A negative cells acquired CD24 from normal cells was not further investigated.

The above data are inconsistent with the evidence that in the disease paroxysmal nocturnal hemoglobinuria, populations of erythrocytes which are entirely GPI anchor negative coexist in the bloodstream for weeks or months with GPI anchor positive erythrocytes, but do not acquire copies of GPI-anchored proteins.[27] It is possible that the conditions of cell culture promote release of GPI-anchored proteins from erythroid cells, either on vesicles or membrane fragments or as single molecules.

Anderson et al[28] infected HeLa cells with an adenovirus carrying cDNA which coded for a fusion protein composed of the extracellular domain of the T cell antigen CD4, fused to a GPI anchor. These HeLa cells, designated JM88 cells, then expressed a GPI-anchored form of CD4. Uninfected HeLa cells incubated with JM88 cells acquired CD4-GPI in a time and temperature-dependent manner, as evidenced by immunofluorescence staining. Acquisition of CD4 immunoreactivity occurred within fifteen minutes and was prevented by formaldehyde fixation of the JM88 cells. Further, cells expressing the viral glycoprotein gp160, which binds CD4, induced syncytia formation of HeLa cells bearing acquired CD4-GPI, providing strong evidence that the CD4-GPI protein had indeed become an integral component of the cell membrane. Erythrocytes acquired CD4 immunoreactivity during incubation with culture supernatants from JM88 cells, demonstrating that in this system, unlike that described by Dunn et al,[26] direct cell-cell contact was not required for acquisition of GPI-anchored protein by the acceptor cells.

Mechanisms by which Cells Acquire Exogenous GPI-Anchored Proteins

Cells might acquire new GPI-anchored proteins in the following ways:

1. By adherence of donor cell membrane fragments or vesicles to the acceptor cell surface;
2. By fusion of donor vesicles with the outer leaflet of the acceptor cell's plasma membrane;
3. By direct transfer of the GPI moiety from donor cell (or vesicle) membrane to acceptor cell membrane during membrane-membrane contact;
4. By transfer of the GPI moiety from a proteolipid or micellar intermediate to the outer leaflet of the acceptor cell membrane.

All of the above mechanisms may operate in vitro and in vivo. In recent years several authors have focused on this question and evidence has accumulated that, at least in some systems, micellar or proteolipid intermediates play an important role in the phenomenon of intermembrane GPI-anchored protein transfer.

Until recently, only anchor negative forms of GPI-anchored proteins had been detected in body fluids. Anchor-intact GPI-anchored proteins, including the complement regulators CD59 and DAF, and the lymphocyte antigen CDw52, have now been shown to occur in amniotic fluid,[29] seminal plasma[30-32] and breast milk.[33-35] In seminal plasma and breast milk these proteins are in part associated with membranous particles. In seminal plasma,

GPI-linked proteins are carried on the membranes of "prostasomes",[31,32] extracellular vesicular organelles which arise from prostatic epithelium,[36] while in breast milk they have been identified on the membranes of milk fat globules,[37] membrane-bound lipid droplets secreted by mammary cells.[35]

The funtion of prostasomes is poorly understood, although some evidence exists that these organelles promote sperm motility[36] and may have an immunosuppressive function.[38] They have been shown to associate with the membranes of spermatozoa in vitro.[39] Rooney et al[32] investigated the interaction of prostasomes with cells using fluorescent dyes and videomicroscopy and demonstrated that prostasomes bind to, but do not fuse with, cell membranes, in a time and temperature dependent manner. Cells incubated with prostasomes acquired prostasome CD59 and prostasome membrane cofactor protein (a transmembrane protein) with comparable efficiency, indicating that cellular acquisition of CD59 was due to uptake of whole prostasomes, and not to intermembrane GPI transfer. Cells to which prostasomes bound showed increased resistance to lysis by human complement, demonstrating that proteins on vesicles which are adherent to cells can function as though expressed on the cell's plasma membrane. Adherence of cell-derived vesicles to acceptor cells likely explains some of the cellular acquisition of GPI-anchored proteins described in the reports cited here, and is an important caveat for researchers in this area.

Cellular acquisition of GPI-anchored proteins from seminal plasma was, however, only partly due to prostasomes. Using density gradient centrifugation, Rooney et al[32] identified a second, membrane-free form of GPI-anchored proteins in seminal plasma. This fraction had higher density than prostasomes and other membranes, as would be expected from complexes with a high protein, low lipid content. GPI-anchored proteins in this fraction partitioned into the detergent phase of solution of Triton X-114, demonstrating that they retained the hydrophobic anchor[40] and were able to incorporate into cell membranes.

The above finding suggested that GPI-anchored proteins may be protected by "carriers", such as lipid-protein complexes or lipoproteins, in some fluids. This possibility is supported by the work of Vakeva et al[41] who, using radiolabeled, purified CD59 demonstrated that this protein incorporated efficiently into high density lipoprotein (HDL) particles (but poorly into low density lipoproteins) of human serum. Further, they isolated HDL and LDL from human serum and demonstrated the presence of CD59 in association with HDL, but not LDL. HDL particles were able to incorporate 25-42% of labeled CD59 which had been pre-incorporated into rabbit erythrocytes, and to donate 7-14% of this acquired CD59 to new rabbit erythrocytes, indicating that these lipoprotein particles can serve as intermediates in the transfer of GPI-anchored proteins between cells.

Mechanisms by which Cells Release GPI-Anchored Proteins

The evidence presented above indicates that GPI-anchored proteins can be released intact from cells and, via the anchor, become incorporated into the membranes of other cells or associate with lipoprotein carriers. The mechanisms by which GPI-anchored proteins could be released from the cell surface are discussed below.

Spontaneous Transfer Between Cell Membranes: Effect of Collision

A small amount of transfer may occur between membranes spontaneously, and this may be increased under certain conditions. Yang and Huestis[42] studied transfer of phospholipids between membranes and demonstrated that, for hydrophobic lipids, percentage transfer is significantly increased when the density of the membrane particles allows frequent collision. Additionally, transfer is promoted at acidic pH. This may explain why PNH erythrocytes do not acquire significant amounts of GPI-anchored proteins from healthy cells in the bloodstream, but can be shown to acquire these proteins in vitro, in

culture conditions. In situations with high cell density, low fluid volume and relative stasis of fluid (e.g., inflammatory foci and body cavities), the amount of transfer which occurs may be sufficient to have a significant biological effect.

Effect of Stress on Cells

Cells may release increased amounts of GPI-anchored proteins from the outer leaflet of the cell membrane's lipid bilayer when under certain stress conditions, for example when the membrane deforms during the process of vesiculation. Further, GPI-anchored proteins are preferentially concentrated in released vesicles,[11-13] and it is possible that such vesicles release their GPI-anchored proteins more easily than do intact cell membranes. In support of this hypothesis, milk fat globules have been shown to be unstable, and to undergo further vesiculation after their release from cells.[43]

Lipid-Protein Intermediates: Effects of Fluid Characteristics

Intact GPI-anchored proteins are abundant in seminal plasma.[30-32] Concentrations of free CD59 in the high density, nonmembrane fraction of the fluid are at least 1 μg/ml (Rooney IA, unpublished data). These unusually high concentrations of free GPI-anchored proteins might be due to characteristics of the fluid itself, or to characteristics of the cells which contribute to it. GPI-anchored proteins in seminal plasma derive from several sources. CD59, which is widely expressed in human tissues,[44] may be released by several tissues; since this protein is present in vasectomy seminal plasma at approximately half the concentration of that seen in fertile seminal plasma, it must derive from organs both above (testis and epididymis) and below (prostate and seminal vesicles) the point of transection of the ductus deferens. CD55 is unlikely to derive from testis which expresses CD55 of lower molecular weight than that seen in seminal plasma,[45,46] and may derive from prostate, epididymis and seminal vesicles. CDw52, which is poorly expressed on prostasomes, although abundant in the nonmembrane fraction, and is absent from vasectomy seminal plasma,[32] must arise from above the point of transection of the ductus deferens, most likely from epididymis, where it is very strongly expressed.[47] Clearly, more than one cell type contributes to the nonmembrane fraction of GPI-anchored proteins in seminal plasma, suggesting that the high concentration of anchor-intact proteins in seminal plasma depends on characteristics of the fluid itself, rather than on characteristics of a specialized cell type.

GPI-anchored proteins are known to associate preferentially with membranes which are rich in cholesterol and sphingolipids.[6,48,49] Seminal plasma is rich in sphingolipids,[50] and prostasomes have an unusually high concentration of cholesterol in their membranes.[51] The presence of these lipids in seminal plasma may support retention of the GPI anchor on released proteins. The fact that this fluid represents the products of several glandular organs contained within a small volume may also be a contributing factor. It is likely that other body fluids contain GPI-anchored proteins in similar complexes to those seen in seminal plasma, albeit in lower concentration.

In summary, it is likely that transfer of GPI-anchored proteins between cell membranes occurs spontaneously at a rate which is low in most physiological situations, and that the rate of transfer is increased under certain conditions, including high cell density, low pH, high lipid content of intercellular fluid and the presence of lipids or lipoproteins with which GPI-anchored proteins preferentially associate. GPI-anchored proteins may be able to transfer between cell membranes with low efficiency and between membranes and lipoprotein "carriers" with higher efficiency. It has been noted that lipoproteins inhibit incorporation of purified GPI-anchored proteins into cell membranes, presumably because the GPI anchor associates preferentially with the lipoprotein.[41] Lipoprotein intermediates may "capture" released GPI-anchored proteins and function thereafter as a "slow-release"

reservoir of these proteins, from which copies of the proteins can incorporate into cell membranes.

Importance of Intercellular GPI-Anchored Protein Transfer

Acquisition of new GPI-anchored proteins by a cell can profoundly alter the characteristics of the cell's surface membrane, including antigenicity, susceptibility to immune attack, surface enzyme and receptor properties.

Infective organisms may benefit by acquisition of complement regulatory proteins and other immunosuppressive proteins, as has been reported to occur in schistosomiasis.[23] Rifkin and Landsberger[24] suggest that incorporation of trypanosome VSG protein into host erythrocytes may precipitate immune attack of these erythrocytes, thus contributing to the anemia associated with trypanosomiasis. Bacteria and viruses may also acquire immunoprotective proteins. It has recently been shown that HIV can incorporate purified CD59 and DAF into its membranous coat and that the acquired proteins render the virus more resistant to lysis by human complement.[52] This phenomenon may be of particular importance in seminal plasma, where the concentration of the GPI-anchored CD59 is high, and may facilitate sexual transmission of HIV.

Intercellular transfer of GPI-anchored proteins may also benefit self cells. Complement regulators in semen may replenish protein lost from spermatozoa during normal membrane turnover or complement attack in the female reproductive tract, and may therefore be important for fertility. GPI-anchored proteins released from damaged or dead cells may become available for incorporation into new cells, thus providing a mechanism by which important surface proteins can be recycled, reducing the demand for synthesis by cells which are already under stress. Vakeva et al[53] observed loss of CD59 from damaged myocardium after infarction, and described removal of CD59 from damaged cells in microparticles. They suggest that released CD59 may be incorporated into HDL particles, and thus become available for incorporation into myocardial cells which, although undamaged by the primary infarction event, are at risk of complement-mediated lysis in the post-infarction period. Thus, complement regulatory proteins released from dead cells at inflammatory sites could contribute to the immune defense of neighboring cells.

Clearly, the phenomenon of intercellular transfer of GPI-anchored proteins is both intriguing and of major biological consequence. Further research in this area will yield data important both in our understanding of the physiology of these proteins and for therapeutic advances.

References

1. Ferguson MAJ, Williams AF. Cell-surface anchoring of proteins via glycosylphosphatidylinositol structures. Ann Rev Biochem 1988; 57:285-320.
2. Low MG. The glycosylphosphatidylinositol anchor of membrane proteins. Biochim Biophys Acta 1989; 988:427-454.
3. Lublin DM. Glycosylphosphatidyl-inositol anchoring of membrane proteins. Curr Top Microbiol Immunol 1992; 178:141-162.
4. Tartakoff AM, Singh N. How to make a glycosylphosphatidyl-inositol anchor. Trends Biochem Sci 1992; 17:470-473.
5. Caras IW, Weddell GN. Signal peptide for protein secretion directing glycosylphospholipid membrane anchor attachment. Science 1989; 243:1196-1198.
6. Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 1992; 68:533-544.
7. Dotti CG, Parton RG, Simons K. Polarized sorting of glypiated proteins in hippocampal neurons. Nature 1991; 349:158-161.

8. Stefanova I, Horejsi V, Ansotegui LJ et al. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science 1991; 254:1016-1019.
9. van den Berg CW, Cinek T, Hallett MB et al. Exogenous glycosylphosphatidyl-inositol anchored CD59 associates with kinasesin clusters on U937 cells and becomes Ca signalling competent. J Cell Biol 1995; 131:669-677.
10. Brown DA. The tyrosine kinase connection: How GPI-anchored proteins activate T cells. Curr Opin Immunol 1993; 5:349-354.
11. Robinson PJ. Phosphatidyl-inositol membrane anchors and T cell activation. Immunol Today 1991; 12:35-41.
12. Hagelberg C Allen D. Restricted diffusion of integral membrane proteins and polyphosphoinositide leads to their depletion in microvesicles released from human erythrocytes. Biochem J 1990; 271:831-834.
13. Johnstone RM, Adam M, Hammond JR et al. Vesicle formation during reticulocyte maturation. J Biol Chem 1987; 261:15368
14. Bulow R Overath PJ. Purification and characterization of the membrane form variant surface glycoprotein hydrolase of *Trypanosoma brucei*. J Biol Chem 1986; 261:11918-11923.
15. Fox JA, Duszenko M, Ferguson MAJ et al. Purification and characterization of a novel glycan-phosphatidylinositol-specific phospholipase C from *Trypanosoma brucei*. J Biol Chem 1986; 261:15767-15771.
16. Hereld D, Krakow JL, Bangs JD et al. A phospholipase C from *Trypanosoma brucei* which selectively cleaves the glycolipid on the variant surface glycoprotein. J Biol Chem 1986; 261:13813-13819.
17. Kinugasa T, Kuroki M, Yamanaka T et al. Nonproteolytic release of carcinoembryonic antigen from normal human colonic epithelial cells cultured in collagen gel. Int J Cancer 1994; 58:102-107.
18. Miotti S, Alberti S, Facheris P et al. Membrane association and shedding of the GPI-anchored Ca-Mov18 antigen in human ovary carcinoma cells. Int J Cancer 1992; 51:499-505.
19. Bouma SR, Drislane FW, Huestis WH. Selective extraction of membrane-bound proteins by phospholipid vesicles. J Biol Chem 1977; 252:6759-6763.
20. Medof ME, Kinoshita T, Nussenzweig V. Inhibition of complement activation on the surface of cells after incorporation of decay accelerating factor (DAF) into their membranes. J Exp Med 1984; 160:1558-1578.
21. Medof ME, Walter I, Rutgers JL et al. Identification of the complement decay accelerating factor (DAF) on epithelium and glandular cells and in body fluids. J Exp Med 1987; 165:848-864.
22. Medof ME, Kinoshita T, Silber R et al. Amelioration of lytic abnormalities of paroxysmal nocturnal hemoglobinuria with decay-accelerating factor. Proc Natl Acad Sci USA 1985; 82:2980-2984.
23. Pearce EJ, Hall BF, Sher A. Host-specific evasion of the alternative complement pathway by schistosomes correlates with the presence of a phospholipase C-sensitive molecule resembling human decay accelerating factor. J Immunol 1990; 144:2751-2756.
24. Rifkin MR, Landsberger FR. Trypanosome variant surface glycoprotein transfer to target membranes: A model for the pathogenesis of trypanosomiasis. Proc Natl Acad Sci USA 1990; 87:801-805.
25. Kooyman DL, Byrne GW, McClellan S et al. In vivo transfer of GPI-linked complement restriction factors from erythrocytes to the endothelium. Science 1995; 269:89-91.
26. Dunn DE, Yu J, Nagarajan S et al. A knockout model of paroxysmal nocturnal hemoglobinuria: PIG-a- hematopoiesis is reconstituted following intercellular transfer of GPI-anchored proteins. Proc Natl Acad Sci USA 1996; 93:7938-7943.
27. Bessler M. Paroxysmal nocturnal hemoglobinuria: The price for a chance. Schweiz med Wochenschr 1996; 126:1912-1921.
28. Anderson SM, Yu G, Giattina M et al. Intercellular transfer of a glycosylphosphatidylinositol (GPI)-linked protein: Release and uptake of CD4-GPI from recombinant adeno-associated virus-transduced HeLa cells. Proc Natl Acad Sci USA 1996; 93:5894-5898.

29. Rooney IA, Morgan BP. Characterization of the membrane attack complex inhibitory protein CD59 antigen on human amniotic cells and in amniotic fluid. Immunology 1992; 76:541-547.
30. Hale G, Rye PD, Warford A et al. The glycosylphoshatidyl-inositol-anchored lymphocyte antigen CDw52 is associated with the epididymal maturation of human spermatozoa. J Reprod Immunol 1993; 23:189-205.
31. Rooney IA, Atkinson JP, Krul ES et al. Physiologic relevance of the membrane attack complex inhibitory protein CD59 in human seminal plasma: CD59 is present on extracellular organelles (prostasomes), binds cell membranes and inhibits complement-mediated lysis. J Exp Med 1993; 177:1409-1420.
32. Rooney IA, Heuser JE, Atkinson JP. GPI-anchored complement regulatory proteins in seminal plasma. An analysis of their physical condition and the mechanisms of their binding to exogenous cells. J Clin Invest; 1996:1675-1686.
33. Meri S, Vakeva A, Laari T et al. Soluble forms of CD59 antigen: Distribution in body fluids and functional activity. Compl Inflamm (abstr) 1991; 8:193.
34. Bjorge L, Jensen TS, Krostoffersen GK et al. Identification of the human complement regulatory protein CD59 in human colostrum and milk. Am J Reprod Immunol. 1996; 35:35-40.
35. Hakulinen J, Meri S. Shedding and enrichment of the glycolipid-anchored complement lysis inhibitor protectin (CD59) into milk fat globules. Immunology 1995; 85:495-501.
36. Ronquist G, Brody I. The prostasome: Its secretion and function in man. Biochim Biophys Acta 1985; 822:203-218.
37. Patton S, Huston GE. A method for isolation of milk fat globules. Lipids 1986; 21:170-174.
38. Kelly RW, Holland P, Sibinski G et al. Extracellular organelles (prostasomes) are immunosuppressive components of human seminal plasma. Clin Exp Immunol 1991; 86:550-556.
39. Ronquist G, Nilsson BO, Hertjen S. Interaction between prostasomes and spermatozoa from human semen. Arch Androl 1990; 24:147-157.
40. Bordier C. Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem 1981; 256:1604-1607.
41. Vakeva A, Jauhianen M, Ehnholm C et al. High density lipoproteins can act as carriers of glycosylphosphatidyl-inositol lipid-anchored CD59 in human plasma. Immunology 1994;82:28-33.
42. Yang E, Huestis WH. Mechanism of intermembrane phosphatidylcholine transfer: Effect of pH and membrane configuration. Biochemistry 1993; 32:12218-12228.
43. Patton S, Huston GE. A method for isolation of milk fat globules. Lipids 1986; 21:170-174.
44. Meri S, Waldmann H, Lachmann PJ. Distribution of protectin (CD59), a complement membrane attack inhibitor in normal human tissues. Lab Invest 1991; 65:532-537.
45. Simpson KL, Holmes CH. Differential expression of complement regulatory proteins decay accelerating factor (CD55) membrane cofactor protein (CD46) and CD59 during human spermatogenesis. Immunology 1994; 81:452-461.
46. Cervoni F, Oglesby TJ, Fenichel P et al. Expression of decay accelerating factor (CD55) of the complement system on human spermatozoa. J Immunol 1993; 151:931-948.
47. Hale G, Xia MQ, Tighe HP et al. The CAMPATH I antigen. Tissue Antigens 1990; 35:118-127.
48. Ilangumaran S, Briol A Hoessli D. Distinct interactions among GPI-anchored, transmembrane and membrane associated intracellular proteins, and sphingolipids in lymphocyte and endothelial cell plasma membranes. Biochim Biophys Acta 1997; 1328:227-236.
49. Sheets ED, Lee GM, Simson R et al. Transient confinement of a glycosylphosphatidylinositol-anchored protein in the plasma membrane. Biochemistry 1997; 36:12449-12458.
50. Vignon F, Koll-Back MH, Clavert A et al. Lipid composition of seminal plasma. J Androl 1989; 22:49-53.
51. Arvidson G, Ronquist G, Wikander G et al. Human prostasome membranes exhibit very high cholesterol/phospholipid ratios yielding high molecular ordering. Biochim Biophys Acta 1989; 984:167.

52. Saifuddin M, Parker CJ, Peeples ME et al. Role of virion-associated glycosylphosphatidyl-inositol-linked proteins CD55 and CD59 in complement resistance of cell line-derived and primary isolates of HIV-1. J Exp Med 1995; 182:501-504.
53. Vakeva A, Lurila P Meri S. Loss of expression of protectin (CD59) is associated with complement membrane attack complex deposition in myocardial infarction. Lab Invest 1992; 67:608.

CHAPTER 11

Engineering GPI-Anchored Proteins

Periasamy Selvaraj, Rebecca S. McHugh and Shanmugam Nagarajan

The majority of cell surface proteins contain three distinct polypeptide segments: a glycosylated extracellular domain, a hydrophobic transmembrane (TM) domain and a hydrophilic cytoplasmic tail. The TM domain anchors the protein to the plasma membrane. Some cell surface proteins do not have a TM hydrophobic peptide sequence, but are anchored to the cell surface via a glycosylated phosphatidylinositol (GPI) moiety, and are widely distributed from parasites to man[1-3] (see chapter 1). It remains difficult to make a general statement regarding the physiological significance of this type of membrane anchoring. However, it is becoming clear that the domain architecture of the plasma membrane influences the physical and functional behavior of GPI-anchored membrane proteins (see chapter 3). Comparative studies using membrane isoforms of receptors which are either naturally occurring or produced by genetic engineering have shed some light on the influence of the membrane anchor on biological and functional properties of some cell surface receptors. The property of GPI-anchored proteins to transfer to foreign cell membranes has evolved into a simple and useful technology to express novel proteins on the cell surface without resorting to gene transfer. In this chapter we discuss the evolution of this technology and the use of engineered GPI-anchored proteins of medical interest in therapeutic applications.

Naturally Occurring Membrane Isoforms of Cell Surface Glycoproteins

At least five well characterized cell surface receptors, neural cell adhesion molecule (NCAM),[4,5] leukocyte function antigen 3 (LFA-3),[6-9] mouse vascular cell adhesion molecule 1 (VCAM-1),[10-13] CD16[14-19] and cadherins[20-22] exist as both TM- and GPI-anchored proteins. The membrane isoforms may be cell type specific or both isoforms may be expressed on the same cell. NCAM is a homophilic adhesion molecule mediating adhesion events during neuronal development.[5] The GPI-anchored form is expressed later in development than the TM-anchored form.[23] The physiological significance of developmental, stage specific expression of a membrane isoform remains elusive. LFA-3 is a counterreceptor for a T lymphocyte adhesion antigen, CD2.[24] Human erythrocytes express only the GPI-anchored form of LFA-3, whereas all nucleated cells express both forms.[6,7] Both membrane isoforms of LFA-3 bind CD2 and mediate cell adhesion.[6,25] VCAM-1 is a cell adhesion molecule expressed on cells of the vasculature. It binds VLA-4, a β1 integrin expressed in many immune cells.[26,27] The VLA-4-VCAM-1 interaction plays a major role in lymphocyte homing. VCAM-1 expressed on human vascular cells are TM-anchored forms,[28] whereas in mouse both TM- and GPI-anchored VCAM-1 have been reported.[10-13] TM-anchored murine VCAM-1 contains seven Ig domains, as does the human VCAM-1. GPI-anchored VCAM-1 is a truncated form with three Ig domains. Molecular cloning

GPI-Anchored Membrane Proteins and Carbohydrates, edited by Daniel C. Hoessli and Subburaj Ilangumaran. ©1999 R.G. Landes Company.

studies show that the NCAM, VCAM-1 and LFA-3 membrane isoforms arise from a single gene by alternative mRNA splicing.[8-11,29,30]

CD16, a low affinity receptor for monomeric IgG, is a glycoprotein of 50-70 kDa expressed on neutrophils, natural killer (NK) cells, eosinophils and tissue macrophages.[31-35] The CD16b molecule expressed on neutrophils is GPI anchored,[14,36] while the NK cell and macrophage CD16a molecules are TM anchored.[15-19] The neutrophil and NK cell CD16 are products of two highly homologous genes[17] differing by only 4 or 6 amino acids in the extracellular domain. TM-CD16 expressed on NK cells can associate with subunits including the γ chain of FcεRI[37,38] and the ζ chain of the T cell receptor (TCR)/CD3 complex.[39]

Cadherins are a group of Ca^{2+}-dependent cell adhesion molecules that mediate homophilic cell-cell interaction.[20,22] A large number of cadherins and cadherin-related proteins are expressed in a variety of multicellular organisms. The cadherin superfamily is subdivided into six groups depending upon the number of “cadherin-repeats” and homology in the cytoplasmic domains. Most of the cadherins are expressed as TM-anchored proteins except the T-cadherin, which is expressed as a GPI-anchored protein.[40] T-cadherin shares the ectodomain of TM-anchored N- and E-cadherins. Most cell types express at least one type of cadherin in a developmentally regulated manner.[21] Cadherin-mediated cell adhesion plays a pivotal role in various developmental functions such as cell layer segregation, axon guidance, cell differentiation and formation and maintenance of tissues.

Transmembrane Proteins Engineered into GPI-Anchored Forms

Although the mature GPI-anchored proteins expressed at the cell surface do not have a transmembrane polypeptide domain, their mRNA sequence predicted that the precursors of GPI-anchored proteins have a hydrophobic stretch of amino acids at the C-terminus resembling TM domains. Site-directed mutagenesis and recombinant DNA techniques revealed that the C-terminal hydrophobic domain and 15-20 amino acids of the extracellular domain proximal to the C-terminus possess the signal for GPI anchor attachment. Analysis of the GPI anchor attachment signal sequences of many cloned GPI-anchored proteins revealed a lack of consensus.[41] In the case of Qa-2 protein, the TM domain carries the signal for the GPI anchor addition.[42] Extensive studies by Udenfriend and coworkers have established the amino acid requirements for GPI anchor attachment.[41,43-45]

TM-proteins are converted into GPI-anchored forms by replacing the TM and cytoplasmic domains with a GPI anchor.[46-53] This manipulation involves ligating the 5′ end of a cDNA encoding the extracellular domain of a TM-anchored, or a secretory, protein to the 3′ end of another cDNA coding for the anchor attachment signal of a GPI-anchored protein (Fig. 11.1). The chimeric construct is transfected into cells, which are then analyzed for surface expression of the protein and its susceptibility to PI-PLC. Initial studies using this recombinant DNA methodology were focused on constructing chimeric molecules to identify the signal sequence for GPI anchor attachment on endogenous GPI-anchored proteins. In a series of experiments, Caras et al[54-56] and Tykocinski et al[57] assigned the GPI anchor addition signal of DAF to the last 37 amino acids at the C-terminus. Subsequently, GPI-anchored forms of type I and type II integral membrane proteins and secretory and viral envelope proteins were constructed to characterize the structural requirements of their extracellular domains to carry out specific functions (Table 11.1).

The immunologically important CD4 protein was converted to a GPI-anchored form to map the domains required for HIV-1 binding and infection.[58,59] These studies demonstrated that GPI-anchored CD4 can bind to HIV-1 envelope protein gp120 via the first two extracellular domains, and TM and cytoplasmic domains are not at all required for HIV-1 infection. Other immunologically important molecules such as CD16a,[38,60] CD8,[61] mouse MHC class I,[49,62,63] MHC class II,[47] TCR,[64] ICAM-1,[48,65] B7-1,[66,67] B7-2[67,68] and the

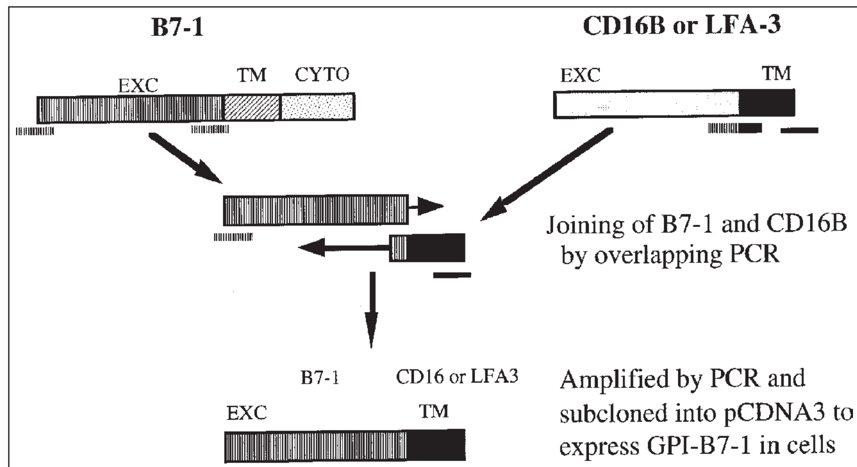

Fig. 11.1. Construction of a GPI-anchored form of B7-1 transmembrane protein. The extracellular domain of B7-1 and the GPI anchor signal domain of CD16b or LFA-3 was amplified by PCR reaction. The N-terminal primer of CD16b or LFA-3 contained an overhang with C-terminal sequences of the extracellular domain of B7-1. To obtain a hybrid product, the two PCR products were mixed together and overlapping PCR was carried our for 5 cycles without primers and then 25 cycles with primers. The amplified product was cloned into the pCDNA-3 expression vector and used for transfection.

LFA-1 I domain[69] were also converted to GPI-anchored forms for investigating the functional consequences of this new mode of membrane association. Studies from our laboratory using GPI-anchored CD16a (natively expressed as a TM-anchored form in NK cells) have shown that the GPI-anchored form can bind to an immune complex and endocytose the bound ligand.[60,70] GPI-anchored T cell receptor efficiently recognized antigen presented by MHC class II molecules.[64] GPI-anchored mouse MHC class I molecule H2-D^d conferred protection from NK cell lysis in vivo and in vitro.[62] However, in other studies GPI-anchored H2-D^b was not able to load endogeneouly processed antigenic peptides, though it bound to exogenously added antigenic peptides as efficiently as its TM counterpart.[63] Recently, we have demonstrated that GPI-anchored B7-1 can bind to CD28 and induce T cell proliferation as efficiently as the transmembranous B7-1.[66] The therapeutic use of GPI-anchored B7-1 in tumor immunotherapy is discussed later in this chapter.

The technology of constructing chimeric GPI-anchored molecules has also been applied to analyze the structure/function relationship of viral envelope proteins such as herpes simplex virus I envelope glycoprotein D (gD1),[71-73] vesicular stomatitis virus glycoprotein (VSVG),[74] influenza virus hemagglutinin (HA),[75-77] rubella virus envelope protein E1,[78] murine leukemia virus envelope protein,[79] HIV-1 gp120[80] and Rous sarcoma virus envelope protein.[81] In these studies, the chimeric GPI-anchored viral proteins were used to map the sites required for virus entry and replication.

Comparative Studies Using Naturally Occurring and Genetically Engineered Membrane Isoforms

GPI-anchored cell surface proteins are implicated in a number of cellular functions such as signal transduction (T lymphocyte activation, e.g., Thy-1, CD73),[82,83] protecting cells from complement-mediated damage (DAF and homologous restriction factor)[84,85] and

Table 11.1 Studies on engineered GPI-anchored proteins

Engineered GPI-anchored proteins	Studies to delineate the functional domains	Ref.
Immunologically important proteins		
CD4	IV-1 viral infection, endocytosis	58,59,133-135
CD8	Ligand binding	61
T-cell receptor	Antigen presentation	64
ICAM1	Rhinovirus internalization Cytoskeletal association	48,65
B7-1	Ligand binding, induction of tumor immunity	66,67
B7-2	Ligand binding, induction of tumor immunity	67,68
MHC class I	Antigen presentation NK cytotoxicity	62,63,136,137
MHC class II	Antigen presentation	47
CD16A	IgG binding and endocytosis Signal transduction	38,60
LFA-1 I domain	Binding to ICAM-1 and ICAM-3	69
Viral envelope proteins		
HSV-1 g-D1	Apical targeting of proteins	71-73
HA	Cell fusion and apical targeting studies	75-77
Murine leukemia virus E1	Subunit association and viral infection	79
HIV-1 gp-120	CD4 binding Viral entry and infection	80
Enzymes		
Neutral endopeptidase	Topology of type II membrane	138

cell adhesion (LFA-3 and NCAM).[7,8,86,87] Some GPI-anchored proteins like 5' nucleotidase and alkaline phosphatase are enzymes.[88,89] However, in all these cases, how the GPI anchor influences the function of the protein does not follow any pattern. Comparative studies using naturally occurring and engineered membrane isoforms suggest that the GPI anchor does influence the physical and functional properties of the protein, which include:

1. Lateral mobility on the plane of the membrane;
2. Apical targeting in polarized epithelial cells; and
3. The nature of the signal transduced.

GPI-anchored Thy-1,[90] DAF[91] and alkaline phosphatase[92] show altered lateral mobilities in the cell membrane compared to TM-anchored proteins. It is predicted that the lipid anchor of the GPI moiety, which does not cross the membrane bilayer, facilitates a less

restricted mobility of GPI-anchored proteins in the cell membrane.[88,92,93] However, a large fraction of GPI-anchored molecules is relatively less mobile[90] due to their association with sphingolipid-rich membrane rafts[94,95] and extracellular interactions with transmembrane glycoproteins.[93] High lateral mobility of the GPI anchor may be physiologically important during cellular adhesion in facilitating the diffusion of adhesion receptors to the contact site. Using glass supported planar membranes reconstituted with LFA-3 isoforms, Chan et al[96] and Tozeren et al[97] demonstrated that mobile GPI-LFA-3 was more efficient than glass immobilized TM-LFA-3 in binding of CD2-expressing Jurkat cells. On the other hand, the TM-HLA class II is more efficient in interacting with TCR than GPI-HLA class II,[47] and TM- and GPI-anchored forms of DAF and membrane cofactor protein are equally efficient in protecting cells from complement-mediated cytotoxicity.[98] It appears that the GPI anchor may cause a subtle change in the conformation or orientation of a receptor on the cell membrane that may influence the affinity of only some receptors, but not others. Other factors, such as interaction with neighboring molecules via extracellular domains, may also influence the lateral mobility of a receptor.[90,93,99]

Role of GPI anchors in apical targeting of membrane receptors is well established in different systems. Endogenous and recombinant GPI-DAF are always expressed on the apical surface of epithelial cells.[73] Conversion by recombinant DNA methods of basolateral transmembrane proteins such as vesicular somatitis virus glycoprotein and herpes simplex glycoprotein D into GPI-anchored forms results in apical sorting.[73,100] Similarly, in polarized epithelial cells, TM-NCAM is found on the basolateral membrane, whereas GPI-NCAM is expressed on the apical surface.[101]

Comparative studies using CD16 membrane isoforms show that signals transduced by GPI- and TM-receptor isoforms could be very different. Crosslinking of TM-CD16 on transfected Jurkat cells induces IL-2 secretion and IL-2 receptor expression, whereas GPI-CD16 is unable to do so.[60] Membrane isoforms of CD16 expressed on CHO cell transfectants also differ in their ability to mediate phagocytosis of IgG-coated particles, as only the TM-CD16 but not GPI-CD16 was able to mediate efficient phagocytosis.[102] CD16 isoforms also differ in triggering tumor cell cytotoxicity. NK cells with TM-CD16 kill tumor targets bearing anti-CD16 surface Ig very efficiently, whereas neutrophils carrying GPI-CD16 are inefficient effectors.[15,103] The GPI anchor of CD16 on neutrophils, however, is not completely signaling incompetent, since neutrophils can be triggered through CD16 to lyse chicken erythrocytes.[104,105] Crosslinking of GPI-CD16 on neutrophils with specific mAbs induces Ca^{2+} mobilization and degranulation, but not the respiratory burst.[106,107] These differences in nature and intensity of signaling demonstrate that receptor isoforms with distinct membrane anchors may associate with different signal transducing molecules and thus influence the receptor function.

GPI-Mediated Protein Transfer

Purified proteins that contain a GPI anchor are able to spontaneously incorporate into the lipid bilayer of many cells.[6,84,108] Reconstitution of GPI-anchored proteins into cell membranes is a specific process, mediated by hydrocarbon chains of the lipid moiety, as chemical or enzymatic removal of the acyl chains completely abolished the incorporation. The GPI-mediated protein transfer (also referred to as GPI painting, see ref. 109) has become an attractive strategy to express new proteins on cell membranes. Both naturally occurring and engineered GPI-anchored proteins transfer equally well. The membrane incorporation process is dependent on temperature and duration of incubation and concentration of the purified protein.[110] Fatty acid binding serum proteins such as BSA and orosomucoid inhibit the transfer.[84] Under serum-free conditions, genetically engineered, affinity purified GPI-B7-1 incorporated maximally at 10-20 μg/ml concentration after 2 h

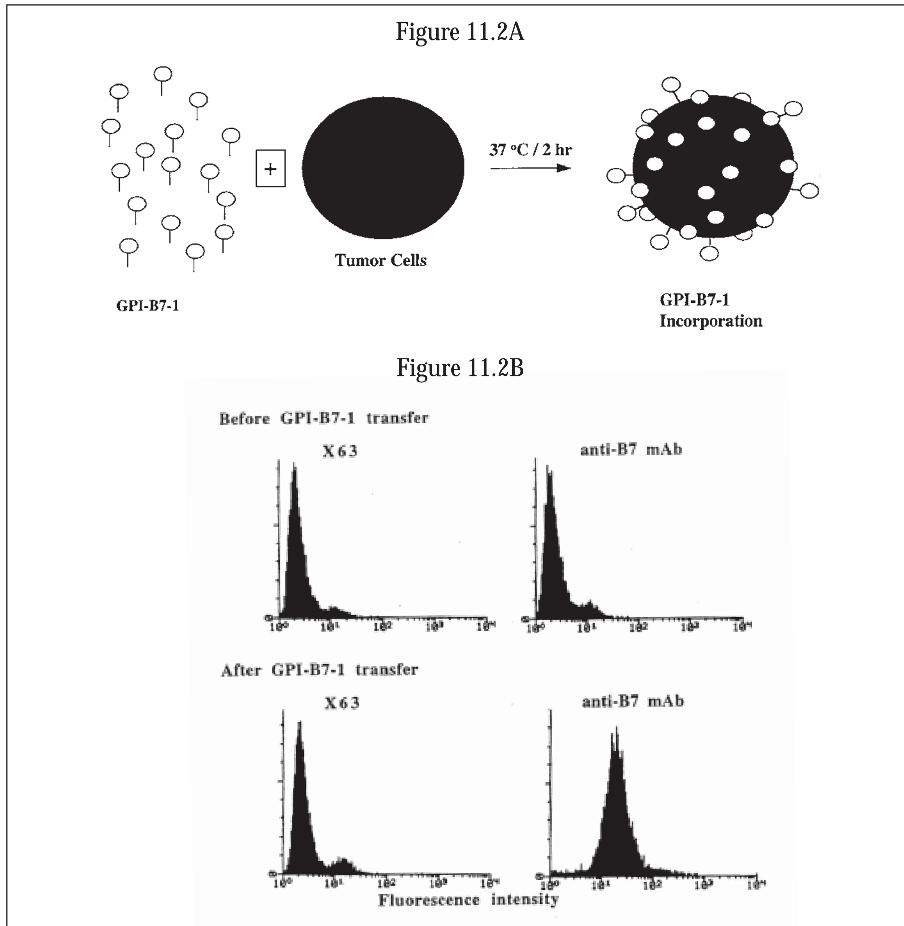

Fig. 11.2. Schematic representation of GPI-mediated protein transfer. (A) Intact live or irradiated tumor cells were incubated with purified GPI-B7-1 molecules for 2 hours at 37°C. The unincorporated proteins were washed out and the tumor cells incorporated with GPI-B7-1 were used for further studies. (B) WM115, a human melanoma cell line, was incubated with 30 μg/ml of purified GPI-B7-1 for 2 h in HBSS/5mM EDTA. The cells were washed and stained with a non-binding control antibody (X63) or with anti-B7 mAb (BB-1 from Becton-Dickinson) and a FITC-conjugated goat anti-mouse antibody. The stained cells were analyzed by flow cytometry.

incubation at 30°C without any significant decrease in cell viability (Fig. 11.2). A number of tumor cell lines including primary breast carcinoma cells could be modified with GPI-B7-1 with similar kinetics of incorporation.[66]

Initially, the GPI-protein transfer was used to correct the defect in paroxysmal nocturnal hemoglobinuria (PNH) patients' erythrocytes. PNH is an acquired abnormality of hematopoietic cells affecting GPI anchor biosynthesis or attachment, thus selectively affecting the membrane expression of GPI-anchored proteins.[1,2] The complement regulatory activity of erythrocytes from PNH patients could be reconstituted by incorporation of GPI-anchored

Table 11.2 Protein transfer of native and engineered GPI-anchored proteins

Molecule	Cells Incorporated	Functions Tested	Ref.
Naturally occuring GPI-anchored molecules			
DAF	Erythrocytes, Endothelial cells	Complement regulation	84,111,112, 128-129
CD59	Erythrocytes, U937, Oligodendrocytes	Complement regulation Signal transduction Complement regulation	112,130,131
LFA-3	Erythrocytes	Cell adhesion	6
CD16B	Leukemic cells	Ligand binding Endocytosis	110
Thy-1	Tumor cells	Lateral mobility	108
Engineered GPI-anchored molecules			
Human B7-1	Tumor cells	Immunoregulation	66
CD4	HeLa cells	Viral infection	132
Mouse B7-1	Tumor cells	Immunoregulation	67
Mouse class I	Tumor cells	CTL target lysis	49

DAF and CD59[84,111,112] by protein transfer. Apart from complement regulatory proteins, PNH erythrocytes also lack LFA-3 and, therefore, do not adhere to T cells expressing CD2, a natural ligand for LFA-3.[24] Expression of LFA-3 by protein transfer reconstituted the ability of PNH erythrocytes to adhere to T cells,[6] suggesting that adhesion function of a cell can be manipulated by protein transfer.

Subsequent studies have shown that not only erythrocytes, but also nucleated cells can be reconstituted with GPI-anchored molecules (Table 11.2). In all these studies, purification and reincorporation of GPI-anchored proteins do not seem to alter their ligand binding capacity: GPI-CD4 retained the ability to bind HIV-1 gp120;[58,59] GPI-MHC class I molecule induced allogenic T cell proliferation like its TM counterpart;[49] and GPI-MHC class I MHC molecules loaded with a viral peptide stimulated CTLs specific for that particular viral peptide.[49] As shown in Figure 11.3, GPI-B7-1 incorporated RCC-1 cells could stimulate proliferation of allogeneic T cells to the same extent as the TM- or GPI-anchored B7-1 expressed by transfection. These observations highlight the potential use of GPI-protein transfer for immunomodulation (see below).

Potential Application of GPI-Protein Transfer in Tumor Immunotherapy

Many murine and human tumor cell lines lack the expression of costimulatory molecules, especially B7-1[113-115] and thus are incapable of providing costimulatory signals to tumor-specific T lymphocytes. However, they can provide the first TCR recognition signal by presenting the tumor antigen on MHC class I molecules. In the absence of a costimulatory signal, these TCR-stimulated tumor-specific T cells become anergic and eventually die. In this way, tumor cells can incapacitate the T cell population specific for their antigens and escape from immune surveillance. Expression of B7-1[114,116-121] and other

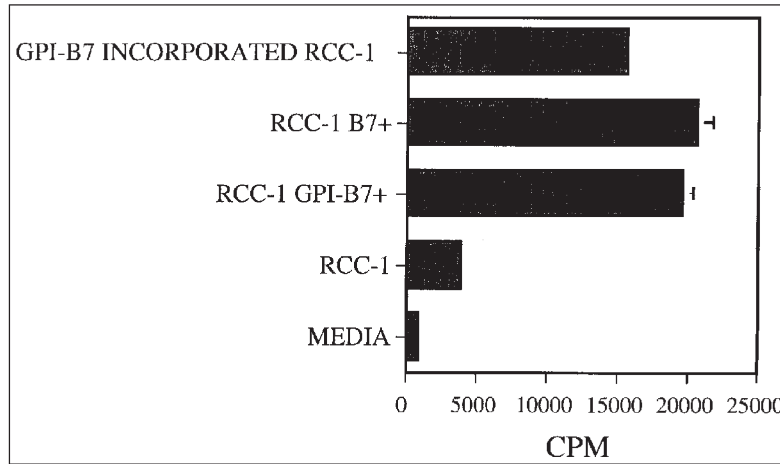

Fig. 11.3. GPI-anchored protein transferred to a foreign cell surface is functional. Human renal carcinoma cell line RCC-1 was either transfected with transmembrane B7-1 or GPI-B7-1 or modified with GPI-B7-1 by protein transfer and irradiated. Human peripheral blood lymphocytes were stimulated with irradiated RCC-1 cells and the proliferation was measured using ^{3}H-thymidine uptake. Reprinted with permission from Vaccines: New Advances in Technologies and Applications, R. Ostriker, Ed. ©1996 International Business Communications, Inc., Southborough, MA.

costimulatory molecules such as ICAM-1[122,123] on the tumor cell by gene transfection induces specific anti-tumor immunity and subsequent tumor rejection in animal models.

Gene transfer by either transfection or viral transduction is the most widely used method for stable expression of novel proteins. This technique has a number of practical limitations and disadvantages for use in patients. Transfection of cells is a time consuming process, requiring weeks for selection of homogeneous cell populations. In addition, transfection of primary tumor cells is difficult, requiring establishment of tumor cell lines. The use of viral vectors to transduce cells has eliminated most time constraints, but this technique has its own disadvantages. Utilization of viral vectors may introduce mutations at the site of DNA integration. Further, the host can develop strong immune responses to viral proteins, making it difficult to immunize more than once with the same vector.[124-126]

Protein transfer provides an alternative method for the introduction of costimulatory molecules onto the surface of tumor cells and eliminates most of the problems encountered with gene transfer.[109,127] GPI-protein transfer is fast, requiring only a short incubation of the cells with the purified protein. This technique also allows for simultaneous incorporation of a number of molecules, virtually on all cell types including primary tumor cells at any stage of their cycle. One of the limitations of GPI-protein transfer is related to the stability of the incorporated molecule on the cell surface. Live cells gradually lose surface expression of the incorporated protein upon multiple cell divisions. However, in clinical settings where live cells are undesirable to use, non-proliferating, irradiated cells or cell membrane preparations are preferred. Live cells and even irradiated cells retained only about 30% of surface incorporated GPI-B7-1 after 24 hours, probably due to membrane dynamics. Stability of GPI-B7-1 on the surface of cell membrane preparations, however, is stable up to

four days after incorporation (unpublished observations). Experimentation with these techniques for tumor immunotherapy in animal models is currently under way.

Summary

Understanding the mechanism of addition of GPI anchor has resulted in techniques to create GPI-anchored forms of transmembrane cell surface glycoproteins.[49,66,67] Comparative studies using TM- and GPI-anchored isoforms of membrane glycoproteins have been used to analyze the structure/function relationship of distinct segments or domains of receptor proteins. The special property of naturally occuring and engineered GPI-anchored molecules to incorporate spontaneously onto cell membranes has been utilized in a simple, rapid technique for transient expression of foreign molecules on virtually any cell type. This technique has overcome several limitations of gene transfer techniques and thus offers many advantages when human clinical trials are considered. Using this technique, we have demonstrated that GPI-anchored B7-1 can spontaneously incorporate onto many tumor cell lines,[66] which regained their capacity to stimulate tumor-specific T cells.

In addition to tumor immunotherapy, many other experimental and therapeutic applications of GPI-protein transfer can be envisioned. As against proteins that stimulate the immune response, proteins that downregulate or modulate the effector immune functions can be expressed on the cell surface. Certain molecules, such as CD8, could be used to downregulate T cell responses after antigen recognition,[127] which may be beneficial in treating autoimmune diseases and transplant rejection. In conclusion, we envisage that engineering and transfer of GPI-anchored proteins is likely to become a method of choice for developing immunotherapeutic reagents to stimulate specific anti-tumor immunity, to treat autoimmune diseases, and to delay/inhibit transplant rejection.

References

1. Low MG. Biochemistry of the glycosyl-phosphatidylinositol membrane protein anchors. Biochem J 1987; 244:1-13.
2. Ferguson MAJ, Williams AF. Cell surface anchoring of proteins via glycosyl-phosphatidylinositol structures. Ann Rev Biochem 1988; 57:285-320.
3. Cross GAM. Eukaryotic protein modification and membrane attachment via phosphatidylinositol. Cell 1987; 48:179-181.
4. He HT, Finne J, Goridis C. Biosynthesis, membrane association, and release of N-CAM-120, a phosphatidylinositol-linked form of the neural cell adhesion molecule. J Cell Biol 1987; 105:2489-2500.
5. Cunningham BA, Hemperly JJ, Murray BA et al. Neural cell adhesion molecule: Structure, immunoglobulin-like domains, cell surface modulation, and alternative splicing. Science 1987; 236:799-806.
6. Selvaraj P, Dustin ML, Silber R et al. Deficiency of lymphocyte function associated antigen-3 (LFA-3) in paroxysmal nocturnal hemoglobinuria: Functional correlates and evidence for a phosphatidylinositol membrane anchor. J Exp Med 1987; 166:1011-1025.
7. Dustin ML, Selvaraj P, Mattaliano M et al. Anchoring mechanisms for LFA-3 cell adhesion glycoprotein at membrane surface. Nature 1987; 329:846-848.
8. Seed B. An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. Nature 1987; 329:840-842.
9. Wallner BP, Frey AZ, Tizard R et al. Primary structure of lymphocyte function associated antigen-3 (LFA-3): The ligand of the T-lymphocyte CD2 glycoprotein. J Exp Med 1987; 166:923-934.
10. Hession C, Moy P, Tizard R et al. Cloning of murine and rat vascular cell adhesion molecule-1. Biochem Biophys Res Commun 1992; 183:163-169.

11. Moy P, Lobb R, Tizard R et al. Cloning of an inflammation-specific phosphatidyl inositol-linked form of murine vascular cell adhesion molecule-1. J Biol Chem. 1993; 268:8835-8841.
12. Terry RW, Kwee L, Levine JF et al. Cytokine induction of an alternatively spliced murine vascular cell adhesion molecule (VCAM) mRNA encoding glycosylphosphatidylinositol-anchored VCAM protein. Proc Natl Acad Sci USA 1993; 90:5919-5923.
13. Kumar AG, Dai XY, Kozak CA et al. Murine VCAM-1. Molecular cloning, mapping, and analysis of a truncated form. J Immunol 1994; 153:4088-4098.
14. Selvaraj P, Rosse WF, Silber R et al. The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal hemoglobinuria. Nature 1988; 333:565-567.
15. Selvaraj P, Carpen O, Hibbs, ML et al. Natural killer cell and granulocyte FcγRIII (CD16) differ in membrane anchor and signal transduction. J Immunol 1989; 143:3283-3288.
16. Lanier LL, Phillips JH, Testi R. Membrane anchoring and spontaneous release of CD16 (FcR III) by natural killer cells and granulocytes. Eur J Immunol 1989; 19:775-778.
17. Ravetch JV, Perussia B. Alternative membrane forms of Fc gamma RIII (CD16) on human NK cells and neutrophils: Cell-type specific expression of two genes which differ in single nucleotide substitutions. J Exp Med 1989; 170:481-497.
18. Scallon BJ, Scigliano E, Freedman VH et al. A human immunoglobulin G receptor exists in both polypeptide-anchored and phosphatidylinositol-glycan-anchored forms. Proc Natl Acad Sci USA 1989; 86:5079-5083.
19. Edberg JC, Redecha PB, Salmon JC et al. Human FcγRIII (CD16): Isoforms with distinct allelic expression, extracellular domains, and membrane linkages on polymorphonuclear and natural killer cells. J Immunol 1989; 143:1642-1649.
20. Takeichi M. Cadherins: A molecular family important in selective cell-cell adhesion. Ann Rev Biochem 1990; 59:237-252.
21. Suzuki ST. Structural and functional diversity of cadherin superfamily: Are new members of cadherin superfamily involved in signal transduction pathway? J Cell Biochem 1996; 61:514-523.
22. Aberle H, Schwartz H, Kemler R. Cadherin-catenin complex: Protein interactions and their implications for cadherin function. J Cell Biochem 1996; 61:514-523.
23. Lyles JM, Linnemann D, Bock E. Biosynthesis of the D2-cell adhesion molecule: Posttranslational modifications, intracellular transport, and developmental changes. J Cell Biol 1984; 99:2082-2091.
24. Selvaraj P, Plunkett ML, Dustin ML et al. The T-lymphocyte glycoprotein CD2 binds the cell surface ligand. Nature 1987; 326:400-403.
25. Hollander NP, Selvaraj P, Springer TA. Biosynthesis and function of LFA-3 in human mutant cells deficient in phosphatidylinositol anchored proteins. J Immunol 1988; 141:4283-4290.
26. Hemler ME. VLA proteins in the integrin family: Structures, functions, and their role on leukocytes. Ann Rev Immunol 1990; 8:365-400.
27. Bergelson JM, Hemler ME. Integrin-ligand binding. Do integrins use a 'MIDAS touch' to grasp an Asp? Current Biol 1995; 5:615-617.
28. Vonderheide RH, Springer TA. Lymphocyte adhesion through very late antigen 4: Evidence for a novel binding site in the alternatively spliced domain of vascular cell adhesion molecule 1 and an additional alpha 4 integrin counter-receptor on stimulated endothelium. J Exp Med 1992; 175:1433-1442.
29. Murray BA, Hemperly EA, Prediger EA et al. Alternatively spliced mRNAs code for different polypeptide chains of the chicken neural cell adhesion molecule. J Cell Biol 1986; 102:189-193.
30. Cybulsky MI, Allan-Motamed M, Collins T. Structure of the murine VCAM-1 gene. Genomics 1993; 18:387-391.
31. Unkeless JC, Scigliano E, Freedman VH. Structure and function of human and murine receptors for IgG. Ann Rev Immunol 1988; 6:251-281.

32. Anderson CL, Looney RJ. Human leukocyte IgG Fc receptors. Immunol Today 1986; 7:264-266.
33. Mellman I. Relationships between structure and function in the Fc receptor family. Curr Opin Immunol 1988; 1:16-25.
34. Fanger MW, Shen L, Graziano RF et al. Cytotoxicity mediated by human Fc receptors for IgG. Immunol Today 1989; 10:92-99.
35. Ravetch JV, Kinet J-P. Fc Receptors. Ann Rev Immunol 1991; 9:457-492.
36. Huizinga TWJ, Van Der Schoot CE, Jost C et al. The PI-linked receptor FcRIII is released on stimulation of neutrophils. Nature 1988; 333:667-669.
37. Hibbs ML, Selvaraj P, Carpen O et al. Mechanism regulating expression of membrane isoforms of FcγRIII (CD16). Science 1989; 246:1608-1611.
38. Kurosaki T, Ravetch JV. A single amino acid in the GPI attachment domain determines the membrane topology of Fc gamma RIII. Nature 1989; 342:805-807.
39. Lanier LL, Yu G, Phillips JH. Co-association of CD3 zeta with a receptor (CD16) for IgG Fc on human natural killer cells. Nature 1989; 342:803-805.
40. Ranscht B, Dours-Zimmermann MT. T-cadherin, a novel cadherin cell adhesion molecule in the nervous system lacks the conserved cytoplasmic region. Neuron 1991; 7:391-402.
41. Berger J, Howard AD, Brink L et al. COOH-terminal requirements for the correct processing of a phosphatidlylinositol-glycan anchored membrane protein. J Biol Chem 1988; 263:10016-10021.
42. Waneck GL, Stein ME, Flavell RA. Conversion of a PI-anchored protein to an integral membrane protein by a single amino acid mutation. Science 1988; 241:697-699.
43. Gerber LD, Kodukula K, Udenfriend S. Phosphatidylinositol glycan (PI-G) anchored membrane proteins: Amino acid requirements adjacent to the site of cleavage, and PI-G attachment in the COOH-terminal signal peptide. J Biol Chem 1992; 267:12168-12173.
44. Udenfriend S, Kodukula K. How glycosylphosphatidylinositol-anchored membrane proteins are made. Ann Rev Biochem 1995; 64:563-591.
45. Kodukula K, Gerber LD, Amthauer R et al. Biosynthesis of glycosylphosphatidylinositol (GPI)-anchored membrane proteins in intact cells: Specific amino acid requirements adjacent to the site of cleavage and GPI attachment. J Cell Biol 1993; 120:657-664.
46. Straus DS, Stroynowski I, Schiffer SG et al. Expression of hybrid class I genes of the major histocompatibility complex in mouse L cells. Proc Natl Acad Sci USA 1985; 82:6245-6249.
47. Wettstein DA, Boniface JJ, Reay PA et al. Expression of a class II major histocompatability complex heterodimer in a lipid-linked form with enhanced peptide/soluble MHC complex formation at low pH. J Exp Med 1991; 174:219-228.
48. Staunton DE, Guar A, Chan P-Y et al. Internalization of a major group human rhinovirus does not require cytoplasmic or transmembrane domains of ICAM-1. J Immunol 1992; 148:3271-3274.
49. Huang J-H, Getty RR, Chisari FV et al. Protein transfer of preformed MHC-peptide complexes sensitizes target cells to T cell cytolysis. Immunity 1994; 1:607-613.
50. Fasel N, Rousseaux M, Schaerer E et al. In vitro attachment of glycosyl-inositolphospholipid anchor structures to mouse Thy-1 antigen and human decay-accelerating factor. Proc Natl Acad Sci USA 1989; 86:6858-6862.
51. Clissold PM, Ebling HJ, Lachmann PJ. Construction, expression and functional analysis of a glycolipid-linked form of CR1. Eur J Immunol 1993; 23:2346-2352.
52. Altmann DM, Hogg N, Trowsdale J et al. Cotransfection of ICAM-1 and HLA-DR reconstitutes human antigen-presenting cell function in mouse L cells. Nature 1989; 338:512-514.
53. Englund PT. The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. Ann Rev Biochem 1993; 62:121-138.
54. Caras IW, Weddell GN, Davitz MA et al. Signal for attachment of a phospholipid membrane anchor in decay accelerating factor. Science 1987; 238:1280-1283.
55. Caras IW, Weddell GN. Signal peptide for protein secretion directing glycophospholipid membrane anchor attachment. Science 1989; 243:1196-1198.

56. Caras IW, Weddell GN, Williams SR. Analysis of the signal for attachment of a glycophospholipid membrane anchor. J Cell Biol 1989; 108:1387-1396.
57. Tykocinski ML, Shu H-K, Ayers DJ et al. Glycolipid reanchoring of T-lymphocyte surface antigen CD8 using the 3' end sequence of decay-accelerating factor's mRNA. Proc Natl Acad Sci USA 1988; 85:3555-3559.
58. Diamond DC, Finberg R, Chaudhuri S et al. Human immuodeficiency virus infection is efficiently mediated by a glycolipid-anchored form of CD4. Proc Natl Acad Sci USA 1990; 87:5001-5005.
59. Jasin M, Page KA, Littman DR. Glycosylphosphatidylinositol-anchored CD4/Thy-1 chimeric molecules serve as human immunodeficiency virus receptors in human, but not mouse, cells and are modulated by gangliosides. J Virol 1991; 65:440-444.
60. Wirthmueller U, Kurosaki T, Murakami MS et al. Signal transduction by Fc gamma RIII (CD16) is mediated through the gamma chain. J Exp Med 1992; 175:1381-1390.
61. Meyerson HJ, Huang J-H, Fayen JD et al. Functional dissociation of CD8α's Ig homologue and connecting peptide domains. J Immunol 1996; 156:574-584.
62. Sentmann CL, Olsson-Alheim MY, Lendahl U et al. Influence of glycosylphosphatidylinositol-linked H-2D^d molecules on target cell protection and natural killer cell specificity in transgenic mice. Eur J Immunol 1996; 26:2127-2132.
63. Cariappa A, Flyer DC, Rollins CT et al. Glycosylphosphatidylinositol-anchored H-2D^b molecules are defective in antigen processing and presentation to cytotoxic T lymphocytes. Eur J Immunol 1996; 26:2215-2224.
64. Lin AY, Devaux B, Green A et al. Expression of T cell antigen receptor heterodimers in a lipid-linked form. Science 1990; 249:677-679.
65. Carpen O, Pallai P, Staunton DE et al. Association of intercellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and alpha-actinin. J Cell Biol 1992; 118:1223-1234.
66. McHugh RS, Ahmed SN, Wang Y-C et al. Construction, purification and functional reconstitution on tumor cells of glycolipid-anchored human B7-1 (CD80). Proc Natl Acad Sci USA 1995; 92:8059-8063.
67. Brunschwig EB, Levine E, Trefzer U et al. Glycosylphosphatidylinositol-modified murine B7-1 and B7-2 retain costimulator function. J Immunol 1995; 155:5498-5505.
68. Groves JT, Wulfing C, Boxer SG. Electrical manipulation of glycan-phosphatidylinositol-tethered proteins in planar supported bilayers. Biophysical J 1996; 71:2716-2723.
69. Knorr R, Dustin ML. The lymphocyte function-associated antigen 1 I domain is a transient binding molecule for intercellular adhesion molecule (ICAM)-1 and ICAM-3 in hydrodynamic flow. J Exp Med 1997; 186:719-730.
70. Nagarajan S, Anderson M, Ahmed SN et al. Purification and optimization of functional reconstitution on the surface of leukemic cell lines of GPI-anchored Fcγ receptor III. J Immunol Meth 1995; 184:241-251.
71. Lisanti MP, Rodriguez-Boulan E. Glycophospholipid membrane anchoring provides clues to the mechanism of protein sorting in polarized epithelial cells. Trends Biochem Sci 1990; 15:113-118.
72. Lisanti MP, Sargiacomo M, Graeve L et al. Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in renal epithelial cell line. Proc Natl Acad Sci USA 1988; 85:9557-9561.
73. Lisanti MP, Caras IW, Davitz MA et al. A glycolipid membrane anchor acts as an apical targeting in polarized epithelial cells. J Cell Biol 1989; 109:2145-2156.
74. Brown DA, Crise B, Rose JK. Mechanism of membrane anchoring affects polarized expression of two proteins in MDCK cells. Science 1989; 245:1499-1501.
75. Melikyan GB, White JM, Cohen FS. GPI-anchored influenza hemagglutinin induces hemifusion to both red blood cell and planar bilayer membranes. J Cell Biol 1995; 131:679-691.
76. Kemble GW, Danieli T, White JM. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. Cell 1994; 76:383-391.

77. Kemble GW, Henis Yi, White JM. GPI- and transmembrane-anchored influenza hemagglutinin differ in structure and receptor binding activity. J Cell Biol 1993; 122:1253-1265.
78. Bernasconi E, Fasel N, Wittek R. Cell surface expression of a functional rubella virus E1 glycoprotein by addition of a GPI anchor. J Cell Sci 1996; 109:1195-1201.
79. Ragheb JA, Anderson WF. Uncoupled expression of Moloney murine leukemia virus envelope polypeptides SU and TM: A functional analysis of the role of TM domains in viral entry. J Virol 1994; 68:3207-3219.
80. Weiss CD, White JM. Characterization of stable Chinese hamster ovary cells expressing wild type, secreted, and glycosylphosphatidylinositol-anchored human immunodeficiency virus type 1 envelope glycoprotein. J Virol 1993; 67:7060-7066.
81. Gilbert JM, Hernandez LD, Chernov-Rogan T et al. Generation of a water-soluble oligomeric ectodomain of the rous sarcoma virus envelope glycoprotein. J Virol 1993; 67:6889-6892.
82. Rock KL, Yeh ETH, Gramm CF et al. TAP, a novel T-cell activating protein involved in the stimulation of MHC-restricted T lymphocytes. J Exp Med 1986; 163:315-333.
83. Kroczek RA, Gunter KC, Germain RN et al. Thy-1 functions as a signal transduction molecule in T lymphocytes and transfected B lymphocytes. Nature 1986; 322:181-184.
84. Medof ME, Kinoshita T, Nussenzweig V. Inhibition of complement activation on the surface of cells after incorporation of decay-accelerating factor (DAF) into their membranes. J Exp Med 1984; 160:1558-1563.
85. Zalman LS, Wood LM, Frank MM et al. Deficiency of the homologous restriction factor in paroxysmal nocturnal hemoglobinuria. J Exp Med 1987; 165:572-575.
86. He HT, Barbet J, Chaix JC et al. Phosphotidylinositol is involved in the membrane attachment of NCAM-120, the smallest component of the neutral cell adhesion molecule. EMBO J 1986; 5:2489-2494.
87. Jesaitis AJ, Naemura JR, Skalar LA et al. Rapid modulation of N-formyl chemotactic peptide receptors on the surface of human granulocytes: Formation of high-affinity ligand-receptor complexes in transient association with cytoskeleton. J Cell Biol 1984; 98:1378-1387.
88. Low MG, Saltiel AR. Structural and functional roles of phosphatidylinositol in membranes. Science 1988; 239:268-275.
89. Thompson LF. 5'-nucleotidase-an overview of the last three years. Adv Exp Med Biol 1991; 309B:145-150.
90. Ishihara A, Hou Y, Jacobson K. The Thy-1 antigen exibits rapid lateral diffusion in the plasma membrane of rodent lymphoid cells and fibroblasts. Proc Natl Acad Sci USA 1987; 84:1290-1293.
91. Thomas J, Webb W, Davitz M et al. Decay accelerating factor diffuses rapidly on HeLa AE cell surfaces. Biophys J 1987; 51:522a-abstr.
92. Noda M, Yoon K, Rodan GA et al. High lateral mobility of endogenous and transfected alkaline phosphatase: A phosphatidylinositol anchored membrane protein. J Cell Biol 1987; 105:1671-1677.
93. Zhang F, Crise B, Bing S et al. Lateral diffusion of membrane-spanning and glycosylphosphatidylinositol-linked proteins: Toward establishing rules governing the lateral mobility of membrane proteins. J Cell Biol 1991; 115:75-84.
94. Brown DA, London E. Structure of detergent resistant membrane domains: Does phase separation occur in biological membranes? Biochem Biophys Res Commun 1997; 240:1-7.
95. Simons K, Ikonen E. Functional rafts in cell membranes. Nature 1997; 387:569-572.
96. Chan PY, Lawrence MB, Dustin ML et al. Influence of receptor lateral mobility on adhesion strengthening between membranes containing LFA-3 and CD2. J Cell Biol 1991; 115:245-255.
97. Tozeren A, Sung KP, Dustin ML et al. Micromanipulatiion of adhesion of a Jurkat cell to a planar bilayer membrane containing lymphocyte function-associated antigen 3 molecules. J Cell Biol 1992; 116:997-1006.

98. Lublin DM, Coyne KE. Phospholipid-anchored and transmembrane versions of either decay-accelerating factor or membrane cofactor protein show equal efficiency in protection from complement-mediated cell damage. J Exp Med 1991; 174:35-44.
99. Phelps BM, Primakoff P, Koppel DF et al. Restricted lateral diffusion of PH-20, a PI-anchored sperm membrane protein. Science 1988; 240:1780-1782.
100. Brown DA, Crise B, Rose JK. Mechanism of membrane anchoring affects polarized expressions of two proteins in MDCK cells. Science 1989; 245:1499-1501.
101. Powell SK, Cunningham BA, Edelman GM et al. Targeting of transmembrane and GPI-anchored forms of N-CAM to opposite domains of a polarized epithelial cell. Nature 1991; 353:76-77.
102. Nagarajan S, Chesla S, Cobern L et al. Ligand binding and phagocytosis by CD16 (Fc gamma receptor III) isoforms. J Biol Chem 1995; 270:25762-25770.
103. Lanier LL, Ruitenberg JJ, Phillips JH. Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. J Immunol 1988; 141:3478-3485.
104. Karpovsky B, Titus JA, Stephany DA et al. Production of target-specific effector cells using hetero-crosslinked aggregates containing anti-target cell and anti-Fcγ receptor antibodies. J Exp Med 1984; 160:1686-1701.
105. Shen L, Guyre PM, Fanger MW. Polymorphonuclear leukocyte function triggered through the high affinity Fc receptors for monomeric IgG. J Immunol 1987; 139:534-538.
106. Kimberly RP, Ahlstrom JW, Click ME et al. The glycosyl phosphatidylinositol-linked FcγRIII PMN mediates transmembrane signalling events distinct from FcγRII. J Exp Med 1990; 171:1239-1255.
107. Huizinga TWJ, Dolman KM, Van Der Linden NJM et al. Phosphatidylinositol-linked FcRIII mediates exocytosis of neutrophil granule proteins, but does not mediate initiation of the respiratory burst. J Immunol 1990; 144:1432-1437.
108. Zhang F, Schmidt WG, Hou Y et al. Spontaneous incorporation of the glycosyl-phosphatidylinositol-linked protein Thy-1 into cell membranes. Proc Natl Acad Sci USA 1992; 89:5231-5235.
109. Medof ME, Nagarajan S, Tykocinski ML. Cell-surface engineering with GPI-anchored proteins. FASEB J 1996; 10:574-586.
110. Nagarajan S, Selvaraj P. Reconstitution of CD16 expression on nucleated cells using purified CD16. FASEB J 1991; 5:A1718.(Abstr.)
111. Medof ME, Gottlieb A, Kinoshita T et al. Relationship between decay accelerating factor deficiency, diminished acetylcholinesterase activity, and defective terminal complement pathway restriction in paroxysmal nocturnal hemoglobinuria erythrocytes. J Clin Invest 1987; 80:165-174.
112. Wilcox LA, Ezzell JL, Bernshaw NJ et al. Molecular basis of the enhanced susceptibility of erythrocytes of paroxysmal nocturnal hemoglobinuria to hemolysis in acidified serum. Blood 1991; 78:820-829.
113. Gimmi CD, Freeman GJ, Gribben JG et al. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. Proc Natl Acad Sci USA 1993; 90:6586-6590.
114. Chen L, Ashe S, Brady WA et al. Costimulation of antitumor immunity by the B7 counter receptor for the T lymphocyte molecules CD28 and CTLA-4. Cell 1992; 71:1093-1102.
115. Baskar S, Ostrand S, Rosenberg N et al. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. Proc Natl Acad Sci USA 1993; 90:5687-5690.
116. Chen L, McGowan P, Ashe S et al. B7-1/CD80-transduced tumor cells elicit better systemic immunity than wild type tumor cells admixed with Corynebacterium parvum. Cancer Res 1994; 54:5420-5423.
117. Townsend SE, Allison JP. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. Science 1993; 259:368-370.
118. Townsend SE, Su FW, Atherton JM et al. Specificity and longevity of antitumor immune responses induced by B7-transfected tumors. Cancer Res 1994; 54:6477-6483.

119. Li Y, McGowan P, Hellstrom I et al. Costimulation of tumor reactive CD4+ and CD8+ T lymphocytes by B7, a natural ligand for CD28, can be used to treat established mouse melanoma. J Immunol 1994; 153:421-428.
120. Chen L, McGowan P, Ashe S et al. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. J Exp Med 1994; 179:523-532.
121. Wang Y-C, Zhu L, McHugh RS et al. Induction of autologous tumor-specific cytotoxic T-lymphocyte activity against a human renal carcinoma cell line by B7-1 (CD80) costimulation. J Immunother 1996; 19:1-8.
122. Chen PW, Shreedhar VK, Ananthaswamy HN. Rejection of murine melanoma cells transfected with the intercellular adhesion molecule-1 gene. Int J Oncol 1995; 6:675-680.
123. Cavallo F, Martin-Fontecha A, Bellone M et al. Coexpression of B7-1 and ICAM-1 on tumors is required for rejection and the establishment of a memory response. Eur J Immunol 1995; 25:1154-1162.
124. Nabel EG, Plautz G, Nabel GJ. Transduction of a foreign histocompatibility gene into the arterial wall induces vasculitis. Medical Sci 1992; 89:5157-5161.
125. Ada GL. Vaccines. In Fundamental Immunology. W.E. Paul, editor. New York:Raven Press, Ltd., 1993:1309-1352.
126. Davis HL, Demeneix BA, Quantin B et al. Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. Human Gene Ther 1993; 4:733-740.
127. Tykocinski ML, Kaplan DR, Medof ME. Antigen-presenting cell engineering. The molecular toolbox. Am J Pathol 1996; 148:1-16.
128. Rodbeck WG, Liu D, Sperry J et al. Localization of classical and alternative pathway regulatory activity within the decay accelerating factor. J Immunol 1996; 56:2528-2533.
129. Dalmasso AP, Vercellotti GM, Platt JL et al. Inhibition of complement-mediated endothelial cell cytotoxicity by decay-accelerating factor: Potential for prevention of xenograft hyper-acute rejection. Transplantation 1991; 52:530-533.
130. van den Berg CW, Cinek T, Hallett MB et al. Exogenous glycosyl phosphatidylinositol-anchored CD59 associates with kinases in membrane clusters on U937 cells and becomes $Ca(2^{+})$-signaling competent. J Cell Biol 1995; 131:669-677.
131. Wing MG, Zajicek J, Seilly DJ et al. Oligodendrocytes lack glycolipid anchored proteins which protect them against complement lysis. Restoration of resistance to lysis by incorporation of CD59. Immunology 1992; 76:140-145.
132. Brodsky RA, Jane SM, Medof ME et al. Purified CD4.DAF can incorporate into $CD4^{-}$ cells and function as a receptor for targeted HIV-mediated gene transfer. Human Gene Ther 1994; 5:1231-1239.
133. Kost TA, Kessler JR, Patel IR et al. Human immunodeficiency virus infection and syncytium formation in HeLa cells expressing glycophospholipid-anchored CD4. J Virol 1991; 65:3276-3283.
134. Keller G-A, Siegel MW, Caras IW. Endocytosis of glycophospholipid-anchored and transmembrane forms of CD4 by different endocytic pathways. EMBO J 1992; 11:863-874.
135. Marshall WL, Mittler SE, Avery P et al. Glycosylphosphatidylinositol-anchored CD4 supports human immunodeficiency virus type 1 replication, but not cytopathic effect, in T-cell transfectants. J Virol 1994; 68:4039-4042.
136. Fayen J, Huang JH, Meyerson H et al. Class I MHC alpha 3 domain can function as an independent structural unit to bind CD8 alpha. Mol Immunol 1995; 32:267-275.
137. Huang JH, Greenspan NS, Tykocinski ML. Alloantigenic recognition of artificial glycosyl phosphatidylinositol-anchored HLA-A2.1. Mol Immunol 1994; 31:1017-1028.
138. Howell S, Lanctot C, Boileau G et al. Expression of an enzymatically active glycosylphosphatidylinositol-anchored form of neutral endopeptidase (EC 3.4.24.11) in Cos-1 cells. Biochem J 1994; 299:171-176.

Index

Symbols

1,10-phenanthroline 171, 173, 176, 178
2-fluoro-2-deoxyglucose 20
5'-nucleotidase 2, 8, 74
α-bungarotoxin 78
ω site 21

A

Acetylcholinesterase 2, 5, 20, 165, 167, 169, 179, 186
ADP-ribosyl cyclase 77
ADP-ribosyltransferase 77
Aerolysin 76
Alkaline phosphatase 1, 2, 5, 31, 56, 69, 79, 165, 170, 171, 175, 176, 198
Alternative pathway 107, 134, 136
Anaphylatoxins 108
Anthrax 1, 2
Antigenic variation 137, 139, 167
Apical sorting 8, 35, 199
Apolipoprotein AI 172
Archaetidylinositol 4, 32
Axonin-1 94, 95

B

BIP/GRP78 21
Breast milk 116, 187, 188
Brefeldin A 103, 178

C

C3/C5 convertase 107, 108
Cadherins 195, 196
Campath-1 73
Carcinoembryonic antigen 74
Caveolae 8, 31, 32, 33, 36, 37, 38, 46, 51, 52, 55, 56, 91, 94, 99
Caveolin 31, 32, 33, 46, 48, 51, 54, 56
Caveolin binding motif 51
CD1 131
CD109 77
CD11b/CD18 69, 72, 75
CD130 76
CD14 46, 57, 69, 71, 131
CD157 77
CD16b 46, 69, 71, 72, 196
CD20 55, 56
CD24 72, 73, 187
CD3 ζ chain 58
CD44 55, 56, 58, 59
CD48 50, 73
CD52 5, 73
CD55 16, 56, 58, 73, 107, 118, 119, 189
CD58 73, 119
CD59 16, 20, 50, 74, 78, 108, 112, 114, 115, 116, 117, 118, 119, 120, 186, 187, 188, 189, 190
CD66 74
CD87 46, 48, 75, 76, 78, 115
CD90 76, 78
CDw108 77
Ceramide 3, 4, 6, 7, 19, 139, 140, 141
Cerebral malaria 146
Cholestasis 175, 176
Cholesterol depletion 36, 37, 50
Ciliary neurotrophic factor 46
Classical pathway 107
Clathrin 31, 36, 37, 94, 99
Coated pits 31, 36, 37, 99
Colloidal silica 32
Compactin 33
CRD epitope 171
Creutzfeldt-Jakob disease 97
Cromer antigens 111
Crossreacting determinant 165
Cruzain 140

D

Decay accelerating factor 16, 73, 107, 186
Detergent-insoluble complex 30, 32, 33, 34, 36
DiIC12 38
DiIC16 38
Diisopropylfluorophosphate 20

E

Echoviruses 73, 113
EGFR 56
Embryonic stem cells 7
Endosome 37, 38, 130, 131, 143, 145
Ethanolaminephosphotransferase 19

F

Fasciculation 91, 93, 95, 102
Fatal familial insomnia 97
Fatty acid remodeling 19
Fatty acyl-CoA 18
FcεRI 55, 56, 196
Fibronectin 74, 93, 178
Flippase 22
Fluid mosaic model 1, 29
Fluorescence quenching 33
Fluorescence recovery after photobleaching 30, 35
Folate receptor 31, 34, 37, 38
Formyl-peptide receptor 72
Fyn 6, 50, 52, 53, 55, 58, 78, 94, 95

G

G-proteins 32
GlcNAc transferase 16, 22
Glial-cell derived neurotrophic factor 43
Glycoinositol phospholipids 131
Glycosylphosphatidylinositol-specific phospholipases D 165
Glypican 5
Gp130 46, 59, 92
GPI-derived second messengers 57
GPI-rich annulus 51

H

Hemagglutinin 95, 97, 197
High density lipoprotein 71, 188
HIV 54, 113, 118, 190, 196, 197, 201
Homologous restriction 108, 114, 119, 197
Homologous restriction factor 108, 114, 119, 197
HTLV-I 119
Hyaluronan 55, 56

I

IL-1 69, 76, 111, 117
IL-10 111
IL-6 69, 72, 76, 111
Inab phenotype 111, 112, 113
Insulin 57, 169, 170
Insulin receptor 56, 57, 95
Insulin-like growth factor type II receptor 76
Integrin 43, 46, 48, 57, 59, 69, 72, 75, 76, 85, 86, 94, 172, 174, 175, 195
Isoprenoids 5

K

Keratinocytes 32, 78, 171
Kuru 97

L

Laminin 139
Lck 6, 52, 53, 54, 55, 58, 77
Lectin pathway 107
Leukemia inhibitory factor 46, 92
Levamisole 117
Lipoarabinomannans 129
Lipomannans 129
Lipophosphoglycan 55, 133, 141
Lipopolysaccharide 57, 69, 117, 131
Liquid-disordered 30, 38
Liquid-ordered 30, 33, 38
Low density lipoprotein 99, 188
LPS binding protein 57
Ly-6 56, 58, 75, 78, 115
Lyn 55

M

MAC inhibiting protein 119
Mannosamine 20
Mannose receptors 131
Mannose-6-phosphate 76
Mannosyltransferase 18, 22, 135
Melanotransferrin 78
Membrane attack complex 107, 114, 119, 142, 144
Membrane cofactor protein 107, 188, 199
Membrane inhibitor of reactive lysis 16, 114
Membrane skeleton fence 30
Merozoite surface protein 146
Microdomains 6, 8, 9, 10, 38, 43, 46, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 59, 69, 76, 78, 175
Microsomal ER 22
Mitochondrion-associated ER 22
Monensin 178
Mucins 19, 138, 139, 140, 141, 142
Muscarinic acetylcholine receptor 55
Myo-inositol 1, 2, 4, 7, 8, 15
Myristoylation 5, 34, 53

N

NAD glycohydrolase 77
Nephritic factors 108
Neural cell adhesion molecule 30, 195
Neurturin 43, 91
Nitric oxide synthase 143, 146

O

Oncostatin M 92

P

P56^{lck} 34, 113
P59^{fyn} 34, 113
P59^{hck} 143, 146
P60^{src} 34
Palmitoylation 5, 34, 53, 56
Paroxysmal nocturnal hemoglobinuria 16, 112, 186, 200
Pattern receptor 71
PDGFR 56
Peptidoglycan 71, 129, 131
Persephin 91
Phosphatidylinositol-specific phospholipase C 2, 109, 131, 165
Phosphatidylinositolmannosides 129
Photobleaching 8, 30, 35
PIG-A 7, 16, 112, 117
PKCε 143, 146
PLCγ1 55
PMSF 18, 20
Potocytosis 37
Prion 38, 91, 94, 97, 99
Procyclin 133, 136
Prostasomes 120, 188, 189
Protein kinase C 145, 146, 176
Protozoa 3, 4, 5, 7, 8, 20, 129, 131, 136, 141

Q

Qa-2 56, 79

R

Rafts 9, 29, 30, 31, 34, 35, 36, 91, 94, 95, 97, 100, 102, 199
Ras 6
Ret 43, 91
Rheumatoid arthritis 77, 108, 111, 117

S

SA85-1 138, 139
Saponin 33, 175
Schistosoma mansoni 113, 118
Scrapie 38
Seminal plasma 116, 187, 188, 189, 190
Single particle tracking 30, 35
Ssp-4 140
Stem cell antigen-2 58
Sulphonylurea 169

T

Tc-85 139
TGF-β 76, 91
Thy-1 2, 3, 8, 31, 32, 33, 48, 58, 76, 77, 78, 94, 95
TNF-α 111, 117
Toxoplasmosis 147
Trans-sialidase 112, 136, 138, 139
Transamidase 20, 21
Transferrin receptor 31, 32, 37, 137, 146
Triton X-114 100
Trk 56

U

Urokinase plasminogen activator receptor 75

V

Variant surface glycoprotein 19, 133, 165, 167, 186
VIP-21 31, 33
VIP-36 32
Vitronectin 75
VLA-4 72

W

ω site 21

Z

Zinc finger 173