Structural and Functional Roles of Glycosyl-Phosphatidylinositol in Membranes

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Glycosylated forms of phosphatidylinositol, which have only recently been described in eukaryotic organisms, are now known to play important roles in biological membrane function. These molecules can serve as the sole means by which particular cell-surface proteins are anchored to the membrane. Lipids with similar structures may also be involved in signal transduction mechanisms for the hormone insulin. The utilization of this novel class of lipid molecules for these two distinct functions suggests new mechanisms for the regulation of proteins in biological membranes.

IN THE LAST 3 YEARS, A NOVEL MECHANISM BY WHICH proteins are anchored to membranes has been elucidated. This mechanism involves a covalent linkage from the protein to an oligosaccharide which is in turn glycosidically linked to phosphatidylinositol. The resulting class of membrane glycophospholipids, termed glycosyl-phosphatidylinositols, have now been detected in a wide variety of eukaryotic cells [reviewed in (*1, 2*)]. For the most part, their precise chemical structures remain unresolved, although certain structural elements are conserved. Additionally, molecules with similar properties have been implicated in the transmembrane signaling function of the hormone insulin (*3*). The involvement of these glycophospholipids in both the anchoring of proteins to membranes and insulin action is somewhat surprising and suggests the potential for a novel mechanism in the hormonal regulation of membrane protein function. In this review we survey the recent work on the occurrence of glycosyl-phosphatidylinositols in biological membranes and attempt to explore their functional significance.

Anchoring of Membrane Proteins by Phosphatidylinositol

Although a specific role for phosphatidylinositol in the attachment of proteins to membranes was first demonstrated for alkaline phosphatase (APase) in 1980 (*4*), preliminary evidence for such a function was available as much as 20 years earlier (*5*). These studies showed that treatment of membranes with highly specific bacterial phospholipases, the phosphatidylinositol-specific phospholipases C (PI-PLC), released a number of hydrolytic enzymes (for example, alkaline phosphatase, 5′-nucleotidase, and acetylcholinesterase) from membranes in a water-soluble, nonaggregated form that retained full activity but was unable to reassociate with the membrane (*4, 6–9*). The release of these proteins was not a consequence of nonspecific alterations in the membrane microenvironment since PI-PLC could also remove the hydrophobic attachment site from proteins solubilized from the membrane by detergents or organic solvents (*4, 9*). Thus, the liberation of these proteins from the membrane was the result of a selective removal of the membrane anchoring domain by PI-PLC, leading to the proposal that membrane attachment was entirely the result of a covalently linked phosphatidylinositol molecule (*4, 9*). This novel concept was supported by the detection of covalently attached *myo*-inositol in acetylcholinesterase (AChE) from the electric organ of *Torpedo* (*10*) and alkaline phosphatase from human placenta (*11*).

The ability of bacterial PI-PLC to release proteins attached to the membrane by this mechanism has permitted the identification of several additional phosphatidylinositol-anchored proteins in the last 3 years (Table 1). This group of proteins is both evolutionarily and functionally diverse. It includes seven distinct hydrolytic enzymes, a complement regulatory protein [decay accelerating factor (DAF)], neural and lymphocyte cell adhesion molecules (N-CAM and LFA-3), a protective coat protein in the parasitic protozoan *Trypanosoma brucei* [variant surface glycoprotein (VSG)], and the scrapie prion protein (PrP), as well as a number of antigens of specific cellular distribution but unknown function. The only known property common to these proteins (with the notable exception of the zymogen granule membrane protein GP-2; see Table 1) is their location at the cell surface. In addition, three membrane proteins with cytoplasmic orientations (namely, myelin basic protein, styrene oxide hydrolase, and ornithine decarboxylase) have been proposed to contain covalently attached phosphoinositides (*12*), but there is no evidence that phosphatidylinositol is involved in attachment of these proteins to the membrane. In the specific case of myelin basic protein, the phosphoinositide may be attached to only a small proportion of the protein molecules (*13*). Although utilization of phosphatidylinositol anchoring by cytoplasmically oriented membrane proteins remains to be established, this possibility should not be excluded, since indirect evidence suggests that hormonally sensitive lipids with similar structures occur in this location (see below).

Structure of the Glycosyl-Phosphatidylinositol Anchor

Knowledge of the detailed structure of the glycosyl-phosphatidylinositol anchors largely derives from studies done with the variant surface glycoprotein of the protozoal parasite *Trypanosoma brucei* (*14–16*). The experimental strategies developed with this protein, which is available in relatively large amounts, have served as a model for similar structural studies of other less abundant proteins that use this mechanism of anchoring. The major features of this structure

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are illustrated in Fig. 1 and can be summarized as follows: (i) an ethanolamine that is amide-linked to the α-carboxyl group of the COOH-terminal amino acid; (ii) a phosphodiester linkage between the hydroxyl of the ethanolamine and the 6-hydroxyl on a mannose; (iii) this mannose is part of a glycan which contains a glucosamine residue with a free amino group at its reducing terminus; the presence of this unusual sugar allows the structure to be selectively cleaved at this point by nitrous acid; and (iv) the glucosamine is glycosidically linked at the C-1 position to the 6-hydroxyl on the inositol ring of a phosphatidylinositol molecule. It has been known for some time that different variants of VSG have different amounts of galactose (up to eight residues), suggesting a certain heterogeneity in the composition of the glycan region. There is evidence that even the structure of the glycan in an individual variant of VSG exhibits microheterogeneity with respect to galactose composition (*15*). Thus, the overall sugar composition for any particular variant is probably the average of several different molecular species. On the basis of detailed analysis of individual molecular species it has been proposed that the anchor has an unbranched core glycan structure of Man6P$(Man)_2$GlcN, which is then further modified with a variable number of galactose residues (*15*). However in another study in which a different variant of VSG was used, a 1–4 linkage between the glucosamine and the inositol, and a branched structure for the three mannose residues, has been proposed (*16*). It is not yet clear whether these differences reflect genuine structural variants or are methodological in origin.

It was proposed (*1*), on the basis of chemical composition studies in other proteins, that elements of this unusual and complex structure would be found in the anchors of all the PI-PLC–releasable proteins. Recent data tend to support this proposition. The presence of ethanolamine, glucosamine, or *myo*-inositol, or sensitivity of the anchoring region to nitrous acid, has been demonstrated either by chemical analysis or biosynthetic labeling techniques for all six of the proteins analyzed so far [VSG (*14, 15*), Thy-1 (*17*), AChE (*11, 18*), DAF (*19*), PrP (*20*), and APase (*11, 21*)]. In spite of the conservation of these structural elements in a wide range of proteins, a number of variants of the anchors have now been identified. A second (and in some cases a third) ethanolamine residue, which is not observed in VSG, has been found in the mammalian proteins Thy-1 (*17*), AChE (*18*), DAF (*19*), and PrP (*20*). The presence of a free amino group suggests that the additional ethanolamine is linked to the remainder of the structure through its hydroxyl group, although the involvement of a phosphodiester linkage (as for the single ethanolamine in VSG) has not yet been demonstrated. A rare inositol isomer, *chiro*-inositol, not normally present in phosphatidylinositol, has been found in purified AChE, APase, and DAF (*10, 11, 19*), although it is not clear whether this is covalently associated with the protein. In addition, significant amounts of *chiro*-inositol have been detected in the purified inositol-glycans produced in response to insulin (see below), suggesting that this isomer may commonly occur in the glycosyl-phosphatidylinositols.

Another apparent structural variation was suggested by the observation that PI-PLC treatment released 100 percent of the AChE from bovine and pig erythrocytes but only 5 to 10 percent from human erythrocytes (*8*). This curious phenomenon was subsequently confirmed when only a fraction of the purified, detergent-solubilized human erythrocyte AChE was hydrolyzed by PI-PLC (*18*). Another human erythrocyte protein, DAF, is similarly resistant to this cleavage (*22*). It is interesting that the principal lipid moiety released from human erythrocyte AChE and DAF by nitrous acid did not copurify with phosphatidylinositol (*19, 23*), and recent studies with AChE suggest that this may be due to substitution of the 2-OH on the inositol ring with an ester-linked palmitic acid residue (*23*). Since the lipid anchors for these human erythrocyte proteins are known to contain certain structural components of the glycosyl-phosphatidylinositol anchor [that is, presence of ethanolamine, inositol, and fatty acids, and the sensitivity to nitrous acid or the anchor-specific phospholipase D (*18, 19, 23, 24*)], it seems most likely that the anchoring lipid is a glycosyl-phosphatidylinositol, but a modification of the inositol ring at the 2-OH with the palmitate renders the lipid insensitive to PI-PLC. Partial resistance to release by PI-PLC has also been observed with other proteins (5′-nucleotidase, Thy-1, Qa, and APase) in a variety of cell types, but at present there is no evidence to suggest that these are due to a similar molecular modification (*7, 25–27*).

Variations in the fatty acid composition have also been identified. The VSG and PrP anchors contain only myristate and stearate, respectively (*14, 20*). By contrast, several fatty acids are present in differing proportions in the anchors of Thy-1, AChE, and DAF (*17–19*). Furthermore, recent evidence suggests that the glycosyl-phosphatidylinositol anchor for bovine and human erythrocyte acetylcholinesterase has a 1,2-alkylacylglycerol rather than a 1,2-diacylglycerol structure, and it should be emphasized that this is the only

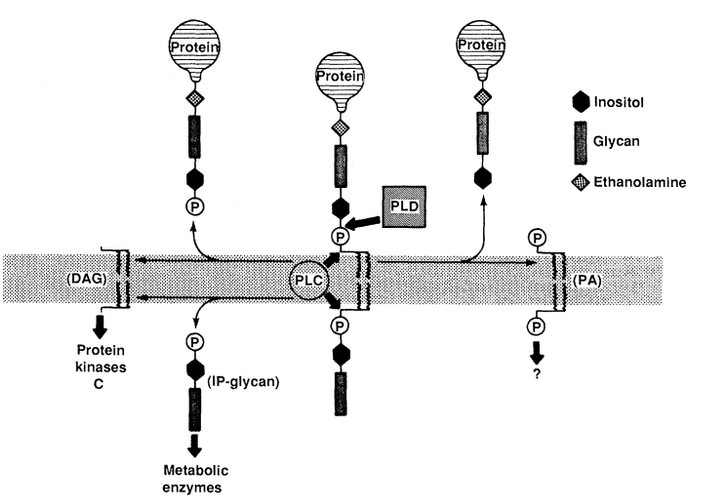

Fig. 1. The probable distribution and metabolic relationships of the two major types of glycosyl-phosphatidylinositol (whose properties are summarized in Table 2) are illustrated schematically. Hydrolysis of the cell-surface form of the molecule can result in the release of proteins anchored by glycosyl-phosphatidylinositol. This reaction may provide a mechanism for regulating the concentration of these proteins at the cell surface and possibly altering their function. A specific phospholipase C (PLC) capable of performing this reaction has been identified in trypanosomal and liver membranes (*49, 50*). The products are a soluble protein containing an exposed glycosyl-inositol-phosphate and a 1,2-diacylglycerol (DAG), remaining in the membrane, which is an activator of protein kinase C (*83*). Additionally, the hydrolysis could be catalyzed by a specific, soluble phospholipase D (PLD), which may be located extracellularly (*24, 46, 51*). The products of this reaction are a soluble protein containing an exposed glycosyl-inositol (without phosphate) and a phosphatidic acid (PA) molecule, which may have additional biological activities through unknown mechanisms (*85–87*). On the cytoplasmic aspect of the plasma membrane, glycosyl-phosphatidylinositols can also be hydrolyzed by a specific phospholipase C that may be insulin-sensitive. The products of this reaction may serve as second messengers for insulin action (*3*). These include phosphorylated forms of inositol-glycans (IP-glycans), which may regulate metabolic enzymes and also 1,2-diacylglycerol (*29, 30, 58*). Although one phospholipase C has been detected (*49, 50*) that can cleave both types of glycosyl-phosphatidylinositol, there is no reason to suppose that a single enzyme is responsible for the hydrolysis of both molecules in vivo. These observations do not preclude the existence of other phospholipases with related specificities that remain to be identified.

mammalian protein for which information on the fatty acid linkages is available (*23*). An alkylglycerol structure has also been observed in the lysophosphatidylinositol anchor of the lipophosphoglycan of *Leishmania donovani* (*28*). The functional significance of these variations in the glycerol lipid moiety of the anchor are not known. However, it is interesting that the insulin-sensitive glycosyl-phosphatidylinositol in BC_3H1 myocytes contains a myristic acid–rich diacylglycerol structure (*29*), whereas in the H35 hepatoma cell a 1,2-alkylacylglycerol structure has been proposed for this lipid (*30*). It is possible that these variations in the fatty acid linkages and compositions of glycosyl-phosphatidylinositols among different cell types are not relevant to their protein-anchoring function but may profoundly influence their sensitivity to endogenous phospholipases C or D, as well as the biological activity of the glycerolipid derivative resulting from these hydrolysis reactions, as discussed below.

Biosynthesis of the Glycosyl-Phosphatidylinositol Anchor

The details of the biosynthesis and assembly of the glycosyl-phosphatidylinositols are largely unknown. In *T. brucei*, attachment of lipid anchor components can be detected within 1 minute of translation of the VSG polypeptide (*31*). The rapidity of this assembly process suggests that the protein is attached to a preformed anchor precursor lipid en bloc, rather than individual components being attached to the protein in a stepwise fashion. Candidates for this anchor precursor lipid with the predicted structural features of the glycosyl-phosphatidylinositols have been identified in *T. brucei* (*32, 33*). These lipids apparently contain glucosamine, mannose, and ethanolamine, but not galactose (*33*). The presence of a free amino group on the ethanolamine suggests a direct insertion of this molecule with the concerted replacement of the COOH-terminal peptide. Additionally the absence of galactose in this precursor indicates that the variable galactose region in the anchor is added after attachment to the protein.

In mammalian cells, lipids with similar properties have been identified that are proposed to be involved in insulin action, possibly as a source of enzyme-modulating second messengers (see below) (*29, 30*). These lipids contain phosphatidylinositol glycosidically linked to an oligosaccharide through glucosamine, but do not appear to contain ethanolamine or any attached protein. Table 2 compares the properties of the free glycosyl-phosphatidylinositols with those utilized for protein anchoring.

Preliminary studies in BC_3H1 cells (*34*) and liver microsomes (*35*) indicate that synthesis of the free glycosyl-phosphatidylinositol follows that of phosphatidylinositol, suggesting the possibility that these lipids arise from the glycosylation of a specific pool of phosphatidylinositol. Although the subcellular site of the insulin-stimulated degradation of these lipids is in the plasma membrane, their transbilayer distribution is uncertain. However, teleological arguments suggest a cytoplasmic orientation, since the water-soluble degradation products (generated in response to insulin) are intracellular. The tentative identification of these different forms of glycosyl-phosphatidylinositol on opposite sides of the plasma membrane has raised questions relating to the topological relationship of the biosynthetic processes that have not been resolved. Presumably the early stages of biosynthesis of glycosyl-phosphatidylinositols occur by glycosylation of phosphatidylinositol on the cytoplasmic aspect of the endoplasmic reticulum. Upon attaining a certain level of glycosylation, molecules destined for protein anchor biosynthesis might then be translocated across the membrane. This translocation step may in fact serve to segregate further biosynthetic modifications of the lipid molecules destined for protein attachment from those that will remain on the cytoplasmic face. Thereafter, transport of these intracellular membranes to the cell surface would result in a cytoplasmically oriented free lipid and a cell surface–oriented lipid protein anchor. Although the membrane translocation of a lipid molecule with a bulky polar head group such as a glycosyl-phosphatidylinositol might seem inherently unlikely, we note the precedent of the translocation of the $(Man)_5(GlcNAc)_2$-lipid precursor for *N*-linked glycosylation of proteins in the endoplasmic reticulum (*36*). At this stage, it is premature to regard the cytoplas-

Table 1. Cell surface proteins with a glycosyl-phosphatidylinositol membrane anchor. Most of the proteins listed are widely distributed in mammalian tissues; information on some proteins with specific cellular distributions that are not obvious from their names is given in parentheses.

Protein	References
Hydrolytic enzymes	
Alkaline phosphatase	(*4–6, 11, 21*)
5′-Nucleotidase	(*7, 27*)
Acetylcholinesterase	(*8–11, 18, 23*)
Alkaline phosphodiesterase	(*88*)
Trehalase	(*89*)
p63 protease (*Leishmania major*)	(*90*)
Renal dipeptidase	(*91*)
Merozoite protease (*Plasmodium falciparum*)	(*92*)
Mammalian antigens	
Thy-1	(*17, 25, 26, 41*)
RT-6 (rat lymphocytes)	(*93*)
T cell–activating protein (TAP) and other Ly-6 antigens	(*26, 94*)
Qa	(*26, 43, 95*)
Carcinoembryonic antigen	(*74*)
Blast-1 (human lymphocytes)	(*96*)
CD-14 (human monocytes)	(*97*)
Cell adhesion	
Neural cell adhesion molecule (N-CAM)	(*98*)
Heparan sulfate proteoglycan	(*81*)
LFA-3 (human lymphocytes)	(*99*)
Contact site A (*Dictyostelium discoideum*)	(*100*)
Protozoal coat proteins and antigens	
Variant surface glycoprotein (*Trypanosoma brucei*)	(*14–16*)
Surface proteins (*Paramecium primaurelia*)	(*78*)
Ssp-4 (*Trypanosoma cruzi*)	(*75*)
Miscellaneous	
Decay accelerating factor	(*19, 22*)
130-kD hepatoma glycoprotein	(*55*)
34-kD placental growth factor	(*80*)
Scrapie prion protein	(*20*)
Tegument protein (*Schistosoma mansoni*)	(*101*)
PH-20 protein (guinea pig sperm)	(*102*)
GP-2 (pancreatic zymogen granule)	(*103*)

Table 2. Comparison of properties of plasma membrane glycosyl-phosphatidylinositols. This table summarizes the principal characteristics of the glycosyl-phosphatidylinositols for the purpose of general comparison only. For more complete details references cited in text should be consulted.

Properties	Protein-linked lipid	Free lipid
Main biological function	Protein anchoring	Signal transduction
Hydrophobic domain	1,2-diacylglycerol or 1,2-alkylacylglycerol	1,2-diacylglycerol or 1,2-alkylacylglycerol
Degradation stimulated by insulin	?	Yes
Phospholipase C sensitivity	Yes	Yes
Phospholipase D sensitivity	Yes	?
Nitrous acid cleavage product	Phosphatidylinositol	Phosphatidylinositol
Glucosamine	Yes	Yes
Ethanolamine	Yes	No
Subcellular location	Cell surface	Intracellular
Approximate size of glycan*	4 to 12	3 or 4

*Number of sugar residues (excludes *myo*-inositol).

mically oriented, insulin-sensitive glycosyl-phosphatidylinositol molecules as direct "precursors" of the protein anchors, but the apparent absence of ethanolamine and the smaller size of the polar head group in the former group of lipids is consistent with this sequence of events (*29, 30*).

Although attachment of the protein to glycosyl-phosphatidylinositol seems to be relatively rapid and probably occurs as an early post-translational event in the lumen of the endoplasmic reticulum (*31*), the enzyme (or enzymes) involved in this process is completely unknown. In the case of at least two of the proteins (VSG and Thy-1) a proteolytic processing event at the COOH-terminus must either precede or be simultaneous with lipid attachment, since complementary DNA (cDNA) and genomic sequences predict the existence of additional amino acid residues at the COOH-terminus that are not present in the mature protein (*37*). It is not known whether this occurs in the other proteins shown in Table 1, since cDNA-predicted sequences and amino acid sequences derived from the mature proteins have not been compared. However, the analyses of the cDNA sequences so far available [(*38–40*); see (*2*) for additional references] predict the existence of similar, short (10 to 20 residues) hydrophobic sequences at the COOH-terminus, suggesting that a proteolytic event occurs during attachment to the anchor. If this proteolysis is simultaneous with attachment to a glycosyl-phosphatidylinositol containing a terminal ethanolamine residue, then the process might not require the input of additional metabolic energy. It has been proposed that a Thy-1$^-$ mutant lymphoma cell line is unable to express Thy-1 on the cell surface as a result of its inability to make the precursor lipid (*41*). These cells also appear to be unable to remove the COOH-terminal peptide from the nascent polypeptide chain. One interpretation of these results is that the protein is attached to the lipid by a concerted mechanism catalyzed by a single enzyme. The enzyme carrying out this reaction (a "transamidase") might even utilize the hydrophobic COOH-terminal sequence to attain the correct orientation of the polypeptide cleavage site close to the bilayer surface and the exposed amino group of the anchor precursor lipid.

Recent evidence also suggests that the 10- to 20-residue hydrophobic COOH-terminal sequence acts as a signal that directs lipid attachment, analogous to the NH_2-terminal cotranslational insertion signals or leader peptides. Transfection with hybrid gene constructs containing the coding region for the 37 amino acid residues at the COOH-terminus of DAF and a truncated form of the herpes simplex virus glycoprotein D gives rise to a lipid-anchored form of this normally secreted protein (*42*). Similar transfection experiments (*43*) with Qa hybrid gene constructs indicate that the presence of the COOH-terminal region from Qa can cause the NH_2-terminal domains of H-2 (a protein normally attached by a COOH-terminal transmembrane and cytoplasmic domain) to become lipid anchored. Conversely, when a Qa construct containing the COOH-terminal region of H-2 was used, the expressed protein was not lipid anchored (*43*). An analogous situation may occur naturally with N-CAM. This molecule may be anchored either by glycosyl-phosphatidylinositol or a transmembrane plus cytoplasmic domain. Recent studies suggest that the lipid-anchored form of N-CAM arises by differential messenger RNA (mRNA) processing that results in a mature mRNA species with a unique 3′ sequence coding for a 25–amino acid residue COOH-terminal peptide, the last 15 residues of which are hydrophobic (*39*). Alternatively, differential mRNA splicing may result in the absence of a COOH-terminal hydrophobic sequence and the production of soluble or "secreted" forms of DAF, Qa, and AChE lacking the lipid anchor (*40, 43*). It has been shown that removal of as few as 11 residues from the COOH-terminus by deletion mutagenesis leads to the production of a form of APase that is secreted rather than lipid anchored (*44*). Although such studies have indicated the importance of a hydrophobic COOH-terminal signal peptide for lipid attachment, the precise nature of the information contained in this sequence will remain uncertain until the exact locations of the processing sites are identified.

Enzymic Hydrolysis of Glycosyl-Phosphatidylinositols

A number of phospholipases with clear specificity for phosphoinositides have been described. These can be divided into four distinct groups. (i) The Ca^{2+}-dependent, phosphoinositide-specific phospholipases C found in the cytosol or associated with membranes of many mammalian tissues. These enzymes are capable of hydrolyzing phosphatidylinositol and its 4-phosphate and 4,5-bisphosphate derivatives to generate the intracellular second messengers 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (*45*) but thus far appear to have no activity towards glycosyl-phosphatidylinositol (*46, 47*). (ii) The phosphatidylinositol-specific phospholipases C that are secreted by several bacteria (*48*). These enzymes are metal ion–independent and can hydrolyze both phosphatidylinositol and glycosyl-phosphatidylinositol structures, but since glycosyl-phosphatidylinositol–anchored proteins have not been described in prokaryotes, their biological role is obscure. (iii) The phospholipases C with specificity for glycosyl-phosphatidylinositol that have been purified from *T. brucei* (*49*) and rat liver plasma membranes (*50*). (iv) A phospholipase D with specificity for glycosyl-phosphatidylinositol that has been identified in mammalian tissues and plasma (*24, 46, 51*). Since the purpose of this section is to discuss the enzymes that might be involved in metabolism of glycosyl-phosphatidylinositols, (i) and (ii) will not be considered further.

Both the mammalian phospholipase D and the trypanosomal phospholipase C were originally discovered as a result of their autolytic anchor-degrading effects on alkaline phosphatase and VSG respectively, observed when cells or tissues were disrupted or extracted prior to purification of these proteins (*4, 52*). The trypanosomal phospholipase C was purified in several laboratories (*49*) and shown to be thiol-dependent but insensitive to inhibition by EGTA. It has a molecular size of 37 to 40 kilodaltons (from SDS gel electrophoresis) and behaves as a monomer in detergent. It has relatively low activity against free phosphatidylinositol but will catalyze the solubilization of several different glycosyl-phosphatidylinositol–anchored proteins. The precise subcellular location of this enzyme is unknown, as is its mechanism of regulation in vivo. In spite of the recent progress made in the characterization of this enzyme, there is as yet no evidence that rapid VSG release by the phospholipase C is relevant to antigenic variation or cellular transformation in the mammalian or insect hosts (*53*). It is possible that the degradation products of the phospholipase C–catalyzed reaction, the released VSG and diacylglycerol, aid in the survival of the organism. The release of VSG from a trypanosome, either in response to an external stimulus or an injury, might interfere with the function, metabolism, or processing of other released proteins in the host organism (see below). In this regard it is interesting that activation of the trypanosomal phospholipase C and degradation of the VSG anchor result from the osmotic lysis of these parasites (*14, 52*).

A phospholipase C has recently been purified from rat liver plasma membranes that has some similarities to the trypanosomal enzyme (*50*). It catalyzed the hydrolysis of both the VSG anchor from trypanosomes and the glycosyl-phosphatidylinositol from BC_3H1 cells. This enzyme has been proposed to be responsible for the intracellular generation of 1,2-diacylglycerol and inositol-glycan in response to insulin (see below). It is thiol-dependent, active as a

monomer in detergent, insensitive to inhibition by EGTA, and specific for glycosyl-phosphatidylinositols. However, it does appear to be distinctly larger than the trypanosomal enzyme (52 kD by SDS–polyacrylamide gel electrophoresis), leading to the speculation that the distinct molecular structures of the mammalian and protozoal enzymes are related to differences in their cellular regulation.

The anchor-specific phospholipase D seems to be quite different from the phospholipases C described above. This enzyme, originally identified as a result of its ability to degrade the anchor of alkaline phosphatase during extraction from the membrane, was thought to reflect the action of an intracellular phosphoinositide-specific phospholipase C (*4, 54, 55*). However, subsequent studies revealed that the anchor-degrading activity observed in tissues and membrane fractions was due to a novel, anchor-specific phospholipase D (*56*). This enzyme has also been demonstrated in high levels in plasma (*24, 46, 51*), and it is possible that the anchor-degrading activity previously observed in the tissues (*4, 54, 55*) is due to contamination with the plasma enzyme. The phospholipase D is thiol-dependent and sensitive to inhibition by EGTA and 1,10-phenanthroline (*24, 46, 51, 56*). Although it has not been purified to homogeneity, a functional molecular size of approximately 500 kD was determined by gel filtration, suggesting that it is present in plasma as a multimer or in a complex with other proteins or lipids (*46*). This enzyme can catalyze the hydrolysis of several different phosphatidylinositol-anchored proteins (APase, 5′-nucleotidase, DAF, VSG, and AChE) and the anchor precursor lipid for VSG, but has little or no activity against other phospholipids.

The physiological role of these three phospholipases has not been determined, but their high degree of specificity and wide distribution is consistent with the proposition that they are important in regulating the functions of the glycosyl-phosphatidylinositols.

Proposed Role in Insulin Action

The molecular mechanisms by which insulin elicits its numerous metabolic responses have been the subject of intense research over the past several decades. Much of this effort has been devoted to the search for a second messenger, a substance produced from the plasma membrane in response to insulin that mediates some of the intracellular effects of the hormone (*3, 57*). Although such insulin-sensitive, enzyme-modulating substances were detected by several laboratories, their precise chemical structures proved difficult to establish. Two such activities were isolated that exhibited the properties of low molecular weight oligosaccharide-phosphates with differing net negative charges (*58*). Their generation by insulin was reproduced by the addition of the *Staphylococcus aureus* PI-PLC to liver plasma membranes, indicating that inositol-phosphate is a component. A phosphatidylinositol-containing glycolipid precursor was identified that gave rise to the enzyme modulators after digestion with this PI-PLC (*58*). The metabolic labeling of both the precursor and products with [^{3}H]inositol and [^{3}H]glucosamine (*29*) suggested that the enzyme-modulating activities were structurally related inositol-glycans derived from a lipid with apparent structural homology to the glycosyl-phosphatidylinositol protein anchor (Table 2).

Structure of inositol-glycans. Studies of the chemical structure of the inositol-glycans and their lipid precursor have so far been limited to evaluation of biological activity and chromatographic behavior after chemical treatments. The enzyme-modulating activities were acid and heat stable, but were lost after methylation, acetylation, periodate oxidation, and nitrous acid deamination (*58*). The presence of inositol in these molecules was validated by their production with the *S. aureus* PI-PLC (*29, 30, 58–60*) and a glycosyl-phosphatidylinositol–specific phospholipase C from liver (*50*), insulin-sensitive [^{3}H]inositol labeling (*29, 30*), and mass spectral analysis after hydrolysis (*60*). Glycosidically linked, nonsubstituted hexosamine was suggested by insulin-sensitive [^{3}H]glucosamine incorporation (*30*) and nitrous acid sensitivity (*30, 58*). The composition and orientation of the remaining monosaccharides distal to the hexosamine remain to be determined, although preliminary metabolic labeling studies indicate the presence of at least one mannose residue (*61*). An inositol glycophospholipid that had identical properties and that was labeled with inositol, glucosamine, inorganic phosphate, and fatty acids was identified in adrenocortical cells (*62*).

Digestion of the glycosyl-phosphatidylinositol precursor from BC_3H1 cells with phospholipase C indicated that the hydrophobic portion of the molecule contained 1,2-diacylglycerol (*29*). However, Mato *et al.* (*30*) suggested a 1,2-alkylacylglycerol structure based on the relative alkaline resistance of a similar glycosyl-phosphatidylinositol isolated from the H35 hepatoma cell line. More recent studies with the glycosyl-phosphatidylinositol derived from this cell line have indicated the presence of variable but significant amounts of *chiro*-inositol (*60*). This observation is of some interest, since variable amounts of this unusual inositol isomer have also been observed in the glycosyl-phosphatidylinositol–anchored proteins (*10, 11, 19*). As was described for the VSG glycosyl-phosphatidylinositol anchor from *T. brucei* (see above), there appears to be some heterogeneity of these lipids in liver and muscle cells, since multiple species of this insulin-sensitive glycosyl-phosphatidylinositol have now been chromatographically resolved (*35*). Whether these distinct substances represent different stages in the biosynthesis of the glycolipid or perhaps molecules with distinct cellular functions remains to be determined.

Regulation of hydrolysis. The mechanism by which insulin regulates the cleavage of the glycosyl-phosphatidylinositols is unclear. It appears that insulin-receptor interaction leads to a rapid, dose-dependent activation of a distinct and selective phospholipase C that hydrolyzes this glycosyl-phosphatidylinositol, since both the inositol-glycan and 1,2-diacylglycerol are produced in response to insulin in BC_3H1 cells (*29*). The glycosyl-phosphatidylinositol–specific phospholipase C from rat liver plasma membranes described above is a good candidate for this putative enzyme. The insulin-sensitive pool of glycosyl-phosphatidylinositol appears to be predominantly localized on the cytoplasmic face of the plasma membrane, since extensive treatment of BC_3H1 cells with PI-PLC does not alter the ability of insulin to stimulate the intracellular accumulation of inositol-glycan (*61*). In addition, the labeling of the glycosyl-phosphatidylinositol identified in adrenocortical cells was stimulated five- to tenfold by serum, further suggesting a role for hormonal regulation in the turnover of these lipids (*62*). While the precise molecular events involved in the activation of the hormone-sensitive phospholipase C are unknown, the regulation of similar hormone-effector systems by guanyl nucleotides and studies of the effects of toxins (*64*) or antibodies to guanine-nucleotide binding proteins (*65*) on insulin action suggest a role for a G-protein as an intermediate in coupling of the insulin receptor to the phospholipase C. Moreover, some G-proteins appear to be relatively good substrates for the insulin receptor kinase (*66*), suggesting a possible high-affinity interaction between the receptor and a guanosine triphosphate–binding, coupling protein. Along these lines, a recent report has suggested that insulin-stimulated [^{3}H]myristate diacylglycerol production can be completely blocked by pretreatment of BC_3H1 cells with pertussis toxin (*67*).

Action of inositol-glycans. The inositol-glycan regulates the activities of several insulin-sensitive enzymes measured in cell-free assays, including cyclic adenosine monophosphate (cAMP) phosphodies-

terase (*58, 68*), adenylate cyclase (*68*), pyruvate dehydrogenase (*68*), and phospholipid methyltransferase (*59*). The precise biochemical mechanism (or mechanisms) involved in the modulation of these enzymes is unclear, but the effects of the inositol-glycan may be mediated by control of protein phosphorylation, perhaps due to activation of specific protein phosphatases (*68*). Although a role for these compounds as second messengers of insulin action remains unproven, they are clearly produced rapidly enough and in sufficient quantity to account for some of the metabolic actions of the hormone, especially those involved in the regulation of lipid metabolism. Further support for this possibility emerges from recent studies demonstrating an insulin-mimetic effect of these compounds on glucose utilization and lipolysis (*63, 69*) but not glucose transport (*69*) in intact adipocytes. It is interesting that the biological activities of these purified substances on intact cells could be blocked with millimolar concentrations of inositol monophosphate, indicating the possibility of a specific cellular transport system that recognizes the inositol phosphate portion of the molecule (*63*). At present, however, there is no evidence that the inositol-glycans are released from cells after hormonal stimulation, so the significance of these findings remains obscure. Establishing a definitive role for these compounds as intracellular second messengers will rely on the determination of their precise chemical structures, their organic syntheses, and reevaluation of their biological activities. However, our knowledge of the mechanisms of insulin action suggests that the inositol-glycans may be involved in mediating only a subset of the pleiotropic actions of insulin.

Possible Functions of Glycosyl-Phosphatidylinositol

The evidence cited above has demonstrated the involvement of glycosyl-phosphatidylinositol in the anchoring of a diverse group of membrane proteins and as a precursor for what may be an important second messenger of insulin action. Although many questions remain, a reasonably consistent picture has emerged concerning the structure, biosynthesis and metabolism of these molecules. In contrast, we can still only speculate about the full biological significance of glycosyl-phosphatidylinositols. While the existence of glycosylated species of phosphatidylinositol on the inner leaflet of the plasma membrane has clear ramifications for insulin action, utilization of similar lipids on the cell surface merely as inert structural anchors seems unlikely, since other membrane protein–anchoring mechanisms exist that do not involve covalently attached lipid. Thus, it is possible that the glycosyl-phosphatidylinositol anchor might confer upon a protein unique physical properties or susceptibility to hormonal regulation.

Mobility. Most cell-surface proteins with a large extracellular domain are anchored by a relatively short, hydrophobic transmembrane region linked to a cytoplasmic domain. By contrast, the glycosyl-phosphatidylinositol anchor is located entirely within the outer leaflet of the bilayer. In the case of many membrane-spanning proteins, interactions between the cytoskeleton and cytoplasmic domains can reduce the lateral mobility [$D \sim 10^{-10}$ cm^2/sec (*70*)]. The glycosyl-phosphatidylinositol–anchored proteins would not be subject to such constraints and should therefore be inherently more mobile. In this regard, fluorescence photobleaching studies have shown that the mobile fractions of Thy-1, DAF, APase, and PH-20, which generally constitute at least one-half of the total, have relatively high diffusion coefficients ($D > 10^{-9}$ cm^2/sec) compared to other cell-surface proteins (*71*). The diffusion coefficient of VSG in *T. brucei* is approximately one-tenth of this value, but this lower mobility does not appear to be due to some peculiarity of the parasite cell surface, since purified VSG exhibited a similarly low diffusion coefficient when implanted into BHK cells (*72*). Although these high diffusion coefficients may confer novel properties on lipid-anchored proteins, the functional significance of increased mobility is not obvious. Furthermore, variations in the size of the mobile fraction for different proteins are observed which have not been explained (*71, 72*). It has been suggested (*22*) that high mobility of DAF may be a prerequisite for it to interact randomly with and inhibit membrane-associated and potentially lytic C3b and C4b complement fragments. Since the precise physiological functions or mechanisms of action of most of the other proteins in Table 1 are not known, it is possible that a requirement for high lateral mobility is a common feature. For example, rapid lateral redistribution of a protein might play a role in cell-cell interaction (for example, as for N-CAM, heparan sulfate proteoglycan, and LFA-3), reception or transduction of extracellular stimuli (for example, by Thy-1, TAP, LFA-3, and RT-6) or genesis of cellular polarity of certain glycosyl-phosphatidylinositol–anchored proteins.

Release of proteins from the cell surface. Identification of glycosyl-phosphatidylinositol hydrolyzing enzymes (see above) suggested that one function of the anchor might be to allow the rapid release of proteins (*1*). This is supported by a number of reports indicating that glycosyl-phosphatidylinositol–anchored proteins can be released in vivo or from intact cells in culture. APase is released into the serum after bile duct ligation, fat ingestion, or during pregnancy, as well as in a variety of diseases (*73*). A hydrophilic form of carcinoembryonic antigen also appears to be released into the serum of colon cancer patients (*74*), whereas Ssp-4, a major, stage-specific protein in amastigotes of the parasitic protozoan *T. cruzi* is released during in vitro transformation (*75*). In some cases release can also be increased by extracellular stimuli. AChE is released from certain areas of the brain and adrenal chromaffin cells in response to a variety of stimuli (*76*), Qa-2 is secreted from concanavalin A–stimulated T lymphocytes (*77*), and surface antigenic variation in the free-living protozoan, *Paramecium primaurelia*, occurs in response to alterations in environmental parameters (*78*). At present, direct involvement of endogenous, anchor-specific phospholipases in the release of these glycosyl-phosphatidylinositol–anchored proteins has not been demonstrated, and the role of these enzymes in the regulation of anchored proteins in vivo remains uncertain.

Hydrolysis of the anchor might be advantageous in the regulation of the concentration of a protein at the cell surface as well as its rate of regulated secretion. In consideration of the former possibility, the release of proteins involved in adhesion or homing of cells (N-CAM$_{120}$, LFA-3, and heparan sulfate proteoglycan), in reception or transduction of extracellular stimuli [Thy-1, TAP, LFA-3, and RT-6 (*79*)], or as a protective coat in parasites (VSG) might provide an effective means for the termination of these cell-surface events. Moreover, this phenomenon may provide a mechanism for homologous or heterologous desensitization, especially relevant to the development of tolerance in immunological systems. Once released, the proteins could acquire altered or enhanced enzymatic function or behave as paracrine or autocrine factors. For instance, preliminary evidence suggests that a placenta-derived 34-kD polypeptide with autocrine growth factor properties is initially anchored to the membrane via phosphatidylinositol (*80*). Similarly, heparan sulfate proteoglycan was released from a hepatocyte line in response to exogenously added PI-PLC or insulin (*81*). Although the concentrations of insulin used were probably not physiologically relevant, these data suggest that this action of insulin may be mediated by activation of an endogenous glycosyl-phosphatidylinositol–specific phospholipase C. It was further suggested that the released form of heparan sulfate proteoglycan behaved as an autocrine growth regulator as a result of its specific internalization, which occurs at

binding sites recognizing the inositol-phosphate moiety of the released proteoglycan. The observation that alkaline phosphatase levels in rat osteosarcoma cells are decreased by insulin treatment, that 5′-nucleotidase levels in adipocytes are elevated in diabetic rats and that AChE can be bound to the cell surface in a dissociable form, is consistent with this general scheme of release, binding, and internalization (*82*). Specific inositol-phosphate glycan binding sites that function in a transport capacity for released proteins may also account for the insulin-mimetic effects of the purified inositol-glycan on glucose utilization in intact cells that can be blocked with inositol-phosphates (*63*).

An additional consequence of the hydrolysis of glycosyl-phosphatidylinositols is the generation in the plasma membrane of diacylglycerol (by phospholipase C digestion) or phosphatidic acid (by phospholipase D digestion). Since 1,2-diacylglycerol is an endogenous activator of the calcium- and phospholipid-dependent protein kinases C (*83*), the activation of the phospholipase C–catalyzed hydrolysis of glycosyl-phosphatidylinositol may result in one or more of the cellular changes associated with the activities of kinase C. The glycosyl-phosphatidylinositols involved in protein anchoring or insulin action appear to have fatty acid compositions that are different from each other and from free phosphatidylinositol (*14, 17–20, 29, 30*). This leaves open the possibility that distinctive 1,2-diacylglycerol species result from the phospholipase C–catalyzed hydrolysis of distinct substrates and thus provide differential regulation of protein kinase C with respect to the extent of activation, substrate specificity, compartmentalization, or susceptibility to proteolytic activation, or are specifically directed to one of the isoforms of the enzyme (*84*). These possibilities might explain the perplexing relationship between protein kinase C and insulin action (*3*), in which protein kinase C activators such as phorbol esters mimic some, but not all, of the effects of insulin.

Phosphatidic acid also has marked effects on the metabolism and growth of cells (*85*) owing to a receptor-mediated action (*86*) or perhaps to a direct stimulation of the phosphoinositide-specific phospholipase C (*87*). It is possible that a biologically active phosphatidic acid might be locally generated at the cell surface by the phospholipase D–catalyzed degradation of protein anchors (*24, 46, 51*). The similarity in some of the effects of phosphatidic acid and serum (a rich source of the phospholipase D) is consistent with this idea (*87*). Furthermore, since several glycosyl-phosphatidylinositol–anchored proteins are involved in the reception of mitogenic signals (*79*) it is possible to speculate that the glycerolipids released by the specific phospholipases C or D play an important role in the transduction of such signals across the plasma membrane (Fig. 1).

Final Comments

It has become increasingly clear over the past 3 years that the glycosyl-phosphatidylinositols play a broad role in the functions of biological membranes. Thus far, these molecules have been demonstrated to serve as the major means of membrane attachment for about 30 distinct proteins from a variety of cell types, and it is likely that this mechanism of anchoring will be identified for additional membrane proteins. Given the functional diversity of these proteins, it seems that the precise contributions of this unusual post-translational modification will only be elucidated as the result of extensive and detailed biochemical investigations of the glycosyl-phosphatidylinositols. The major challenges include (i) the isolation and structural analysis of different forms of glycosyl-phosphatidylinositol; (ii) elucidation of their biosynthetic pathways, including the isolation of intermediates and identification of the relevant enzymatic steps involved; (iii) evaluation of the means by which glycosyl-phosphatidylinositol is covalently attached to protein—these efforts may entail the search for specific peptide sequences and conformations which regulate the action of the putative lipid-protein condensing enzyme; and (iv) further studies on the enzymology of glycosyl-phosphatidylinositol hydrolysis, especially regarding the hormonal regulation of the specific phospholipases C and D, and their role in signal transduction. These investigations may help to determine the precise contribution of these molecules to the actions of insulin and other hormones and indicate new possibilities relating to the regulation of membrane protein function.

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104. We thank P. Sherline for critical reading of the manuscript and many other colleagues for supplying us with preprints and unpublished manuscripts. M.G.L. is supported by grants from the American Heart Association and NIH (GM 35873); A.R.S. is supported by grants from the Irma T. Hirschl Trust, the Diabetes Research and Education Foundation, the Juvenile Diabetes Foundation, and NIH (DK33804).