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GLYCOSYLPHOSPHATIDYL INOSITOL MEMBRANE-ANCHORED PROTEINS

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For stable association with the plasma membrane, proteins and other cell surface macromolecules require a hydrophobic anchor. For the majority of proteins in the higher eukaryotes, this anchor is provided by a sequence of amino acids incorporated into the polypeptide during translation. For a subset of proteins however, a glycolipid anchor is added rapidly following translation; the glycolipid is based on phosphatidyl inositol, hence glycosylphosphatidyl inositol or GPI anchor. The GPI anchor is widespread, being a component of hundreds of proteins, and is present in all eukaryotic lineages. Lymphocytes in particular have a large number of GPI-anchored proteins on their surface. Consequences of this alternate means of membrane attachment include differences in mechanisms for export to the cell surface, alteration in behavior of the protein on the plasma membrane, incorporation into glycolipid-rich microdomains with the potential for engagement of novel signal transduction pathways and entry into nonclassical endocytic pathways. Several of these aspects remain incompletely defined at this time. In addition, GPI anchor fragments demonstrate signaling properties of their own, particularly in parasitic infections. Among the lower eukaryota there is a heavy emphasis on GPI anchors as the mechanism for cell surface attachment, whereas yeasts use this pathway in construction of the cell wall.

STRUCTURE

The glycosylphosphatidyl inositol (GPI) membrane protein anchor is a complex glycolipid that is attached to the C-terminus of a vast range of cell surface proteins and provides the sole means of membrane attachment for this class of protein. These molecules include exoenzymes (e.g., alkaline phosphatase), adhesion molecules (e.g., NCAM), and complement regulatory proteins [e.g., decay accelerating factor (DAF)]. The GPI anchor has the generalized structure EthN-P-Man_3GlcN-PI, where EthN is ethanolamine, P is a phosphodiester, Man designates mannose, GlcN glucosamine and PI phosphatidyl inositol (Fig. 1). In many cases the core structure is modified by additional monosaccharides (e.g., mannose, N-acetylgalactosamine) and ethanolamine phosphate residues; modification is cell-type and species specific, and two GPI-linked proteins expressed in the same cell will usually have similar or identical anchor structures. Covalent linkage is obtained between the GPI and the polypeptide via an amide bond between the carboxyl terminus of the protein and the primary amino group of ethanolamine. GPI-anchored proteins are classified as intrinsic membrane proteins as their membrane association is of sufficient stability to require detergent for extraction, but a key facet is that the polypeptide can, in many (but not all) cases, be released experimentally from cell surfaces by treatment with phospholipases, in particular PI-specific phospholipase C. These proteins also show an increased propensity for shedding or migration from the cell where they were synthesized either into the extracellular fluid or onto other biological membranes.

As all GPI-anchored proteins are expressed at the cell surface, or in the topologically equivalent interior of secretory organelles, they contain a signal sequence directing translocation into the endoplasmic reticulum (ER) typically at the N-terminus. Additionally, GPI-anchored proteins also contain a C-terminal signal that specifies processing by the GPI-anchoring apparatus. Addition of the GPI moiety is rapid, and occurs essentially immediately following emergence of the GPI signal into the ER lumen; the signal is rapidly removed and replaced in a transamination reaction by the GPI anchor (1). The signal is composed of approximately 25 to 30 amino acids that can be subgrouped into three domains; overall physicochemical properties are important, whereas precise sequence is less critical, in common with other classes of signal sequence. At the very C-terminus a hydrophobic stretch of 15 to 20 amino acids is present that may serve to retain the protein within the ER membrane (Fig. 1). N-terminal to this membrane association signal is a spacer region of approximately 10 amino acids with amphipathic composition. The addition site of the GPI anchor is located at the N-terminus of this region and is composed typically of three small amino acids, although some tolerance has been observed for bulky residues; cleavage and GPI addition takes place at the amide bond between the first and second amino acids in this region. The basic structure and sequence requirements for the GPI signal are remarkably well conserved throughout eukaryotic evolution and can be accurately predicted using computer algorithms (2,3). Significantly, many proteins are expressed as both GPI-linked and either soluble or transmembrane anchored forms brought about by differential spicing, resulting in inclusion or exclusion of an exon encoding a GPI signal from the mature mRNA. The production of such spliceoforms serves to increase functional diversity and may also be developmentally linked as in the case of NCAM (neural cell adhesion molecule), where GPI-anchored forms predominate during myogenesis but not following formation of the myotube.

BIOSYNTHESIS

Similarly, to the pathway for N-glycosylation, the GPI anchor is performed prior to addition to the protein acceptor. However, there is no energy requirement for the anchoring process, and the GPI lipid is not thermodynamically activated. Construction of the anchor is initiated on the cytoplasmic face of the endoplasmic reticulum (4–6) by addition of N-acetylglucosamine from UDP-GlcNAc (uridine diphosphate-N-acetylglucosamine) onto phosphatidyl inositol. Studies suggest that only a subset of PI is selected for GPI construction. At least four proteins that form a complex (PIG-A (phosphatidyl inositol glycan A), PIG-C, PIG-H and GPI-1) are involved in this first step; PIG-A has distant sequence similarity to glycosyltransferases, suggesting that it contributes the catalytic component of the holoenzyme. The GlcNAc-PI is de-N-acetylated by the product of the *PIG-L* gene, and three mannose residues are subsequently added. The deacetylation step is largely confined to a subcompartment of the ER that appears to be associated with mitochondria and is also enriched in enzymatic activities involved in

the conversion of GlcN-PI to a singly mannosylated GPI structure, H5, containing one phosphoethanolamine side chain (7). H5 is acylated on the inositol residue via a reaction that both precedes and is required for addition of the first mannose. The efficient synthesis of H5 may be a result of substrate channeling through a multienzyme complex, containing activities catalyzing de-N-acetylation, inositol acylation, and the first mannosylation reaction. Mannose is donated from dolichyl-phosphorylmannose (8,9). Of the three enzymes that are presumed to be needed for the mannosyltransfer reactions, only one—responsible for the addition of the third mannose residue—has been tentatively

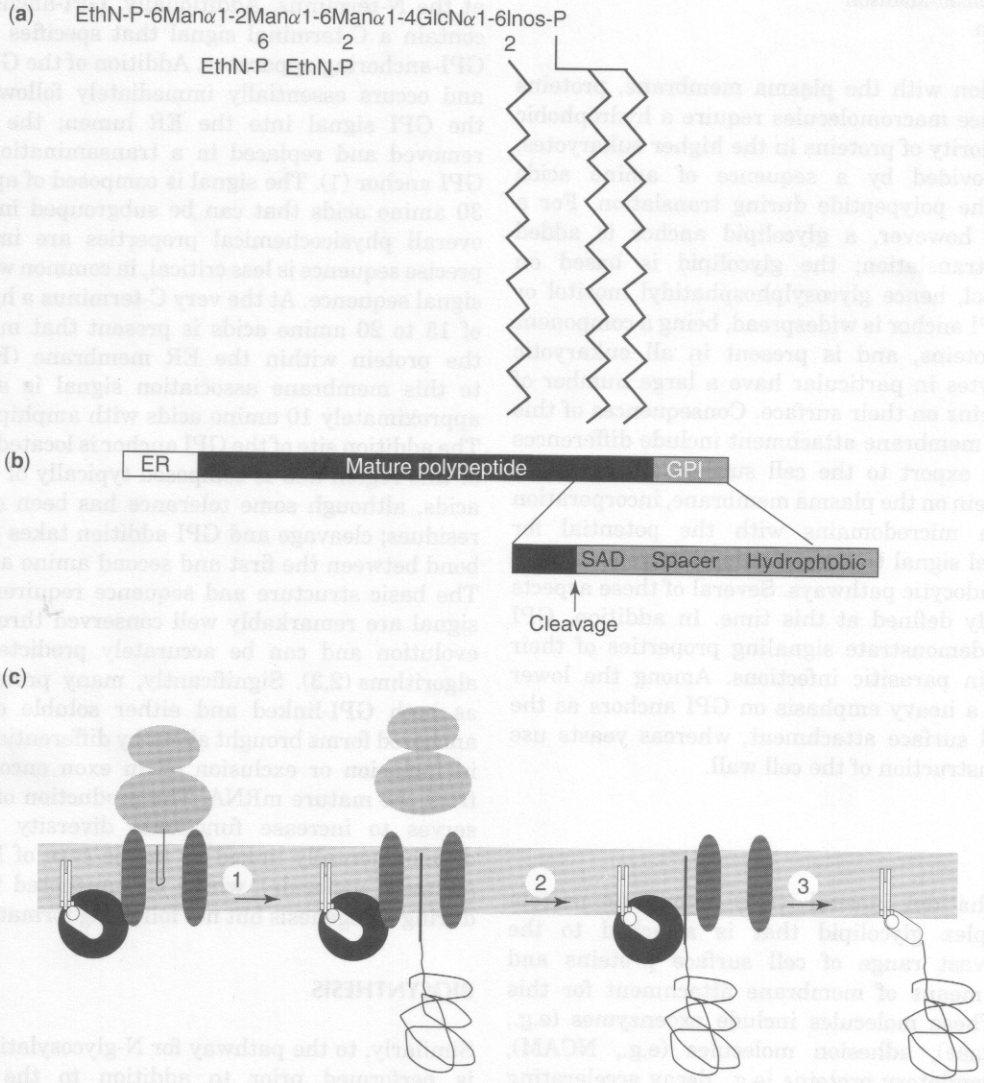

Figure 1. Generalized structure of the GPI anchor signal and attachment to protein. (**a**) Structure of the GPI anchor core from a mammalian cell. The ethanolaminylphosphoglycan is shown schematically and the positions of the acyl moieties are indicated. (**b**) The nascent polypeptide chain for a GPI-anchored protein contains two signal sequences, one at the N-terminus for directing the protein to the endoplasmic reticulum, and a second signal at the C-terminus for GPI-addition. The expanded region shows this latter signal in more detail. The cleavage site is followed by two small side-chain amino acids, and is separated from a hydrophobic C-terminus by an amphipathic spacer of 12 to 15 residues. Typically, both the ER- and GPI-signals are cleaved and degraded. (**c**) Mechanism for addition of the GPI lipid to a nascent polypeptide. ER-localized ribosomes import proteins through the Sec61 translocon. An associated transamidase activity composed of at least the GAA1 and GPI8 gene products, is positioned within the ER membrane for transfer of a GPI-lipid to the nascent chain. Step 1: translation is completed, the ribosome disengages from the translocon. A hydrophobic C-terminus causes the nascent chain to pause within the membrane. Step 2: the nascent chain is released from the translocon and interacts with the transamidase. Step 3: the GPI-lipid is transferred to the acceptance site during a coupled transamidation reaction, resulting in a GPI-anchored protein. ER membrane is in light shade; the Sec61 translocon is represented by two dark ovals. The transamidase is shown as a black circle, and the precursor GPI lipid is drawn in white. The ribosome is represented as a figure of eight structures. Cytosol is at the top, and ER lumen at the bottom of the figure.

identified as the product of the *PIG-B* gene (10). Addition of the terminal ethanolamine phosphate is achieved by headgroup donation from phosphatidyl ethanolamine (11). In mammals, up to two additional ethanolamine phosphate moieties may be added to the core and also by headgroup donation from PE (phosphoethanolamine). The enzymes responsible for these phosphoethanolamine transfers have been tentatively identified as products of a gene family including *MCD4, GP17*, and *YLL031c* (6); a fourth gene product, the PIG-F protein may also play a role. At some stage in the assembly pathway, possibly coincident with mannose addition, a GPI lipid is flipped from the cytoplasmic face of the ER to the lumenal leaflet where assembly is completed. It is also possible that the flip is deferred until a complete phosphoethanolamine-containing GPI structure is assembled on the cytoplasmic face of the ER (6). The mechanism by which flipping occurs is unknown but is presumed to involve a transport protein.

Addition of the GPI lipid to protein takes place within the ER lumen. There is reasonable evidence to indicate that GPI anchoring involves a transamidation reaction mechanism (1,12). Both the polypeptide acceptor and the GPI precursor must be present for addition to take place, although small nucleophiles, such as hydrazine, can substitute for GPI in cell-free assays of GPI anchoring. Components of the transamidase enzyme responsible for catalyzing the reaction have been identified in genetic screens in yeast (13,14). These components, termed *Gaa1p* and *Gpi8p*, most likely form a complex in the ER. Gpi8p has similarity to a plant vacuolar transamidase involved in the maturation of concanavalin A (14), indicating that it may be the catalytic subunit. The relationship of the transamidase to the general ER translocation system or chaperone-folding system in the ER is not known. When GPI anchor addition is prevented by a deficiency of GPI lipid precursors, the unmodified proproteins fail to exit the ER and are retained and diverted into a degradation pathway that involves reexport from the ER into the cytosol for hydrolysis into peptide by the proteasome (15). A minority of unprocessed proteins are exported where they are unstably associated with the plasma membrane.

Following GPI addition, the protein exits the ER. At least two maturation mechanisms for GPI anchors have been characterized; elaboration of the core glycan and remodeling of the lipid moiety. Glycan modification is a Golgi-mediated event and is characterized by addition of further monosaccharide substituents. Except in a number of specialized cases (specifically some reactions observed to occur in protozoan parasites and *Saccharomyces cerevisiae*), lipid remodelling is not well characterized, but in essence a proportion of the diacyl or alkylacyl PI anchors are modified to ceramide-based structures. This is presumed to occur via a reaction similar to headgroup exchange processes, whereby the inositol phosphate of the anchor migrates from a glyceride to a sphingosine. The functional significance of glycan elaboration or lipid remodeling is unknown.

DISEASES OF GPI ANCHORING

GPI deficiency is an extremely rare occurrence and is never observed in the neonate mammal. This most probably reflects the critical importance of the large number of GPI-anchored proteins that are expressed in the developing mammalian embryo. Complete GPI deficiency would result in catastrophic developmental defects. In experimental systems like yeast, defective alleles of the *GPI-8* gene result in a severe growth defect and incomplete cell wall biosynthesis (14). However, the magnitude of GPI deficiency is most likely far stronger in multicellular organisms, where GPI-linked molecules are involved in cell–cell interactions and migration behavior. In humans, a haemolytic disorder, paroxysmal nocturnal hemoglobinuria (PNH), is accompanied by a deficiency of GPI-anchored proteins on the cell surface of erythrocytes, myeloid cells, and occasionally lymphocytes (16). PNH maps to Xp22.1 and the *PIG-A* gene required for the transfer of N-acetylglucosamine to phosphatidyl inositol. Because of X-inactivation in females, PIG-A is always hemizygous, and hence somatic mutations in this gene are far more frequently observed than for the other GPI biosynthetic genes that are all on somatic chromosomes. PNH is characterized by complete or partial deficiency of GPI lipid biosynthesis and increased hemolysis, probably due to loss of surface expression of GPI-linked complement regulatory proteins, in particular decay accelerating factor.

INTRACELLULAR TRANSPORT

The clearest consequence of lipid as opposed to transmembrane polypeptide anchoring is in interaction with the lipid bilayer. This has been demonstrated to have profound effects in the way that GPI-anchored proteins behave and are handled by the cell, and is particularly important as different species or cell types utilize GPI anchors with differing acyl (or other lipidic) compositions, which also influences the properties of the protein. In particular, the GPI anchor influences rate and route through the secretory system (Table 1). The presence of the GPI anchor can accelerate export of a protein, resulting in more rapid arrival at the surface. Packaging into transport vesicles at the ER exit region is also selective, and in *S. cerevisiae* GPI proteins occur in a subset of vesicles only (17).

In some polarized cells, GPI-anchored proteins have a restricted distribution. On the Madin-Darby canine kidney epithelial cell line, GPI proteins are localized exclusively to the apical compartment. Similarly, in neuronal cells GPI proteins are restricted to the axonal plasma membrane and excluded from the cell body. Hence the GPI anchor functions as a sorting signal. Absence of the transmembrane domain prevents any potential for direct interaction with cytoplasmic vesicle coat proteins or other components of the cellular sorting machinery. Despite this, evidence suggests that the GPI functions as a true sorting signal.

The manner in which the GPI acts as a sorting determinant is likely achieved via the physical partitioning of GPI-anchored proteins during transit through the Golgi complex into glycolipid-rich microdomains (18). These domains were originally identified due to their comparative resistance to solubilization with commonly used detergents at certain conditions. Under such conditions, GPI-anchored proteins become detergent insoluble during transit through the Golgi apparatus. Importantly, the solubility of GPI proteins is influenced by ongoing sterol and sphingolipid biosynthesis. Both sterols and glycosphingolipids (GSLs) begin synthesis in the ER, but assembly of GSLs is a Golgi function, and hence the concentration of polar lipid species may only attain a critical threshold in the Golgi complex. Correlation between detergent resistance and glycolipid rafts has been achieved

Table 1. Functions of the GPI Anchor

Function	Example
Selective packaging during ER exit	Gas1p in *S. cerevisiae*
Incorporation into glycolipid rafts	General property of in higher eukaryotes
Decreased transit time to cell surface	GPI-deficient VSG in *T. brucei*
Association with surface microdomains	General property, numerous examples
Involvement in signal transduction	Ly6, Thy-1, NCAM
Altered surface mobility	Numerous examples
Endocytosis by clathrin and caveolin	Numerous examples, prion protein, folate receptor
Cell wall component in yeasts	Gas1p
Migration to other membranes	VSG, NCAM

General functions ascribed to either all GPI-anchored proteins or specific-marker proteins that are supported by experimental data are given in the table.

by demonstrating that the detergent-resistant membranes are cholesterol- and GSL-rich, and that inhibition of GSL and sterol biosynthesis prevents formation of detergent-resistant domains, acquisition of insolubility of GPI proteins, and mistargetting of GPI proteins in polarized cells. Selective packaging of the rafts into apical transport vesicles effects a high level of sorting. Although rafts provide an efficient sorting mechanism, they are also present in nonpolarized cells, indicating that the transport of GPI-anchored proteins by this process is constitutive.

Importantly, GPI protein association with membrane microdomains persists on the cell surface. By indirect immunofluorescence, GPI-anchored proteins can be observed in small clusters on the cell surface, and spectroscopic measurements suggest that these microdomains are small, of the order of 70 to 300 nm in diameter, and contain a limited number of proteins. GPI proteins preferentially associate with these domains but are also in equilibrium with the bulk fluid phase membrane, and hence exist in an equilibrium between lower and higher motility populations. Partitioning behavior is influenced by acyl chain length, and hence GPI proteins anchored with short acyl chains remain more soluble than long chain GPI proteins.

Cell surface raft microdomains are also enriched in a number of signal transduction molecules, including several Src tyrosine kinases, nitric oxide synthases, focal adhesion kinase, Gα subunits and Ras isoforms, and some members of the integrin family. The association of these cytoplasmic proteins with the GSL- and cholesterol-rich microdomain is promoted by acylation, whereas the integrins associate via a number of mechanisms. The concentration of these important surface molecules into microdomains suggests that these regions may function as specialized platforms for signal transduction. In addition, the concentration of GPI-anchored proteins into rafts may have been exploited by a number of pathogens, which secrete toxin molecules that bind preferentially to this class of molecule. For example, Thy-1(thymine) is targeted by the aerolysin of *Aeromonas* spp., and possibly association with rafts may be important to function.

The presence of a GPI anchor also influences endocytic transport processes. GPI-anchored proteins can be endocytosed by a variety of mechanisms (clathrin-dependent and clathrin-independent), although the rate at which these proteins are taken up into endosomes is lower than that for transmembrane proteins that make use of receptor-mediated endocytosis (19). In the case of the folate receptor, a well-studied GPI-anchored protein is recycled from the cell surface, the rate of recycling is dependent on cholesterol, such that in normal cells recycling is slower than transmembrane-anchored proteins, but in cholesterol-depleted cells the rates are more equivalent. This result is consistent with the idea that GPI-anchored proteins are transported as constituents of cholesterol- and sphingolipid-rich microdomains. Such domains may coalesce in certain cells to form morphologically identifiable invaginations termed *caveolae*. Caveolins, a group of proteins that associate with caveolae membranes by virtue of binding cholesterol (20), have the property of self-assembly and function in an analogous manner to clathrin in prompting endocytosis but by a distinct route.

GPI FRAGMENTS AS SIGNALING MOLECULES

Both GPI lipids and fragments of GPI anchors generated by hydrolysis of mature proteins have been implicated as signaling moieties. Early work suggested that the isolated GPI phosphoinositolglycan could function as an insulinomimetic, but a lack of structural characterization of the GPI species and clear physiological relevance of the response has left this proposal as contentious. More recently, studies have shown that GPI fragments derived from a number of protozoan parasites are capable of engaging signal transduction systems, and these moieties may account for a large component of the cytokine production that accompanies these infections. In particular, the phosphoinositolglycan fragment from *Plasmodium, Trypanosoma brucei* and *Leishmania* act as agonists for signaling through protein tyrosine kinase p59(hck) in macrophages. Additionally, the diacylglycerol fragment acts synergistically via PLCε (porcine leukocyte cysteine) with the phosphoinositolglycan to activate NFκB (nuclear factor kappa B) and to stimulate TNFα (tumor necrosis factor), IL-1α (interleukin–1) and iNOS (inducible nitric oxide synthase) production. The GPI fragments appear to function as coagonists as they cannot stimulate quiescent macrophages, but only those already primed with IFNγ. Similar observations have been made for *Trypanosoma cruzi* protein GPI anchors. Significantly, the related but structurally distinct glycosylphosphoinositol lipids (GIPL) of *Leishmania* are not capable of stimulating macrophages in this manner.

NONMAMMALIAN SYSTEMS

GPI-anchored proteins have been identified throughout the eukaryota, including plants, algae, yeasts, and protozoa. The best understood systems are S. cerevisiae and several representative parasitic protozoa.

In yeast, GPI-anchored proteins have an important role in cell wall biogenesis. GPI-anchored yeast proteins are released from the cell membrane and become cross-linked within the cell wall glycocalyx; the GPI glycan provides an initiation point for the elaboration of mannans for the construction of the cell wall. Interference with this process has been suggested to be a novel point for therapeutic intervention for treatment of pathogenic fungi (e.g., *Candida albicans).* In the case of protozoa, particularly among the apicomplexa and kinetoplastida, use of the GPI anchor appears favored over the transmembrane polypeptide anchor mechanism, and the predominant surface proteins are all GPI-linked. Significantly, *Leishmania* and *Trypanosoma cruzi,* pathogenic members of the kinetoplastida, express on their surfaces a range of nonprotein GPI-anchored molecules (21), some of which are implicated in virulence and transmission. This is in contrast to higher eukaryotes where nonprotein GPI molecules are present at very low-levels on the exoplasmic leaflet of the plasma membrane. These parasite nonprotein GPI species can be small glycolipids referred to as *GIPLs,* similar but not identical to free GPI anchors, or more elaborate phosphopolymers belonging to the lipophosphoglycan (LPG) family. Several related biosynthetic pathways for these novel GPI molecules, distinct to that giving rise to the protein anchor precursor, have been described. The importance of these molecules and the GPI-anchored proteins present on the pathogen surface has suggested that the GPI biosynthetic pathway is a potential chemotherapeutic target. Detailed comparative studies of several steps in GPI biosynthesis suggest sufficient divergence between parasite and host enzymes is a real possibility.

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