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Topical Review

Emerging Functional Roles for the Glycosyl-Phosphatidylinositol Membrane Protein Anchor

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Introduction

Most glycoproteins are anchored to the cell surface by hydrophobic interactions of the protein with the membrane bilayer. In the conventional mechanism of anchoring, one or more stretches of relatively hydrophobic amino acids span the membrane, resulting in a firm attachment to the cell surface (Blobel, 1980; Adams & Rose, 1985). In addition, this hydrophobic domain provides a stop transfer signal during translocation of the protein to the lumen of the endoplasmic reticulum. An alternative mechanism of anchoring involves the covalent linkage of the C-terminal amino acid of the protein to a glycosylated form of phosphatidylinositol, termed glycosyl-phosphatidylinositol (GPI). This mechanism of anchorage has been detected in a wide variety of cell types. Over 40 proteins of considerable functional and evolutionary diversity utilize this mechanism, from cell adhesion molecules, hydrolytic enzymes, and mammalian surface antigens to parasitic coat proteins (Low & Saltiel, 1988). A complete list is provided in Table 1. The basic structural features of the GPI linkage indicate important biophysical differences between this and the conventional mechanism of protein anchoring, suggesting a role for this structure in cellular regulation.

Several reviews have recently appeared that describe in detail the distribution and structure of GPI-anchored proteins (Low et al., 1986; Cross, 1987; Low, 1987, 1989; Ferguson & Williams, 1988; Low & Saltiel, 1988). We discuss here the novel structural features of the GPI anchor that may play a role in determining the distribution, concentration or function of proteins anchored to the plasma membrane by this mechanism.

Key Words phospholipase · cell surface polarity · lateral mobility · hormone action

The GPI Anchor Possesses a Unique Structure

Elucidation of the basic structure of the GPI moiety has resulted largely from studies using bacterial PI-specific phospholipase C's from *Staphlococcus aureus, Bacillus cereus*, or *B. thuringiensis* (Ikezawa & Taguchi, 1981; Low, 1981). The release of anchored proteins by treatment with PI-PLC, by removal of the glycerolipid anchoring domain, has been used as the major diagnostic tool for identification of this anchoring mechanism (Low & Saltiel, 1988). In general, an amide linkage between ethanolamine and the C-terminal amino acid provides the bridge between the protein and the glycan of GPI. The glycan, exhibiting a non-(N)-acetylated glucosamine at the reducing end, is glycosidically linked to the 6-hydroxyl of inositol in PI (Fig. 1). The detailed structure of the glycan portion has been elucidated for only three GPI-anchored proteins, the variant surface glycoprotein (VSG) of *Trypanosoma brucei* (Schmitz et al., 1987; Ferguson et al., 1988), rat brain Thy-1 (Homans et al., 1988) and human erythrocyte acetylcholinesterase (AChE) (Roberts et al., 1988*b*) (Table 2). These studies revealed a highly conserved core structure, consisting of ethanolamine-phosphate, three mannose residues, and glucosamine linked to PI [EtN-P-$(Man)_3$-GlcN-PI]. The presence of non-N-acetylated glucosamine is one of the most unusual features of the GPI anchor, rarely detected in other mammalian carbohydrates. This sugar is conserved in all forms of the anchor identified thus far and provides another key site for identification of the GPI anchor due to its unique sensitivity to deamination by nitrous acid.

In addition to the conserved "core" structure described above, variations occur in side chain composition and linkage. For example, the VSG anchor of *T. brucei* contains a galactose antennae [$(Gal)n = 2–4$] on the hexose adjacent to glucos-

Table 1. Proteins with a glycosyl-phosphatidylinositol anchor

Hydrolytic enzymes
Alkaline phosphatase
Acetylcholinesterase
5′-Nucleotidase
Trehalase
Alkaline phosphodiesterase I
p63 protease *Leishmania*
Renal dipeptidase
Merozoite protease *Plasmodium*
Aminopeptidase P
Lipoprotein lipase
Mammalian antigens
Thy-1
RT-6
Qa
Ly-6
Carcinoembryonic antigen
Blast-1
CD14
Nonspecific crossreacting antigen
Protozoal antigens
Ssp-4 *Trypanosoma*
Variant surface glycoprotein *Trypanosoma*
Surface proteins *Paramecium*
195-kDa antigen *Plasmodium*
Cell adhesion
LFA-3
Neural cell adhesion molecule
Heparin sulfate proteoglycan
Contact site A *Dictyostelium*
PH-20 guinea pig sperm
Miscellaneous
Decay-accelerating factor
130-kDa hepatoma glycoprotein
34-kDa placental growth factor
Scrapie prion protein
GP-2
Folate receptor (Rothberg et al., 1990)
Tegument protein *Schistosoma*
FcIII receptor
Oligodendrocyte-myelin protein
Antigen 117 *Dictyostelium*
125-kDa glycoprotein *Saccharomyces*
Homologous restriction factor
Elongation factor EF-1 alpha (Hayashi et al., 1989)

See Low (1989) for specific references not listed here.

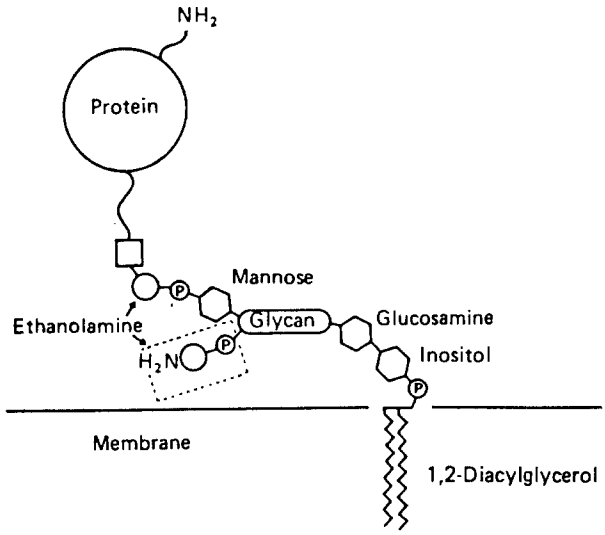

Fig. 1. Structure of the GPI Protein Anchor. The basic structure of this membrane protein anchor is illustrated. The C-terminal amino acid is linked by an amide bond to ethanolamine, which is in turn connected through a phosphodiester linkage to an oligosaccharide of variable composition and structure. The terminal monosaccharide of this glycan is non-N-acetylated glucosamine, which is linked at the C-1 position to the C-6 hydroxyl of the inositol ring on phosphatidylinositol. The glycerol lipid moiety serves as the membrane-anchoring domain

amine, whereas both the Thy-1 and AchE anchors contain an extra ethanolamine-phosphate (with a free amino group) at this position (Homans et al., 1988). Based on compositional analyses, the presence of an extra ethanolamine appears to be a common feature of other mammalian anchors (Ferguson & Williams, 1988). The functional significance of variations in glycan composition is not known, nor is it clear whether different proteins contain distinct anchors within a single cell type. The fatty acid composition of the glycerolipid moiety may also vary. The VSG anchor exclusively uses dimyristoylglycerol (Ferguson, Low & Cross, 1985), whereas most of the mammalian anchors examined thus far contain an unsaturated fatty acid in the Sn-1 position with a saturated fatty acid in the second position (Ferguson & Williams, 1988; Low & Saltiel, 1988). A 1,2 alkylacylglycerol structure has also been commonly observed (Roberts & Rosenberry, 1985). Another structural variation of potential functional significance is the acylation of the inositol ring. The presence of palmitate on the inositol ring of the AchE anchor confers resistance to enzymatic cleavage by PI-specific phospholipase C, although this anchor remains sensitive to the GPI-phospholipase D(PLD) (Roberts et al., 1988*a*). This modification may prevent the formation of a cyclic 1,2 inositol phosphate intermediate necessary for phosphodiester cleavage by PLC. However, removal of this fatty acid by acetolysis permits the action of PI-PLC.

A glycophospholipid with a number of structural similarities to the GPI anchor has been described in several cell types. This lipid shares the core structure of PI-glucosamine, linked to additional monosaccharides, but lacks ethanolamine, and is not attached to protein (Saltiel & Cuatrecasas, 1988). Numerous studies in tissue culture cells have demonstrated that this lipid is hydrolyzed by a

Table 2. Structural analysis of three GPI-anchored proteins

Protein	C-terminal amino acid	Core glycan	Additional modifications	Glycerolipid fatty acid composition
VSG	asp	Et-P-$(Man)_3$ GlcN	(Gal) n = 2–4	Myristate
Thy-1	cys	Et-P-$(Man)_3$ GlcN	Et-P, Man, Gal NAc Beta	?
AChE	gly	Et-P-$(hex)_3$-GlcN	Et-P, palmitoylation of inositol	?

PLC mechanism in response to insulin and related hormones. The resulting hydrolytic products, diacylglycerol and an inositol phosphate glycan, are thought to mediate some of the actions of insulin in fat, liver and muscle cells (Saltiel & Cuatrecasas, 1986; Saltiel et al., 1986; Saltiel, Sherline & Fox, 1987).

The GPI Anchor is Attached to Proteins Rapidly after Translation

The anchoring of proteins to GPI appears to occur by the attachment of the proteins to a preformed lipid precursor. This attachment requires the removal of a relatively short, C-terminal hydrophobic domain. The formation of this linkage appears to occur cotranslationally in the ER, as judged by sensitivity to cleavage by PI-PLC or reactivity with antibodies that recognize the glycan moiety of the anchor (Bangs et al., 1985, 1986; Ferguson et al., 1986; Conzelmann, Spiazzi & Bron, 1987; He, Finne & Goridis, 1987; Zamze et al., 1988; Bailey et al., 1989). The rapid kinetics of anchor addition suggest that cleavage of the hydrophobic C-terminal peptide and GPI-attachment may be catalyzed by the same enzyme (transpeptidase or transamidase) and that a presynthesized GPI-moiety is added *en bloc*.

The detection of GPI precursors in *T. brucei* (Krakow et al., 1986; Menon et al., 1988) with structures identical to those of the VSG GPI anchor provided strong evidence for a transamidase mechanism. One of these lipids contains the core PI-GlcN-$(Man)_3$-P-EtN structure. Other GPI molecules were identified with modifications to the core structure. Moreover, the *in vitro* synthesis of this lipid was detected in a cell free system (Masterson et al., 1989; Doering et al., 1990). These studies indicate that GPI synthesis proceeds via the sequential glycosylation of PI. First glucosamine is added to PI via a UDP-GlcNAc donor, followed by deacetylation, mannosylation and presumably attachment of ethanolamine-phosphate (Masterson et al., 1989). The *T. brucei* precursor lipids detected thus far are devoid of galactose, consistent with the idea that the galactose side chain for the VSG anchor is added after conjugation to protein, possibly at the level of the Golgi (Grab, Webster & Verjee, 1984). Previous reports (Conzelmann et al., 1986, 1988; Fatemi & Tartakoff, 1986, 1988) suggested that dolichol-P-mannose is the donor for addition of at least one of the mannose residues, since a mutant thyoma cell line (complementation group E) that is defective in GPI-addition is also defective in the synthesis of dolichol-P-mannose (Chapman, Fujimoto & Kornfeld, 1980).

The protein signals that direct attachment to GPI appear to be localized to C-terminal hydrophobic regions. Initial studies comparing the amino acid sequence predicted from cDNAs of Thy-1 or trypanosomal VSG with the actual sequence demonstrated the loss of the predicted C-terminal peptide and replacement with the GPI moiety (Boothroyd et al., 1980; Tse et al., 1985). Several GPI-anchored proteins are alternatively expressed with transmembrane hydrophobic peptide regions [e.g., N-CAM (Hemperly, Edelman & Cunningham, 1986), FcR III (Selvaraj et al., 1988; Simmons & Seed, 1988), CEA (Barnett et al., 1989), and LFA-3 (Dustin et al., 1987)], or with sequences that produce secreted proteins [e.g., DAF (Caras et al., 1987*a*), Qa-2 (Stroynowski et al., 1987), and N-CAM (Gower et al., 1988)]. Each of these variations is thought to be derived from differential mRNA splicing, due to differences in the C-terminal regions.

A number of studies have focused on the identification of the precise signal sequence responsible for the attachment of proteins to GPI. The 37 C-terminal amino acid sequence of decay accelerating factor (DAF) was transferred to a secreted protein, gD-1, which is a truncated form of the herpes simplex envelope glycoprotein. The resulting fusion protein was both GPI-anchored and targeted to the

Table 3. Known GPI-anchored proteins which are apically distributed in polarized epithelia

Proteins	References
Alkaline phosphatase	Simons & Fuller, 1985; Brown et al., 1989
5′ Nucleotidase	Simons & Fuller, 1985
Trehalase	Takesue et al., 1986
Renal dipeptidase	Littlewood, Hooper & Turner, 1989
Aminopeptidase P	Hooper & Turner, 1988
Thy-1	Kollias et al., 1987
Carcinoembryonic antigen	Lisanti et al., 1990
Decay-accelerating factor	Lisanti et al., 1989*a*
Folate receptor	Rothberg et al., 1990

cell surface (Caras et al., 1987*b*). In separate experiments, linkage of the C-terminal domain of DAF to the extracellular domain of the cell surface antigen, CD8, also produced a GPI-anchored fusion protein (Tykocinski et al., 1988).

Further studies have defined two different regions within the C-terminal GPI-attachment "signal" of DAF: a 17 amino acid C-terminal hydrophobic region and the 20 amino acids located just upstream. Removal of the 17 amino acid C-terminal hydrophobic segment of DAF prevented GPI-anchoring, although the addition of this hydrophobic domain to a second protein (human growth hormone) did not confer GPI-anchoring, indicating that this hydrophobic segment is necessary, but not sufficient to direct lipid attachment (Caras, Weddell & Williams, 1989). Additional information (cleavage and attachment sites) must reside within the adjacent 20 upstream amino acids. Moreover, it is likely that the signal nature of the 17 amino acid C-terminal hydrophobic region depends upon on overall hydrophobicity, since replacement with a random hydrophobic sequence or by the N-terminal ER signal sequence of human growth hormone still allowed GPI attachment (Caras & Weddell, 1989). Similar deletion or chimeric gene experiments employing GPI-anchored placental alkaline phosphatase, Qa-2 and Thy-1 have also localized the signal to C-terminal hydrophobic regions (Berger et al., 1988; Waneck et al., 1988*a*, Crise et al., 1989).

GPI-anchored placental alkaline phosphatase may be converted to a transmembrane protein by replacement of its C-terminal domain with the transmembrane segment of an integral membrane protein, the vesicular stomatitis virus G protein (Berger et al., 1989). Similarly, a single amino acid substitution (from *asp* to *val*) in the C-terminal region of GPI-anchored Qa-2 leads to anchoring via a hydrophobic transmembrane domain (Wanek, Stein & Flavell, 1988*b*). The latter finding suggests that the C-terminal region must remain weakly hydrophobic for its recognition and proper removal. In further support of this hypothesis, conversion of a critical C-terminal ser residue to phe converts the GPI-anchored Fc receptor to a transembrane protein (Hibbs et al., 1989; Kurosaki & Ravetch, 1989; Lanier et al., 1989).

The GPI-Anchor Exhibits Diverse Functional Roles

Although the basic structural features of the GPI anchor are known, the broad diversity of the anchored proteins makes assignment of a function for the anchor quite difficult. Nevertheless, the unusual properties of this anchor, and the evolution of a complex mechanism to remove a hydrophobic peptide domain for lipid attachment suggests that the utilization of this mechanism of anchoring merely as an inert structural entity is unlikely. Four possible functional roles for the GPI anchor are discussed.

The GPI Anchor Plays a Role in the Cellular Distribution of Proteins

Most of the GPI-anchored proteins identified thus far are located at the cell surface and face the extracellular space. A few GPI-anchored proteins exhibit intracellular localizations, such as GP-2 in the zymogen granules of the exocrine pancreas (Le Bel & Beattie, 1988), 82- and 68-kDa proteins in chromaffin granules (Fouchier et al., 1988), elongation factor EF-1α in the endoplasmic reticulum of V79-UF Chinese hamster fibroblasts (Hayashi et al., 1989), a fraction of PH-20 in the acrosome of guinea pig sperm (Phelps et al., 1988), and a fraction of DAF in the neutrophil (Berger & Medof, 1987). Each of these is distributed in the lumen of intracellular vesicles, rather than on the cytoplasmic face. Interestingly, several of these proteins undergo regulated release to the cell surface, suggesting a potential mechanism for regulated secretion involving the GPI anchor. The observed asymmetric orientation of all GPI-anchored proteins is compatible with their incorporation into the lumen of the ER via N-terminal signal sequences with subsequent processing and transfer to GPI.

In experiments with COS cells (Caras et al., 1987*b*, 1989), attachment of the C-terminal peptide of a GPI-anchored protein to a secreted protein resulted in GPI-addition and targeting to the plasma membrane. These and similar experiments suggested that GPI-anchored proteins must require ad-

ditional signals for intracellular retention and targeting to a pathway for regulated secretion, as has been shown for regulated secretory proteins, such as insulin, growth hormone, pre-pro-somatostatin (Chung et al., 1989). Although this observation may seem trivial, since in most cells transport to the cell surface is by default and retention at specific compartments along the secretory pathway is signal mediated (Pfeiffer & Rothman, 1987; Wieland et al., 1987), it is especially relevant to protein transport in polarized cells that contain multiple plasma membrane domains of distinct lipid and protein composition. In the case of polarized epithelial cells, transport to at least one domain must be signal-mediated, whereas transport to another may occur by default (Rodriguez-Boulan & Nelson, 1989; Wandinger-Ness & Simons, 1989). In a variety of polarized epithelial cell lines (MDCK I and II, LLC-PK_1, Caco-2, and SK-CO15), GPI-anchored proteins are selectively enriched in the apical domain, while depleted or absent from the basolateral cell surface (Lisanti et al., 1988, 1990). This striking correlation between GPI-anchoring and apical localization is highly conserved across species (pig, dog and human) and tissue type (renal and intestinal), suggesting that GPI might act as an apical transport signal to target the attached protein to the apical cell surface (*see* Table 3 for a complete list of known apical GPI-linked proteins).

A molecular biological approach has recently been employed to determine whether GPI-anchoring confers upon a protein apical localization. Recombinant transfer of GPI-attachment signals to proteins known to exhibit basolateral distribution on polarized cells, such as the viral envelope glycoproteins, HSV gD-1 (Lisanti et al., 1989*a*) or VSV G protein (Brown, Crise & Rose, 1989) or to a regulated secretory protein (Lisanti et al., 1989*a*), such as human growth hormone, resulted in GPI-anchoring and targeting to the apical membrane. Recognition of the GPI-moiety as a sorting signal may require components other than the conserved core structure (EtN-P-$(Man)_3$-GlcN), since the apical polarity of certain GPI-anchored proteins is disrupted in a lectin-resistant mutant epithelial cell line (MDCK-Con A^r) (Lisanti et al., 1990). Although the precise genetic defect in this mutant is not known, it may involve mannosylation. In this light, modification of the core structure with mannose, ethanolamine-phosphate, or acylation of the inositol ring may be required for efficient apical targeting. Thus, by several independent criteria, GPI-anchorage behaves as a "dominant" apical targeting signal (Lisanti & Rodriguez-Boulan, 1990).

These observations also provide suggestive evidence for the hypothesis that glycosphingolipids (i.e., glucosyl-ceramide) might act as epithelial sorting "receptors" (van Meer et al., 1987; van Meer & Simons, 1988). After clustering in the trans-Golgi network, these lipids might promote the incorporation of apical glycoproteins (both transmembrane and GPI-linked) into glycolipid-rich apical carrier vesicles. Clustering of certain GPI-linked proteins is supported by the detection of a significant immobile fraction by fluorescence recovery after photobleaching (FRAP) (Ishihara, Hou & Jacobson, 1987*a*; Noda et al., 1987; Phelps et al., 1988). In further support of this hypothesis, a florescent precursor of glycosphingolipids (NBD-6-ceramide) specifically stained the trans-most compartment of the Golgi, the putative site for the spacial segregation of apical and basolateral glycoproteins into distinct populations of carrier vesicles (Pagano, Sepanski & Martin, 1989).

The apical distribution of GPI-linked proteins may have clinical significance in determining the serum levels of GPI-anchored tumor antigens, such as carcinoembryonic antigen (CEA). Elevations in serum CEA levels occur in diseases of epithelial origin, either carcinomas (colon, liver, lung, breast and pancreas) or benign disease [such as inflammatory bowel disease, colorectal polyps, pulmonary inflammatory disease, collagen disorders, heavy smokers and other benign liver or renal disease (Virji, Mercer & Heberman, 1988)]. In CEA-positive colorectal or breast carcinomas, serum CEA levels correlate with tumor stage, are indicative of residual disease if levels do not decline post-therapy, and rising levels may indicate tumor recurrence (Virji et al., 1988). The apical polarity of CEA is only maintained in normal tissues or well-differentiated adenocarcinoma of the colon, but is lost in poorly differentiated adenocarcinoma (Hamada et al., 1985). The loss of polarity allows expression of CEA on the basolateral membrane, contact with the basement membrane, and access to the serum. Thus, loss of polarity, coupled with the observation that GPI-anchored proteins may be released by GPI-anchor degrading enzymes (protease, glycosidase or phospholipase), may determine the circulating levels of CEA in pathogenic states. Release of apical CEA would not contribute to serum levels, since the apical surface faces the external environment and is separated from the blood by the tight junctional barrier, accounting for the low serum levels of CEA observed in normal subjects (<2.5 ng/ml).

Other polarized cells also exhibit an asymmetric distribution of GPI-anchored proteins. For example, neural cell surface molecule F3, (Gennarini et al., 1989), 5′-nucleotidase (Grondal & Zimmerman, 1987) and acetylcholinesterase (Rotundo &

Carbonetto, 1987) are localized to neuronal outgrowths and neuronal ramifications. The distribution of PH-20 varies with the degree of sperm maturation (Phelps et al., 1988). This protein is randomly distributed in testicular sperm, localized to the posterior head region in epididymal sperm, and undergoes redistribution to the anterior head region after the acrosome reaction. Paradoxically, PH-20 is "immobile" when randomly distributed, but freely diffusible when localized to the head region (posteriorly or anteriorly). Thus, linkage to GPI may convey specific patterns of cell surface localization in different polarized cell types or membrane microdomains.

GPI-Anchored Proteins Exhibit Increased Lateral Mobility

An obvious consequence of lipid anchoring is an inherent increase in mobility in the plane of the membrane. Diffusion coefficients on the order of $1–4 \times 10^{-9}$ cm^2/sec are observed for Thy-1 (Dragsten et al., 1979; Ishihara et al., 1987*a*), alkaline phosphatase (Noda et al., 1987), DAF (Thomas et al., 1987), and PH-20 (Phelps et al., 1988). These are lower than values determined for freely diffusing lipid probes ($0.5–1 \times 10^{-9}$), but much higher than those for transmembrane glycoproteins ($0.5–6 \times 10^{-10}$) (Derzko & Jacobson, 1980; Gall & Edelman, 1981; McCloskey & Poo, 1984; Vaz, Goodsaid-Zaluondo & Jacobson, 1984). Paradoxically, some GPI-anchored proteins possess significant immobile fractions, up to 50% in the case of Thy-1 (Ishihara et al., 1987*a*). Certain GPI-anchored proteins may require increased lateral mobility for function, as in the case of DAF (for rapid inhibition of the complement cascade) or AChE (for deactivation of acetylcholine at the synapse). Other GPI-linked proteins require a high degree of immobility, such as the trypanosomal VSG (diffusion constant 1×10^{-10}) and PH-20. Interestingly, the low lateral mobility of VSG is not a result of some other factor in the trypanosomal cell surface, since implantation of the protein in the BHK cell membrane did not increase its mobility (Bulow, Overath & Davoust, 1988). The diffusion of PH-20 is dependent on the state of differentiation of guinea pig sperm (*see* above).

Differences in the mobility of membrane proteins may relate to the submembrane cytoskeleton. The relative mobility of transmembrane glycoproteins might be affected by the interaction of a cytoplasmic tail with the cytoskeleton. However, these factors would not be expected to affect their GPI-anchored counterparts which lack cytoplasmic tails. Thus, regulation of the diffusion of GPI-anchored proteins is likely to be influenced by interactions with solely the extracellular protein domain or the GPI portion.

GPI-Anchored Proteins are Excluded from Clathrin-Coated Pits

It is now well established that the cytoplasmic domains of receptors function to promote clustering into clathrin-coated pits and subsequent receptor-mediated endocytosis. Mutant receptors, such as the polymeric Ig receptor (Mostov, de Bruyn-Kops & Deitcher, 1986), LDL receptor (Davis et al., 1986, 1987), EGF receptor (Prywes et al., 1986) and transferrin receptor (Rothenberger, Iacopetta & Kuhn, 1987), that lack cytoplasmic domains, or certain molecules that possess short cytoplasmic tails (e.g., influenza HA) are generally excluded from coated pits and poorly endocytosed, whereas wild type receptors with intact cytoplasmic domains generally undergo efficient endocytosis (Brodsky, 1988). Additional evidence suggests that the signal for coated pit localization resides in the cytoplasmic domain, since site-directed mutagenesis of proteins normally excluded resulted in incorporation into coated pits and efficient endocytosis/recycling (Roth et al., 1986; Lazarovits & Roth, 1988).

Since GPI-anchored proteins lack cytoplasmic domains, another consequence of lipid anchoring should be exclusion from coated pits. Early studies demonstrated that both Thy-1 and 5′nucleotidase were excluded from coated pits (Bretscher, Thomson & Pearse, 1980; Matsuura et al., 1984). Similarly, the folate receptor (another GPI-anchored protein) is excluded from clathrin-coated pits, but associated with small membrane invaginations (caveolae) [Rothberg et al., 1990]. In accordance with these studies, GPI-anchored proteins might undergo endocytosis via a clathrin-independent pathway, as appears to be the case for 5′-nucleotidase (Widnell et al., 1982; van den Bosch et al., 1988). Perhaps increased lateral mobility also contributes to the exclusion of GPI-anchored molecules from clathrin-coated pits by decreasing the average time spent in coated areas of the membrane.

In VSV-infected leukemic cells, transmembrane glycoproteins such as H-2, Pgp-1, and T-200, are excluded from the envelopes of budding virions by the close packing of viral envelope glycoproteins, while Thy-1 (a GPI-anchored protein) appears to be selectively incorporated (Calafat et al., 1983). Biochemical studies combining cell surface labeling of Vero cells with VSV viral infection demonstrated the selective incorporation of only two cellular membrane antigens of 110 and 20 kDa (Lodish &

Porter, 1980). Whether these represent GPI-anchored proteins remains unknown. The selective incorporation of GPI-anchored proteins into budding virions may facilitate subsequent viral infections of a specific host (adaptation), since several known GPI-anchored proteins function in cell-cell attachment. Thus, in either case (coated pit clustering *vs.* viral budding) GPI-anchored proteins appear to be "sorted" differently from their transmembrane counterparts.

The Release of GPI-Anchored Proteins May Be Regulated

One of the unique features of the structure of the GPI anchor is the presence of sites for enzymatic modification. Within the glycolipid attachment domain, there are a number of sites that represent potential substrates for GPI-specific anchor-degrading enzymes (proteases, glycosidases and/or phospholipases), the actions of which may result in release of the attached protein from the cell surface. Such an enzyme-mediated release mechanism could be regulated or constitutive. Regulated degradation of the GPI anchor, perhaps under hormonal control, could potentially provide a unique mechanism for downregulation of the concentration of the anchored protein at the cell surface or for upregulation of the protein in the circulation, for its actions at a local or downstream target tissue. Similarly, a constitutive degradation of the GPI anchor could provide a mechanism for secretion of proteins such as GP-2 after translocation to the cell surface.

A number of GPI-anchored proteins such as alkaline phosphatase (Sykes et al., 1987; Romero et al., 1988; Sorimachi & Yasumura, 1988), 5'nucleotidase (Spychala, Madrid-Marian & Fox, 1988; Stochaj et al., 1989), DAF (Medof et al., 1987), lipoprotein lipase (Chan et al., 1988; Vannier et al., 1989), GP-2 (Le Bel & Beattie, 1988), CEA (Jean et al., 1988), Qa-2 antigen (Soloski et al., 1986; Robinson, 1987), Thy-1 (Almqvist & Carlsson, 1988), and 34 kDa placental growth factor (Roy-Choudhury et al., 1988) have been detected in soluble forms. These soluble forms may result from GPI-anchor degradation or differential splicing of mRNA's to produce non-GPI anchored proteins, as documented for N-CAM (Gower et al., 1988), Qa-2 (Stroynowski et al., 1987), and DAF (Caras et al., 1987*a*). The detection of free GPI molecules that are hydrolyzed in response to insulin suggested the possibility that the GPI protein anchor might undergo a similar hormone-sensitive hydrolysis reaction. The acute release of certain GPI-anchored proteins from tissue culture cells in response to insulin has been observed, including lipoprotein lipase (Chan et al., 1988), heparin sulfate proteoglycan (Ishihara, Fedarko & Conrad, 1987*b*), 5' nucleotidase (Klip et al., 1988), and alkaline phosphatase (Romero et al., 1988). Interestingly, the cell surface concentrations of a number of these proteins is known to be altered in diabetes (Skillen, Hawthorne & Turner, 1987; Karnieli et al., 1987). A GPI-anchored form of the Fc receptor (FcR III) was released in response to chemotactic peptide (fmet-leu-phe) (Huizinga et al., 1988). A survey of the total cell-surface GPI-anchored proteins in insulin-sensitive BC_3H1 myocytes indicated that a number of the PLC-releasable proteins were depleted by prior treatment of cells with insulin or serum (Lisanti et al., 1989*b*). Interestingly, exposure to insulin caused the loss of only some of the GPI-anchored proteins, while others remained unchanged. These observations suggest the existence of hormone-sensitive and insensitive "structural" pools of the GPI-anchor (Lisanti et al., 1989*b*). One intriguing possibility is that structural modifications of the GPI moiety play a role in dictating susceptibility to enzymatic degradation. As discussed above, the presence of an ester-linked fatty acid on the inositol ring renders the anchor insensitive to cleavage by PI- or GPI-specific PLC (Roberts et al., 1988*a*).

Although the experiments described above are indirect, they suggest that hormonal treatment results in the activation of an anchor-degrading enzyme(s). However, the nature of this enzyme remains unknown, and could be a specific phospholipase, protease or glycosidase. GPI-specific phospholipases C with similar properties have been isolated from *Trypanosoma brucei* (Fox et al., 1986) and mammalian liver (Fox, Soliz & Saltiel, 1987) and brain (G. Zu & A.R. Saltiel, *unpublished observations*). Both the *T. brucei* and liver enzymes exhibit similar peptide maps, are membrane associated, calcium independent and specifically catalyze the hydrolysis of GPI, but not other phospholipids (Fox et al., 1987). The cDNA for the trypanosomal enzyme has been cloned and sequenced (Hereld, Hart & Englund, 1988). This predicted sequence revealed no homology to other phospholipases, and gave no indication of a signal sequence or glycosylation sites that would be indicative of an extracellular or transmembrane protein. In addition, immunohistochemical studies indicated an intracellular localization (Bulow et al., 1989). Thus, it seems unlikely that the mammalian GPI PLC is involved in anchor degradation under normal conditions. However, the possibility remains that other GPI-specific phospholipases exist. A GPI-specific phospholipase D was found in plasma derived from a number of

sources (Davitz et al., 1987; Davitz, Hom & Schenkman, 1989; Low & Prasad, 1988). This enzyme specifically hydrolyzes the GPI anchor from purified membrane-bound proteins, although it cannot remove cell surface GPI-anchored proteins from intact cells.

In further support of regulated release, the cell surface activity of a PI-specific PLC in Swiss 3T3 fibroblasts has recently been visualized using a fluorescent analog of PI (Ting & Pagano, 1990). After cleavage of PI, free inositol phosphate is released into the medium (as would be expected for release of GPI-anchored proteins), while diacylglycerol flip-flops and redistributes to internal membranes, possibly activating protein kinase C. In addition, the activity of this PI-PLC increases as the cells reach confluency, suggesting that it may play a role in regulating cell growth through surface release of putative growth regulators anchored via GPI.

Conclusion

Experimental evidence has accumulated over the past few years to suggest that the GPI protein anchor may play a broad role in the regulation of membrane protein function. The significant changes in the biophysical properties of proteins that are membrane-anchored through GPI in lieu of a hydrophobic transmembrane peptide indicates a variety of potential new functions served by the anchor structure itself. Moreover, the number of structural variations within the family of GPI molecules indicates a further opportunity for subspecialization of such anchored proteins, especially regarding cellular localization, mobility, metabolism and susceptibility to enzymatically-induced release. It is likely that further exploration of the structure and function of the GPI anchor may reveal additional roles for this unusual mechanism of membrane-protein attachment.

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