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Structure, Biosynthesis, and Function of Glycosylphosphatidylinositols

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Glycosylphosphatidylinositols (GPIs) are a recently discovered class of glycolipids that have been proposed to anchor either protein, polysaccharide, or small oligosaccharides to cellular membranes through covalent linkage. Only recently has the precise chemical nature of these molecules become apparent, and so far the complete structures of only a few have been elucidated. Nevertheless, there is a large body of relevant literature, and several comprehensive reviews of the field have been published (Cross, 1987; Ferguson & Williams, 1988; Low & Saltiel, 1988; Low, 1987, 1989).

Proteins from a diversity of eukaryotic organisms have been found to have GPI membrane anchors, and the list is surely to grow. They have been found in a wide variety of mammalian cells and tissues, squid brain, the slime mold *Dictyostelium*, the yeast (fungus) *Saccharomyces*, and protozoa. There have been no reports of GPIs in prokaryotes, algae, or plants, but neither have there been descriptions of attempts to look at these potential sources. GPIs, either unsubstituted or with polysaccharide rather than protein attached, have been described thus far only in the parasitic protozoan *Leishmania*. Despite the widespread occurrence of GPIs, and the large number of proteins that have GPI membrane anchors, few generalizations or conclusions can be made about their biosynthesis or function. At present only the chemical structures of GPIs can be described with any certainty.

Structures of GPIs and Related Molecules

The complete structures of only two protein-attached GPI membrane anchors [*Trypanosoma brucei* variable surface glycoprotein (VSG) and rat brain Thy-1] and the partial structure of a third, human erythrocyte AChE, have thus far been determined. The VSG and Thy-1 structures were determined by a combination of glycosyl-linkage and compositional analyses by GC–MS, specific chemical cleavages, sequential digestion with purified exoglycosidases combined with Bio-Gel P-4 column chromatography, and one- and two-dimensional ^{1}H NMR spectroscopy (Ferguson et al., 1988; Homans et al., 1988). Comparison of the VSG and Thy-1 anchor structures (Table I) reveals a conserved, linear 6-*O*-(ethanolamine-PO_4)-α-Man*p*-(1→2)-α-Man*p*-(1→6)-α-Man*p*-(1→4)-α-GlcNH_2*p*-(1→6)-*myo*-inositol-1-PO_4 core region. A structure of the VSG anchor from the *T. brucei* variant MITat.1.6 has been published in which the three mannosyl residues form a branch (Schmitz et al., 1987). This structure differs from that proposed for the anchor of VSG from variant MITat.1.4 (Ferguson et al., 1988), in which the mannosyl residues form a linear sequence (Table I). However, recent analysis of the anchor of VSG from the MITat.1.6 variant using one- and two-dimensional ^{1}H NMR has shown that its structure is in fact the same as that of VSG MITat.1.4 (Strang & van Halbeek, 1989).

Analysis by FAB-MS suggests that the human erythrocyte AChE anchor has the same core structure as the VSG and Thy-1 anchors (Roberts et al., 1988b) (Table I). Compositional analyses have indicated the presence of mannose as the only neutral hexose [see Roberts et al. (1988b)], *myo*-inositol (Roberts et al., 1987), and glucosamine (Haas et al., 1986). However, since the identities of potential phosphate-substituted monosaccharides, as well as the anomeric configurations and positions of substitution of the component glycosyl residues in the AChE anchor, are not yet known, it can only be presumed that the core of the human AChE anchor is identical with those of the VSG and Thy-1 anchors.

Heterogeneity in carbohydrate of the VSG anchor structure arises from a variable number of α-linked galactosyl side-chain residues linked to the core region. About 70% of the VSG anchor glycans are accounted for by structures bearing from two to four galactosyl residues, while another 15% most likely consist of structures bearing none, one, or five (or more) galactosyl residues (Ferguson et al., 1988). The two mammalian anchors that have been characterized (Thy-1 and AChE) both lack side chains composed of α-linked galactosyl residues (Homans et al., 1988; Roberts et al., 1988b). Instead, the rat brain Thy-1 anchor contains an additional mannose residue and an *N*-acetylgalactosamine (GalNAc) residue. About 30% of the rat brain Thy-1 structures do not contain the extra

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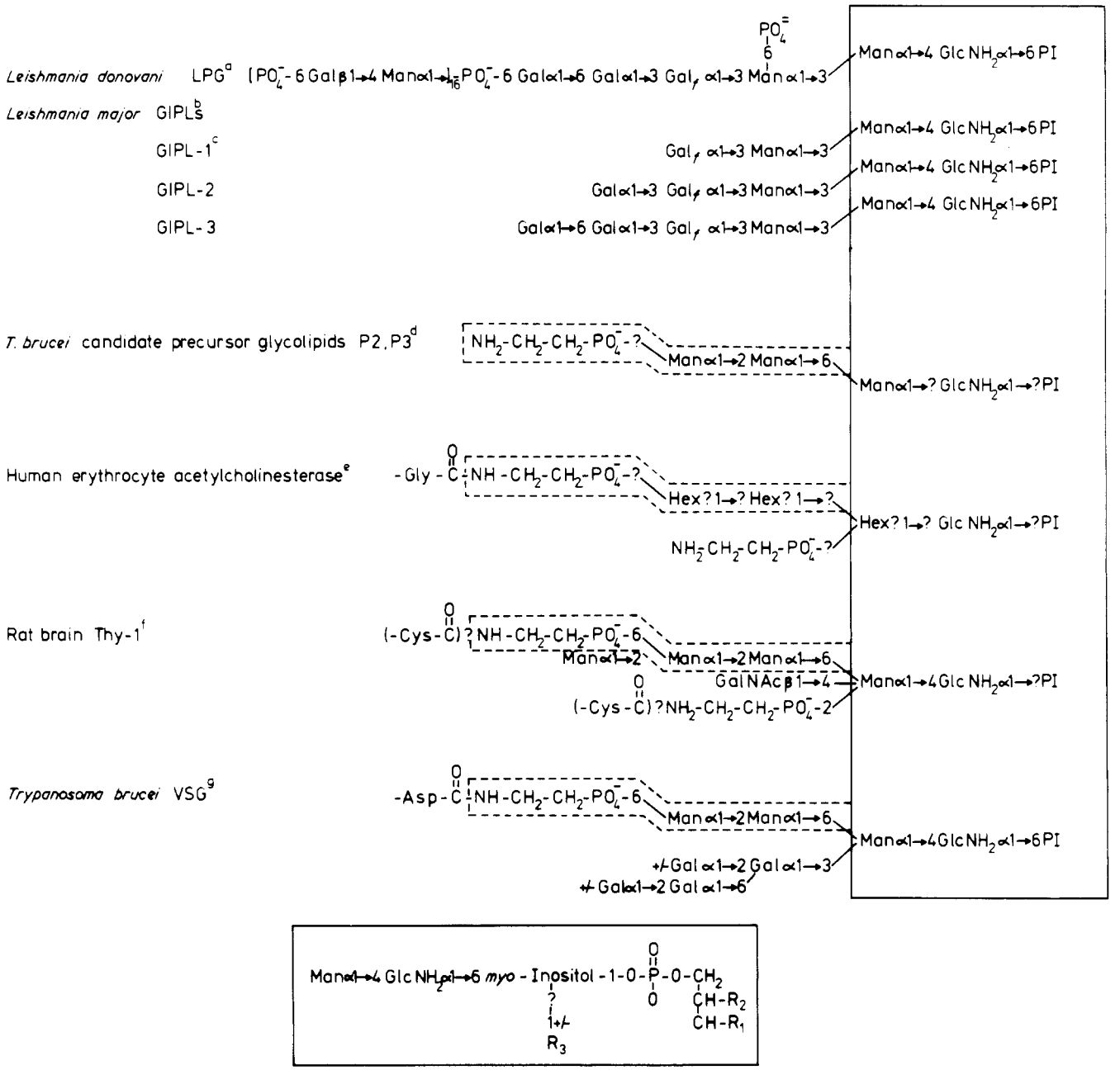
Table I: Structure of GPIs for Which Data Have Been Published[h]



[a] See Turco et al. (1987, 1989). [b] *L. major* GPIs [see Rosen et al. (1989)] have also been reported to bear zero to two galactosyl residues attached to a Man*p* residue rather than the Gal*f* residue found in GIPLs by McConville et al. (1990) (see text for a discussion of these structures). [c] See McConville and Bacic (1989) and McConville et al. (1990b). [d] See Mayor et al. (1990a,b). [e] See Roberts et al. (1989a,b). Mannose is the only hexose detected by compositional analysis. An actylcholinesterose anchor bearing three ethanolamine residues has been observed, but the location of the third residue could not be determined (Roberts et al., 1989b). [f] See Homans et al. (1988). The ethanolamine through which the Thy-1 protein is linked to its anchor has not been determined. Compositional analysis of the rat thymocyte Thy-1 anchors (Tse et al., 1985) suggests that some have one less mannose and one less GalNAc residue than the brain structure. [g] See Ferguson et al. (1988). [h] All of the structures shown have in common a mannoglucosaminyl-PI (areas within solid lines) but can possess different aliphatic side chains R_1 and R_2 (see Table II). R_3 = palmitate in the acetylcholinesterase anchor (Roberts et al., 1988a,b) and probably in the *T. brucei* glycolipid P3 (Mayor et al., 1990b). Glycosyl residues are in the pyranose ring form, unless otherwise indicated, and in the D configuration on the basis of digestion with exoglycosidases. Unknown substitution positions are denoted by (?), glycosidic linkages by (→), and heterogeneity resulting from the presence or absence of residues by (+/−).

mannose residue, giving rise to heterogeneity within structures isolated from the same tissue. Tissue-specific differences in side-chain structure can also occur, as suggested by compositional analysis of rat thymocyte Thy-1 (Tse et al., 1985), which indicates that the extra mannose residue (in brain) is unlikely to be present at all and that only about one third of the anchors contain GalNAc.

Covalent linkage of the protein to the GPI-anchor core region via an ethanolamine–phosphate bridge has been demonstrated for *T. brucei* VSG (Holder, 1983), human AChE (Haas et al., 1986), and Thy-1 (Williams & Tse, 1985). Analysis of the human AChE anchor by FAB-MS revealed that the C-terminal glycine of the polypeptide is linked to the ethanolamine that is located on the nonreducing-terminal hexosyl residue (Roberts et al., 1988b). It has not been determined which, or indeed whether both of the two ethanolamines, bridges the Thy-1 anchor to the protein (Homans et al., 1988). Assuming the core regions of the VSG and human

AChE anchors are the same (see Table I), the proteins would then be linked via a bridging ethanolamine to their respective anchors at the same mannosyl residues in the glycan sequence. It is not known, however, whether linkage of the bridging ethanolamine–phosphate is through O-6 of a mannose residue in the AChE anchor.

In addition to at least one putative bridging ethanolamine, all GPI anchors for which data exist, except that from VSG, contain additional ethanolamine residues. Human red cell decay accelerating factor (DAF) (Medof et al., 1986; Walter et al., 1987) and scrapie prion (Stahl et al., 1987) GPI anchors contain about three ethanolamine residues per mole, while anchors from human and bovine AChE (Roberts et al., 1985; Haas et al., 1986), human placental alkaline phosphatase (Low et al., 1987; Takami et al., 1988), squid brain Sgp2 (Williams et al., 1988), and rat brain and thymus Thy-1 (Tse et al., 1985; Fatemi et al., 1987) contain about two ethanolamine residues per mole. These values may well be averages, since evidence obtained by FAB-MS analysis of the human AChE anchor indicates that it exists in two forms, one with two ethanolamine phosphate residues per molecule and one with three residues per molecule (Roberts et al., 1988b). No information about the location of the third ethanolamine phosphate residue in the AChE anchor was obtained, but the susceptibility of both the second and third ethanolamine residues to reductive methylation prior to Pronase treatment of the purified protein suggests that neither is substituted (Roberts et al., 1988b). The second ethanolamine phosphate residues of the Thy-1 and AChE anchors appear to be linked to analagous hexosyl residues in their respective core glycans (Table I), but it is not known whether the linkage is through O-2 of a mannosyl residue in the AChE anchor as it is in the Thy-1 anchor.

An interesting feature of the human erythrocyte AChE anchor is palmitoylation of the *myo*-inositol residue at a yet to be identified ring position (Roberts et al., 1988b). It was also demonstrated (Roberts et al., 1988a) that this unusual acylation of inositol is responsible for the resistance to PIPLC of a palmitoylated alkylacyl-PI derived from the intact protein by nitrous acid deamination. This result and the observation that the purified protein is resistant to PIPLC (Roberts et al., 1987) strongly suggest that it is indeed palmitoylation of inositol that confers PIPLC resistance to human erythrocyte AChE. Various acylated forms of dimannosyl-PI that contain extra fatty acids (Brennan & Ballou, 1967, Ballou 1972) have been reported in mycobacteria.

Palmitoylation of inositol might also explain the observed resistance to conversion by PIPLC from membrane-bound to soluble forms of other GPI-anchored proteins, which include human erythrocyte DAF (Davitz et al., 1986; Medof et al., 1986) and *Dictyostelium* antigen 117 (Sadeghi et al., 1988). However, no detailed information on the structures of the GPI anchors of these PIPLC-resistant proteins is available. Nevertheless, the TLC mobility of the nitrous acid deamination product of DAF is identical with that of the corresponding product derived from human erythrocyte AChE (Medof et al., 1986; Walter et al., 1987), suggesting that inositol acylation is responsible for PIPLC resistance in both cases (Roberts et al., 1988a). Interestingly, mouse and human erythrocyte AChE are resistant to PIPLC, but bovine, pig, ox, and rat erythrocyte AChE are not resistant (Low & Finean, 1977; Futerman et al., 1985a,b; Roberts et al., 1987; Haas et al., 1986). The functional significance of differences in the sensitivity of GPI-anchored proteins to enzymes that convert membrane-bound forms to soluble forms is unknown.

Table II: Summary of the Aliphatic Side Chains Found in Selected GPI Membrane Anchors, Related Glycolipids, and *Leishmania* Glycolipids[a]

GPI	R_1		R_2		R_3
	acyl	alkyl	acyl	alkyl	acyl
T. brucei VSG anchor[b]	14:0	–[h]	14:0	–	–
T. brucei P3 and glycolipid C[c]	14:0	–	14:0	–	16:0
T. brucei P2 and glycolipid A[c]	14:0	–	14:0	–	–
human erythrocyte AChE anchor[d]	–	18:0, 18:1	22:4, 22:5, 22:6	–	16:0
Leishmania GPIs A-C[e] and GIPLs **1–4**[f]	–	18:0, 24:0	14:0, 16:0, 18:0	–	–
Leishmania LPG[g] and GIPLs[f] **5** and **6**	–	24:0, 26:0	–	–	–

[a] See Table I for the structure of phosphatidylinositol showing the positions of R_1, R_2, and R_3. More abundant residues are underlined. [b] See Ferguson and Cross (1984) and Schmitz et al. (1986). [c] See Menon et al. (1990), Krakow et al. (1986, 1989), Mayor et al. (1990a,b), and the text. The presence of palmitate as R_3 is based solely on biosynthesis labeling (Mayor et al., 1990b). [d] See Roberts et al. (1988a,b). [e] See Rosen et al. (1989). [f] See McConville and Bacic (1989). Lyso forms of GIPLs 2 and 3 have also been found [McConville et al., (1990) and McConville and Bacic (1990)]. [g] See Orlandi and Turco (1987). [h] Indicates not present.

Treatment of GPI-anchored proteins with *T. brucei* GPI-specific PLC or bacterial PIPLC exposes a carbohydrate epitope known as the cross-reacting determinant (CRD). This epitope has been detected by Western blotting on a number of proteins following PIPLC digestion, including *T. brucei* VSG, *Leishmania major* gp63 surface protease, *Torpedo* and human erythrocyte AChE, the scrapie prion protein, Thy-1, and squid brain Sgp2 glycoprotein [see Zamze et al. (1988) and references cited therein], and cross-reaction is a criterion used for GPI anchor identification. Results of selective chemical and enzymatic modification, combined with a competitive ELISA assay, indicate that three overlapping epitopes are involved: (1) the inositol 1,2-cyclic phosphate (produced by PLC cleavage), (2) the non-N-acetylated glucosamine, and (3) the α-galactose branch (Zamze et al., 1988). Western blotting with anti-CRD antibody gives a positive reaction with anchors lacking α-galactose residues (Zamze et al., 1988), but immunoprecipitation may (Krakow et al., 1986) or may not (Zamze et al., 1988) be successful.

It is obvious from the foregoing discussion that failure to observe solubilization, changes in detergent binding, and/or exposure of the CRD upon PIPLC treatment does not necessarily rule out the presence of a GPI membrane anchor. In this event, where inositol acylation may be inhibiting the enzyme, deacylation with mild base may increase PIPLC susceptibility (Roberts et al., 1988a; Clayton & Mowat, 1989). Biosynthetic labeling with *myo*-inositol and ethanolamine or detection of these diagnostic components of GPIs by chemical analysis also provides compelling evidence of their presence. An additional and very useful criterion is the release of a fatty acid label upon deamination with nitrous acid; non-N-acetylated glucosamine is extremely rare and therefore diagnostic of GPIs. The various fragments that can be generated by chemical methods and have been used by various investigators for the structural analysis of GPI anchors are summarized in Figure 1.

GPI anchors display a great deal of variation in the structures of their glycerol-linked lipid components (Table II). Only myristic acid is found in the PI of the *T. brucei* VSG anchor (Ferguson & Cross, 1984; Gurnett et al., 1986), and the glycerol is in the *sn*-1,2-diacyl 3-phosphate configuration

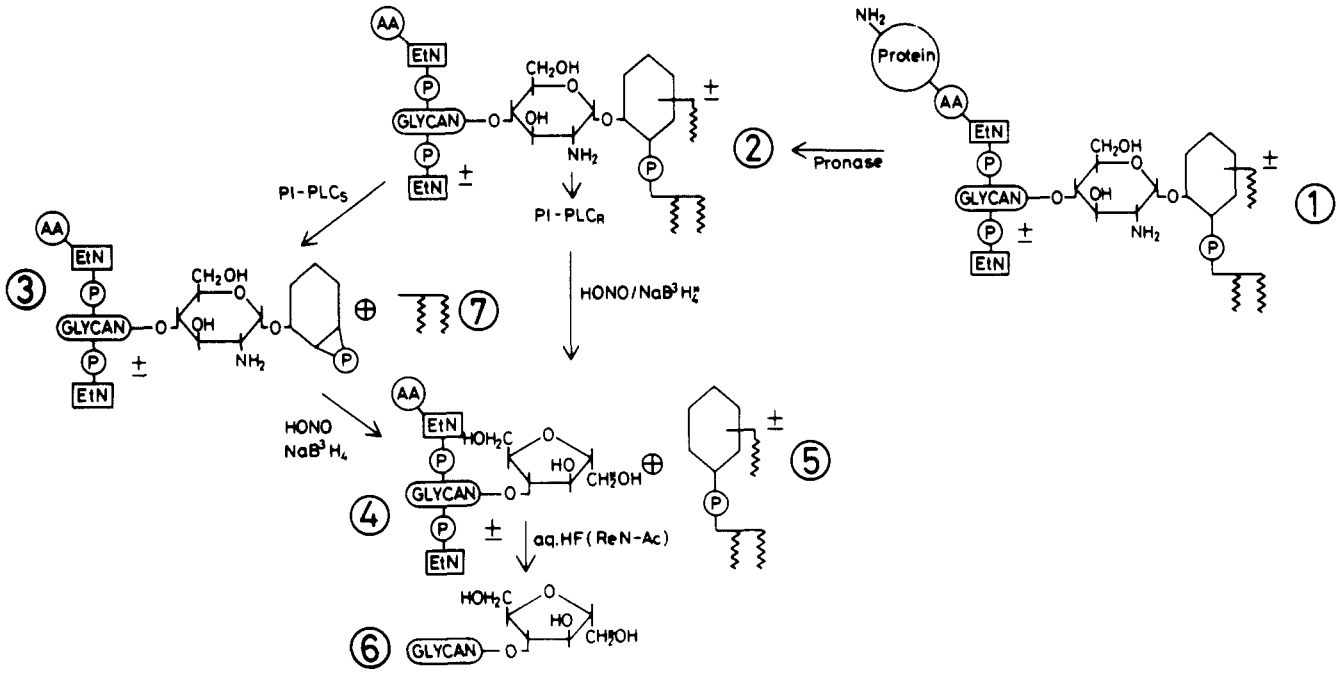


FIGURE 1: Summary of various fragments that have been used for structural determination of GPI. Compound 1 can be metabolically labeled at either the ethanolamine, glucosamine, inositol, or acyl moieties (Brodbeck & Bordier, 1988). Roberts et al. (1988b) used reductive methylation prior to Pronase treatment to label the amine groups of the glucosamine and the extra ethanolamine [see also Brodbeck and Bordier (1988)]. The lipid moieties can also be photolabeled with [^{125}I]TID [3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine] (Roberts et al., 1988a). The reductive methylation however makes the GPI resistant to nitrous acid fragmentation. Compound **2** can be treated with nitrous acid to cleave off the inositol phospholipid moiety (compound **5**). The presence of an extra acyl group on the inositol ring makes the GPI anchor resistant to PI-PLC (Roberts et al., 1988b). If the inositol is not acyl substituted, PI-PLC treatment will give compound **3** plus the diacylglycerol moiety **7**. Radiolabeling of compound **2** and **3** can be achieved by treatment with nitrous acid and subsequent Na^3BH_4 reduction to convert the glucosamine to radiolabeled 2,5-dianhydromannitol **4** (Ferguson et al., 1988). The glycan portion **6** can be generated by treatment of compound **4** with HF. If acid-sensitive residues are present such as galactofuranose, then the HF treatment will also cleave at these points (Turco et al., 1989). Compound **3** contains the anti-CRD reactive epitopes for Western blotting of proteins containing a PI-PLC-sensitive GPI (Zamze et al., 1988). PI-PLC-resistant GPI anchors can be blotted after base treatment to cleave the acyl group, making the protein PI-PLC sensitive (Roberts et al., 1988a). Other chemical treatments useful in the structure analysis of GPI anchors include acetolysis, base hydrolysis, deacylation, and reacylation [see Brodbeck and Bordier (1988)]. A variety of analytical techniques have been used in the structural characterization of GPIs. Sequential exoglycosidase digestion combined with Bio-Gel P-4 chromatography is perhaps the most sensitive method (when radiolabeled oligosaccharides are used) and can, by virtue of the exquisite specificity of the exoglycosidases, define the identities (mannose, galactose, glucose, etc.), ring forms (furanose or pyranose), absolute configurations (D or L), anomeric configurations (α or β), in many cases the sequence, and in some cases the position(s) of substitution of glycosyl residues. The appropriate, highly purified enzymes are required for unambiguous results. Combined gas chromatography–mass spectrometry can yield the identity, molar ratios, absolute configurations, ring forms, and position(s) of substitution of most monosaccharide components, as well as the identities and molar ratios of lipid moieties, but provides only very limited information on the arrangement of the components with respect to each other. Fast atom bombardment mass spectrometry (FAB-MS) analysis provides the molecular weight and information on the sequence of glycosyl residues and both the nature and position of non-carbohydrate substituents; however, the identities of glycosyl residues and stereochemical information cannot be obtained by this method alone. NMR spectroscopy can also provide structural information, from composition to solution conformation, but has the drawback of a relative lack of sensitivity (about $^1/_{100}$ that of MS techniques). Combinations, or even all, of these techniques are necessary for a complete and unambiguous structural assignment of GPIs, due to their structural complexity and the different types of components that may be present (carbohydrate, ethanolamine, phosphate, protein, and lipid). Finally, structural analysis on heterogeneous mixtures is the greatest cause of incorrect structural assignments, necessitating separation of the various forms prior to analysis.

(Ferguson et al., 1985). VSGs of *Trypanosoma equiperdum* and *Trypanosoma congolense* can be biosynthetically labeled with [^{3}H]myristic acid but not [^{3}H]palmitic acid (Lamont et al., 1987), which suggests that they have the same fatty acid composition as their *T. brucei* counterpart. On the other hand, both the gp63 surface acid protease of *L. major* and the 195-kDa merozoite antigen of *Plasmodium falciparum* can be labeled with both fatty acids (Haldar et al., 1985). A variety of saturated and unsaturated fatty acids are found [see Ferguson and Williams (1988) and references cited therein]. 1-Alkyl-2-acylglycerol, rather than 1,2-diacylglycerol, is found in human and bovine erythrocyte AChE (Roberts et al., 1987, 1988b) and probably human erythrocyte DAF (Medof et al., 1986). The alkyl substituents are predominantly 18:0 and the acyl substituents 22:4 and 22:5 (Roberts et al., 1988a). Interestingly, an alkylacylglycerol structure has also been proposed for the insulin-sensitive GPI from H35 hepatoma cells (Mato et al., 1987), whereas the insulin-sensitive GPI from BC_3H1 monocytes contains diacylglycerol (Saltiel et al., 1986; Saltiel & Cuatrecasas, 1986). The LPGs and GPIs/GIPLs of *Leishmania* parasites (see below) also contain long-chain alcohols in ether linkage to glycerol. Finally, there is a report that the contact site A glycoprotein of *Dictyostelium* contains a ceramide-based phospholipid anchor (Stadler et al., 1989).

Some species of the parasitic protozoan *Leishmania* synthesize glycolipids with core structures resembling those of the GPIs that anchor membrane proteins. The GPI core of the *Leishmania donovani* LPG (Turco et al., 1989) resembles the GPI membrane protein anchors by virtue of possessing the sequence α-Man*p*-(1→4)-α-GlcNH_2*p*-(1→6)-*myo*-inositol-PO_4 (Table I). Beyond this conserved monosaccharide sequence, however, the carbohydrate portions of this protozoan glycolipid and the protein-linked GPI membrane anchors diverge completely, with the next mannosyl residue linked α(1→3) and α(1→6), respectively. The LPG of *L. donovani* also contains a polysaccharide made up of a repeating phosphorylated disaccharide unit (average of 16 repeats), 6-*O*-PO_4-β-Gal*p*-(1→4)-α-Man*p*-(1→ (Turco et al., 1987), that

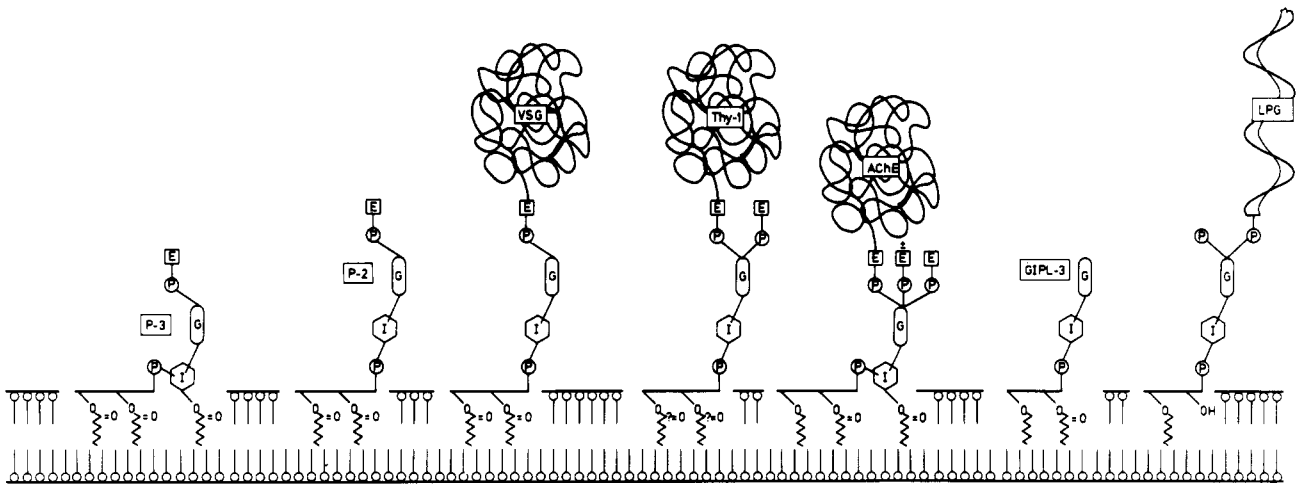


FIGURE 2: Schematic representation of the various forms of GPI anchors and related glycolipids so far reported. No inferences should be made as to the exact orientation of the GPI anchor in the membrane nor the conformational relationship between the protein (or polysaccharide) and the GPI anchor. The glycans (G) differ between the various structures and are tabulated in Table I. The lipids present on each of the molecules are presented in Table II. For Thy-1 it is not known whether the aliphatic side chains are alkyl or acyl. AChE exists in two forms, one with three ethanolamine phosphates and the other with two. The points of attachment of the phosphates (P) are represented schematically, and it should not be inferred that they are linked to the glycans (G) at a single residue (see Table I).

is linked to the glycolipid consisting of 6-*O*-PO_4-α-Gal*p*-(1→6)-α-Gal*p*-(1→3)-α-Gal*f*-(1→3)-α-Man*p*-(1→3)-α-Man*p*-(1→4)-α-GlcNH_2*p*-(1→6)-lysoalkyl-PI (Turco et al., 1989). The mannosyl residue to which the galactofuranosyl residue is linked in the LPG core bears a phosphate attached to O-6 (S. J. Turco, J. R. Thomas, J. Thomas-Oates, R. A. Dwek, and T. W. Rademacher, unpublished results). *L. major* also produces an LPG that contains mannose, galactose, arabinose, and glucose as phosphorylated tri- and tetrasaccharides (McConville et al., 1987) rather than the phosphorylated β-Gal-(1→4)-α-Man disaccharide repeats of the *L. donovani* LPG (Table I); however, the *L. major* LPG contains an internal galactofuranosyl residue in its glycan core (McConville & Bacic, 1990) and the same lipid moieties (see below) as its *L. donovani* counterpart.

L. major also produces glycolipids, termed GPIs (Rosen et al., 1989) or GIPLs (glycoinositol phospholipids) (McConville & Bacic, 1989, 1990), that contain an oligosaccharide of variable size linked to PI (Table I). The published structures of the GPIs from *L. major* strain LRC-L137 (Rosen et al., 1989) differ most notably from the *L. donovani* LPG structure in two respects: the absence and presence, respectively, of a 3-substituted galactofuranosyl residue and the replacement of the 6-substituted α-galactosyl residue in the LPG with a 3-substituted α-galactosyl residue at the homologous position in the GPI. Structures of the GIPLs from *L. major* strains V121 and LRC-L119 (McConville & Bacic, 1989, 1990; McConville et al., 1990) are shown in Table I. All of the GIPLs contain a 3-substituted galactofuranosyl residue, and the terminal α-galactosyl residue of GIPL-3 is linked α(1→6), which makes it structure homologous to that of the *L. donovani* LPG core. It remains to be determined whether the differences in the structures of *L. major* GPIs and GIPLs result from the use of different subclones of the same variant or from the interpretation of results.

Further divergence between the protozoan glycoconjugates (LPGs and GIPs/GPILs) and GPI anchors of membrane proteins is seen in their lipid portions (Table II). The *L. major* GPIs (Rosen et al., 1989) and some of the *L. major* GIPLs contain 1-alkyl-2-acylglycerol (Rosen et al., 1989; McConville & Bacic, 1989). As noted above, some protein GPIs have been found to contain 1-alkyl-2-acylglycerol. The lipid portions of both *L. donovani* LPG and some of the *L. major* GIPLs are even more unusual, containing a lyso-1-*O*-alkyl-PI (Orlandi & Turco, 1987; McConville et al., 1987; McConville & Bacic, 1989). These novel lipids contain only one ether-linked alkyl chain and no ester-linked fatty acid (Table I). Di-*O*-alkyl-PIs have been isolated from *Leishmania mexicana mexicana* (Singh et al., 1988); however, no such lipids bearing either oligosaccharide, polysaccharide, or protein have been isolated.

In summary, a variety of glycolipid structures based on PI have been characterized (Table I and Figure 2). The GPI membrane protein anchors contain a conserved core glycan to which are attached various carbohydrate side chains and ethanolamine phosphate(s). One of the ethanolamine phosphates links the protein to the glycolipid via amide and phosphodiester linkages. Acylation of the inositol in GPI anchors confers resistance to digestion by bacterial PIPLC. *Leishmania* glycolipids (LPGs, GPIs/GIPLs) appear to share a portion of the conserved core glycan of reported GPI membrane protein anchors, but beyond this α-Man*p*-(1→4)-α-Glc*N*p-(1→6)-*myo*-inositol sequence, the structures diverge. *L. donovani* LPG contains a small, highly phosphorylated polysaccharide attached to its glycan core, while *L. major* GPIs/GIPLs contain only a few glycosyl residues. Further investigation is required before any conclusion concerning the roles of the *L. major* GPIs/GIPLs as biosynthetic intermediates in either LPG or GPI membrane protein anchors can be inferred. It has been suggested (McConville & Bacic, 1990; McConville et al., 1990) that GIPL-3 may function as the biosynthetic precursor of LPG after undergoing selective deacylation to give the lyso-1-*O*-alkyl-PI analogue present in LPG. Since the structure of a GPI anchoring a membrane protein from *L. major* or *L. donovani* has yet to be reported, no conclusions can be made concerning the relationship between GPI anchors, GPIs, GIPLs, and LPGs. Preliminary data, however, suggest that the GIPLs are not precursors for membrane anchors of cell surface proteins (McConville et al., 1990).

Biosynthesis of GPIs

Fully processed proteins containing GPI anchors lack a C-terminal sequence that is present in the immature, anchorless form [see Ferguson and Williams (1988) and references cited therein]. For example, nascent VSG contains a 20 amino acid C-terminal peptide, which is replaced with the

GPI anchor (Boothroyd et al., 1981). It is not known whether removal of the C-terminal sequence occurs before or simultaneously with addition of the anchor, but presumably this sequence functions as the signal for GPI addition. Fusion of the C-terminal domains of GPI-anchored Qa-2 antigen (Stroynowski et al., 1987; Waneck et al., 1988) or DAF (Caras et al., 1987) to proteins that are normally secreted or transmembrane results in the addition of GPI to the fusion proteins. All known GPI-anchored proteins are synthesized with a 10–20-residue hydrophobic domain at the C-terminus, which appears to be a requisite component of the addition signal. The hydrophobic domain must be a minimum length (Berger et al., 1988), but there seem to be no stringent sequence requirements, as replacement with other hydrophobic domains results in correct processing (Caras & Weddell, 1989). On the other hand, the degree of hydrophobicity may be important, since a single base change resulting in substitution of Asp with Val changes GPI-anchored Qa-2 to an apprently integral membrane protein (Waneck et al., 1988). In addition to a hydrophobic domain, information within the adjacent 20 residues is also important. Caras et al. (1989) found that either residues 291–310 or 311–330 of DAF could direct GPI addition but that other, nonspecific sequences could not. The cleavage/attachment site of DAF is not known; so far, Ala, Asn, Asp, Cys, Gly, and Ser have been identified as sites in other proteins [see Low (1989) and references cited therein]. Thus, the exact nature of the required signal within the 20 amino acid sequence adjacent to the hydrophobic domain is not known.

The rapidity with which GPI is added to proteins (1–5 min) (Bangs et al., 1985; Krakow et al., 1986; Ferguson et al., 1986; Conzelman et al., 1987; Takami et al., 1988) suggests that at least a portion of it exists in a preassembled form that is added en bloc. Two putative precursor glycolipids, termed glycolipid A (Krakow et al., 1986) and P2 (Menon et al., 1988), have been isolated from trypanosomes. Glycolipid A can be labeled by [^{3}H]myristrate but not by [^{3}H]palmitate and liberates PI when treated with nitrous acid and dimyristylglycerol when treated with PIPLC, and [^{3}H] mannose label can be immunoprecipitated by anti-CRD antibodies after PIPLC treatment (Krakow et al., 1986). P2 contains mannose, glucosamine with a free amino group, phosphate, and myristic acid, releases dimyristyl-PI upon nitrous acid deamination, and is susceptible to PIPLC (Menon et al., 1988). The structure of the deaminated, reduced, and phosphorylated glycan from P2 has been found to be α-Man-(1→2)-α-Man-(1→6)-α-Man-(1→?)-2,5-anhydromannitol (Mayor et al., 1990a), which suggests that its structure is the same as that of the conserved core region of the VSG and Thy-1 anchors (Table I). It is highly likely that glycolipids A and P2 are the same molecule, but detailed structural information on the glycan of glycolipid A is needed to confirm the identity of the two molecules. Although the two glycolipids possess structural features consistent with a precursor role and the kinetics of labeling of glycolipid A suggest that it is a metabolic intermediate (Krakow et al., 1986), there is no direct evidence that they are involved in GPI anchor formation.

A pathway for the biosynthesis of the putative glycolipid precursor of the membrane anchor of VSG has been proposed on the basis of results obtained with trypanosome cell free systems. The putative precursor is built up from PI by the sequential addition of a GlcNAc residue, three mannosyl residues, and finally ethanolamine phosphate to give ethanolamine-P-Man$_3$GlcNH$_2$-PI (Masterson et al., 1989; Menon et al., 1990). Doering et al. (1989) have obtained evidence suggesting that the GlcNH$_2$ residue is derived from GlcNAc, which is donated to PI by UDP-GlcNAc and subsequently de-N-acetylated. Addition of one or more mannosyl residues may involve dolichol-P-Man as the donor species. Treatment of trypanosomes with 2-fluoro-2-deoxy-D-glucose, an inhibitor of dolichol-P-Man synthesis in chick fibroblasts, inhibits incorporation of radiolabeled precursors into the putative precursor (Schwarz et al., 1989). Furthermore, a mutant lymphoma cell line that is deficient in dolichol-P-Man synthesis is also deficient in cell-surface expression of GPI-anchored Thy-1 (Conzelmann et al., 1986; Fatemi et al., 1987). Preliminary evidence (Conzelmann et al., 1990) indicates that the only available secretion and glycosylation mutant of *Saccharomyces cerevisiae* that does not produce GPI-anchored proteins is *sec* 53, which does not produce phosphomannomutase; although this suggests that GDP-mannose is also required for the biosynthesis of GPI anchors, it may only be needed for dolichol-P-Man synthesis.

Preliminary results (Masterson et al., 1989; Doering et al., 1990; Menon et al., 1990) suggest that the putative glycolipid precursors of GPI anchors undergo fatty acid remodeling. Myristate present in the mature glycolipid species is thought to replace more hydrophobic fatty acids at a late stage in the biosynthesis. Consistent with this scheme, the PI substrate for GlcNAc addition appears to be a heterogeneously acylated lipid rather than dimyristyl-PI (Menon et al., 1990), and [^{3}H]myristrate label is incorporated only into the mature glycolipids (Masterson et al., 1989; Menon et al., 1990). Myristoyl coenzyme A appears to be the donor for replacement at the *sn*-2 position (Doering et al., 1990), but little else is known of the remodeling system.

In addition to the PIPLC-sensitive glycolipids (glycolipids A and P2) described above, trypanosomes synthesize similar glycolipids that are resistant to the enzyme, termed either glycolipid C (Krakow et al., 1986) or glycolipid P3 (Menon et al., 1988). P3 and probably glycolipid C have the same glycan linked to PI as their enzyme-sensitive counterparts, and acylation of the inositol moiety appears to be the sole difference between the sensitive and resistant molecules (Mayor et al., 1990a,b; Krakow et al., 1989). Biosynthesis of the inositol-acylated, PIPLC-resistant glycolipids in trypanosomes has been investigated by Cross and co-workers using a cell-free system (Menon et al., 1990). The donor of the inositol-linked fatty acid does not appear to be a fatty acyl coenzyme A, and inositol-acylated GlcNH$_2$-PIs were observed. If inositol-acylated glycolipids are important in the assembly of GPI anchors, they could (1) make PI-containing glycolipids better substrates for glycosyltransferases, (2) protect mannosyl-PIs from digestion by GPI-specific PLC, and/or (3) effect the transbilayer distribution of mannosyl-PIs (Menon et al., 1990). In the case of P3, biosynthetic labeling suggests that the inositol is palmitoylated (Mayor et al., 1990b). Inositol palmitoylation is the same modification that renders the GPI anchor of human erythrocyte AChE resistant to PIPLC (Roberts et al., 1988a,b; see above). A palmitoylated, PIPLC-resistant glycolipid could serve as the precursor of a PIPLC-resistant, GPI-anchored cell-surface glycoprotein synthesized by procyclic (insect stage) trypanosomes (Clayton & Mowatt, 1989). It remains to be determined whether PIPLC-resistant glycolipids that could serve as precursors to proteins with resistant GPI anchors are synthesized by other eukaryotes.

Bangs et al. (1988) have obtained evidence suggesting that the galactosyl residues in the VSG anchor are added subsequent to GPI as an addition to the protein. They showed that the C-terminal glycopeptide from a mature 59-kDa form of

VSG can be converted by treatment with α-galactosidase to a form that coelutes from Bio-Gel P-6 with the glycopeptide isolated from an immature 58-kDa form. Although VSG appears to travel to the cell surface via the classical eukaryotic transport route through the Golgi apparatus (Duszenko et al., 1988), the intracellular site at which galactosylation occurs is not known. Since biosynthetic studies have been carried out only with trypanosomes, the timing and site of addition of the GalNAc and fourth mannosyl residues to the Thy-1 anchor are not known. Likewise, no conclusions can be made about the addition of further ethanolamine phosphates to those authors with more than one such residue (see above), since trypanosome VSG contains only one ethanolamine phosphate.

Fasel et al. (1989) have recently demonstrated selective incorporation of phospholipase-sensitive anchoring moieties into Thy-1 and DAF during in vitro translocation experiments. This indicates that rough microsomes are able to support and regulate GPI anchor incorporation and that all components for GPI-anchor processing are contained in this cell fraction. The availability of this cell-free translation system containing all the ingredients necessary for GPI addition will be useful as a experimental model to define the precursors and enzymes involved in the mammalian biosynthetic pathway.

Function of GPIs

No generalization can be made so far about the roles or properties of proteins that have GPI anchors. Most seem to be located at the cell surface, but one (the major protein of the pancreatic zymogen granule membrane; LeBel & Beattie, 1988) has been detected elsewhere in the cell. They vary in size from 10 to 300 kDa (Low, 1989) and fall into diverse functional groups, including hydrolytic enzymes (e.g., alkaline phosphatase, AChE, and the *Leishmania* 63-kDa surface protease), cell adhesion molecules (e.g., N-CAM, LFA-3, and *Dictyostelium* contact site A), and protective coat proteins (e.g., *Trypanosoma* VSG). The physiological roles of many GPI-anchored proteins are not yet known (e.g., scrapie prion protein, *Saccharomyces* 125-kDa glycoprotein, and mammalian antigens such as Thy-1, Qa-2, and carcinoembryonic antigen).

Phospholipid-containing membrane proteins from *S. cerevisiae* are >90% susceptible to the action of PIPLC (Conzelmann et al., 1990). However, there is no loss of the detergent-binding moiety upon PIPLC treatment for a large number of the proteins. These authors have proposed that many of the GPI-containing membrane proteins may have other hydrophobic, detergent- (membrane-) binding domains. These observations may be fundamental to our current perception of the role of GPI anchors as the sole membrane anchor of the proteins to which they are attached. The lack of hydrophobic amino acids near the C-terminal in proteins containing GPI anchors has been put forward as evidence that membrane attachment of these proteins is due entirely to the acyl chains of the phosphatidylinositol (Low & Kincade, 1985). Obviously other hydrophobic protein domains may still be present to interact with the lipid bilayer. Flow-cytometry reduction of intensity after PIPLC treatment of intact cells cannot be interpreted as release of a protein from the cell surface. Both internalization following PIPLC cleavage and a change in reactivity to the fluorescently labeled antibody to the protein under study would give a similar experimental result. Further, unless absolute calibration of fluorescent intensity and copy number is performed, these flow-cytometry experiments cannot be interpreted. Such multipoint attachment to membrane has been previously proposed for acylated (palmitoylated) proteins such as rhodopsin and the β_2-adrenergic receptor (Ovchinnikov et al., 1988; O'Dowd et al., 1989). In both of these molecules detachment from the acyl group leads to conformational and functional changes in the protein without release from the membrane. Figure 3 illustrates how such an arrangement may be useful in maintaining either an active or inactive conformation. Phospholipase action would be an on/off switch in this model. A further mechanism of multipoint anchoring can be envisaged for proteins that have their hydrophobic amino acid sequence (membrane spanning) near the N-terminus rather than near the C-terminus (i.e., the C-terminus is extracellular).

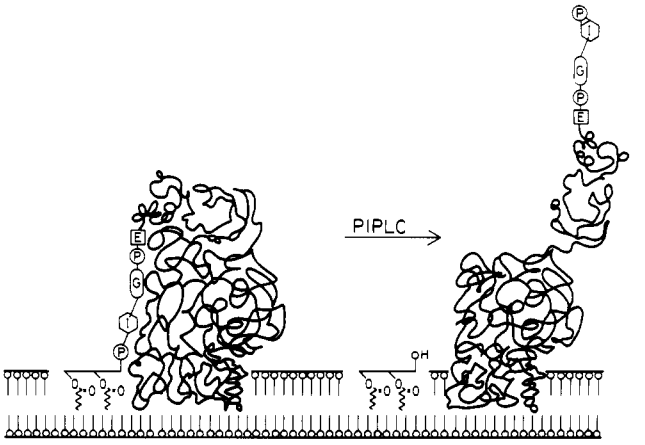


FIGURE 3: Hypothetical alternative function of GPI anchors. The inability to remove the detergent-binding (hydrophobic) domains of some GPI-containing proteins (see text) suggests that they may not be their sole membrane anchor and that the GPI moiety may function to tie down domains that can conformationally be modulated by the cleavage of the GPI anchor by PIPLC.

The physical properties imparted to proteins via GPIs may be crucial to their functioning. For example, it is easy to envisage that the mediation of cell adhesion by surface molecules such as N-CAM, LFA-3, and *Dictyostelium* contact site A is dependent upon, or at least affected by, their mode of membrane attachment. Proteins with GPI anchors do have higher lateral diffusion coefficients and different solubilities in detergents than do proteins that span the membrane [see Low (1987, 1989b) and Ferguson and Williams (1988) and references cited therein]. The GPI glycan of VSG has been proposed to serve a space-filling role, on the basis of the determination of its average solution conformation (Homans et al., 1989). The VSG glycan is proposed to lie along the plane of the membrane and span an area of 600 Å^2, which is comparable to the cross-sectional area of the protein's N-terminal domain (Figure 4), and could, therefore, be important in dimerization and/or the maintenance of the VSG coat as a diffusion barrier. Several proteins [e.g., the IgG receptor (CD16), LFA-3, N-CAM, AChE, and Leu 8/TQI] have been found to exist as GPI anchored, as well as transmembraneous [Camerini et al., (1989), Scallon et al. (1989), and Ferguson and Williams (1988) and references cited therein]. It remains to be determined whether functional differences result from the expression of alternatively anchored forms of the same protein.

The existence of phospholipases that can cleave GPI membrane anchors has led to speculation that release of membrane-bound proteins via this mechanism is of physiological significance. For example, degradation of the GPI anchor of GP-2 in the pancreatic zymogen granule has been proposed to lead to its release from the granule membrane (LeBel & Beattie, 1988); however, the enzyme responsible has yet to be identified. A GPI-PLC (so-called because of its specificity for GPI anchors and related molecules) has been isolated from

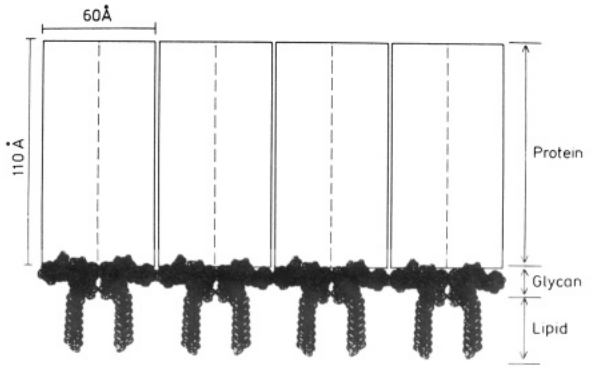


FIGURE 4: Two VSG glycan anchors can be accommodated by a rectangle of 30 Å × 50 Å (diagonal of 56 Å). The N-terminal two-thirds of a VSG protein (dimer) can be approximated as a 110-Å columnar structure (max diameter 60 Å) including a cap region which can be accommodated with a rectangle of 30 Å × 50 Å. No structural data are available concerning the C-terminal portion of the molecule. The figure was drawn with the dimensions given above to illustrate the relative widths of the VSG glycoprotein (monomer and dimer) relative to the GPI anchors. It should be noted, however, that a considerable amount of surface of the protein may still interact directly with the membrane. Preliminary data on the interaction between the Thy-1 peptide and its glycan anchor suggest that the glycan part of the GPI anchor may fit up into a pocket in the protein with only the terminal part of the lipids extending, thus allowing for extensive direct interactions between the Thy-1 peptide and the membrane surface (C. Edge, R. A. Dwek, and T. W. Rademacher, unpublished results).

T. brucei and rat liver, and a GPI-PLD is found in serum [see Ferguson and Williams (1988) and Low (1989) and references cited therein]. The *T. brucei* enzyme could function in the shedding of VSG during conversion of the bloodstream form to the coatless, insect-dwelling form of the organism (Bulow & Overath, 1985), and a model involving GPI-PLC-containing vesicles and both endo- and exocytosis has been proposed (Ferguson & Williams, 1988).

While VSG is being shed from the trypanosome surface, another surface protein (termed either procyclin or PARP) is expressed, and both VSG and PARP are for a time present simultaneously on the cell surface (Roditi et al., 1989). PARP is PIPLC resistant, and the differential susceptibility of PARP and VSG anchors could allow the selective release of VSG and retention of PARP during the life-cycle transformation (Clayton & Mowatt, 1989). Many GPI anchors are resistant to bacterial PIPLC (discussed above). Resistance to specific phospholipases could be a general mechanism whereby the release of GPI-anchored proteins is controlled. No unequivocal data, however, have been put forward that demonstrate that these proteins are in fact released by the action of a phospholipase.

The successful invasion and infection of hosts by *Leishmania* may depend on the presence of LPG and GIPLs on its surface. These parasites must survive in the harsh environments of the alimentary tract within the sandfly vector and of the phagolysosomes of infected mammalian host macrophages. LPG is the major cell-surface glycoconjugate of *Leishmania*, and protection probably is afforded by the presence or large amounts of LPG serving as a protective barrier against hydrolytic enzymes. In addition, both LPG and GIPLs from *Leishmania* have been shown to be efficient inhibitors of protein kinase C (McNeely et al., 1989) and may therefore prevent induction of the microbicidal oxidative burst within macrophage phagolysosomes (Turco, 1988a). LPG may also inhibit the oxidative burst by chelating intracellular calcium or other divalent cations and/or by scavenging oxygen free radicals [Chan et al. (1989) and Turco (1988b) and references cited therein]. It has been suggested that, besides having a protective role, LPG is also involved in attachment to and penetration of the host macrophage (Handman & Goding, 1985; Puentes et al., 1988). It is not known at present whether GPI anchors also have roles similar to those of LPGs and GIPLs.

Results from a large number of studies suggest that both GPI-anchored proteins and glycolipids that are structurally related to GPI anchors are involved in cell activation. Two of the more interesting cases where GPIs may be involved in transduction of extracellular signals are (1) the mitogenic effect of antibodies directed against GPI-anchored proteins on T-lymphocytes [see Robinson and Spencer (1988) and references cited therein; Robinson et al., 1989] and (2) the possible role of a GPI anchor or related glycolipids as a mediator of insulin action (Low & Saltiel, 1988). Unfortunately, more information is needed before any meaningful conclusions about the importance of GPIs in these processes can be made.

GPI membrane anchors appear to act as a targeting signal in Madin–Darby canine kidney (MDCK) cells, which form a polarized monolayer at confluency with an apical and a basolateral surface. Digestion with PIPLC released six proteins from the apical surface but none from the basolateral surface (Lisanti et al., 1988). Results of experiments in which fusion proteins are expressed in transfected cells show that a GPI anchor can direct proteins to the apical surface. Thus, GPI anchoring of a normally basolateral protein led to its apical expression, and replacement of the GPI anchor of an apical protein with the membrane-spanning domain of a basolateral protein led to its basolateral expression (Brown et al., 1989). Likewise, transfer of the GPI-addition signal of an apical membrane protein to either a basolateral or secreted protein resulted in apical expression of the fusion proteins (Lisanti et al., 1989). The mechanism by which GPIs influence intracellular trafficking of proteins in MDCK cells is unknown, but it has been suggested (Brown et al., 1989; Lisanti et al., 1989) that the sorting of glycosphingolipids and GPI-anchored proteins may be linked.

Summary

The last few years have witnessed an explosion in our knowledge of GPI membrane anchors and related glycolipids and molecules where structure details are available, as illustrated in Figure 2. There is now sufficient information on a handful of these molecules to allow a detailed comparison of their chemical structures (Table I). Despite a common structural theme, i.e., the presence of mannoglucosaminyl-PI, a great deal of diversity exists in both the glycan structures and the glycerol-linked aliphatic substituents. The complexities of the structures clearly show that a multitechnique approach is required in the elucidation of their structures. The anticipated publication of more structures from a wider range of organisms may reveal even greater diversity, as well as suggesting possible biosynthetic pathways. The details of a potential biosynthetic pathway in *T. brucei* are becoming apparent, but confirmation of its importance awaits the isolation and characterization of the enzymes involved. *Leishmania*, in which LPG, GPIs, GIPLs, and GPI membrane anchors are produced, may also provide an interesting system for biosynthetic studies. The recent description of a GPI biosynthetic system in yeast may provide the crucial breakthroughs necessary in unraveling the enzymes and sugar donors involved in the biosynthetic pathway and possibly the role of the GPI membrane anchor in the functions of proteins containing these moieties.

Knowledge of the solution structure (conformation), in addition to the complete chemical structure, of the *T. brucei*

VSG anchor has led to speculation that the glycan fulfills a space-filling role in the VSG coat. Many other possible roles of GPI membrane anchors have been suggested, including the shedding and turnover of membrane proteins, signal transduction, and intracellular targeting. Nevertheless, the only function of GPIs that we can so far be certain of is that they anchor proteins or polysaccharide to a membrane. Regardless of the roles GPIs may or may not ultimately be shown to play, the fact that such a widely occurring structure has only recently been characterized serves as a reminder of the incompleteness of our knowledge of biological phenomena and the constant possibility of finding novel molecules in obvious places.

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