GLYCOLIPID ANCHORING OF CELL SURFACE PROTEINS

Mark C. Field and Anant K. Menon

Laboratory of Molecular Parasitology, The Rockefeller University, New York, NY 10021, USA.

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I. Introduction

Over the last decade a number of new covalent modifications of proteins have been identified and characterised. Several of these involve the attachment of hydrophobic moieties to the polypeptide chain, e.g. myristoylation, isoprenylation, palmitoylation, and glypiation. The first three of these modifications are dealt with elsewhere in this volume. Glypiation,[1] the attachment of a phosphatidylinositol-containing glycolipid (GPI) is perhaps the most complex in structural and biosynthetic terms.

In this chapter we shall discuss several aspects of GPI biology. We shall briefly outline the data which suggested the existence of this type of protein modification, and then describe the elucidation of the structure of the GPI-anchor of the variant surface glycoprotein of (VSG) of *Trypanosoma brucei*. We shall also document the current structural data base of GPI (and related glycoconjugate) structures, and briefly describe the empirical approaches towards identification and elucidation of the structures of GPI species. We shall then describe the biosynthesis of GPI-anchors, and finally consider possible functional aspects of GPI-anchoring as opposed to transmembrane polypeptide-anchoring. The reader is referred to a number of recent reviews for more detailed discussions of particular aspects of this field; general overviews[2,3,4], details of structure and elucidation[1,5,6], summary of C-terminal extension and processing site sequences,[1] possible functions[7], and precursor biosynthesis[8,9,10].

II. Discovery of GPI

1. Release of Surface Proteins From Cells with PI-PLC

Increased levels of alkaline phosphatase (AP) activity were detected in the serum of experimental animals infected with *Bacillus anthrax*[11]. The effect could be reproduced with culture filtrates from other *Bacillus* species, notably *B. cereus* and *B. thuringiensis*[12,13] and it was proposed that the release of AP was most probably due to the action of a novel phosphatidylinositol specific phospholipase C (PI-PLC).[14,15] These observations were corroborated in the 1970s by the demonstration that highly purified PI-PLCs from *B. cereus, Staphylococcus aureus,* and *B. thuringiensis* could specifically release AP from cell membranes.[16,17,18] Phospholipases with other specificities were not able to duplicate this effect. The generality of the phenomenon was demonstrated when it was found that PI-PLC could release 5'-nucleotidase and acetylcholinesterase (AChE) [19,20,21] from intact erythrocytes in the absence of hemolysis. The process was specific, as certain other cell surface markers were not released.[20]

Characterisation of AP before and after treatment with PI-PLC demonstrated that the physical properties of the enzyme were altered. PI-PLC

released AP was of low molecular weight, i.e. not aggregated or associated with lipid vesicles,[20,21] and was unable to bind to phospholipid vesicles. Organic solvent extracted AP however could bind phospholipid,[22] but this property was lost on PI-PLC treatment suggesting that the lipophilic properties of AP were intimately associated with AP itself, rather than being some property of the lipid bilayer with which it was associated, and could be explained by the covalent linkage of a PI molecule to the protein. Similar observations made for *Torpedo marmorata* AChE,[23] coupled with analyses showing that *T. marmorata* AChE and AP contained *myo*-inositol in stoichiometric amounts,[24,25] provided strong evidence of a covalent PI-protein linkage.

2. Analysis of Membrane Proteins Lacking Hydrophobic Peptide Anchors (Thy-1 and VSG)

Investigations into the mechanism of anchoring of two further proteins, the rat neural and T cell antigen Thy-1 and the variant surface glycoprotein (VSG) from *Trypanosoma brucei* provided further evidence for a novel form of lipid-mediated anchoring. Thy-1, the smallest member of the immunoglobulin superfamily[26] was considered to use an enigmatic method for membrane anchoring by virtue of the absence of a clear transmembrane region from the primary structure of the isolated glycoprotein. Thy-1 could also be released from thymocytes by PI-PLC,[27] suggesting an anchor mechanism similar to that of AP and AChE. Chemical studies of the protein showed that a hydrophobic structure attached to the C-terminal cysteine contained EthN, GlcN and Gal,[28] as well as *myo*-inositol, phosphate, glycerol, mannose and stearate.[29,30] Subsequent to the identification of the complete structure of the VSG glycosylphosphatidylinositol (GPI)-anchor, Thy-1 was the first mammalian glycoprotein to have the structure of it's GPI glycan solved (see below). The structure of the lipid portion of the Thy-1 anchor remains unknown at the present time.

VSG provided an excellent paradigm for the clarification of the structure of the GPI anchor, and proof that the lipid was the sole mechanism of membrane anchoring. Comparison of the complete amino acid sequence and the cDNA sequence for the VSG from *T. brucei* variant 117[31,32] led to the prediction that hydrophobic peptide sequences from both the N and the C-terminus were removed during maturation of the glycoprotein. The N-terminal extension is the classical secretion signal sequence. The C-terminal extension (17-23 amino acids, depending on the VSG type)[33] was of sufficient hydrophobicity to be a membrane insertion sequence, and was highly conserved between otherwise diverse VSG sequences.[33]

As isolated by cell disruption, VSG is a soluble glycoprotein (sVSG).[34,35] However, lysis of trypanosomes in boiling detergent or in the presence of 10mM Zn^{2+} produces an amphipathic membrane form

(mfVSG).[36] Conversion of mfVSG to sVSG during osmotic shock was due to the action of an endogenous enzyme,[35,37] which removed myristic acid from the VSG[38] while generating the 'cross reacting determinant' (CRD), the only common immunogenic structure observed between different VSG variants.[39,40] The CRD itself is associated with a complex oligosaccharide that was attached to the C-terminus of the VSG polypeptide.[39,40,41,42] The linkage between the oligosaccharide and the C-terminal amino acid was shown to involve ethanolamine in amide linkage to the α-carboxyl group.[43] In order for this addition to be made, removal of the C-terminal extension would have to take place. All these data came together when it was shown that the activity converting the mfVSG to sVSG was in fact an endogenous phospholipase C.[44] The similarities between the biochemical data for VSG, Thy-1, AP and the other PI-PLC released enzymes suggested that a common mechanism for membrane anchoring was involved. Other proteins were also soon identified based on their susceptibility to PI-PLC, that were proposed to be GPI-anchored, e.g. *Leishmania* gp63.[45]

III. Structure of GPI

1. Elucidation of the Structure of the VSG GPI-anchor

As discussed in the previous section, data from several directions converged in the mid 1980s, and suggested that a PI containing glycolipid was responsible for the anchoring of a group of membrane glycoproteins. As an example we shall briefly discuss the elucidation of the complete structure of the VSG GPI-anchor.

Ferguson and coworkers showed that the lipase activity converting mfVSG to sVSG released *sn*-1,2-dimyristoylglycerol from the mfVSG.[44] The C-terminal domains from all VSGs, generated by protease treatment, contained mannose, ethanolamine, glucosamine and varying amounts of galactose.[46] Unusually, the glucosamine was not N-acetylated.[47] Nitrous acid treatment of the sVSG did not release any carbohydrate (which cleaves glycan chains by deamination of non-N-acetylated amino-sugars), suggesting that the glucosamine was located at the reducing terminus of the glycan.[48] It was then shown that deamination of ^{3}H-myristate-labelled mfVSG released PI,[48] and that treatment with *S. aureus* PI-PLC released 100% of the radiolabel, demonstrating that all fatty acid substituents were contained within the PI moiety. Composition analysis of the C-terminal glycopeptide following treatment with mild acid and alkaline phosphatase identified *myo*-inositol in this VSG fragment, as well as galactose and mannose.[48] The inability to detect inositol in previous work was ascribed to the unusual acid stability of the GlcN-Inositol linkage. Recovery of inositol-1-phosphate following alkaline phosphatase treatment of the deaminated glycopeptide, suggested formation of a cyclic inositol phosphate product from the PI-PLC cleavage,

which was confirmed by mild acid treatment.[48] This feature is important in generation of the CRD epitope.[49]

The complete glycan structure for VSG117 and the linkage of ethanolamine-phosphate to the glycan was determined by chemical analysis, utilising nuclear magnetic resonance (NMR), gas chromatography mass spectrometry (GCMS), chemical and enzymatic degradation (Figure 1). The C-terminal glycopeptide was prepared from mfVSG by pronase digestion, and a neutral glycan prepared by deamination, reduction and dephosphorylation.[50] Proton NMR and methylation analysis provided linkage positions, anomericity and composition, whilst gel filtration and exoglycosidase treatments provided an estimate of glycan microheterogeneity as well as confirmation of the NMR/GCMS assignments. Periodate oxidation and GCMS of the reaction products was used to provide data on the Man-GlcN-Inositol linkage positions. The residue to which EtN-P was linked was determined by the resistance of the mannose core of the glycan to α-mannosidase digestion, and subsequent sensitivity following dephosphorylation.

Similar approaches have been used to deduce the structures of additional GPI-anchors, e.g. acetylcholinesterase,[51,52] gp63 (PSP) from *Leishmania major*[53] and the glycan from the *Saccharomyces cerevisiae* 125kDa glycoprotein (C. Fankhauser, personal communication), although in the former case more extensive characterisation by fast atom bombardment mass spectrometry (FABMS) was utilised. The structure of the GPI-anchor of the scrapie form of the prion protein PrP^{Sc} has been determined by a novel approach utilising electrospray mass spectrometry and laser desorption mass spectrometry[54] but these data have not been confirmed by an independent method. Proteins with a GPI anchor are listed in Table 1.

2. Structures of Glycosylphosphatidylinositol-Containing Species

Many workers have described the structures of a variety of GPI-containing molecules. These essentially fall into three classes, the GPI-protein anchors (Tables 1 and 2), the lipophosphoglycans and glycosylinositolphospholipids (LPGs, GIPLs, Table 3) and the free GPI lipids (Table 2), some of which are protein anchor precursors.

Protein GPI-Anchors; All protein-linked GPI anchors defined to date contain a consensus core glycan, which is conserved to the level of linkage position. Table 2 summarises known GPI-anchor structures and the structures of homologous free GPI-lipids, which are presumed to be biosynthetic intermediates in the construction of the protein anchor. Apart from conservation of the glycan core sequence, and, by definition, the presence of PI, the GPI-anchors represent a diverse set of stuctures.

Lipid Structures; Diacyl,[44,48,50] monoacyl,[55] alkylacyl,[51,53] and ceramide-based PIs (Fankhauser, personal communication)[56] have been reported as PI structures of protein GPI-anchors. So far, *lyso*-alkyl PI has only been reported for the lipophosphoglycan of leishmanial parasites (see below).[57] In addition, the presence of a fatty acid substituent on the inositol ring has been reported for a number of proteins and GPI-anchor precursors, including protozoan antigens (PARP/procyclin)[55] and GPI precursors[58,59,60] and mammalian proteins (acetylcholinesterase)[51] and precursors.[61,62] The inositol fatty acid is palmitate in all cases so far determined, which is in marked contrast to the heterogeneity displayed by the rest of the PI moiety (see Table 2).

The functional significance of palmitoyl-inositol is not understood, but an important consideration is that the presence of this modification renders the GPI-anchor resistant to cleavage by bacterial PI-PLCs,[51,55,59,60] and therefore will not allow release of proteins linked to the cell membrane by such anchors, and also will not allow the generation of the CRD epitope. As both of these observations are important criteria for the detection of a GPI, it is important to be aware that such variants of GPI-anchor structure exist, and may be quite common (see section III). Interestingly, the GPI-anchor precursors recently identified in T cells are PI-PLC resistant,[61,63] probably due to inositol acylation, whilst the anchors of the surface glycoproteins are PI-PLC sensitive, e.g. Thy-1 (see biosynthesis, section IV.3).

Glycan Decorations; A number of additional substituents have been described that are added to the GPI core. It is believed that many of these substituents are added after initial transfer of the glycolipid to the protein (see section IV.3, Table 2). As with structural microheterogeneity in other glycosylation reactions, i.e. N and O-linked glycans, modification of the core is non-stoichiometric. For example, substitution of the VSG 117 GPI-anchor with α-galactose produces four major structures, containing 2 to 4 α-Gal residues,[50] with the Gal_3 species consisting of two isomers. Other minor species are probably also present. Different VSG classes contain either no galactose or up to 8 galactose residues.[5,46] For Thy-1, the fourth mannose residue is present in only 80% of the structures recovered from the protein.[63]

The functional significance of any of the additional substituents has not been determined. Factors controlling maturation of the GPI-anchors have also not been assessed. From the types of substituents present it is most likely that the majority of these additions take place within the Golgi compartment. In the case of VSG, GPI-anchor processing occurs with a half-life of approximately 15 minutes,[65] at a similar time as the processing of the N-linked glycans to an endoglycosidase H resistant form, implying a *cis* or *medial* Golgi location. However, our knowledge of the compartmentalisation of the trypanosome secretory pathway is rudimentary and therefore extrapolation to the mammalian system is not completely valid.

Lipophosphoglycan and Glycoinositolphospholipids; In *Leishmania* spp. (a group of South American trypanosomatids) a second class of GPIs have also been identified[66,67,68,69] and extensively characterised. Structural information and citations for this class of molecules are summarised in Table 3. These lipids (GIPLs) are homologous to the protein-linked GPIs and their precursors, but identity is restricted to the core sequence Manα1-4GlcNα1-6PI. Some GIPLs are expressed at the cell surface and are immunogenic.[67] GIPLs may serve as precursors for a major cell surface glycoconjugate termed lipophosphoglycan (LPG)[57,70,71,72,73] that may be involved in the infection of macrophages by *Leishmania*.[74,75] These organisms also synthesise the protein anchoring GPI series.[53,76] Interestingly, the presence of an acyl-inositol-PI has not been seen in the *Leishmania* GIPL/LPG series, and therefore this feature of GPI decoration may be restricted to the protein-anchor series, or these organisms may lack the enzyme neccessary for addition of the acyl moiety to inositol.

Species specific expression of different GIPLs has been reported. In *L. major*, the GIPLs contain terminal αGal residues, whereas in *L. donovani* the GIPLs are structurally more closely related to the GPI-protein anchor precursors identified in other organisms.[69] In addition the structures of the glycan headgroups of the *L. donovani* lipids are developmentally regulated, with a Manα1-3 only being seen in the promastigote stage and not in the amastigote (Table 3). Interestingly, expression of LPG is also shut off in the amastigote stage.[69]

A large glycoconjugate, lipopeptidophosphoglycan, (LPPG), with a similar structure to LPG, has been reported in *Trypanosoma cruzi* (Table 3).[77,78] The LPPG structure is more closely related to the protein-anchor series, but interestingly contains galactose-furanose substituents, which have also been seen in LPG. Little is known about the biosynthesis and function of this species beyond its structure. The precise relationship of the LPG/LPPG/GIPL series to the protein-linked GPI series remains to be fully evaluated and they will not be discussed further.

3. Analysis of GPI Structures

Methodology for the study of GPI-anchors and their precursors has been well documented over the past few years, and most information can be obtained in the original papers themselves. Given below is a brief overview of the methods that are available, and the information that can be obtained by each one. The reader is directed to other articles,[79,80] and a forthcoming volume in the 'Practical Approach' series published by IRL Press on 'Lipid Modifications of Proteins' (Hooper and Turner, eds) for detailed protocols.

Essentially the methods can be considered as three units, (i) identification of a GPI-anchored protein or lipid, (ii) dealing with the protein

and releasing the GPI moiety or extracting and purifying the lipid, and (iii) analysing the glycan headgroup, and lipid moiety.

Identification of GPI-Anchored Proteins: Methods depend on either composition, incorporation of GPI components in metabolic-labelling experiments, or on the sensitivity of the protein to enzyme cleavage. The methods used will depend on factors such as abundance, availability of a rigorous purification protocol, and of monospecific antibodies.

Composition analysis for ethanolamine can be performed using an amino acid analyser, and is a useful method available to most laboratories. For inositol or fatty acid analysis, GCMS is required, which necessitates greater investment both financially and in terms of time. Composition data are only valid for extensively purified material, which in most cases will not be available.

Metabolic-labelling with ethanolamine or inositol is informative as these compounds are not incorporated into many other protein-linked structures, and therefore is quite specific. Fatty acid labelling (usually palmitate or myristate) coupled with another criterion is also useful, but many proteins are acylated which do not contain GPI-anchors (see other chapters in this volume). Incorporation of sugars is less specific as the labelling must be performed in the presence of tunicamycin (to inhibit addition of N-linked sugars), but inhibition may be incomplete or the protein could be O-glycosylated, complicating analysis. Treatment with N-glycanase or endoglycosidase H can be used to discriminate between GPI and N-linked glycosylation. Under some circumstances labelling with particular compounds will be unsuccessful for obscure reasons, for example in *T. brucei*, GPI lipids and proteins are not labelled with [^{3}H]-inositol, whereas *S. cerevisiae* and *Leishmania* GPIs are labelled efficiently.[66,81,82] Use of inhibitors, e.g., D-mannosamine or PMSF, may also be helpful (section IV.6). These compounds prevent synthesis of GPI-anchor precursors, and can be used to inhibit the incorporation of ^{3}H-ethanolamine into GPI-anchored proteins (see section IV.6).[83,84]

Alteration of biochemical properties by treatment with bacterial PI-PLC is a powerful method for the detection of GPI-anchored proteins. Incubation of intact cells with PI-PLC can result in the selective release of GPI-anchored proteins into the culture medium. Fluorescent activated cell sorter analysis of endogenous GPI-anchored proteins requires the availability of good antibodies, but is an extremely powerful method. This procedure is also of use in demonstrating GPI-anchoring of cloned protein sequences that have potential GPI-anchor addition signals that can be expressed in heterologous eukaryotic systems. Commonly, products of PI-PLC digests are analysed by detergent-phase partitioning, using TX-114.[85,86] PI-PLC sensitive GPI-anchored proteins are converted from amphipathic proteins (which are recovered in the detergent-enriched phase) to hydrophilic proteins (recovered in the aqueous phase). Samples can then be fractionated by gel

electrophoresis. In addition, PI-PLC generates the CRD,[49] which contains a cyclic 1,2-inositol-phosphate, generated by the action of the enzyme. Therefore, immunoprecipitation or western-blotting following PI-PLC treatment can be used as a further demonstration of the presence of a GPI-anchor. The CRD epitope is destroyed by mild acid treatment, which can be incorporated as an additional control for the assay.[53] It should be noted that the structure of the CRD epitope is complex, so that negative reactions (when there is other information supporting the possibility of GPI-anchorage) should be treated with caution.[49] A very real problem with PI-PLC cleavage is the occurence of acyl-inositol. This modification will prevent PI-PLC action. One possible method is to perform a partial base treatment, to remove some of the acyl groups from the inositol. Alternatively the protein could be treated with mammalian serum GPI-PLD. In order for this enzyme to work the protein of interest must be fully solubilised, as incorporation of the protein into a lipid bilayer prevents the action of the enzyme.[87a] In this case, the PLD releases a phosphatidic acid from the GPI-anchor, which can be analysed by thin-layer chromatography.[51,55]

Obtaining a GPI Fragment from an Intact Glycoprotein: The most obvious and simplest method to produce a GPI fragment is exhaustive proteolysis, with, for example, pronase.[50,53] This method works in most cases to produce a C-terminal glycopeptide, containing a one or a few amino acids. The hydrophobic GPI can be purified by extraction into organic solvent and further processed. In the case of a glycoprotein containing a convenient protease site, a specific protease can be used to release the GPI (e.g. papain cleavage of AChE).[87] The amino acid composition of the C-terminal glycopeptide, or even N-terminal sequencing can be used at this stage to determine the GPI attachment site. The glycan can at this point be released by deamination and reduction, to cleave off the PI moiety, and HF treatment to remove the ethanolamine phosphate. A radiolabel can be introduced by reduction with ^{3}H-sodium borohydride. Direct analysis of the GPI moiety by mass spectrometric techniques can be utilised, as exemplified for acetylcholinesterase.[52] Alternatively the protein can be treated with PI-PLC at the begining of the procedure. Differential sensitivity of the glycan to exoglycosidase digestion before and after release from the residual peptide and ethanolamine-phosphate can be used to determine the position of attachement of the phosphate to the core glycan.[50]

Purification of Lipid Species: For GPI-anchor precursors, which are free lipids, removal of the protein is eliminated. However, the glycolipids must be extracted and purified from the cells of interest. This is most conveniently performed by using metabolically labelled material, as the quantity of GPI lipids is small compared to cellular phospholipid. Standard extraction protocols with chloroform methanol-containing solvents are suitable.[79,80,88] Subsequent fractionation and purification is most easily achieved by thin-layer chromatography, but other methods involving Iatrobead silica liquid-

chromatography[80,89] or octyl-sepharose[66] can also be used. The glycan can then be released following the same procedures as described for proteins.

Analysing the GPI-Glycan: The methodology employed will depend on the amount of material available. For plentiful samples (>50nmoles), NMR and mass spectrometry analysis are the methods of choice. As several GPI-glycans have already been studied, interpretation of NMR spectra is now somewhat easier. Composition and linkage analysis by GCMS, followed by exoglycosidase and chemical degradation will provide additional information, allowing a complete assignment to be made in most cases. At present, the number of structures that have been sequenced is too small to allow complete reliance on exoglycosidase digestion and chromatographic properties for a full assignment, but this may become possible in the near future.

Analysis of the precursors (with the exception of the GIPLs from *Leishmania*) relies on metabolically-labelled material. There are now a large number of GPI lipids which have been characterised. Essentially, headgroup analysis is performed by liquid chromatography (most commonly utilising the Dionex anion exchange HPLC system) coupled with exoglycosidase digestion of the deaminated glycan.[90] Gel filtration and ion exchange are also useful additional methods to consider. Linkage analysis has been performed on some of the *T. brucei* precursors by methylation of the glycan before hydrolysis.[90,91,92] Because of the smaller amounts of material available, analysis of free GPIs will remain less rigorous than that of the protein-linked GPI-anchors themselves.

Analysis of Lipid Substituents: For the GPI-anchored proteins, preliminary information can be obtained by treatment of the protein with PI-PLC, GPI-PLD, or sphingomyelinase.[56] Analysis of both the released fragment and the efficiency of release are important in assigning the architecture of the GPI-anchor. For example, an inositol-acylated anchor releases only a fraction of lipid following GPI-PLD digestion.[55,60] Analysis of fragments can be performed by GCMS or by TLC with radiolabelled material. Base hydrolysis can be used to determine the presence of ester-linked fatty acids, and phospholipase A_2 to probe the individual fatty acid substituents. Fatty acid composition data can be obtained by conversion of the fatty acid to a methylester, followed by fractionation by TLC or GCMS. Alkyl-acyl structures are usually analysed as fatty acid methylesters and methylalkylglycerols.[53]

IV. Biosynthesis of GPI Anchors

1. GPI anchor addition is a rapid post-translational event

Studies from many laboratories have shown that GPI anchor addition is signalled by a COOH-terminal amino acid sequence that is rapidly removed upon completion of protein synthesis and replaced by a preformed GPI

(Figure 3). The rapidity of COOH-terminal processing and GPI addition was first discovered in pulse-chase experiments with *T. brucei* which showed that VSG was already GPI-anchored after a short pulse (1 min) and chase (0 and 2 min).[65,93,94] In JEG-3 human choriocarcinoma cells GPI addition to AP occurs within 5 minutes of protein synthesis.[95] Rapid addition of GPI to protein has also been demonstrated for Thy-1 glycoprotein,[96] carcinoembryonic antigen (CEA),[97] and the neural cell adhesion molecule, N-CAM-120.[98]

In addition to the rapid kinetics of processing, other data indicate that GPI addition occurs early in the secretory pathway, probably in the endoplasmic reticulum. (i) GPI anchoring proceeds normally in the *sec18* secretion mutant of *Saccharomyces cerevisiae* when vesicular transport between the ER and Golgi is blocked at the non-permissive temperature.[99] (ii) Newly synthesized decay accelerating factor (DAF) in HeLa cells can be metabolically labelled with [^{3}H]ethanolamine before O-linked glycans are added[100] indicating that GPI addition occurs before the protein enters the Golgi apparatus. (iii) GPI-anchored proteins can be isolated with immature N-linked glycans, indicating that the GPI anchor is added prior to N-linked glycan processing in the Golgi.[65,94,95,97] (iv) COOH-terminal cleavage and GPI-addition activity can be detected in ER microsomal preparations from tissue culture cells[101,102,103] and from canine pancreas (J. Vidugiriene, unpublished observations).

2. Identification and Characterization of GPI Anchor Precursors

African Trypanosomes; The rapid processing of proteins to GPI-anchored forms suggested that the GPI anchor was pre-assembled. This proposal was stengthened when a polar lipid, P2 (also called Lipid A,[104] or Glycolipid A[89]), isolated from extracts of bloodstream form *T. brucei* metabolically labelled with [^{3}H]ethanolamine, [^{3}H]mannose, [^{3}H]glucosamine, [^{3}H]myristic acid, or [^{32}P], was shown to have the essential structural features of a GPI anchor.[104,105] Metabolic labelling of P2 was not affected by tunicamycin, a potent and specific inhibitor of the first step in the synthesis of dolichol-linked oligosaccharides.[90,106] Labelling in the presence of cycloheximide, a protein synthesis inhibitor, showed that P2 was still synthesised, indicating that the lipid was not produced by adventitious cleavage of GPI-anchored VSG.[105] Estimates based on inositol analysis[90] and metabolic incorporation of radioactive precursors of defined specific activity[105] indicated a pool size of ~10^4 molecules of P2 per cell, compared with ~10^7 molecules of VSG per cell.

P2 was characterized as ethanolamine-P-Manα1-2Manα1-6Manα1-GlcN-Inositol-P-*sn* 1,2-dimyristylglycerol by a variety of methods[59,90,105] (see section III). The linkages not defined in the structure are likely to be

identical to those in the VSG GPI-anchor since glycan fragments prepared from metabolically labelled P2 were found to be indistinguishable from similar preparations derived from the core of the VSG GPI anchor (Table I). However, unlike the VSG-linked GPI, the ethanolamine residue in P2 contains a free amino group.[105]

A second polar lipid termed P3[105] (also called Lipid C or Glycolipid C)[89,104] was also found in bloodstream form trypanosome extracts. P3 was less polar than P2, but, like P2, could be metabolically labelled via [^{3}H]ethanolamine, [^{3}H]mannose, [^{3}H]glucosamine, [^{3}H]myristic acid, and [^{32}P]. Although P3 appeared to be a GPI by compositional criteria, it was not cleaved by PI-PLC, a primary diagnostic of GPI structures. Subsequent to the elucidation of a PI-PLC-resistant inositol acylated GPI-anchor structure for human acetylcholinesterase,[51] P3 was shown to be identical to P2 except for the presence of palmitic acid esterified to one of the inositol hydroxyl groups.[58,59] P3 could be converted to P2 by selective removal of the palmitate residue.[59] The significance of P3 in trypanosomes is the subject of some speculation and will be discussed later in this chapter (section IV.9).

The GPI anchors of trypanosome VSGs are galactosylated to different extents, the average number of galactose residues per anchor depending on the particular trypanosome variant (table 2). Addition of galactose residues to the VSG anchor appears to occur in the Golgi apparatus.[65] Galactosylated forms of P2 and P3, structurally homologous to the galactosylated VSG GPI anchor, are also synthesized by bloodstream trypanosomes.[107] Curiously, P2 galactosylation is extensive but only the monogalactosylated form of P3 has been detected.

More recently, GPI lipids have also been identified in insect-stage (procyclic) *T. brucei*, and in a variety of mammalian cells and yeast. The single abundant GPI species (PP1)[60] identified in procyclic trypanosomes is a variant of P3: it is PI-PLC resistant and contains one palmitic acid residue esterified to inositol, and one stearic acid residue esterified to the 1-position of glycerol (Table 2). An identical arrangement of fatty acids was subsequently found in the GPI anchor of the major procyclic surface protein[55] consistent with PP1 as the GPI anchor precursor in procyclic trypanosomes. Furthermore, PP1 is synthesised from a diacyl structure, homologous to P3.[108]

Other systems; GPI anchor precursors in mammalian cells are being studied in several laboratories, and, although the structural data are incomplete, some general points may be made. Contrary to original expectations based on the abundance of GPI-anchored proteins in mammalian cells versus *T. brucei*, the mammalian GPIs appear to be about as abundant as the trypanosome GPI precursors. In a variety of mammalian cells, the most abundant [^{3}H]ethanolamine-labelled GPI was PI-PLC resistant and more polar than trypanosome P2. The GPIs were inositol acylated and most probably contained one or two extra phosphoethanolamine moieties

attached to the mannose residue proximal to glucosamine.[61,62] Side-chain phosphoethanolamine moieties have been described in the GPI anchors of Thy-1 and AChE[64,52] and their presence on GPI anchor precursors indicates that they are added to the GPI structure early, rather than as a late event during transit of the protein through the secretory pathway. An alternative explanation is that a large proportion of the metabolically labelled GPIs are exported to the cell surface as well, and are modified with phosphoethanolamine side-chains en route (section IV.5). Curiously, only PI-PLC-resistant GPI anchor precursors were found in the cells examined, even though the cells expressed PI-PLC-sensitive GPI-anchored proteins. This observation is consistent with the hypothesis that many GPI-anchored proteins are initially PI-PLC-resistant and aquire sensitivity following removal of the inositol-linked fatty acid later in the secretory pathway before the protein reaches the cell surface.

3. Assembly of GPI Anchor Precursors

Glycan Biosynthesis; Much of our present information on GPI anchor biosynthesis is derived from investigations in the trypanosome system, beginning with the demonstration of the cell-free synthesis of P2 and P3.[89,91,109] Metabolic labelling experiments indicated that tunicamycin had no effect on GPI biosynthesis,[59,90,94] which allowed the use of this inhibitor prior to lysis[89] and in the labelling reactions in order to suppress labelling of lipid intermediates in the N-glycosylation pathway. It was clear that the radiolabelled sugar nucleotides UDP-[^{3}H]GlcNAc and GDP-[^{3}H]mannose could be used as sugar donors *in vitro* to generate [^{3}H]P2, [^{3}H]P3, and a range of [^{3}H]glycolipids characterized as GlcNAc-PI, GlcN-PI, $Man_{1\text{-}3}$GlcN-PI, and $Man_{2\text{-}3}$GlcN-acylPI.[89,91,110] Although the identification of partially constructed GPI species suggested that, like dolichol-PP-oligosaccharide biosynthesis,[111,112] GPI assembly involved sequential addition of monosaccharides to lipid acceptors (Figure 4), it remains possible that the assembly pathway is more complex.

The first committed step in GPI assembly is the transfer of GlcNAc from UDP-GlcNAc to PI to form GlcNAc-PI. GlcNAc-PI is then rapidly de-acetylated to form GlcN-PI.[91,110,113,114] Three murine T cell mutants (complementation classes A, C, and H) originally selected for their inability to express cell surface Thy-1[115,116] are in fact defective in GlcNAc-PI synthesis.[63,113] Since each of the three mutants belongs to a separate complementation class, it is clear that three genes are directly involved in GlcNAc-PI synthesis. One explanation is that the glycosyltransferase responsible for GlcNAc-PI synthesis is a three-subunit enzyme, and all three subunits are required for function. An alternative possibility is that another protein involved in GlcNAc-PI synthesis, e.g. the UDP-GlcNAc transporter, responsible for

transport of UDP-GlcNAc from the cytosol into the lumen of the ER[111,112,112a] is defective. It remains to be established whether GlcNAc-PI is synthesized in the lumenal leaflet of the ER membrane, and therefore, if a defect in UDP-GlcNAc transport would affect GPI assembly (see below).

Although GDP-mannose is able to support GPI assembly *in vitro*,[89,91,109] it is not the direct donor of the three core mannose residues in the GPI structure. Dolichol-P-mannose (DPM), a lipid metabolite synthesized in the ER from GDP-mannose and dolichol-P, was implicated as the mannose donor for at least one if not all the GPI mannose residues when a Thy-1 negative murine T cell mutant (Class E)[115,116] known to be defective in DPM synthesis[117,118] was shown to be unable to synthesize the Thy-1 GPI anchor.[119,120,121,122] Also GPI-anchoring is defective in yeast mutants generated by disruption of the dolichol-P-mannose synthase DPM1 gene,[82] and defective Thy-1 and Ly-6A surface expression in a DPM synthase deficient T cell hybridoma mutant (M38/20)[123] could be restored by transfecting the cells with the yeast DPM1 gene.[61] The involvement of DPM in GPI synthesis was also suggested by experiments showing that a DPM synthesis inhibitor 2-deoxy-2-fluoroglucose (FGlc, see section IV.6), could suppress the synthesis of P2 and P3 in bloodstream trypanosomes.[124] FGlc has been used more recently to inhibit the synthesis of GPI-anchored alkaline phosphatase in tissue culture cells.[125] Similar data were obtained from *in vitro* labelling experiments using GDP-[^{3}H]mannose: incorporation of [^{3}H]mannose into P2 and P3 was stimulated by dolichol-phosphate and inhibited by amphomycin, an antibiotic inhibitor of DPM synthesis[92] (see section IV.6).

The role of DPM in GPI assembly was established firmly by direct *in vitro* labelling using dolichol-P-[^{3}H]mannose, followed by methylation linkage analysis of neutral glycans derived from labelled P2, Man_2GlcN-PI, and Man_3GlcN-PI. The results demonstrated conclusively that the assembly of all three mannose residues in the GPI core required DPM.[92] These data also raised the possibility that elongation may not occur by sequential transfer of mannose residues[9,92] and were consistent with a scheme in which Man_1GlcN-PI is elongated by the direct transfer of Manα1-2Man from a DPM-derived donor, bypassing Man_2GlcN-PI. The Man_2GlcN-PI species detected in the labelling may be adventitiously derived by α-mannosidase trimming of Man_3GlcN-PI. However, these biosynthetic alternatives remain to be established.

Phosphoethanolamine; The terminal phosphoethanolamine residue in the GPI structure appears to be derived from phosphatidylethanolamine (PE).[10,126,127] Direct experimental proof of phosphoethanolamine transfer from PE to a Man_3 GPI acceptor has been difficult to obtain, but several lines of evidence are consistent with this model. *In vitro* labelling with CDP-

[^{3}H]ethanolamine and [β-^{32}P]CDP-ethanolamine shows that phosphoethanolamine is transferred as a group, and *in vitro* pulse-chase studies clearly rule out CDP-[^{3}H]ethanolamine as the direct donor of the GPI phosphoethanolamine moiety. Furthermore, metabolic labelling of trypanosomes with [^{3}H]serine labels GPIs from radiolabelled PE synthesized via decarboxylation of labelled phosphatidylserine (PS). Related experiments with yeast mutants unable to synthesize PE from CDP-ethanolamine due to disruption of the genes encoding the ethanolaminephosphotransferases show that, when incubated with [^{3}H]ethanolamine, the mutant cells can synthesize [^{3}H]ethanolamine-phosphate and CDP-[^{3}H]ethanolamine but not [^{3}H]PE, and they also cannot incorporate [^{3}H]ethanolamine into GPI anchors. However the ability of the mutant cells to synthesize GPI anchors, as assessed by [^{3}H]inositol labelling, is unaltered because they make PE by decarboxylation of PS.

Phosphatidylinositol Specificity; In bloodstream trypanosomes, the PI molecular species selected for GPI biosynthesis contains stearic acid in the 1-position and an as yet undefined fatty acid in the 2-position of the glycerol moiety.[128] Once the GPI structure is assembled the glycerol-linked fatty acids are sequentially replaced with myristic acid.[89,128] Re-modelling of the Man_3 GPI intermediate can occur if phosphoethanolamine addition is inhibited.[84] PP1, the GPI anchor precursor in procyclic stage trypanosomes contains only one glycerol-linked stearic acid at the analogous position to the unremodelled bloodstream form GPI, and is the consequence of partial re-modelling of a diglyceride-containing precursor.[108] Similar fatty acid re-modelling reactions might occur in other systems giving rise to unique molecular species (glycerol fatty acids) in GPI anchors that are not representative of the molecular species spectrum found in total cellular PI. However, it is difficult to see how this process could occur with ether-linked fatty acids found in many GPI anchors (table 1). Only a minor subset of the spectrum of PI molecular species available in cells of the electric organ of the electric eel *Torpedo marmorata*, appears in the GPI anchor of *Torpedo* acetylcholinesterase.[129] These data could be explained by postulating fatty acid re-modelling reactions, or by suggesting that only a particular subset of PI molecular species is available to the first GPI glycosyltransferase. Analysis of the PI labelled with [^{3}H]fatty acid in procyclic trypanosomes suggests that the GPI species are derived from a minor fraction of the cellular PI pool.[55] The inability to label GPIs via [^{3}H]inositol in trypanosome lysates[91] or in culture (Field and Menon, unpublished) despite efficient labelling of PI, suggests that the PI required for GPI assembly is a very minor fraction of the labelled PI. This may reflect targetting of specific molecular species of PI to the domains of the ER, e.g., the inner leaflet of the ER membrane bilayer, where GPI biosynthesis is initiated.

Inositol Acylation; The biosynthetic pathway outlined above ignores the relationship between PI-PLC-resistant (i.e., inositol acylated) GPI species, and PI-PLC-sensitive forms. The role of inositol acylation in GPI assembly is not understood, and it remains to be established whether inositol acylation merely represents a GPI structural variation, or whether it is biosynthetically important. Since the original characterization of P3 in bloodstream trypanosomes, several inositol acylated GPI intermediates have been detected in extracts from *in vitro* labelling reactions using trypanosome membrane preparations. Although analyses of GPI biosynthetic intermediates in bloodstream trypanosomes show that a only minor proportion are inositol acylated, similar experiments with procyclic stage trypanosomes which express a major inositol acylated protein[55,130] show that the degree of inositol acylation increases with the size of the GPI glycan chain to the point where the mature phosphoethanolamine-containing GPI anchor precursor, PP1, is exclusively inositol acylated.[60] The smallest inositol acylated GPI intermediate detected was Man_1GlcN-acylPI in procyclics.[108] In other experiments in bloodstream trypanosomes, GlcN-acylPI may also have been synthesized[91] suggesting that the inositol-linked fatty acid is added very early in the assembly pathway. Pulse-chase experiments in procyclic trypanosomes suggest that the inositol-linked fatty acid is not derived directly from fatty acyl Coenzyme A, but instead from a relatively metabolically stable donor such as a phospholipid.[55] However these results need to be re-examined in the context of recent data indicating that inositol acylation in yeast membrane preparations is dependent on fatty acyl CoA.[131] In all cases where the fatty acid esterified to the inositol has been identified, it has been shown to be palmitic acid (Table 2). In the case of *T. brucei* inositol acylation in the insect-dwelling form may be important to guard against the effects of PI-PLC on surface antigens as certain *Bacillus* species are insect pathogens.[132]

Studies of GPI biosynthesis in mammalian cells provide a clearer indication of the potential functional significance of inositol acylation. The analyses have been facilitated by the availability of a series of T-cell mutants which are blocked at different stages of GPI biosynthesis, and which therefore accumulate GPI biosynthetic intermediates.[115,116,123] Possibly unlike the sequence of events in bloodsteam trypanosomes, inositol acylation in mammalian cells occurs at the GlcN-PI stage and persists through all the subsequent mannosylated structures, including the GPI anchor precursor.[61,62,63,133,134] Thus the initial assembly sequence is GlcNAc-PI-> GlcN-PI-> GlcN acylPI. GlcN-acylPI accumulates in the absence of GPI mannosylation, and is therefore easily detectable by metabolic labelling in mammalian and yeast mutants that are defective in DPM synthesis.[82,131,134] With the exception of GlcNAc-PI and GlcN-PI, no PI-PLC-sensitive (non-inositol-acylated) GPI intermediates or anchor precursors have been detected in wild-type T-cells or in a spectrum of T-cell mutants suggesting that

inositol acylation is important for mannosylation. This effect may be a consequence of the profound stereochemical constraint imposed on the GPI structure by the inositol-linked fatty acid.[10,91]

A number of reports are consistent with a hypothesis that GPI anchor precursors are initially PI-PLC-resistant and are processed to a PI-PLC-sensitive form after transfer to protein. As discussed above, GPI anchor precursors in a variety of mammalian cells appear to be exclusively PI-PLC-resistant, even though GPI-anchored proteins in the same cells are PI-PLC-sensitive suggesting either conversion of the GPI from a PI-PLC-resistant to a sensitive form after attachment to protein, or rapid processing of the precursor just prior to transfer. In two sublines of human K562 cells, mature AChE is either PI-PLC-sensitive or resistant, but in both lines AChE that has not been sialylated is PI-PLC-resistant, suggesting that all newly synthesized AChE contains an inositol acylated GPI anchor, which is subsequently processed during transit to the cell surface in one of the two cell lines.[135] PI-PLC sensitivity or resistance of GPI-anchored proteins appears to be cell-type and not protein specific. In several murine L cell lines, PI-PLC-resistance of GPI-anchored proteins behaves as a recessive trait in cell fusion experiments,[136] consistent with the presence of an inositol-specific acyl hydrolase in the cells with PI-PLC-sensitive GPI-anchored proteins. The sub-cellular location of the inositol-specific acyl hydrolase remains to be determined, as does its specificity for GPI-anchored-proteins versus non-protein-linked GPIs. However, the 110kDa precursor of the yeast gp125 is PI-PLC sensitive, even when studied in *sec 18* cells at the non-permissive temperature (a block in ER to *cis*-Golgi trafficking), suggesting that, if GPI-anchors are added as inositol-acylated forms, then deacylation must occur in the ER compartment.[99] This topic is discussed further elsewhere.[137]

4. Membrane Topology of GPI Biosynthesis

The model that GPI anchors are constructed via the sequential glycosylation of PI is further complicated by membrane topological issues. The end products of the assembly process are presumed to be associated with the luminal leaflet of the ER in order to be transferred to a newly synthesized polypeptide. However, the starting material, PI, is synthesized from CDP-diacylglycerol and inositol on the cytoplasmic face of the ER[138] suggesting that at some stage of the assembly process a cytosolically disposed lipid intermediate flips to the luminal face of the ER. There are several other indications that GPI assembly is likely to involve flipping of intermediates between the cytoplasmic and luminal leaflets of the ER accompanied by the transport of cytosolically synthesized precursors into the lumen of the ER. The three components required for the synthesis of GPI anchors from PI are glucosamine, mannose, and phosphoethanolamine. The most immediate (identified) donors of these forms of all three are available

on both faces of the ER membrane. UDP-GlcNAc can be transported into the ER,[111,112,112a] whilst synthesis of DPM from cytosolic GDP-Man followed by translocation of the lipid into the luminal leaflet[111,112,139] provides a source of activated mannose on both sides of the ER. Also the phosphoethanolamine donor, PE, is available in both leaflets of the ER,[140] so that no indication of the step at which the presumed translocation of the nascent GPI occurs can be gleaned from donor location.

5. Sub-cellular Distribution of Non-Protein-Linked GPIs

There is no direct information concerning the sub-cellular location of GPI biosynthesis. As discussed above, the lipids are presumed to be assembled in the ER and located in the lumenal leaflet of the ER membrane bilayer in order to be transferred to newly translated and translocated proteins. However, some observations suggest that a proportion of the anchor precursors may be found in other sub-cellular locations, indicating that they are exported from the ER after synthesis. (i) Pulse-chase labelling of procyclic trypanosomes with [^{3}H]glucosamine showed that ca. 60% of labelled PP1 was resistant to the chase, and could still be detected after several hours. The most plausible explanation is that this fraction corresponds to PP1 that is no longer in the ER, and therefore unavailable for transfer to protein. However, as it is still recoverable as PP1 no processing of the majority of the lipid occured.[55] (ii) Galactosylated forms of P2 and P3 are readily labelled *in vitro*, via UDP-[^{3}H]galactose, in the absence of other sugar nucleotides, rather than via GDP-[^{3}H]mannose (in the presence of non-radioactive UDP-galactose), implying galactosylation of pre-existing rather than *in vitro de novo* synthesized P2 and P3, probably in a different sub-cellular compartment.[107] Since galactosylation of the VSG GPI anchor apparently occurs as the protein traverses the Golgi apparatus en route to the cell surface,[65] P2 and P3 are most likely galactosylated in the Golgi. More elaborate GPI lipid glycans have also been detected in the procyclic form trypanosome.[60] (iii) GPI related molecules (primarily GIPL-2) have been detected on the surface of *Leishmania major* promastigotes using specific antibodies[66,67] and are immunogenic to their mammalian hosts.

6. Inhibitors of GPI Biosynthesis

Several compounds have been shown to affect GPI biosynthesis *in vivo* and *in vitro* (Figure 4). Except PMSF all affect other types of glycosylation as well. The inhibitors and their properties are summarized below.

2-fluoro-2-deoxy-D-glucose (FGlc) - FGlc prevents the synthesis of GPI anchors by inhibiting the formation of DPM (but not GDP-mannose) *in vivo*.[124,125,141,142,143,144] The mechanism of the DPM synthesis inhibition is unknown, but it is clear that FGlc (like other fluorosugar inhibitors of

glycosylation) has to be metabolized in order to exert its effect, and therefore will not function in cell free assays. It should be noted that FGlc treatment has to be carried out in cell culture medium that is essentially glucose-free, and that the inhibitor (used at ~1mM) affects protein synthesis (considerable inhibition in bloodstream trypanosomes, ~30% inhibition in JEG-3 choriocarcinoma cells[125]).

Amphomycin - Amphomycin, a lipopeptide antibiotic, forms a complex with dolichol-P and prevents it from participating in cell-free reactions leading to the synthesis of DPM, dolichol-P-glucose, and dolichol-PP-GlcNAc.[145,146] As with FGlc, GPI synthesis is blocked because DPM synthesis is inhibited.[92] Amphomycin inhibition can be overcome by adding excess dolichol-P.[92]

Mannosamine (2-amino-2-deoxy-D-mannose) (ManN) - ManN blocks the formation of α1,2 mannose linkages in N-linked sugars,[147] and inhibits GPI synthesis *in vivo* possibly by inhibiting the α1,2 mannosyl transferase responsible for the conversion of Man_2- to Man_3-GPI intermediates, or by acting as a chain terminator.[83] Although the mechanistic details remain to be elucidated, it is known that ManN is ineffective in cell-free systems since it probably needs to be converted to an active metabolite in order to act (Field and Menon, unpublished data).[147] Also, unlike FGlc, ManN has very little effect on protein synthesis at concentrations at which it effectively inhibits GPI biosynthesis. ManN also is able to inhibit biosynthesis of the LPG species in *Leishmania* (Medina-Acosta and Field, unpublished data).

Phenylmethanesulphonyl fluoride (PMSF) - The serine esterase inhibitor, PMSF, suppresses incorporation of the terminal phospho-ethanolamine moiety into the GPI chain, resulting in the accumulation of a Man_3 GPI species.[84] Inhibition can be achieved both *in vitro* and *in vivo*. The same effect can also be achieved *in vitro* with diisopropyl fluorophosphate, a reagent which phosphorylates activated serines.[84]

7. Defects in GPI Anchoring

Several GPI biosynthesis mutants have been described in the previous sections. Many of these are rare naturally occurring mutants selected from populations of wild-type cells. Others were selected after mutagenesis of wild-type cells.[123,148,149,150] Commonly used L-cell fibroblast cell lines are unable to synthesize GPI-anchored proteins for reasons that remain to be defined.[151,152] Chinese hamster ovary (CHO) cell line (B421) with a minimal capacity to synthesize DPM are also defective in GPI anchoring; however, the defect can be corrected by exposing the cells to tunicamycin[153] which presumably diverts the residual DPM from the N-glycosylation pathway to the GPI pathway.

Mutant thymoma cell lines derived from rare Thy-1$^-$ cells isolated originally by using cytotoxic immunoselection procedures on Thy-1$^+$ T cell lymphomas adapted to tissue culture[115,116] have been mapped into nine complementation classes. Six of the mutant classes define genes acting at points in the GPI assembly pathway[62,63,113,116,117,118,119,121,122,154]: classes A, C, and H do not synthesize GlcNAc-PI (see above), class E cells cannot synthesize DPM and therefore accumulate GlcN-acylPI, class B GPI synthesis is blocked at the Man_2 stage, class F cells cannot add the terminal phosphoethanolamine group and are therefore blocked at the Man_3 stage (class F cells also have an ether lipid defect which appears to be unrelated to GPI synthesis)[155]. For unknown reasons, the aminoglycoside G418 can reversibly induce class F and B cells to express some cell surface Thy-1.[156]

The human disease, paroxysmal nocturnal hemoglobinuria (PNH) correlates with defects in GPI-anchor synthesis. PNH is an acquired hemolytic anemia in which variable proportions of circulating blood cells, arising from clonal expansion of a somatically mutated pluripotent stem cell, show increased sensitivity to autologous complement lysis owing to the absence of the GPI-anchored complement regulatory proteins DAF[157,158] and homologous restriction factor.[159] In addition, the cells lack a number of other GPI anchored proteins including LFA-3,[160] AP,[161] CD14,[162] and CD16.[163] The absence of several GPI-anchored proteins coupled with the failure of affected cells to express cell-surface DAF despite the presence of a normal DAF gene, mRNA transcripts, and nascent DAF polypeptides[164,165] indicates that PNH is due to a defect(s) in some stage of GPI assembly or transfer. The precise identification of the defect has been hindered by the absence PNH-derived cell lines; however cloned natural killer cells carrying the PNH defect have recently been described.[165a]

8. Protein COOH-terminal sequences signalling GPI attachment

Signals for GPI Addition; A recognition/signalling event is clearly involved in directing attachment of GPI anchors to some proteins and not others, since only a restricted set of cell surface proteins are GPI-anchored. Indications of the nature of the GPI attachment signal came originally from comparisons of the cDNA-predicted and actual amino acid sequences of a trypanosome VSG and Thy-1 (see section II). Both the proteins had been processed at the N- and at the C-terminus.[26,28,31,32,166] The N-terminal processing was identified as the normal removal of an N-terminal signal sequence by ER signal peptidase, after translocation of the polypeptide into the lumen of the ER. The cleaved, relatively hydrophobic C-terminal sequence is now known to be the signal for GPI attachment. Transfection experiments using a variety of gene constructs, showed that addition or substitution of the C-terminal sequence could convert water-soluble or

transmembrane proteins to GPI-anchored forms in mammalian cells.[151,152,67,167,167a,168,169,170,171]

Dissection of the C-terminal signal sequence by construction of chimeras and site-specific mutants, showed that the signal was composed of two distinct elements, a short (generally 15-20 residues) hydrophobic domain, and a processing site consisting of 2-3 small amino acid residues positioned 10-12 amino acids N-terminal to the hydrophobic domain (Figure 3). Although the hydrophobic domain had to be of a certain length, the specific sequence was unimportant as it could be replaced with a random hydrophobic peptide without affecting GPI anchoring.[169,172,173] Curiously, the hydrophobic signal was found to be effective even when positioned at an internal location rather than at the C-terminus.[174] Site-specific mutagenesis of the cleavage/attachment site of DAF and placental AP, and identification of the attachment site in a number of other proteins indicated that only small amino acids (Ser, Gly, Ala, Asp, Asn, Cys) would function at the attachment site.[1,175,176] Small amino acids are also generally found in the +1 and +2 positions relative to the attachment site, i.e., the first two amino acids of the cleaved C-terminal peptide. Mutagenesis experiments by Gerber et al.[177] showed that while a spectrum of substitutions at the +1 site were permissible (Asp, Ser, Cys, Met, Thr, Glu; reduced processing with Trp; no processing with Pro) the requirements at the +2 site were far more stringent. Of the 11 amino acid substitutions examined at the +2 site, only Gly and Ala permitted normal processing (although only trace activity was detected with Ser or Cys in these assays, it should be noted that Ser occurs naturally in the +2 position in trypanosome VSGs sequenced to date !). In separate experiments with a series of chimeras consisting of sequences from DAF, the LDL receptor, and human growth hormone, the positioning of the small amino acid domain relative to the hydrophobic stretch appeared to be critical since GPI anchoring efficiency dropped sharply if the spacing was changed from the optimum of 10-12 amino acids.[178] However, these experiments may need to be examined further since the precise attachment site was not determined for the processed mutants.

Differential Splicing/Expression; Proteins such as LFA-3[179] and N-CAM[180,181,182,183] are naturally expressed in both GPI-anchored and transmembrane forms on the same cell. The different forms probably arise from a single gene by differential processing of the primary mRNA transcript. Other mechanisms for the expression of transmembrane versus GPI-anchored forms also exist. Alternate forms of CD16 [Fcγ receptor III] have been found on different cell types through cell-specific expression of similar but distinct genes, rather than alternative mRNA processing.[184]

9. Transfer of GPI precursors to protein

Despite rapid GPI-anchoring of newly synthesized VSG in trypanosomes, membranes prepared from bloodstream trypanosomes contain a small amount of non-glycolipid-anchored VSG which can accept glycolipid precursors synthesized *in situ* or added exogenously.[185] Although the membranes synthesize roughly equal quantities of radiolabelled P2 and P3, VSG labelled under the conditions of the *in vitro* assay is exclusively PI-PLC-sensitive, indicating that only P2 is transferred to the protein. The membranes also catalyze the transfer of exogenously added GPIs to VSG, demonstrating that the trypanosome GPIs P2, P3, and PP1, are all *bona fide* GPI anchor precursors. Transfer does not require ongoing protein synthesis and efficiency is unaltered in the presence of non-hydrolyzable ATP analogues or an ATP regenerating system,[185] consistent with a transpeptidation reaction mechanism (see below). Curiously, when radiolabelled P3 is added to the membranes, the processed (labelled) VSG is found to be PI-PLC-resistant[185] suggesting a lack of specificity under the assay conditions that is not reflected in living cells or when the precursors are synthesized in situ in membranes. In these situations P2 may have a competitive advantage over P3 resulting in exclusively PI-PLC-sensitive VSG. Alternatively, both lipids may be transferred to VSG acceptors but P3-anchored VSG may be rapidly inositol-deacylated to give PI-PLC-sensitive VSG, and this process is not supported by the *in vitro* system.

Non-GPI-anchored protein precursors can be detected in mammalian cells, particularly in mutants incapable of synthesizing complete GPI structures[119,120,154] and in normal cells after inhibition of GPI biosynthesis with FGlc[125] or ManN.[83] The unprocessed proteins could not be detected in cells metabolically labelled with [^{3}H]ethanolamine or [^{3}H]fatty acids, consistent with the lack of a GPI anchor. The proteins clearly retained the C-terminal hydrophobic tail since they partitioned into the detergent phase in Triton X-114 phase partitioning experiments, and unprocessed Thy-1 in five thymoma mutants (Classes A, C, E, F and H) could be metabolically labelled with [^{3}H]tryptophan (the only tryptophan residue in Thy-1 is located in the C-terminal tail).[119,154] In the absence of GPI synthesis, the nascent proteins may persist for several hours before finally being cleaved at or near the GPI attachment site, and secreted into the medium.[83,125] Cells of the Class B mutant secrete Thy-1 after removal of some or all of the C-terminal domain.[154] However, some thymoma mutants (Classes A, B, C, and E), apparently accumulate uncleaved Thy-1 (detected in metabolic labelling over a few hours) with immature N-linked glycans, suggesting that the peptide tail impairs exit from the ER or cis-Golgi.[119,154]

Curiously, in cells where GPI synthesis is not completely suppressed, but nevertheless reduced considerably to the point where detection of GPI anchor precursors is difficult, efficiency of transfer of GPI anchors appears to be selective so that some proteins are processed to a greater degree than others. For example in a T-cell hybridoma clone M43/8 which synthesizes

anchor precursors at about 1% the level of wild-type, the surface expression of GPI-anchored Ly-6A was ~2% of wild-type, whereas Thy-1 was ~20%.[186] Similar results were obtained from analyses of neutrophils from patients with PNH: expression of GPI-anchored CD16 (Fcγ receptor III) expression was preserved, although reduced, whereas other GPI-anchored proteins (DAF (CD55) and CD59) were completely absent.[187]

It has been difficult to demonstrate the transfer of GPIs to proteins using mammalian *in vitro* translation-translocation systems, although the C-terminal cleavage event has been clearly described and indirect proof of glycolipid-anchoring has been obtained.[101,102,103,188,189] The C-terminally processed protein was at least partly susceptible to PI-PLC.[103] One difficulty has been to obtain GPI precursors of sufficiently high specific activity for use in *in vitro* assays where only femtomole amounts of protein are translated and processed. Nevertheless, considerable progress has been made, and it is clear that the GPI-anchoring event can be reproduced and studied *in vitro*. Recent experiments have used an engineered protein (derived from placental AP) that is of sufficiently low molecular weight so as to easily detect molecular weight changes caused by N- and C-terminal processing events.[102] When the protein was synthesized *in vitro* in the presence of CHO cell microsomes, C-terminal processing lagged behind N-terminal signal sequence cleavage. C-terminal cleavage did not occur if microsomes derived from a GPI-mutant cell line (M38/20, defective in dolichol-P-mannose synthesis) were used instead, but was restored when polar lipids (presumably including GPI anchor precursors) from wild-type cells were added to the mutant cell microsomes (Kodukula, Yeh and Udenfriend, unpublished data).

The *in vitro* system described provides a basis for elucidating details of the GPI anchoring event, and possibly identification of the enzyme(s) involved. Mechanistically, GPI addition to protein appears to be a transpeptidation reaction possibly analogous to the penicillin-sensitive crosslinking of peptidoglycan strands during the synthesis of bacterial cell walls.[190] The amino terminus of a pentaglycine side-chain on one peptidoglycan strand attacks the terminal peptide bond in another strand to crosslink the two strands and release the terminal D-Alanine.[191,192] The reaction sequence includes formation of an R-D-Ala-enzyme complex, followed by transfer of R-D-Ala to the amino terminus of glycine. The reaction does not require an external source of energy since the energy of the D-Ala-D-Ala bond is conserved in the R-D-Ala-enzyme complex. Transpeptidation reactions are also carried out by a variety of peptidases[193,194] and γ-glutamyl transpeptidases.[195] Recently described examples of protein splicing[196,197,198] may also involve transpeptidation, but in these cases definite mechanistic proof remains to be established.

V. BIOLOGICAL CHARACTERISTICS AND SIGNIFICANCE OF GPI ANCHORS

1. Mobility of GPI-anchored proteins: lateral diffusion in the plane of the membrane

Lateral diffusion coefficients of membrane-spanning proteins are typically substantially lower than those for lipids (membrane proteins D ~10^{-10} cm^2/s, lipids D ~10^{-8} cm^2/s) presumably because of constraints imposed on protein diffusion by interactions with the extracellular matrix and the cytoskeleton.[199,200] These interactions presumably also account for the immobile fraction (usually 20-50%, D < 10^{-12} cm^2/s) of protein molecules consistently detected in fluorescence photobleaching recovery (FPR) measurements. Since the GPI anchor does not cross the membrane bilayer, GPI-anchored proteins might be expected to be relatively highly mobile compared to membrane-spanning proteins, and possess a small, possibly zero, immobile fraction. However, diffusion measurements for several GPI anchored proteins show that the GPI-anchor does not necessarily confer a high degree of mobility, and that mobility may be determined primarily through interactions of the extracellular domain with other cell surface components.[201] It should be noted that the GPI-anchored folate receptor is highly clustered and presumably immobile in the plasma membrane of epithelial cells, a state dependent on cholesterol and therefore attributed to lipid-lipid rather than protein-protein interactions.[202,203] One difference between otherwise similar GPI-anchored versus transmembrane proteins (such as the Class I major histocompatibility molecules $H\text{-}2D^b$ (membrane-spanning) and Qa-2 (GPI-anchored)) is that membrane-spanning proteins appear to be confined to micrometer scale membrane domains defined by dynamic cytoplasmic barriers which do not restrict the long range (several micrometers) lateral movement of GPI-anchored proteins.[204,205]

2. Secretion, sorting, and endocytosis of GPI-anchored proteins

The plasma membrane of polarized epithelial cells is divided into two membrane domains (apical and basolateral) by a circumferential ring of tight junctions formed between adjacent cells. The unique composition of each plasma membrane domain is apparently achieved, at least in some cell types (such as the Madin-Darby canine kidney (MDCK) cell line), by packaging of apical and basolateral components into separate carrier vesicles derived from the *trans*-Golgi network (TGN).[206,207] GPI anchored proteins (such as AP, folate receptor, trehalase, and aminopeptidase P) are located preferentially on the apical surface of polarized epithelia,[202,208,209,210] suggesting that the GPI anchor may contain apical targeting information.[208,212,213,214] This possibility was explored in transfection experiments in MDCK cells using a variety of fusion proteins. Conversion of normally basolaterally targeted membrane-spanning or secreted proteins to

GPI-anchored forms resulted in apical expression of the proteins in MDCK cells.[208,215] These results were extended in other experiments in which the GPI signal in AP was replaced with the transmembrane-cytoplasmic domain of a normally basolaterally targeted membrane protein resulting in basolateral expression.[208] Inhibition of GPI synthesis with ManN (see above) resulted in accumulation of an unprocessed protein precursor which was eventually cleaved and secreted without polarity.[83] Although the GPI anchor may compete with other - possibly conflicting or redundant - targeting signals in the ectodomains of GPI-anchored proteins it appears to act as a "dominant" apical sorting signal in renal (e.g., MDCK) and intestinal (e.g., CaCo-2) epithelial cell lines.[212,213] Interestingly, polarized sorting of GPI-anchored proteins also occurs in neurons: Thy-1 is expressed on axonal membranes but not on the neuronal cell bodies or dendrites.[216]

The mechanism by which GPI-anchored proteins are delivered to the apical surface is not known. The most attractive hypothesis[207] is that GPI-anchored proteins cluster with glycosphingolipids and relevant accessory proteins in the cytosolic leaflet of the TGN membrane. These patches eventually form vesicles containing the necessary markers and machinery for specific delivery to the apical plasma membrane. Evidence for such aggregates has come from analyses of changes in detergent solubility of GPI-anchored proteins (specifically AP) as they traverse the secretory pathway.[217] AP became insoluble in ice-cold Triton X-100 before reaching the TGN, whereas transmembrane proteins were completely solubilized. The detergent insoluble material was enriched in sphingolipids relative to glycerophospholipids, consistent with the notion that GPI-anchored proteins and glycolipids aggregate in the Golgi as a first step toward sorting and delivery to the apical plasma membrane.

GPI-anchored proteins appear to be excluded from the clathrin-mediated endocytic pathway. Thy-1, Ly-6A, 5'-nucleotidase, and the folate receptor are poorly represented in clathrin coated pits when compared with transmembrane proteins such as the transferrin receptor and the LDL receptor.[202,218,219,220] The folate receptor mediates the transport of 5-methyltetrahydrofolic acid into the cytoplasm by using an unusual non-clathrin-coated pit pathway.[202,203,221] The receptor is distributed in remarkably dense clusters in the apical plasma membrane of a kidney epithelial cell line (MA104), typically near non-clathrin coated membrane invaginations called caveolae.[221] Clustering diminishes in the absence of cholesterol.[203] Rothberg et al.[202,222] propose that the receptor mediates 5-methyltetrahydrofolic acid transport into the cytoplasm by partitioning into the caveolae which are capable of transiently closing leaving the receptor inaccessible to antibody probes. The closed caveolar compartment may be acidifed to release the folate, which then enters the cytoplasm via a transmembrane transporter present in the caveolae. The folate is trapped in

the cytoplasm after being covalently modified. The caveolae then unseal and the receptors become available for another cycle.

3. Signal transduction

A number of GPI-anchored proteins are implicated in signalling events in T lymphocytes and in other hematopoietic cells.[223] Activation of T cells resulting in lymphokine production and cell proliferation can be achieved by antibody-mediated cross-linking of GPI-anchored proteins such as Thy-1,[224] Qa-2,[225] or Ly-6A (also known as TAP).[123,226] Activation via GPI-anchored proteins seems to require a functional T-cell receptor, since mutant cell lines deficient in the receptor cannot respond to antibodies against Thy-1 or Ly-6.[227] Transmembrane forms of Qa-2 and Ly-6A/E cannot mediate T-cell activation,[225,226] suggesting that the GPI anchor is a critical component of the stimulus.

The precise role of GPI-anchored proteins in T-cell signalling is far from clear, and it is not possible at this stage to reconcile all the available data. Recent results indicate that GPI-anchored proteins are not required in the early stages of T-cell activation leading to production of interleukin-2 (IL-2), since a T-cell hybridoma mutant defective in GlcNAc-PI synthesis responded normally to mitogenic stimuli.[228] An explanation for these apparently conflicting results may lie in the differences between normal T-cells and T-cell hybridomas, particularly in the requirement for cellular adhesion for activation. Removal of the majority of GPI-anchored proteins from the T-cell surface by PI-PLC treatment eliminates the ability of the cells to respond to mitogens such as Concanavalin A,[229,230] despite apparently undiminished binding of fluorescent Con A to the cell surface. Curiously, activation of T-cells rendered the GPI-anchored proteins resistant to PI-PLC,[229,231,232] possibly because of protective interactions with other proteins or membrane components necessary for or produced by the activation process. PI-PLC treatment may remove a protein critical for T-cell-accessory cell interaction, or, possibly eliminate critical high affinity Con A binding sites. In this context it should be noted that a T-cell hybridoma mutant expressing only intermediate affinity Con A binding sites (high affinity sites corresponding to high mannose glycans were not synthesized owing to a dolichol-P-mannose synthase defect) was shown to be unresponsive to antigen, superantigen, and Con A.[228]

What, if any, is the role of the GPI-anchor in cell signalling? It is possible that the signalling mechanism involves the phospholipase-mediated degradation of the GPI anchor resulting in diacylglycerol which could flip into the inner leaflet of the plasma membrane bilayer and activate protein kinase C. In this context it is interesting to note the identification of a PI-specific phospholipase C activity at the surface of Swiss 3T3 cells.[233] Proteolytic or other cleavage of the anchor, coupled with the action of a

phospholipase, could result in a phosphoglycan moiety with second messenger properties as proposed for the insulin system[2,234] - the glycan could be delivered to the cytosol via a caveola-based mechanism as proposed for the delivery of folate.[222] Interestingly, early investigations of the effect of *B. anthrax* suggested that the toxin (subequently identified as PI-PLC) had insulinomimetic properties.[12]

Alternatively, GPI-anchored proteins may mediate activation signals through an interaction between the GPI structure and one or more membrane proteins, such as the T-cell receptor-CD3 complex.[223] Interactions between GPI anchors and membrane proteins have been suggested previously. For example, 10-20% of cell-surface Thy-1 was shown to be in a detergent-insoluble complex involving a protease-sensitive 55kDa membrane protein, minor amounts of Qa-2 and a variety of membrane associated proteins that could not be labelled by surface iodination.[235] Recent data describing co-immunoprecipitation of the cytosolic protein tyrosine kinase $p56^{lck}$ with some GPI-anchored proteins in human and mouse T-cells,[236] provides another example of a GPI anchor-protein interaction. Again, interaction of the GPI anchor with $p56^{lck}$ must be mediated by one or more intermediary proteins since the kinase is a myristylated cytosolic protein and the GPI is confined to the opposite (external) leaflet of the plasma membrane bilayer.

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Figure Legends

Figure 1; Isolation of VSG GPI-glycan fragment for structural analysis. The figure is redrawn from Ferguson *et al.* (1988)[50], and shows the route to production of a neutral glycan from the GPI-anchor suitable for structural analysis. See text for a detailed discussion. Abbreviations used are; CO; carboxyl-terminus of the VSG polypeptide, EthN; ethanolamine, P; phosphate, P'; cyclic phosphodiester, GlcN; glucosamine, PI; phosphatidylethanolamine, DAG; diacylglycerol, AA; amino acid remaining following exhaustive pronase digestion, HONO; nitrous acid, AHM; anhydromannitol. Structures in bold are ^{3}H-labelled.

Figure 2; Schematic diagram of a GPI-anchor or lipid to show the positions of action of various reagents. The shaded region represents the neutral glycan core, which can be studied by liquid chromatography or exoglycosidase digestion. The figure is redrawn from Field and Menon (1992)[88]. See text for detailed discussion of individual reactions. R1 in GPI-anchored proteins is the C-terminal amino acid, and in precursor lipids is H. R4 is either H or palmitate.

Figure 3; Attachment of a GPI-anchor to protein. The reaction probably occurs in the lumenal leaflet of the endoplasmic reticulum (ER) membrane bilayer, after translation of the protein on a membrane-bound ribosome, translocation of the polypeptide across the ER membrane, and cleavage of the the N-terminal signal sequence by ER signal peptidase. GPI attachment is signalled by a COOH-terminal peptide that is removed and replaced by a GPI, probably via a transpeptidation reaction mechanism. The GPI anchor precursor is shown to be inositol acylated (i.e., PI-PLC-resistant) as in mammalian cells. In some cells, the structure is processed (*) to a PI-PLC-sensitive form after attachment to protein, possibly after the protein leaves the ER (see section IV.3 for a complete discussion).

Figure 4; Biosynthesis of GPI. The figure shows an outline of GPI biosynthesis indicating the immediate identified donors (boxed) of individual components of the structure, and sites of action (❄, ❄, ✳) of various inhibitors. Inositol acylated (PI-PLC resistant) structures are not shown, nor is the membrane topology of the assembly process (see sections IV.3 and IV.4 for details).

Table 1: Proteins With A Glycosylphosphatidylinositol Anchor.

The list does not necessarily include all proteins that are believed to utilise the GPI anchor. Note that the data for some molecules are more rigourous than others. We have included those proteins which are believed to be GPI-anchored solely on the basis of primary structure, as this criterion is becoming more reliable. The list includes those from the review by Low[2]. These proteins are not referenced for reasons of brevity, but references for all proteins *additional* to the 1989 compilation are provided.

Protein	Source	Reference
Protozoal antigens		
Ssp-4	*T. cruzi*	
90kDa glycoprotein	*T. cruzi*	
gp50-55	*T. cruzi*	238
gp85 family	*T. cruzi*	239-242
Fl-160 (160kDal)	*T. cruzi*	243
SAPA (shed acute phase antigen)	*T. cruzi*	244
Sialidase (gp120-200kdal complex)	*T. cruzi*	245
Variant surface glycoprotein	*T. brucei*	
Procyclin/PARP	*T. brucei*	
Procyclic Sialidase	*T. brucei*	246
Transferrin binding protein [ESAG6]	*T. brucei*	247

Surface antigens	*P. primaurelia*	
p76 proteinase	*P. falciparum* merizoites	
195kDal antigen	*P. falciparum*	
Transferrin receptor	*P. falciparum*	
P30	*T. gondii*	
gp42/M-2	*L. amazonensis*	248
gp63 (PSP)	*Leishmania major, mexicana*	249
PSA-2	*L. major*	250
GP49	*Giardia lamblia*	251
Cell-Cell Interactions		
LFA-3	human blood cells	
Leu8/TQ1	human endothelia	252
Heparan sulphate proteoglycan	rat hepatocytes	
Neural cell adehesion molecule	mammalian, chicken brain and muscle	
Fibronectin receptor	human and avian tissues	253
TAG-1	mammalian neurons	254, 254b
Opioid binding protein	mammalian neural tissue	255
PH-20	guinea pig sperm	
F11	chicken brain	

Fasciculin I	*D. melanogaster*	256
Chaoptin	*D. melanogaster*	257
AGα1	*S. cerevisiae*	258
AGA1	*S. cerevisiae*	259
Contact site A	*D. discoideum*	
Miscellaneous		
Decay accelerating factor (DAF)	human blood cells	
Urokinase Receptor	mammalian tissues	260
NB-1	murine neutrophils	261
130kDal hepatoma glycoprotein	rat hepatoma	
34kDal growth factor	human placental trophoblast	
scrapie prion (PrP^{Sc}, PrP^{C})	mammalian brain, lymphocytes	262
GP-2	pig, rat zymogen granules	
Tamm-Horsfall glycoprotein (Uromodulin)	mammalian tissues (kidney)	263
CD-16 (Fcγ Receptor III)	human neutrophils	
Oligodendrocyte-myelin protein	human brain	
CNTF Receptor	mammalian brain	264
C8 binding protein	human lymphocytes and monocytes	

GP42	rat NK cells	265
Folate binding protein	rat kidney, KB cells	
26kDal glycoprotein	rat sperm	
150kDal glycoprotein [GPIb]	human platelets	266
high molecular weight mucins	human colon	267
82, 68kDal glycoproteins	bovine adrenal chromaffin granule	
I-antigenic glycoprotein GP-3	calf thyroid	
Heat stable antigen	murine hematopoietic lineage	268
Sgp-1, Sgp-2	squid neural tissue	
Surface antigens	*S. mansoni*	
Antigen 117	*D. discoideum*	
PsA	*D. discoideum*	269
125kDal glycoprotein [GAS1]	*S. cerevisiae*	
115kDal glycoprotein	*S. cerevisiae*	270
Protective antigen	*B. microplus*	271

Hydrolytic enzymes (non-protozoan)

Alkaline phosphatase (APase)	mammalian, *S. mansonii*, salamander

5'-Nucleotidase [CD73]	mammalian	
Acetylcholinesterase	*T. marmorata*, insect brain mammalian erythrocytes, *Schistosomula*	
Trehalase	mammalian	
Alkaline phosphodiesterase I	rat tissue	
Dipeptidase	pig kidney, sheep lung	
Aminopeptidase P	pig and human kidney	
Lipoprotein lipase	3T3-L1 adipocytes	
Carboxypeptidase M	MDCK cells	272
Carbonic anhydrase II and IV	human kidney	273,274
Mammalian antigens		
Thy-1	brain, lymphocytes	
Thy-3	murine lymphocytes	
RT-6	rat lymphocytes	
Qa	murine lymphocytes	
Ly-6 (complex)	murine lymphocytes	
MEM-43	human leukocytes and erythrocytes	
Carcinoembryonic antigen	human tumour cells	
NCA	human	

Blast-1	human lymphocytes		
MRC OX-45	rat lymphocytes		
CD14	human monocytes		
Mo3	human monocytes		
CD48	human leukocytes		
CD24	human granulocytes	275	
CA-MOv18 (38kDal antigen)	human ovarian carcinoma	276	
sgp-60	murine T and B cells	277	
B7 (12kDal antigen)	mammalian lymphocytes		278
Choline-O-Acetyltransferase	rat hippocampus		279
PI-GP150	rat telencephalon		280

Table 2: Structures of Protein GPI-anchors and their Precursors

(ia) Proteins; Core Glycans and Modifications

Name	*Structure*	*Reference*
Trypanosoma brucei VSG 117	Asp-**E-P-Mα1-2Mα1-6Mα1-4GlcNα1-6**PI[a] | $^{+/-}$Galα1-2($^{+/-}$Galα1-2Galα1-6)Galα1-3	50
Trypanosoma brucei VSG 118	Asn-**E-P-Mα1-2Mα1-6Mα1-4GlcNα1-6**PI	46
Trypanosoma brucei VSG 221	Ser-**E-P-Mα1-2Mα1-6Mα1-4GlcNα1-6**PI (+ 8 α-Gal, not fully defined)	46
Trypanosoma cruzi 1G7	**E-P-Mα1-2Mα1-6Mα1-4GlcNα1-6**PI ? | Mα1	281
Leishmania major gp63 (Acid protease)	Asn-**E-P-Mα1-2Mα1-6Mα1-4GlcNα1-6**PI	53
Saccharomyces cerevisiae gp125	**E-P-Mα1-2Mα1-6Mα1-4GlcNα1-6**PI 2 | Mα1	281

Human erythrocyte Acetyl-cholinesterase[a]	Gly-**E-P-Mα1-2Mα1-6Mα1-4GlcNα1-6**PI 2 | E-P	51,52
Rat Brain Thy-1	Cys-**E-P-Mα1-2Mα1-6Mα1-4GlcNα1-6**PI 2 2 4 | | | Mα1 E-P GalNAcβ1	64

The conserved core structure for the protein-linked GPI series is shown in bold.

a. Linkage positions and the identity of the hexoses in the human acetylcholinesterase not determined, but assumed to be homologous.
Abbreviations M = mannose, E = ethanolamine, P = phosphate.

☎ = Phosphatidylinositol (PI) = ***myo*Inositol1-P-CH_2**

?
|
R^3

|
CH-R^1
|
CH_2-R^2

Linkage position of the R^3 substituent has not been determined.

(ib) Proteins; Lipid Substituents

Protein	*R*^{1}	*R*^{2}	*R*^{3}	Reference
Trypanosoma brucei VSG	**$C_{14:0}$**	**$C_{14:0}$**	H	44
Trypanosoma brucei PARP	**$C_{18:0}$**	H	**$C_{16:0}$**	55
Leishmania major gp63[a]	$C_{18:0\text{-}23:0}$, **$C_{24:0}$**, $C_{25:0\text{-}26:0}$	**$C_{16:0}$**, $C_{14:0}$ $C_{12:0}$, $C_{18:0}$	H	53
Human erythrocyte AChE[a]	**$C_{18:0}$**, $C_{18:1}$	**$C_{22:4}$**,**$C_{22:5}$** $C_{22:6}$	**$C_{16:0}$**	51,52
Torpedo AChE	**$C_{16:0}$**	**$C_{16:0}$**	ND	129
Human Folate Binding Protein[a]	**$C_{20:0}$**	**$C_{22:0}$**	ND	252

a For these proteins, substituent at R^1 is in ether linkage. Major species shown in bold. ND= not determined

(ii) GPI-Anchor Precursors

Only mature precursors that have been well identified by chromatography and analytical methods are include in this table. A large number of other species have been identified, and are discussed in the text under biosynthesis.

GPI[a]	*Protein*	*Organism*	*Lipid Substituents* *sn*-1	*sn*-2	*Inositol*	*Reference*
P2	VSG	*T. brucei* BSF	$C_{14:0}$	$C_{14:0}$	-	59, 89
P3	VSG?	*T. brucei* BSF	$C_{14:0}$	$C_{14:0}$	$C_{16:0}$	59, 89
PP1	PARP	*T. brucei* Procyclic	$C_{18:0}$	H	$C_{16:0}$	60
LP-1[b]	gp63	*L. major* Promastigote	Not determined			76
Lipid C	Various	Murine T cells	Not determined[c]			61,63
[d]	Various	*Tetrahymena*	Not determined			283

a. Data are consistent with all the precursors containing the core ethanolaminylphosphoglycan. All linkages have not been determin for P2, P3 and PP1, but are assumed to be the same based on co-chromatography on HPLC. For LP-1, HPLC co-chromatography data available only.

b. Lipid composition not determined, but substituent at *sn*-1 is base stable, and proposed to be alkyl, and homologous with the gp63 GPI-anchor. LP-1 is PI-PLC sensitive, and therefore does not contain a fatty acid inositol substituent.

c. Lipid composition not determined, but resistance to PI-PLC indicates the probable presence of a fatty acid substituent on the inositol.

d. The glycan sequence of this lipid is different from all other putative GPI-precursors. The Manα1-6Man linkage is replaced by Manα1-3Man. This lipid has not been definitively shown to be a GPI-precursor.

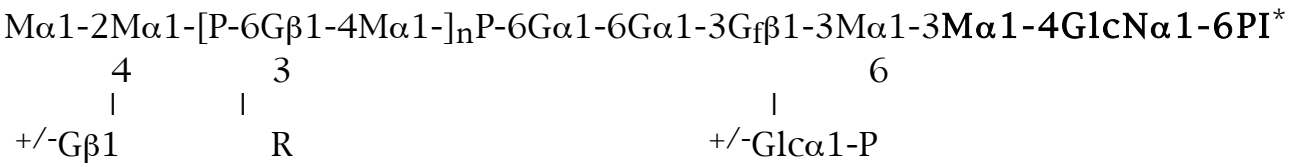
Table 3; Structures of the Lipophosphoglycan and Glycosylinositolphospholipid Series GPI Molecules

The conserved core structure for the LPG/GIPL GPI series is shown in bold. Note that this sequence is also common to the protein GPI-anchor series.

(i) Glycans

(a) Lipophosphoglycan (LPG)

Structure;



Species	R =	Reference
L. donovani	H	71
L. mexicana	H or Glcβ1-	73
L. major	H, $[G\beta1\text{-}3]_{0\text{-}3}G\beta1$-, or $Ara_p1\text{-}2[G\beta1\text{-}3]_{0\text{-}2}G\beta1$-	73

Lipopetidophosphoglycan (LPPG)

AEP

6

Structure; $G_f\beta$1-3Mα1-2Mα1-2Mα1-6**Mα1-4GlcN-Inositol-**PO_4-Ceramide

3

G_f1

Major surface glycoconjugate from epimastigotes of *Trypanosoma cruzi*

Data from 77,78. Note that for this molecule, homology to the protein-anchor series is more extensive and includes the first three mannose residues attached to the GlcN.

n (average number of repeats) = 10-30.
* PI in all cases is a *lyso*-alkylPI
AEP = 2-aminoethylphosphonate.

(b) Glycoinositolphospholipids (GIPLs)

***L. major* promastigote.** Core structure; R-G$_f$α1-3Mα1-3**Mα1-4GlcNα1-6PI**

Lipid Species	R =	Abundance (Strain)/Mol% V121	L119
GIPL-1	H	9	2
GIPL-2[a]	Gα1-3	33	26
GIPL-3	Gα1-6Gα1-3	26	8
GIPL-A	Gα1-3Gα1-3	4	ND
GIPL-4, 5, 6	ND[b]	ND	+

L. donovani. Core structure; R'-6[R''-3]**Mα1-4GlcNα1-6PI**

Lipid Species	R'	R''
Promastigote stage		
IsoM2	H	Mα1-
IsoM3	Mα1-	Mα1-
IsoM4	Mα1-2Mα1-	Mα1-
Amastigote stage		
M2	Mα1-	H
M3	Mα1-2Mα1-	H

a *Lyso* forms of GIPLs 2 and 3 also present.
b GIPLs 4-6 detected in the L119 strain, and possibly LPG precursors.
ND Not determined.
Data from 67, 68, 69, 73.

(ii) Lipid Moieties

Structure/Spp	*sn-1(Alkyl)*	*sn-2(Acyl)*	*Ref*
L.major			
GPI A,B,C/	**$C_{24:0}$**, $C_{20:0}$,	**$C_{18:0}$**, **$C_{16:0}$**,	283
GIPL1-4	**$C_{18;0}$**, $C_{16:0}$	$C_{14:0}$	67
GIPL5,6	**$C_{24:0}$**, $C_{18:0}$, $C_{26:0}$	Not present	67
LPG	**$C_{24:0}$**, $C_{26:0}$	Not present	73
L. donovani			
M2,3, IsoM2,3,4	**$C_{18:0}$**	**$C_{18:0}$**, **$C_{16:0}$** $C_{14:0}$	69
LPG	**$C_{24:0}$**, $C_{26:0}$	Not present	57

Major species are shown in bold.