REVIEW ARTICLE

The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes

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INTRODUCTION

The protozoa are the most diverse and amongst the most ancient group of organisms in the eukaryotic kingdom (Sogin et al., 1989). Many of their members are parasitic and some, like those belonging to the family Trypanosomatidae (African trypanosomes, *Trypanosoma cruzi*, *Leishmania*. spp.) and the genera *Plasmodium*, *Eimeria*, *Babesia*, *Theileria*, *Toxoplasma* and *Entamoeba*, are the cause of important diseases in humans and their domestic livestock. Glycoconjugates on the cell surface of these organisms frequently play a crucial role in determining parasite survival and infectivity. It has become clear over the last 5 years that many of these molecules are anchored to the plasma membrane via glycosyl-phosphatidylinositol (GPI) anchors. This type of anchor is not unique to the protozoa, but it does appear to be used with a much higher frequency in these organisms than in higher eukaryotes. In this article we review the structure, function and biosynthesis of GPI anchors in protozoan parasites and in higher eukaryotes. These data suggest that there may be significant differences in the function of GPI protein anchors in unicellular versus metazoan organisms. In addition, some of the parasitic protozoa, particularly those belonging to the Trypanosmatidae, synthesize a number of exotic GPI-related structures which are not attached to proteins. The structure, biosynthesis and role of these major cell surface glycoconjugates in parasite survival and infectivity are discussed, together with some speculations on the evolutionary aspects of the GPI-family. Previous general reviews on the subject of the GPI anchors and related structures can be found in Ferguson and Williams (1988), Low (1989), Cross (1990a), Thomas et al. (1990), Ferguson (1991, 1992b), McConville (1991) and Turco and Descoteaux (1992).

STRUCTURE OF PROTEIN GPI ANCHORS

Although some of the plasma membrane proteins of the parasitic protozoa use transmembrane polypeptide anchors, most of the major cell-surface proteins of these organisms are GPI-anchored (Table 1). These proteins are functionally diverse and include coat proteins, surface hydrolases and receptors. Some of these are known to be directly involved in parasite protection [e.g. *T. brucei* variant surface glycoprotein (VSG)] or specific host–parasite interactions (e.g. *T. cruzi* trans-sialidase and 35/50 kDa antigen, *P. falciparum* MSA-1 and *Leishmania* gp63).

Structures of parasite anchors

The complete or partial structures of the GPI anchors of four parasite proteins, all from the Trypanosomatidae, have been determined (Figure 1). In each case, the C-terminus of the protein is linked via ethanolamine phosphate to a glycan with the conserved backbone sequence Manα1–2Manα1–6Manα1–4GlcNH_2, which in turn is linked to the 6-position of the *myo*-inositol ring of phosphatidylinositol (PI). The tetrasaccharide backbone may be substituted with other sugars in a species- and stage-specific manner, with the most elaborate side chains being found in the GPI anchors of the two *T. brucei* proteins; the VSG and the procyclic acidic repetitive protein (PARP or procyclin) (Table 1). The VSG anchor is substituted with branched side chains of α-galactose (Ferguson et al., 1988), while the PARP anchor has a large and complex side chain containing *N*-acetylglucosamine, galactose and sialic acid residues (Ferguson et al., 1993) (Figure 1). Preliminary studies on the latter side chains suggest that they contain sequences of poly(*N*-acetyllactosamine) that are substituted with sialic acid. In contrast, the GPI anchors of the *L. major* and *T. cruzi* antigens are either unsubstituted or only substituted with a single α-Man residue, respectively (Schneider et al., 1990; Güther et al., 1992) (Figure 1).

The lipid moieties of the parasite anchors can also vary in a species- and stage-specific manner. These anchors have been found to contain dimyristoylglycerol (in VSG), lyso-1-*O*-stearoylglycerol (in PARP) and alkylacylglycerol (in *Leishmania* gp63 and *T. cruzi* 1G7 antigen) (Figure 1). Some of these anchors may also contain an additional fatty acid (palmitate) on the inositol ring (Figure 1). In the PARP anchor, this fatty acid (acyl group) is attached to either the C-2 or C-3 position of the inositol (Field et al., 1991b; Ferguson, 1992a). This feature, first described by Rosenberry and colleagues, renders the anchor resistant to PI-specific phospholipase C (PI-PLC) hydrolysis, probably by blocking the formation of the inositol 1,2-cyclic phosphate during cleavage of the phosphodiester bond (Roberts et al., 1989a). Inositol acylation may be under developmental control in *T. cruzi*, as PI-PLC resistance of the 1G7 antigen increases as the cells develop from a non-infectious to an infectious 'metacyclic' stage (Schenkman et al., 1988).

Comparison with the anchors of other eukaryotes

All the GPI anchors which have been characterized to date (from protozoal, yeast, slime-mould, fish and mammalian sources) contain an identical ethanolamine-phosphate–Manα1–2Manα1–6Manα1–4GlcNα1–6*myo*-inositol backbone (Figure 1), suggesting that this sequence is likely to be conserved in all GPI anchors. These studies also indicate that the elaborate carbohydrate side chains and the distinctive mono- and diacylglycerol moieties of the *T. brucei* anchors are unique to this parasite. In contrast, the presence of additional α-mannose residues, as occurs in the *T. cruzi* anchor, also occurs on the anchors of yeast, slime-mould and mammalian proteins and appears to be a common substituent. It is of interest that all the anchors of higher eukaryotes (from slime moulds upward) are substituted

Abbreviations used: GPI, glycosyl-phosphatidylinositol; GIPL, glycoinositolphospholipid; LPG, lipophosphoglycan; LPPG, lipopeptidophosphoglycan; PARP, *Trypanosoma brucei* procyclic acid repeat protein; PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; VSG, *T. brucei* variant surface glycoprotein.

Table 1 Occurrence of GPI-anchored proteins in the parasitic protozoa

Species	Protein	Properties	Key references
Trypanosoma brucei	Variant surface glycoprotein	Coat glycoprotein of bloodstream trypomastigotes	Ferguson et al., 1988
	Transferrin-binding protein	Surface of bloodstream trypomastigotes	Schell et al., 1991
	PARP/procyclin	Coat glycoprotein of procyclic forms	Clayton and Mowatt, 1989
	Procyclic trans-sialidase	Surface of procyclic forms	Engstler et al., 1992
T. congolense/*T. equiperdum*	Variant surface glycoprotein	Coat glycoprotein of bloodstream trypomastigotes	Lamont et al., 1987; Ross et al., 1987
Trypanosoma cruzi	Ssp-4	Major surface glycoprotein of amastigotes	Andrews et al., 1988
	90 kDa 1G7 antigen	Major surface glycoprotein of metacyclics	Schenkman et al., 1988; Güther et al., 1992
	gp50-55	Epimastigote/trypomastigote/amastigote antigen	Hernandez-Munain et al., 1991
	35/50 kDa/10D8 antigen	Epimastigote/metacyclic antigen; major acceptor of sialic acid	Schenkman et al., 1993
	TCNA/Shed Acute Phase Antigen (SAPA)	120–200 kDa, trans-sialidase/sialidase family (inactive	Periera et al., 1991; Pollevick et al., 1991; Schenkman et al., 1992
	GP85 family	Trypomastigote trans-sialidase/sialidase family (inactive)	Fouts et al., 1991; Takle and Cross, 1991
	F1-160 (160 kDa)	160 kDa flagella antigen	van Voorhis et al., 1991
Leishmania spp.	Promastigote surface protease/gp63	Major surface glycoprotein on promastigote surface	Bouvier et al., 1985; Etges et al., 1986
	GP46/M2	Promastigote surface	Lohman et al., 1990
	PSA-2 (promastigote surface antigen-2)	Promastigote surface	Murray et al., 1989
Crithidia fasiculata	Protease	Homologue of *Leishmania* gp63	Zaretskia et al., 1989; Inverso et al., 1993
Herpetomonas samuelpessoai	Protease	Homologue of *Leishmania* gp63	Schneider and Glaser, 1993
Plasmodium falciparum	MSA-1 (merozoite surface antigen 1)	195 kDa, major merozoite surface glycoprotein	Haldar et al., 1985
	Transferrin binding protein	Merozoite surface	Haldar et al., 1986
	MSA-2 (merozoite surface antigen-2)	Merozoite surface	Smythe et al., 1988
	p76 proteinase	Merozoite/schizont surface	Braun-Breton et al., 1988
Toxoplasma gondii	P22, P23, P30, P35, P43	Major surface proteins on tachyzoite	Nagel and Boothroyd, 1989; Tomavo et al., 1992a
Giardia lamblia	GP49	Trophozoite surface	Das et al., 1991
Eimeria	Undefined antigens	Surface of sporozoites	Gurnett et al., 1990

with one or two additional ethanolamine phosphate residues. These residues have not been detected in the protozoan or yeast anchors, suggesting that they may be specific to metazoan eukaryotes. The nature of the lipid moieties in the non-protozoan anchors is also variable. Alkylacylglycerols and diacylglycerols have been found in the mammalian and fish anchors, whereas ceramide is the most common lipid in the anchors of yeast (Conzelmann et al., 1992; Fankhauser et al., 1993) and *Dictyostelium discoideum* (Stadler et al., 1989; Haynes et al., 1993). Many of the mammalian anchors also contain palmitate on the inositol ring (Roberts et al., 1989a; Luhrs and Slomiany, 1989; Walter et al., 1990; Lee et al., 1992).

GPI ANCHOR BIOSYNTHESIS

A protein destined to receive a GPI anchor must contain two pieces of information in its primary translation product. Firstly, it must contain an N-terminal signal sequence for entry into the lumen of the endoplasmic reticulum via the signal recognition particle and, secondly, it must contain a GPI-signal sequence at the C-terminus. This GPI-signal sequence is extremely degenerate but it is quite easily identified from cDNA sequences (reviewed in Ferguson and Williams, 1988; Cross, 1990a; Undenfriend et al., 1991; Caras et al., 1991; Kodukula et al., 1992). The most common feature is a run of 12–20 hydrophobic residues at the very C-terminus of the primary translation product, but there are exceptions. The GPI-signal sequence is cleaved and replaced by a preassembled GPI precursor in what appears to be an ATP- and GTP-independent (Mayor et al., 1991) transamidation reaction (Gerber et al., 1992, and references therein). However, both ATP and GTP appear to be required in some way immediately prior to the transamidation event (Amthauer et al., 1992). From kinetic and genetic data the addition of GPI to proteins is believed to occur in the endoplasmic reticulum (Bangs et al., 1985; Ferguson et al., 1986; Conzelmann et al., 1988). The biosynthetic pathways of GPI precursor formation are summarized in Figure 2. Previous reviews on this subject include Doering et al. (1990), Menon (1991), Tartakoff and Singh (1992), and Englund (1993).

The GPI biosynthetic pathway of bloodstream form African trypanosomes

The surface membrane of the *T. brucei* bloodstream form is covered by a dense coat of VSG molecules which protects the parasite from both non-specific and specific components of the host immune system (Cross, 1990b). The VSG coat is essential for the survival of the parasite and consequently a considerable amount of the parasite's metabolic effort is directed towards the synthesis and processing of VSG glycoprotein. Each trypanosome expresses about 10^7 copies of an individual VSG, all of which bear a GPI membrane anchor. For these reasons *T. brucei* has proved to be a convenient system for the analysis of GPI anchor biosynthesis. The pathway has been delineated by the use of a trypanosome lysates which, when fed with appropriate radiolabelled donor molecules such as UDP-[^{3}H]GlcNAc or GDP-[^{3}H]Man, produce the complete spectrum of intermediate species, all of which have been structurally characterized.

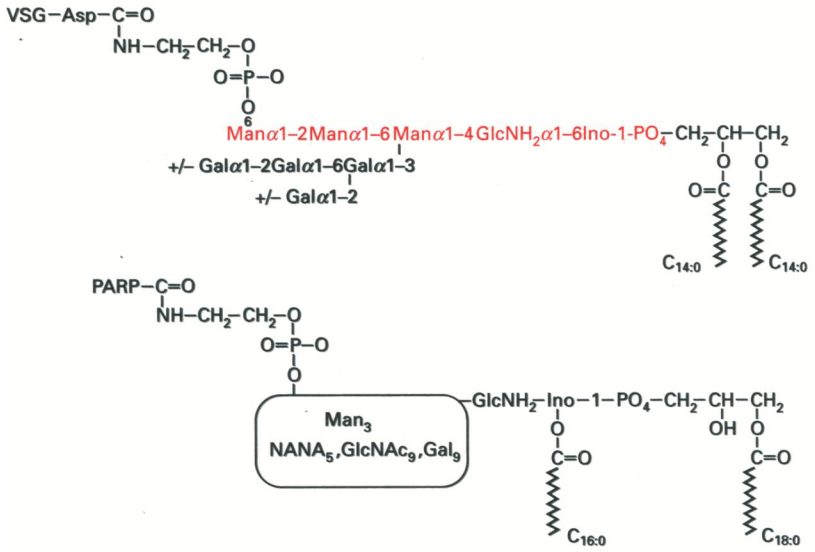
The GPI biosynthetic pathway in bloodstream form *T. brucei* parasites (Figure 2) may be summarized as follows: α-GlcNAc is transferred from UDP-GlcNAc to PI to form GlcNAc-PI, which is then de-N-acetylated to GlcN-PI (Doering et al., 1989). Subsequently, three α-Man residues are transferred in single steps from dolichyl phosphate mannose (Dol-*P*-Man) to form Man_3-GlcN-PI (Masterson et al., 1989; Menon et al., 1990a,b). Ethanolamine phosphate is transferred from phosphatidylethanolamine to the terminal Man residue to form EtN-*P*-Man_3-GlcN-PI (known as glycolipid A′) (Menon et al., 1993). This species then undergoes a complex series of fatty acid remodelling reactions (Masterson et al., 1990) as follows: the *sn*-2-fatty acid of glycolipid A′ is removed to form a *lyso*-species called glycolipid θ. Glycolipid θ is myristoylated to form glycolipid A″ (which contains *sn*-1-stearoyl-2-myristoylglycerol). The *sn*-1-stearoyl group is removed from glycolipid A″ and replaced by myristic acid to form glycolipid A. The donor molecule for the two myristoyltransferase steps is myristoyl-CoA. Concomitant with the formation of glycolipid A is the formation of glycolipid C (the inositol-palmitoylated version of glycolipid A). The structures of glycolipids A and C (also known as glycolipids P2 and P3) have been rigorously determined (Krakow *et al.*, 1989; Mayor et al., 1990a,b). Both of these species have been shown to be competent for transfer to VSG polypeptide when added exogenously to a trypanosome cell-free system (Mayor et al., 1991), but there is no evidence of the transfer of glycolipid C (P3) *in vivo*. It has been suggested that glycolipid C is an obligate intermediate on the pathway to the glycolipid A GPI precursor (Doering et al., 1990; Masterson et al., 1990; Menon et al., 1990b; Menon, 1991), but recent data suggest that glycolipid C could be an end-product of the pathway (M. L. S. Güther, W. J. Masterson and M. A. J. Ferguson, unpublished work). Glycolipid C might therefore represent a reversible reservoir for glycolipid A and/or an intermediate in the catabolism of excess GPI precursors.

The transfer of GPI precursor to VSG polypeptide involves the removal of a hydrophobic C-terminal GPI signal sequence of 17–23 amino acids, depending on the VSG variant. The rapid kinetics of this reaction suggests that this occurs in the endoplasmic reticulum (Bangs et al., 1985; Ferguson et al., 1986). Finally, the GPI anchor becomes α-galactosylated. The first α-Gal residue is probably attached in the endoplasmic reticulum (Mayor et al., 1992) and the subsequent residues are added some 10–15 min later, most likely in the Golgi apparatus (Bangs et al., 1988). The α-galactosyltransferases involved in this processing appear to be unique to the African trypanosomes and may play a role in VSG coat function (see below). Recently a cell-free assay for VSG GPI α-galactosylation has been reported using UDP-[^{14}C]Gal as the donor (Pingel and Duszenko, 1992). Interestingly, small amounts of glycolipid A containing several α-Gal residues are observed in trypanosomes (Mayor et al., 1992), which suggests that some mono-galactosylated glycolipid A escapes the endoplasmic reticulum by bulk flow and becomes further galactosylated in the Golgi apparatus. The physiological significance of these structures is unknown.

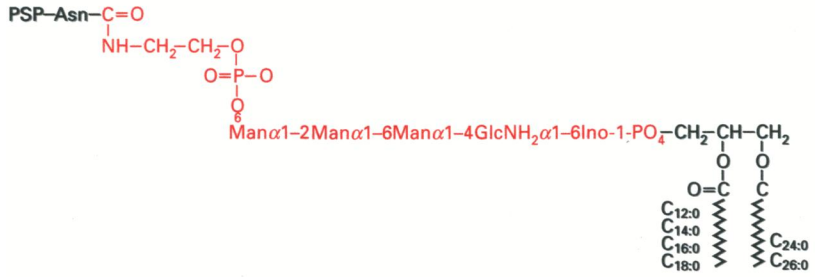
GPI anchor biosynthesis in *T. brucei* procyclic forms

A structurally distinct GPI precursor (called PP1) has been isolated from the procyclic (insect-dwelling stage) of *T. brucei* and shown to have the structure EtN-*P*-Man_3-GlcN-*sn*-1-stearoyl-2-*lyso*-(palmitoyl)PI (Field et al., 1991b). These data suggest that the large side-chain found on the PARP anchor (Figure 1) is probably added after transfer of the anchor to the PARP polypeptide, possibly in the Golgi apparatus. The origin of the novel PI moiety is thought to arise from incomplete fatty acid remodelling such that, like the bloodstream form trypanosomes, the *sn*-2 fatty acid is removed by a specific phospholipase A_2 to yield a lyso species. However, unlike bloodstream forms, the *sn*-2 position is not subsequently reacylated with myristate nor is the *sn*-1-stearoyl group replaced by myristate. Experiments using a procyclic cell-free system suggest that the inositol

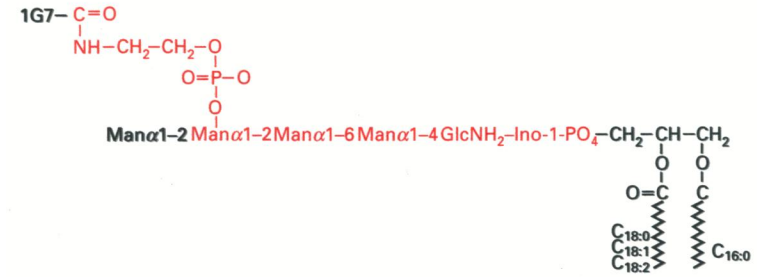
Trypanosoma brucei



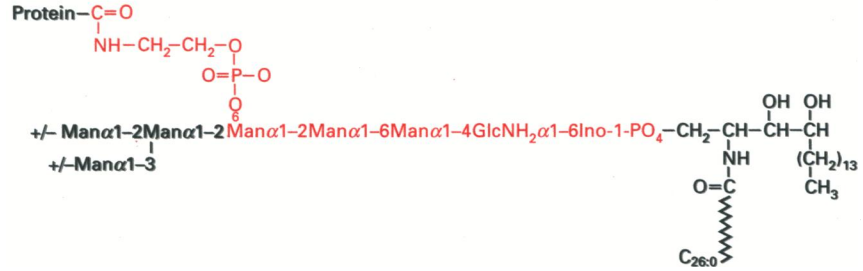
Leishmania major



Trypanosoma cruzi



Yeast



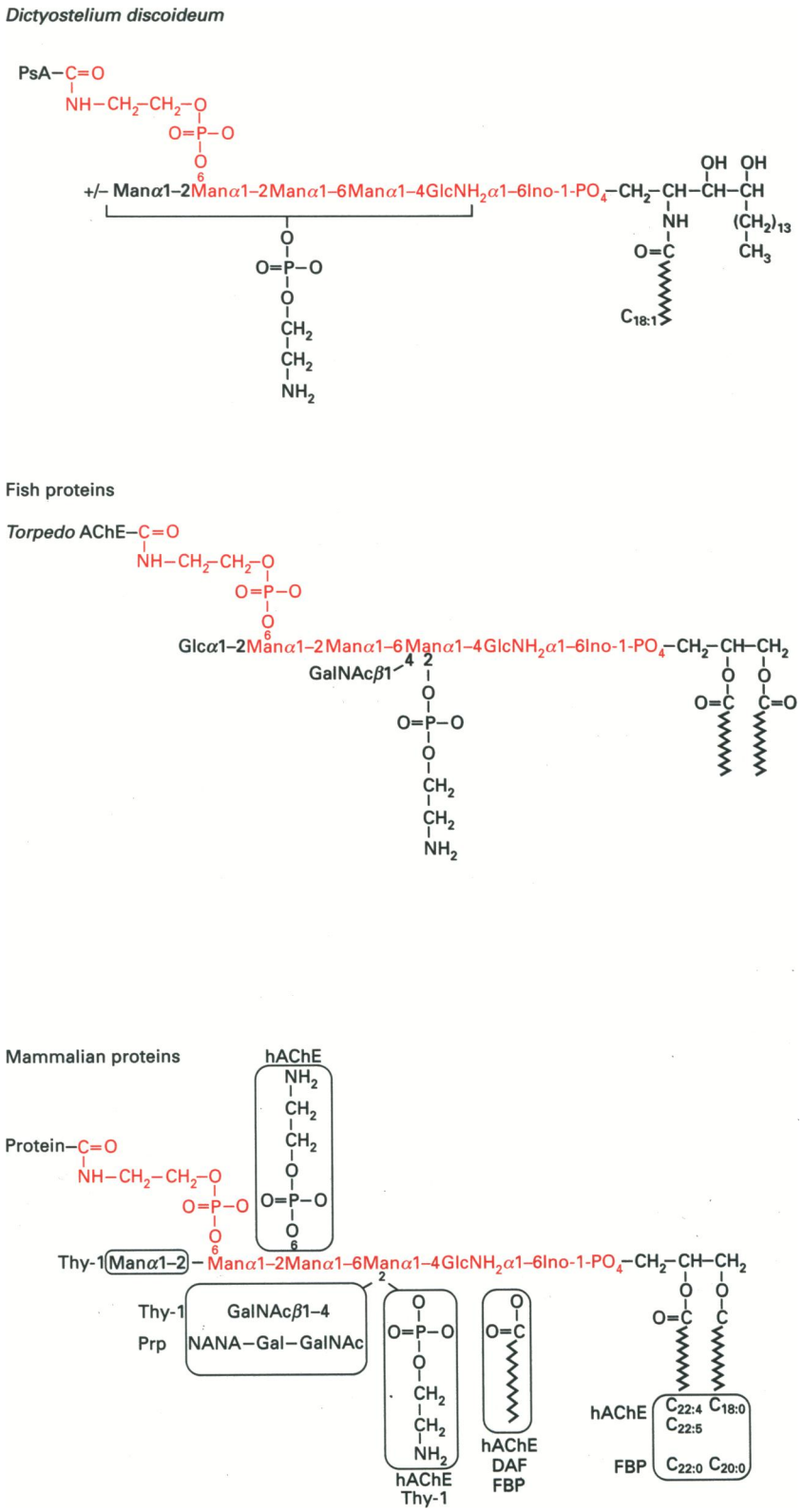


Figure 1 Structures of GPIs

The figure shows a comparison of the structures of the GPIs from four parasite proteins [*T. brucei* VSG (Ferguson et al., 1988) and PARP (Field et al., 1991a; Ferguson et al., 1992a, 1993), *Leishmania major* Gp63 (Schneider et al., 1990) and *T. cruzi* 1G7 antigen (Güther et al., 1992; N. Heise, M. L. Cardoso de Almeida and M. A. J. Ferguson, unpublished work)], a mixture of yeast glycoproteins (Conzelmann et al., 1992; Fankhauser et al., 1993), *Dictyostelium discoideum* PsA (Haynes et al., 1993), *Torpedo* acetylcholinesterase (AChE; Mehlert et al., 1993) and a number of mammalian proteins [rat brain Thy-1 (Homans et al., 1988), human erythrocyte acetylcholinesterase (hAChE; Roberts et al., 1989b; Deeg et al., 1992), scrapie prion protein (PrP; Stahl et al., 1992) and human folate binding protein (FBP; Luhrs and Slomiany, 1989; Lee et al., 1992)]. Components of the conserved GPI backbone, i.e. ethanolamine-phosphate–Manα1–2Manα1–6Manα1–4GlcNα1–6*myo*-inositol, are shown in red.

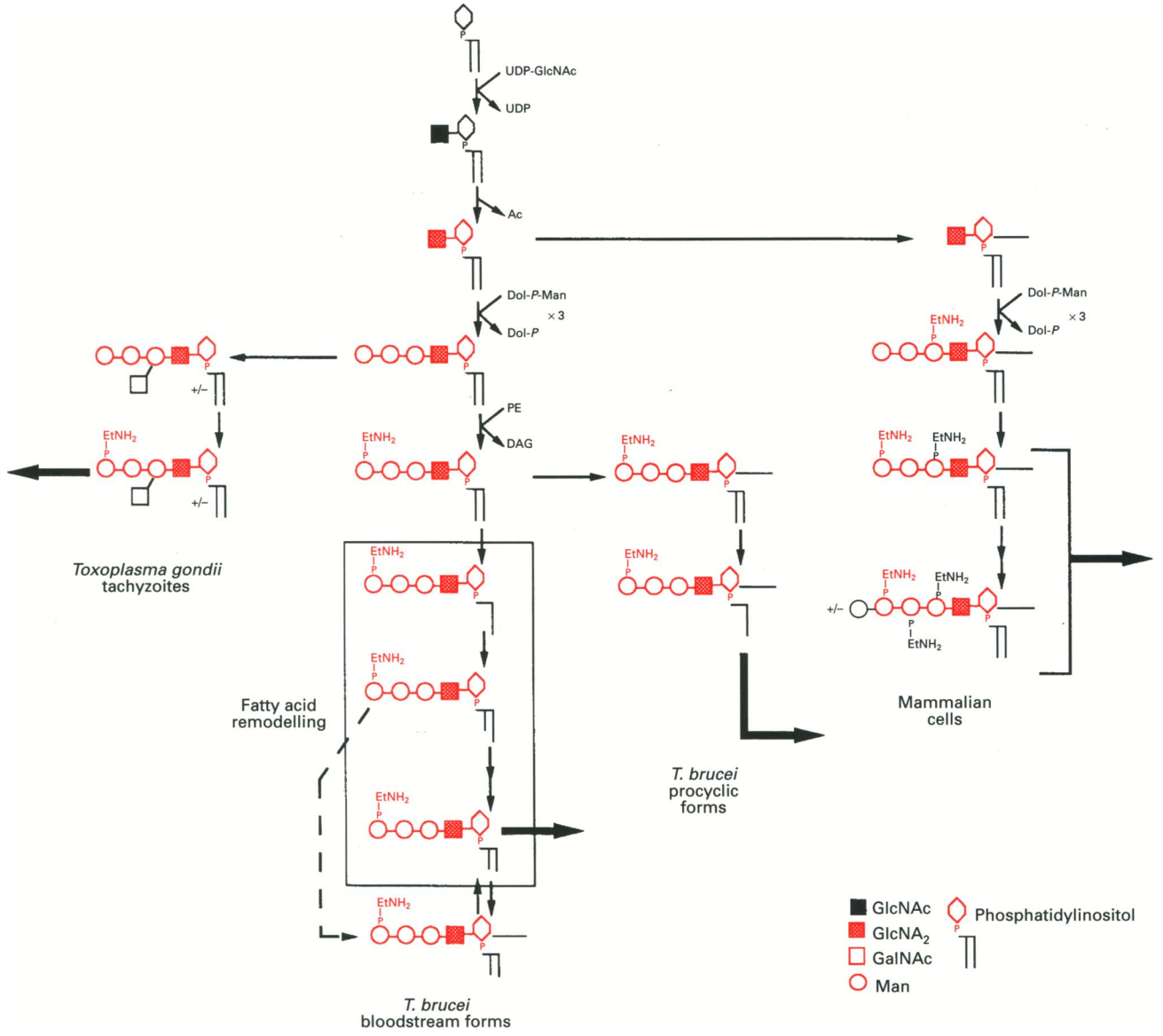


Figure 2 The GPI-anchor biosynthetic pathways in protozoan parasites and mammalian cells

The bold arrows indicate species which are transferred to protein. The donor species of the biosynthetic steps are shown where they are known. The boxed area represents the fatty acid remodelling reactions unique to bloodstream form *T. brucei*. The dashed arrows indicate alternative pathways. Components of the conserved GPI backbone, i.e. ethanolamine-phosphate–Manα1–2Manα1–6Manα1–4GlcNα1–6*myo*-inositol, are shown in red. Abbreviations: PE, phosphatidylethanolamine; $EtNH_2$, ethanolamine; DAG, diacylglycerol; P, phosphate.

palmitoylation event occurs earlier in the pathway than in bloodstream form trypanosomes (Field et al., 1992) (Figure 2). It is interesting that the GPI pathway in *T. brucei* appears to be under developmental control such that the timing of inositol palmitoylation, the extent of fatty acid remodelling and nature of protein-GPI carbohydrate processing are quite distinct. Presumably this represents developmental regulation of several GPI biosynthetic and processing enzymes during procyclic to bloodstream form differentiation, and vice versa.

GPI anchor biosynthesis in *Plasmodium* and *Toxoplasma*

The sporozoan parasites *Plasmodium* and *Toxoplasma* diverged from the kinetoplastid line at the earliest stage of eukaryotic evolution. Nevertheless, their GPI biosynthetic pathways are broadly similar to those of the African trypanosomes. In *Toxoplasma* the pathway produces exclusively diacyl- and *lyso*-acyl-PI GPI precursors (Tomavo et al., 1992b). A novel feature in this organism is the addition of a carbohydrate branch of β-GalNAc prior to the addition of the ethanolamine phosphate (Figure 2). Thus processing of the conserved trimannosyl backbone occurs before GPI transfer to protein. In the malaria parasite, *Plasmodium falciparum*, the majority of the GPI-intermediates are PI-PLC resistant and one of the major precursor species contains four Man residues (Gerold et al., 1992; P. Gerold et al., unpublished work). There is no evidence of fatty acid remodelling in either of these organisms. *Plasmodium* GPI anchors (or related structures) have been implicated in tumour necrosis factor and interleukin-1 production by macrophages during malarial infection (Taverne et al., 1990; Schofield and Hackett, 1993).

GPI biosynthesis in mammalian cells

Because of the emphasis of this article on parasite systems the data on the pathway in mammalian cells will be only briefly reviewed here. Essentially the pathway is the same as that found in parasites (i.e. sequential glycosylation and processing of PI). However, most but not all (Puoti and Conzelmann, 1993), of the intermediates (from GlcN-PI onwards) are inositol-palmitoylated (Urakaze et al., 1992; Hirose et al., 1992). Thus this reaction appears to be an early step in GPI biosynthesis in mammals. The occurrence of PI-PLC-sensitive mature protein-linked GPI anchors is, however, common in mammalian cells. The expression of either PI-PLC resistant or PI-PLC sensitive anchors appears to be cell-type specific (Toutant et al., 1990; Richier et al 1992) and the PI-PLC-resistant phenotype appears to be recessive in cell-fusion experiments (Singh et al., 1991). This suggests that mammalian cells transfer the mature inositol-palmitoylated GPI precursor to protein and then (in some cells) de-palmitoylate the inositol ring. Another notable difference in metazoan GPI anchors (from slime-moulds upwards) is the presence of at least one extra ethanolamine phosphate group (Figure 1). Recent studies show that these groups are added during GPI biosynthesis and not after GPI transfer to protein (Hirose et al., 1992; Kamitani et al., 1992; Puoti and Conzelmann, 1992) (Figure 2). The nature of the donor species for these extra groups is unknown.

The disease paroxysmal nocturnal haemoglobinuria (PNH) is caused by a defect in GPI anchor biosynthesis in blood cells as a result of a somatic mutation in one or more bone marrow progenitor cells. This leads to destruction of their progeny by autologous complement-mediated lysis since the GPI-anchored proteins decay accelerating factor (DAF) and CD59, which are responsible for preventing such autolysis, are not expressed on the cell surface. This phenotype appears to be due to a defect in the formation of GlcNAc-PI (Armstrong et al., 1992; Takahashi et al., 1993).

GPI biosynthesis in yeast

The mature protein-linked GPI anchors in yeast contain mostly ceramide-PI, with a phytosphingosine long chain base and an unusually long C_{26} fatty acid, although in one case (Gp125) the anchor is a *lyso*-acyl-PI (Frankhauser et al., 1993). The mechanism for this differential expression of lipid types is unknown. However, studies on newly synthesized GPI-anchored proteins show a time-dependent shift from base labile (acylglycerol-) GPIs to base stable (ceramide-) GPIs. This suggests that yeast may perform a novel form of lipid remodelling involving the exchange of glycerolipid for ceramide after transfer of the GPI precursors to protein (Conzelmann et al., 1992). Consistent with this, the putative early intermediates in the yeast GPI biosynthetic pathway, partially characterized as GlcNAc-PI, GlcN-PI and inositol-palmitoylated GlcN-PI, also appear to be base-labile (Costello and Orlean, 1992). These authors also present data to suggest that in yeast, palmitoyl-CoA is the donor for the inositol-palmitoylation event. However, this is in contrast to the situation in *T. brucei* procyclics where the palmitate donor appears to be a large pool of membrane-stable lipid, possibly phospholipid (Field et al., 1991b). The donor molecule for the addition of the ethanolamine phosphate bridge in yeast has been shown to be phosphatidylethanolamine (Menon and Stevens, 1992).

The topology and enzymology of the GPI pathway

Recent data, using sealed thymoma endoplasmic reticulum vesicles and permeabilized cells, strongly suggest that the first steps of the pathway (PI → GlcNAc-PI → GlcN-PI) occur on the cytoplasmic face of the endoplasmic reticulum (Vidugiriene and Menon, 1993). The topologies of the subsequent reactions are unknown, but it seems likely that one of the intermediates is able to 'flip' across the endoplasmic reticulum membrane such that the final GPI precursor can become attached to protein in the lumen of the endoplasmic reticulum (Amthauer et al., 1993). Such a translocation event is likely to be protein-mediated; a similar 'flip' has been postulated for the translocation of the Man_5-$GlcNAc_2$-*PP*-Dol intermediate involved in N-glycosylation (Abeijon and Hirschberg, 1992).

None of the GPI biosynthetic enzymes have been completely purified to date. However, the availability of thymoma mutants deficient in various stages of the pathway, and belonging to different complementation groups (Hyman, 1988), suggest that several of the genes may be cloned in the foreseeable future. So far only the cDNA which rescues the class A mutant has been cloned and sequenced (Miyata et al., 1993). The predicted protein of 484 amino acids has no homology with any known protein and its primary structure suggests that it is a transmembrane protein with a large cytoplasmic N-terminal domain (Miyata et al., 1993). The thymoma class A, C and H mutants are deficient in the synthesis of GlcNAc-PI (Stevens and Raetz, 1991), the class B mutant is deficient in the addition of the third Man residue (Puoti et al., 1991) and the class F mutant fails to add the ethanolamine phosphate bridge (Hirose et al., 1992; Kamitani et al., 1992; Puoti and Conzelmann, 1992). Some of these mutations may be in the genes encoding the respective transferases, or in genes encoding proteins involved in regulation and/or supply of donors. However, the possibility that several of the biosynthetic enzymes might form a complex requiring protein and/or RNA structural elements cannot be ruled out. Preliminary gel-filtration studies on partially purified GlcNAc-PI de-*N*-acetylase from *T. brucei* suggest that this enzyme is part of a high-molecular-mass complex or aggregate (Milne et al., 1993). A requirement for structural elements might explain the need for at least three gene products (A, C and H) for the formation of the first GPI intermediate GlcNAc-PI.

Although purified enzymes are not available, whole cell and cell-free system biosynthetic labelling experiments have been successful in identifying a few mechanistic aspects of the pathway. The inhibition of the addition of the ethanolamine phosphate bridge to the Man_3-GlcN-PI intermediate in *T. brucei*, by phenylmethanesulphonyl fluoride (PMSF) and di-isopropyl fluorophosphate, suggests that this enzyme has an active site serine residue which may form a serine–phosphoethanolamine covalent intermediate in the transfer reaction (Masterson and Ferguson, 1991). This is consistent with the data of Menon et al. (1993) who have shown that the ethanolamine and phosphate group of the phosphatidylethanolamine donor are transferred together. PMSF has also been shown to inhibit the formation of glycolipid C in *T. brucei* (Masterson and Ferguson, 1991; M. L. S. Güther, W. J. Masterson and M. A. J. Ferguson, unpublished work), presumably by inhibiting the palmitoyltransferase which adds the palmitate residue to the inositol ring.

The α-GlcNAc transferase, which forms GlcNAc-PI, is inhibited by thiol alkylating reagents such as *p*-chloromercuriphenylsulphonic acid, iodoacetamide and *N*-ethylmaleimide. The inhibition can be prevented by prior incubation with UDP-GlcNAc and UDP, indicating that there is a cysteine residue at or close to the UDP-GlcNAc donor binding site (Milne et al., 1992). In contrast, none of the downstream Dol-*P*-Man-dependent α-mannosyltransferases of the pathway are affected by thiol alkylating agents.

Mannosamine was first shown by Lisanti et al. (1990) to

Table 2 Functions of GPI in mammalian and protozoan cells

Function	Mammalian cells	Protozoan cells
1. Attachment of protein to plasma membrane	+	+
2. Association in membrane microdomains	+	−
3. Intracellular sorting	+	−
4. Transmembrane signalling via GPI clusters	+	−
5. Endocytosis via non- clathrin-coated pits (potocytosis)	+	+
6. High surface expression/low turnover rates	+	+
7. Selective release of protein by GPI-PLC	+	+
8. High surface packing	−	+
9. Contribution to surface glycocalyx	−	+

inhibit GPI biosynthesis in mammalian cells and procyclic trypanosomes . Experiments using mammalian cells have shown that this monosaccharide becomes incorporated into GPI intermediates and inhibits the formation of Man_3-GlcN-PI-containing species (Pan et al., 1992). Recent studies using bloodstream form trypanosomes have shown that the inhibition is less complete (about 80%) in these cells. However, an accumulation of an intermediate with the structure $ManNH_2$-Man-GlcN-PI was observed. Trypanosome lysates made from cells preincubated with mannosamine (i.e. pre-loaded with $ManNH_2$-Man-GlcN-PI) were found to be significantly reduced in their capacity to make GPI precursors, suggesting that the $ManNH_2$-Man-GlcN-PI species may act as a competitive inhibitor of the Manα1–2Man α-mannosyltransferase (Ralton et al., 1993).

Certain myristic acid analogues (with oxygen replacing methylene groups in the acyl chain) become incorporated into GPI precursors and into the VSG GPI anchor in *T. brucei*, presumably via the fatty acid remodelling reactions. These compounds are toxic to bloodstream form trypanosomes but not to procyclic trypanosomes or to mammalian cells, which lack the fatty acid remodelling myristoyltransferases (Doering et al., 1991). These studies also suggest that the specificity for myristate shown by the remodelling enzymes is dependent on acyl-chain length rather than hydrophobicity of the acyl-CoA donor. *T. brucei* bloodstream forms are deficient in *de novo* fatty acid synthesis and they rely on exogenous (host serum) myristate. Recent studies have shown that they selectively incorporate myristate into GPI precursors and VSG, and not into other phospholipids, suggesting that this organism has developed an efficient system for directing this low abundance fatty acid specifically into the GPI pathway (Doering et al., 1993).

GPI ANCHOR FUNCTION

Various functions for GPI protein anchors have been described, particularly in mammalian and protozoan systems (reviewed in Cross, 1990b; Ferguson, 1991, 1992b); see Table 2. These data suggest that some basic functions are common to higher and lower eukaryotes, whereas others may represent specific adaptations that are advantageous to either unicellular or metazoan organisms, respectively.

Basic GPI functions and properties

The most fundamental function of GPI anchors is to afford the stable association of proteins with the lipid bilayer. The GPI anchor is an efficient and stable anchor, comparable with a hydrophobic polypeptide domain. Most GPI-anchored proteins are expressed at high levels on the outer leaflet of the plasma membrane and exhibit low turnover rates. This is true of both mammalian (Lemansky et al., 1990) and protozoan (Bülow et al., 1989b; Seyfang et al., 1990) cells. However, there are exceptions; for example, GP-2 is a luminally disposed pancreatic secretory granule protein (Paul et al., 1991). One of the reasons for the low turnover rate of GPI-anchored proteins in mammalian cells is their exclusion from the clathrin-mediated endocytic pathway (Bretscher et al., 1980; Lemansky et al., 1990). In protozoan cells such as *T. brucei* and *T. cruzi*, which appear to lack clathrin-coated pits (Shapiro and Webster, 1989; Soares et al., 1992), GPI-anchored proteins are successfully endocytosed (Webster and Grab, 1988; Webster et al., 1990) and recycled (Seyfang et al., 1990). Clathrin-independent endocytosis also occurs in mammalian cells (see below).

Specialized GPI functions in higher eukaryotes

Membrane microdomains and transport

Over the past few years, GPI-anchored proteins in mammalian cells have been shown to perform some unexpected functions. These phenomena include intracellular targeting, transmission of transmembrane signals and clathrin-independent endocytosis. Recent data suggesting that GPI-anchored proteins become sequestered in specialized membrane microdomains in mammalian cells (reviewed by Brown, 1992), may be a unifying property that accounts for these functions. Following their transport from the endoplasmic reticulum to the Golgi, GPI-anchored proteins become insoluble in non-ionic detergents. This well-described phenomenon of neutral-detergent insolubility (Hooper and Turner, 1988) appears to correlate with the association of GPI-anchored proteins with sphingolipids and glycosphingolipids to form microdomains (Brown and Rose, 1992). The self-assembly of these microdomains may explain how GPI-anchored proteins are sequestered into specialized transport vesicles, for vectoral delivery exclusively to the apical membrane of polarized epithelial cells (Lisanti et al., 1990; Rodriguez-Boulan and Powell, 1992). Upon arrival at the plasma membrane the GPI-anchored proteins are immobile but become mobile with time (Hannan et al., 1993). It is possible that the delivered microdomains break up into smaller microdomain units with particular functions and properties, depending on their components.

Potocytosis

A good example of functional GPI-rich microdomains are the caveolae; these are membrane pits devoid of clathrin that are responsible for potocytosis (reviewed by Anderson et al., 1992 and Hooper, 1992). Potocytosis is a specialized type of pseudo-endocytosis, involving the occlusion of caveolae as 'tethered

vesicles' to scavenge and concentrate small ligands such as folate. Caveolae are known to contain several GPI-anchored proteins (Ying et al., 1992), a cytoplasmic 'coat' of a 22 kDa protein called caveolin (Rothberg et al., 1992) and, probably, cholesterol (Rothberg et al., 1990). In some mammalian cells, as in the protozoa, GPI-anchored proteins are seen to be completely endocytosed and delivered to intracellular compartments in clathrin-free vesicles (Keller et al., 1992; Bamezai et al., 1992). Whether or not potocytosis and the latter endocytic events are variations on the same theme remains to be determined.

Transmembrane signalling

More evidence for GPI microdomains in mammalian cells has come from co-precipitation studies. Monoclonal antibodies against a variety of GPI-anchored proteins were shown to co-precipitate the intracellular protein tyrosine kinases $p56^{lck}$ and $p60^{fyn}$ from detergent lysates of T-cells (Stefanova et al., 1991; Thomas and Samuelson, 1992). Glycolipids and a 100 kDa membrane protein have also been associated with these microdomains (Cinek and Horejsi, 1992; Lehuen et al., 1992). These microdomains may have functional significance. It is known that the ligation of any GPI-anchored protein by a first antibody followed by a second antibody (i.e. the clustering of GPI-anchored proteins and their respective microdomains) produces a transmembrane signal, which in the presence of phorbol ester leads to mitogenesis in T-cells (Robinson, 1991). The nature of the signal is unclear, but it could involve the stimulation of the aforementioned intracellular tyrosine kinases and/or an increase in intracellular Ca^{2+} levels (Robinson, 1991). With regard to the latter effect, it is of interest that the cross-linking of surface glycosphingolipids with antibody (Dyer and Benjimins, 1990; Lund-Johansen et al., 1992) or pentavalent cholera toxin B-subunit (Masco et al., 1991) also leads to a rise in intracellular calcium in several different cell types. It is tempting to speculate that these transmembrane signalling events, brought about by artificially clustering GPI-anchored proteins and/or gangliosides, have some physiological counterpart. However, as yet, there are no physiological events which are known to operate this way.

GPI functions common to the lower and higher eukaryotes

Insulation of the cytoplasm

In the case of membrane proteins where their primary function resides exclusively in the ectoplasmic domain, such as hydrolases (e.g. alkaline phosphatase, 5'-nucleotidase, dipeptidase, etc) and cell adhesion molecules (e.g. contact site A, LFA-3, $N\text{-}CAM_{120}$), we find GPI anchors quite well represented in both lower and higher eukaryotes. This makes sense since the mode of anchorage is secondary to ectoplasmic domain function. However, the correct targeting of hydrolases to the apical membrane of epithelial cells, for example, could constitute a selective advantage to GPI usage in the higher eukaryotes. Another possible advantage, both in the protozoa and in epithelial cells, could be the physical isolation of the ectoplasmic domain from the cytoplasm and cytoskeleton. Thus proteins which are operating in a 'harsh environment' can be left to get on with their job in the correct location without compromising the intracellular environment. This may be a major functional advantage of GPI anchors to the unicellular protozoa, both free-living and parasitic, and a useful vestigial function for the apical surfaces of epithelia.

Fine tuning of protein function

It may be significant that some proteins are expressed in both transmembrane form and GPI-anchored form, either by differential mRNA splicing of a single gene transcript or encoded by distinct genes (reviewed by Ferguson, 1991). In this way a GPI-anchored version of a particular protein could be left to perform a purely ectoplasmic function (protozoa and higher eukaryotes), or specialized functions such as potocytosis or transmembrane signalling through microdomain effects (higher eukaryotes only). In contrast, the transmembrane version of the same protein could interact with a completely different set of cytoplasmic components, via the cytoplasmic domain. Thus ectoplasmic domain function could be fine-tuned to intracellular function via the alternative anchoring mechanisms. An example of this might include the FCγRIII receptor, which contains a transmembrane polypeptide domain in macrophages, where it can directly elicit cellular responses when ligated with immune complexes. This same receptor is found as a GPI-anchored form in neutrophils where it binds the same immune complexes, but only potentiates cellular responses via other receptors (Perussia and Ravetch, 1991; Anderson et al., 1990; Salmon et al., 1991).

In *Leishmania* parasites, the transcription of genes for GPI-anchored and non-GPI-anchored versions of the abundant metalloproteinase, gp63, is developmentally regulated (Ramamoorthy et al., 1992; Medina-Acosta et al., 1993). In the insect-dwelling promastigote stage, most or all of the protein contains a GPI anchor and is surface expressed, while in the amastigote stage, a low level of a non-GPI-anchored protein is expressed which is localized to the lysosomes (Medina-Acosta et al., 1989, 1993; Bahr et al., 1993). These results suggest that differences in the anchor type may regulate the subcellular localization of some proteins.

Surface release by phospholipases

The presence of a GPI anchor may allow the selective release of some surface proteins via the action of an endogenous PI-specific phospholipase. However, clear evidence for this type of release has been demonstrated in only a few instances, both in higher eukaryotes (Paul et al., 1991; Vogel et al., 1992) and in the protozoa. PI-PLC mediated release of surface proteins has been reported in *Trypanosoma cruzi*, for the major antigens SSp-4 and the polymorphic family of trans-sialidase/sialidases (Andrews et al., 1988; Rosenberg et al., 1991; Hall et al., 1992) and in *Plasmodium falciparum* and *P. chaboudi*, for the 76 kDa serine protease (Braun-Breton et al., 1988, 1992). In the latter case, cleavage by the phospholipase is apparently required for activation of the protease. In contrast to the situation in these parasites, there is no evidence for enzyme-mediated release of GPI-anchored molecules in either *Leishmania* or in the African trypanosomes, despite the identification and characterization of a GPI-PLC in the latter organism. The *T. brucei* enzyme has been cloned, sequenced and expressed in *E. coli* (Hereld et al., 1986; Carrington et al., 1989; Mensa-Wilmot and Englund, 1992) and localized by immuno-electron microscopy (Bülow et al., 1989b). It has no identifiable signal sequence and was localized to the cytoplasmic face of intracellular vesicles. The role of this enzyme therefore remains obscure since it is not on the same side of the membrane as its putative VSG substrate. One possibility is that it may be involved in the catabolism of excess GPI precursors in bloodstream form trypanosomes.

Specialized GPI anchor functions in the parasitic protozoa

There is no direct evidence that GPI-anchored proteins associate with membrane microdomains in the lower eukaryotes. The major GPI-anchored proteins of *T. brucei*, *Leishmania* spp. and yeast are completely soluble in neutral detergents such as Triton

X-100 and X-114 (Bouvier et al., 1985; Das et al., 1991; Schell et al., 1991; Frankhauser et al., 1993). This may reflect the radically different molecular architecture of the surfaces of these organisms, compared with higher eukaryotes. In the case of the kinetoplastid parasites (trypanosomes and *Leishmania*) the surfaces are dominated by GPI-anchored proteins and/or GPI-related glycolipids (see below). The need for membrane targeting in these organisms is obviated by the fact that there is only one small region of the plasma membrane, the flagellar pocket, that appears to be capable of supporting membrane fusion events and pinocytosis. In addition, the need for transmembrane signalling in these organisms is presumably relatively limited and probably served by small numbers of transmembrane proteins. Thus the GPI anchor has probably been widely adopted by these organisms because of its ability to insulate the cell interior from the harsh world of the insect vector gut and the mammalian host bloodstream (*T. brucei*) or the macrophage phagolysosome (*Leishmania*). Indeed, GPI anchors were probably common in their free-living ancestors for the same reason, i.e. protection rather than communication.

In considering the parasitic protozoa then, GPI anchors may be the predominant form of protein anchorage for the following reasons.

1. They isolate proteins with purely extracellular function, such as proteases and coat proteins, from the interior of the cell.
2. They allow very high levels of protein packing without using up membrane space for the inclusion of nutrient transporters. This is particularly relevant for the VSG coat of the African trypanosomes, where 10 million copies of VSG must occupy the cell surface. It may also be relevant for the circumsporozoite antigen, which forms a dense coat on the sporozoite form of *Plasmodium* parasites. Sporozoites are the form of the parasite which are first injected into the host by a mosquito bite, and which subsequently invade the liver. Although the circumsporozoite antigen has not been shown to contain a GPI anchor biochemically, the C-terminus predicted from cDNA sequencing looks like a GPI signal sequence (Ozaki et al., 1983).
3. They can be modified by complex oligosaccharide side chains to form 'glycocalyx' structures.

Space-filling 'glycocalyx' roles for the GPI side chains

The unique oligosaccharide side chains of the *T. brucei* anchors may themselves contribute to the surface glycocalyx of this parasite. Studies on the three-dimensional conformation of the galactosylated VSG anchor (Homans et al., 1989) suggests that the glycan portion lies plate-like along the plasma membrane and has a cross-sectional area of 6 nm^2. This is approximately the same cross-sectional area as the N-terminal domain of VSG (Metcalf et al., 1987), suggesting that the GPI glycan may itself contribute to the macromolecular diffusion barrier properties of the VSG coat (Figure 3). The extent of α-galactosylation may be influenced by the three-dimensional structure of the C-terminal domain of the VSG to which it is attached as there is a correlation between the size of the galactose side chains and VSG subtype (Ferguson and Homans, 1989). Most of the αGal side chain residues are added to the VSG anchor in the Golgi apparatus (Bangs et al., 1988), where the VSG molecules are packaged into coat arrays for transport to the cell surface. This raises the possibility that the α-galactosyltransferases act as spatial probes, filling space close to the membrane according to the steric constraints imposed by the three-dimensional structure of the VSG C-terminal domain. Similarly, the extensive side chains of the GPI anchor of PARP may influence the surface properties of the insect stage of *T. brucei*. These side chains are substituted with charged sialic acid residues and probably form a protective glycocalyx over the surface of the procyclic (insect) stage, which is otherwise notable in lacking a significant glycolipid component (Ferguson et al., 1993; Figure 3). The sialic acid residues are probably added to the PARP anchor by a surface trans-sialidase that transfers sialic acid from serum sialoglycoconjugates (Pontes de Carvalho et al., 1993). These residues may have the more specific function of preventing complement activation on the procyclic surface (Tomlinson et al., 1992) when it is exposed to a blood meal in the insect gut.

PROTEIN-FREE GPIs

Several protozoa also synthesize free GPIs, which are not covalently linked to protein and which appear to be metabolic end-products. These structures are members of the GPI family by virtue of containing the core sequence Manα1–4GlcNα1–6-*myo*-inositol, but may diverge from the protein anchors beyond this sequence. In several trypanosomatid parasites these glycolipids are the major cellular glycoconjugates.

Protein-free GPIs in *Leishmania* spp.

All *Leishmania* synthesize two distinct classes of free GPI, the polydisperse lipophosphoglycans (LPGs) and the low-molecular-mass glycoinositolphospholipids (GIPLs) (reviewed in McConville 1991; Turco and Descoteaux, 1992). The expression of the LPGs is largely restricted to the promastigote (insect-dwelling) stage where it forms a major component in the densely organized surface glycocalyx (Handman et al., 1984; Tolsen et al., 1989; Pimenta et al., 1991) (Figure 3). It is present in very low or undetectable levels in the amastigote stage that infects mamalian macrophages (McConville and Blackwell, 1991; Moody et al., 1993; Bahr et al., 1993). In contrast, the GIPLs are abundant in both major developmental stages of the parasite and are expressed predominantly on the cell surface (McConville and Bacic, 1990; McConville and Blackwell, 1991; Rosen et al., 1989) (Figure 3).

Lipophosphoglycan structure

The primary structures of the LPGs from three species of *Leishmania* have now been elucidated (see Figure 4). They all contain a polydisperse phosphoglycan moiety of 4–40 kDa which is linked to the membrane via a complex GPI anchor. The phosphoglycan is made up of linear chains of repeat units, all of which contain the backbone sequence, *P*-6Galβ1–4Manα1–, where the 3-position of the galactose residue is either unsubstituted (as in *L. donovani* LPG) (Turco et al., 1987), partially substituted with βGlc residues (as in *L. mexicana* LPG) (Ilg et al., 1992) or highly substituted with monosaccharide or oligosaccharide side chains containing βGal, βGlc, or β-D-Ara*p* (as in *L. major* LPG) (McConville et al., 1990a). The ends of the phosphoglycan chains, distal to the membrane, are capped by a number of neutral oligosaccharides, all of which contain the sequence Manα1–2Man (McConville et al., 1990a) (Figure 4). The GPI anchor of LPG is distinct from the protein GPI anchors in containing an unusual hexasaccharide core linked to *lyso*alkyl-PI with either $C_{24:0}$ or $C_{26:0}$ alkyl chains (Orlandi and Turco, 1987; Turco et al., 1989; McConville et al., 1987, 1990a; Ilg et al., 1992). The phosphoglycan chain is attached to the terminal Gal of this core, while one of the core Man residues is usually substituted with Glc-1-*P* (McConville and Homans, 1992; Thomas et al., 1992). The presence of both shared (i.e. the caps, the backbone disaccharide sequence and the core) and species-specific (i.e. the side chains of the repeat units) structures is

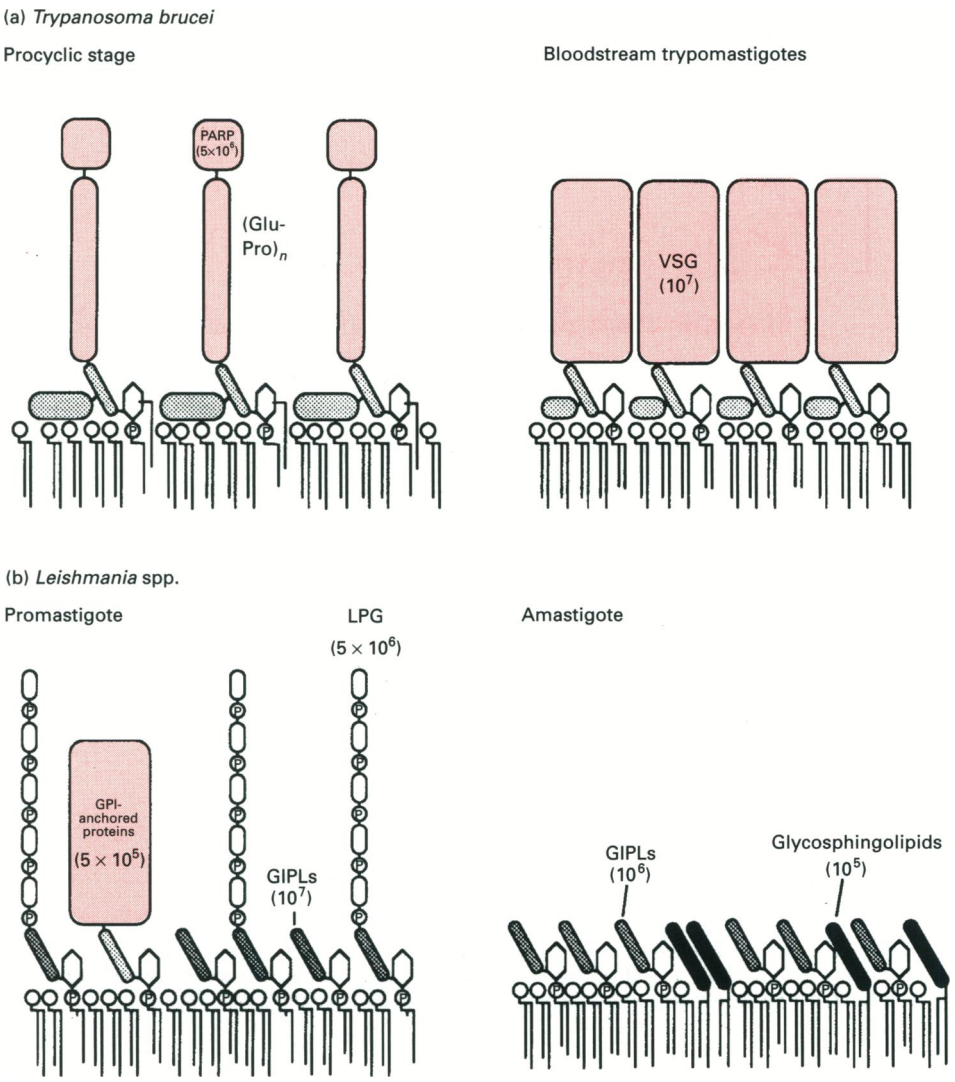


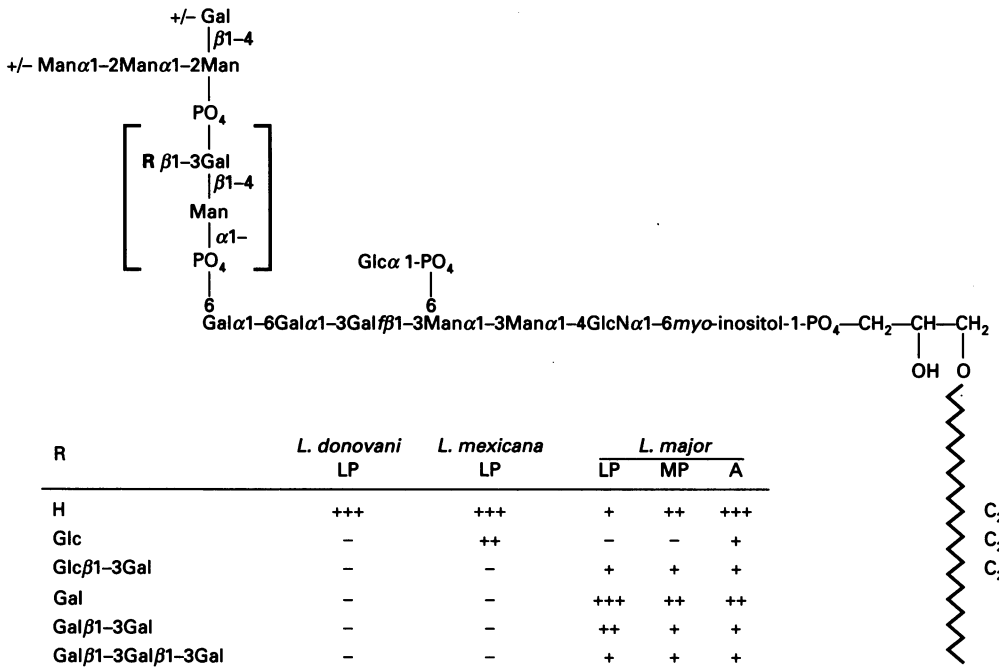
Figure 3 Schematic representations of the cell surfaces of different developmental stages of *T. brucei* and *Leishmania* spp.

Only the major cell surface molecules are depicted (approximate copy numbers per cell are indicated in parentheses). The GPI anchors of the predominant cell surface proteins of *T. brucei* bloodstream and procyclic stages, VSG and PARP respectively, contain complex side chains (stippled area) which may form a glycocalyx over the plasma membrane. The cell surface of *Leishmania* parasites is coated by a more complex glycocalyx which also differs in the different developmental stages. The surface glycocalyx of the insect-dwelling promastigote stage contains several abundant GPI-anchored proteins, the polydisperse lipophosphoglycans (LPG) and the low-molecular-mass glycoinositolphospholipids (GIPLs). The surface expression of LPG and the major surface glycoprotein is greatly down-regulated in the intracellular amastigote stage. The plasma membrane of this stage also contains a number of glycosphingolipids which are apparently aquired from the mammalian host (McConville and Blackwell, 1991).

consistent with serological studies which predicted the presence of both conserved and species-specific epitopes (Greenblatt et al., 1983; Handman et al., 1984; Tolsen et al., 1989).

Marked variation in the size of the phosphoglycan and in the nature of the repeat unit side chains can occur in different developmental stages. During the development of *L. major* promastigotes in the insect midgut from a non-infective procyclic form to an infective 'metacyclic form', there is (1) an approximate doubling in the number of repeat units per molecule and (2) a decrease in the relative abundance of repeat unit side chains which terminate in βGal and a concomitant increase in unsubstituted repeat units or repeat units with side chains that terminate in βAra (Sacks et al., 1990; McConville et al., 1992) (Figure 3). The expression of metacyclic LPG coincides with an increase in the thickness of the surface glycocalyx (Pimenta et al., 1989) and a loss in the binding of the Gal-specific lectin peanut agglutinin to metacyclic promastigotes (Sacks, 1989). Similar developmental changes occur *in vivo* in the sandfly vector (Davies et al., 1990; Lang et al., 1991), and may be involved in regulating anterior migration of the parasite (see below). The intracellular amastigotes of *L. major* produce yet another form of LPG (Glaser et al., 1991; Turco and Sacks, 1991), albeit in much lower levels than in promastigotes. The majority of the repeat units in the amastigote LPG are unsubstituted, while the remainder are substituted with side chains of one to ten β-Gal residues (Moody et al., 1993).

A compelling image of the tertiary structure of LPG was provided by homo- and hetero-nuclear n.m.r. and molecular dynamics modelling of *L. donovani* LPG (Homans et al., 1992). These results suggest that the phosphoglycan chains have an extended helical conformation, although the length of the chains may vary considerably (from 180 Å to 320 Å, assuming 32 repeat



R	*L. donovani* LP	*L. mexicana* LP	*L. major* LP	*L. major* MP	*L. major* A
H	+++	+++	+	++	+++
Glc	–	++	–	–	+
Glcβ1–3Gal	–	–	+	+	+
Gal	–	–	+++	++	++
Galβ1–3Gal	–	–	++	+	+
Galβ1–3Galβ1–3Gal	–	–	+	+	+
Galβ1–3(Galβ1–3)$_{4-10}$Gal	–	–	+	–	+
Arapβ1–2Gal	–	–	+	+++	–
Arapβ1–2Galβ1–3Gal	–	–	+	+	–
Arapβ1–2Galβ1–3Galβ1–3Gal	–	–	+	+	–
n	16	30	14	30	36

Figure 4 Generic structure of *Leishmania* LPG, showing common and species-specific structural features

The major species-specific differences (shown in inset) occur in the nature and frequency of side chain substitution (R) of the phosphorylated disaccharide repeat unit backbone. In the *L. major* LPG, these side chains also vary in different developmental stages (see text for references). LP, promastigotes in exponential (log) growth phase; MP, metacyclic promastigotes; A, amastigotes. The abundances of the different side chains [+++ (> 50%); ++ (10–50%); + (< 10%)] are indicated. It should be noted that the D-arabinopyranose residues in *L. major* LPG have been reassigned the β-configuration (S. W. Homans, unpublished work) and not the α-configuration as published (McConville et al., 1991a).

units per molecule), reflecting the presence of several stable conformers that arise due to rotation around the flexible phosphodiester linkages. Transitions between these stable conformers may occur at physiological temperatures, allowing the LPG chains to contract or expand, somewhat like a slinky spring. The 3-position of the repeat unit galactose is always exposed in the different conformers, allowing the substitution of these residues without major conformation changes to the repeat backbone. These side chains would not only increase the cross-sectional area of the LPGs but would also be accessible for interaction with host receptors.

Lipophosphoglycan function

LPG-deficient strains of *Leishmania* are unable to survive in the sandfly vector (Schlein et al., 1990) or infect mammalian macrophages (Handman et al., 1986; Elhay et al., 1990; McNeely and Turco 1990), although both functions can be partly restored if exogenous LPG is inserted into the plasma membrane of these strains. These data suggest that LPG is an essential virulence factor for *Leishmania* parasites and that it is likely to perform a variety of functions (Table 3). The primary role of LPG is to form a coat over the promastigote surface which protects the plasma membrane from insect and mammalian hydrolases (El-On et al., 1980; McConville et al., 1990a; Schlein et al., 1990; Pimenta et al., 1991) and attack from the complement cascade. The resistance to complement-mediated lysis varies in different developmental stages and appears to be critically dependent on the average chain length of the LPG. Only the infective metacyclic promastigotes, which contain the long (approximately 30 repeat units per molecule) LPG chains are resistant, while the non-infective procyclic forms which express shorter LPG chains (average 15 repeat units per molecule) are susceptible to complement lysis (Sacks, 1989; McConville et al., 1992). Although both forms activate complement to the same extent (Puentes et al., 1988), the longer LPG chains of metacyclic promastigotes appear to sterically hinder the stable insertion of the final C5–C9 complex into the plasma membrane and prevent cell lysis (Puentes et al., 1990). In addition, the LPG coat may mask underlying surface proteins from being opsonized by antibodies in either the insect bloodmeal or in the mammalian bloodstream (Karp et al., 1991). LPG also mediates a number of specific host–parasite interactions. In particular, it is required for binding of *L. major* promastigotes to the insect midgut during the early stages of insect colonization, where it is bound by receptors on insect epithelial cells that recognize the βGal terminating LPG side chains (Pimenta et al., 1992). Most of these side chains are capped with β-D-Arap residues on the LPG of metacyclic promastigotes (McConville et al., 1992) allowing this infective stage to detach from the gut wall and migrate to the insect foregut. These oligosaccharide side chains are unique to the LPG of *L. major* (see Figure 4) and may represent a specialized adaptation to allow this species to colonize the sandfly vector, *Phlebotiminae papatasi*, which is not a host for most other

Table 3 Structure/function relationships of *Leishmania* LPG

Function	Probable LPG domain involved	References
Surface coat		
(i) Protection against complement-mediated lysis		Puentes et al., 1990
(ii) Protection against insect/phagolysosomal hydrolases		El-On et al., 1980; Schlein et al., 1990
(iii) Masking of protein antigens		Karp et al., 1991
Cell–cell recognition and attachment		
(i) Binding to receptors in insect midgut	Repeat unit side chains	Pimenta et al., 1992
(ii) Binding to macrophage receptors	Repeat unit side chains	Handman and Goding, 1985; Talamas-Rohana et al., 1990; Kelleher et al., 1992
(iii) Activation of complement and binding to macrophages via the complement receptors CR1 or CR3	Repeat units/mannose cap structures	Puentes et al., 1988; da Silva et al., 1989; Mosser et al., 1992
Survival in mammalian macrophage		
(i) Scavenger of oxygen radicals	Repeat units	Chan et al., 1989
(ii) Chelation of Ca^{2+}	Repeat units	Eilam et al., 1985
(iii) Modulation of macrophage functions		
Inhibition of protein kinase C	Lipid and repeat units	McNeely et al., 1989; Descoteaux et al., 1992
Inhibition of oxidative burst	Lipid and repeat units	McNeely et al., 1990
Inhibition of chemotactic locomotion		Frankenburg et al., 1990
Down-regulation of tumour-necrosis-factor receptors		Descoteaux et al., 1991
Inhibition of IL-1 production		Frankenburg et al., 1990

Leishmania spp. LPG may also mediate the direct binding of promastigotes to cell surface receptors on the macrophage (Handman and Goding, 1985; Talamas-Rohana et al., 1990; Kelleher et al., 1992). Alternatively, opsonization of the LPG coat with complement components (particulary C3bi) may lead to the binding and uptake of promastigotes via the macrophage complement receptors CR1 and CR3 (da Silva et al., 1989; Mosser et al., 1992). This latter interaction seems to predominate with the physiologically relevant metacyclic forms. LPG also appears to be functionally important in protecting the parasite from hydrolyases and oxygen radicals (El-On et al., 1980; Chan et al., 1989) during the differentiation of the promastigote stage to the amastigote stage within the macrophage phagolysosome. Moreover, there is evidence that LPG, delivered exogenously or from an intracellular parasite, is able to prevent activation of the oxidative burst (McNeely and Turco, 1990), expression of the c-*fos* gene (Descoteaux et al., 1991) and chemotactic locomotion (Frankenburg et al., 1990) by macrophages. These functions are mediated by protein kinase C-dependent pathways, consistent with the finding that LPG is a potent inhibitor, both *in vitro* and *in vivo*, of mammalian protein kinase C (McNeely et al., 1989; Descoteaux et al., 1992). It is not known whether LPG released in the phagolysosome is able to gain access the host cell cytoplasm and directly inhibit the regulatory subunit of the kinase as a competitive inhibitor of the cofactor, diacylglycerol (McNeely and Turco, 1987), or whether it indirectly inhibits the kinase by chelating Ca^{2+}, another cofactor for the enzyme.

Glycoinositolphospholipids

The GIPLs are the major glycolipids synthesized by *Leishmania* parasites. Three distinct lineages of GIPLs have been identified, which are expressed in markedly different levels in different species or developmental stages (Figure 5). The type-1 and type-2 GIPLs have glycan headgroups which are structurally related to the GPI protein anchors and the LPG anchor, respectively (McConville et al., 1990b; McConville and Blackwell, 1991), while the hybrid-type GIPLs have branched glycan headgroups which share features in common with both types of GPI anchor (McConville and Blackwell, 1991; Sevlever et al., 1991) (Figure 5). Some of the hybrid-type GIPLs in *L. mexicana* are substituted with an ethanolamine phosphate residue which is linked, unusually, to the core GlcN (McConville et al., 1993). The addition of this substituent appears to be restricted to these GIPLs as it is not present on either the type-2 GIPLs or protein anchors in the same strain. The lipid moieties of the *Leishmania* GIPLs are exclusively alkylacyl-PI or *lyso*alkyl-PI. Interestingly, the lipid compositions of the hybrid and type-1 GIPLs are distinct from those found in either the protein anchors or LPG in containing predominantly $C_{18:0}$ alkyl chains. In contrast, the type-2 GIPLs contain a more heterogeneous alkyl chain composition, which includes the longer alkyl chains ($C_{24:0}$ and $C_{26:0}$) found in the LPG anchor (McConville and Bacic, 1989; McConville et al., 1990b; McConville and Blackwell, 1991).

The GIPLs may coat a significant proportion of the plasma membrane and, in the case of the type-2 GIPLs, are highly immunogenic (McConville and Bacic, 1989; McConville et al., 1990b; Rosen et al., 1988; Avila et al., 1991). Although the function on these glycolipids is unknown, it is possible that, together with LPG, they are involved in protecting the parasite in the insect midgut and macrophage phagolysosome (McNeely et al., 1989; Descoteaux et al., 1992) and in mediating host–parasite interactions. Evidence that the GIPLs may play a role in parasite invasion of macrophages is suggested by the finding that *L. donovani* promastigotes and amastigotes, which both express mannose-terminating GIPLs, are able to utilize the mannose receptor on the macrophage surface (Blackwell et al., 1985). These functions may be crucial to the intracellular amastigote stage, which dramatically down-regulates the surface expression of the major macromolecules, LPG and gp63, leaving the GIPLs as the major components in the surface glycocalyx (Medina-Acosta et al., 1989; McConville and Blackwell, 1991; Schneider et al., 1992; Bahr et al., 1993).

Biosynthesis and metabolism of the GIPLs and LPG

Each of the GIPL lineages form a natural biosynthetic series suggesting that, like the protein GPI anchors, they are synthesized by the sequential addition of monosaccharides to PI (Figure 6). This is supported by metabolic labelling experiments (L. Proudfoot, P. Schneider, M. A. J. Ferguson and M. J. McConville, unpublished work), which also suggest that some of the type-2

Name	Structure	*L. major* (P)	*L. mexicana* (P)	*L. donovani* (P)	*L. donovani* (A)
M1	Manα1–4GlcN1–6PI	+	+	–	–
Type-1 GIPL					
M2	Manα1–6(Manα1–4GlcN1–6PI)	–	–	–	++
M3	Manα1–2Manα1–6(Manα1–4GlcN1–6PI)	–	–	–	++
Type-2 GIPLs					
iM2	Manα1–4GlcNα1–6PI; Manα1–3	+	++	++	–
GIPL-1	Manα1–4GlcNα1–6PI; Galβ1–3Manα1–3	++	–	–	–
GIPL-2	Manα1–4GlcNα1–6PI; Galα1–3Galβ1–3Manα1–3	++	+	–	–
GIPL-3	Manα1–4GlcNα1–6PI; Galα1–6Galα1–3Galβ1–3Manα1–3	++	+	–	–
GIPL-A	Manα1–4GlcNα1–6PI; Galβ1–3Galα1–3Galβ1–3Manα1–3	++	–	–	–
LPGp	Glcα1—PO_4—6; Manα1–4GlcN1–6*lyso*-PI; Galα1–6Galα1–3Galβ1–3Manα1–3	+	++	–	–
GIPL-4*	Manα1—PO_4—6Galα1–6Galα1–3Galβ1–3Manα1–3; Manα1–4GlcNα1–6*lyso*-PI	++	–	–	–
GIPL-6*	Glcα1—PO_4—6; Manα1—PO_4—6Galα1–6Galα1–3Galβ1–3Manα1–3; Manα1–4GlcNα1–6*lyso*-PI	++	–	–	–
Hybrid-type GIPLs					
iM3	Manα1–6(Manα1–3); Manα1–4GlcN1–6PI	–	++	++	–
iM4	Manα1–2Manα1–6(Manα1–3); Manα1–4GlcN1–6PI	–	++	++	–
EPiM3	$NH_2CH_2CH_2$—PO_4; Manα1–6(Manα1–3); Manα1–4GlcN1–6PI	–	++	–	–

Figure 5 Structures of the protein-free GPIs of *Leishmania* spp.

PI refers to alkylacylphosphatidylinositol. The predominant alkyl chain in type-1 and hybrid GIPLs is $C_{18:0}$, while type-2 GIPLs have a more heterogeneous lipid composition with $C_{18:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$ alkyl chains being the most abundant (from McConville et al., 1990a; McConville and Blackwell, 1991; McConville and Homans, 1992; McConville et al., 1993). Species marked with an asterisk have only been identified in an LPG-deficient *L. major* strain and probably represent truncated forms of LPG. –, not detected; +, minor species (< 5%); ++, major species. P, promastiogote; A, amastigote stage.

GIPLs may act as biosynthetic precursors to LPG (Figure 6). The cellular levels of the type-2 GIPLs varies greatly in different species, from less than 10^3 copies per cell in *L. donovani* to 10^5 and 10^7 copies per cell in *L. mexicana* and *L. major*, respectively (McConville and Blackwell, 1991; McConville et al., 1990a, 1993). As the levels of LPG synthesis are comparable in all these species, it is likely that only a minor population of these GIPLs (< 1% in the high-expressing strains) will be utilized as LPG precursors, consistent with the finding that they are expressed in high copy number on the cell surface (McConville and Bacic, 1990). Recent studies have identified a polar GIPL species in *L. mexicana* which has the expected properties of an LPG precursor (LPGp in Figure 5; McConville et al., 1993). In particular it has a *lyso*alkyl-PI lipid moiety which is highly enriched for long alkyl chains ($C_{24:0}$ and $C_{26:0}$) and a glycan moiety which is substituted with Glc-1-*P*, suggesting that processing of the LPG anchor is

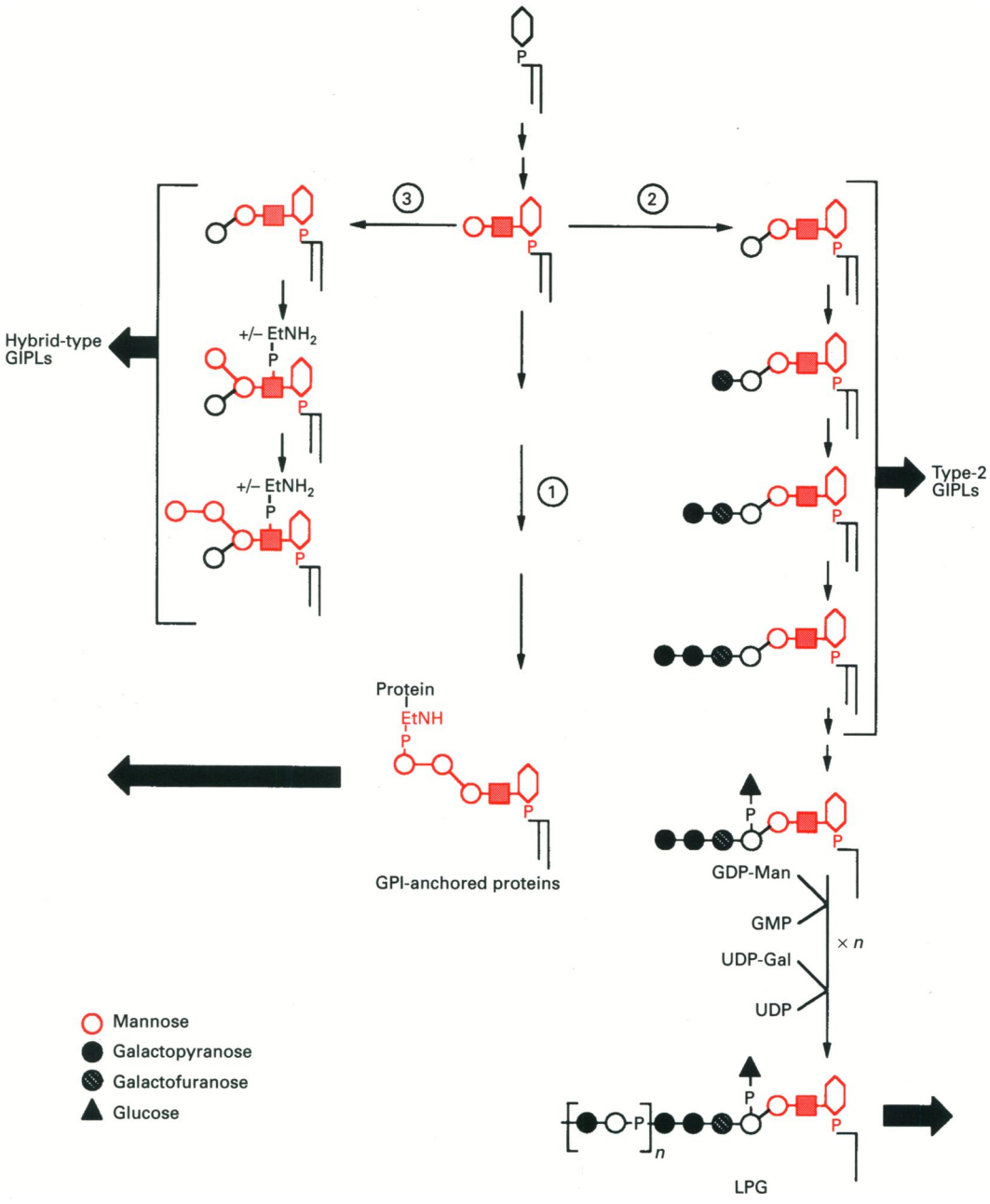


Figure 6 Pathways of GPI biosynthesis in *Leishmania* promastigotes

Pathways 1 and 2 lead to the formation of the protein and LPG GPI anchors, respectively, and probably occur in all species. Intermediates in pathway 1 are present in very low levels and have not been characterized. A putative protein anchor precursor species, identified in *L. mexicana* (Field et al., 1991c), probably corresponds to one of the ethanolamine phosphate-substituted hybrid-type GIPLs. Pathway 2 predominates in *L. major* promastigotes, while pathway 3 predominates in *L. donovani* and *L. mexicana* promastigotes. Large arrows indicate species that are expressed at the cell surface. Components of the conserved GPI backbone, i.e. ethanolamine-phosphate-Manα1–2Manα1–6Manα1–4GlcNα1–6*myo*-inositol, are shown in red. Symbols are defined in the keys to this Figure and to Figure 2.

finished prior to the addition of the repeat units (Figure 6). Studies by Turco and colleagues, using a cell-free system, suggest that the phosphoglycan chains of LPG are built up on this preformed anchor by the sequential addition of Man-1-*P* and Gal from GDP-Man and UDP-Gal, respectively (Carver and Turco, 1991,1992). An LPG-deficient strain of *L. major* has been identified which is unable to form the first repeat unit due to a defect in the transfer of the Gal residue (McConville and Homans, 1992). This strain expresses two highly truncated LPG structures on its cell surface (GIPL-4 and -6 in Figure 5) and is unable to survive in mammalian macrophages (Handman et al., 1986).

The major GIPL species appear to have a relatively low turnover rate (L. Proudfoot, P. Schneider, M. A. J. Ferguson and M. J. McConville, unpublished work), in contrast to LPG, which is actively shed from the cell surface and has a high turnover (Handman et al., 1984; King et al., 1987). The rate of LPG shedding is increased when parasites are grown in the presence of serum albumin, which contains a hydrophobic pocket

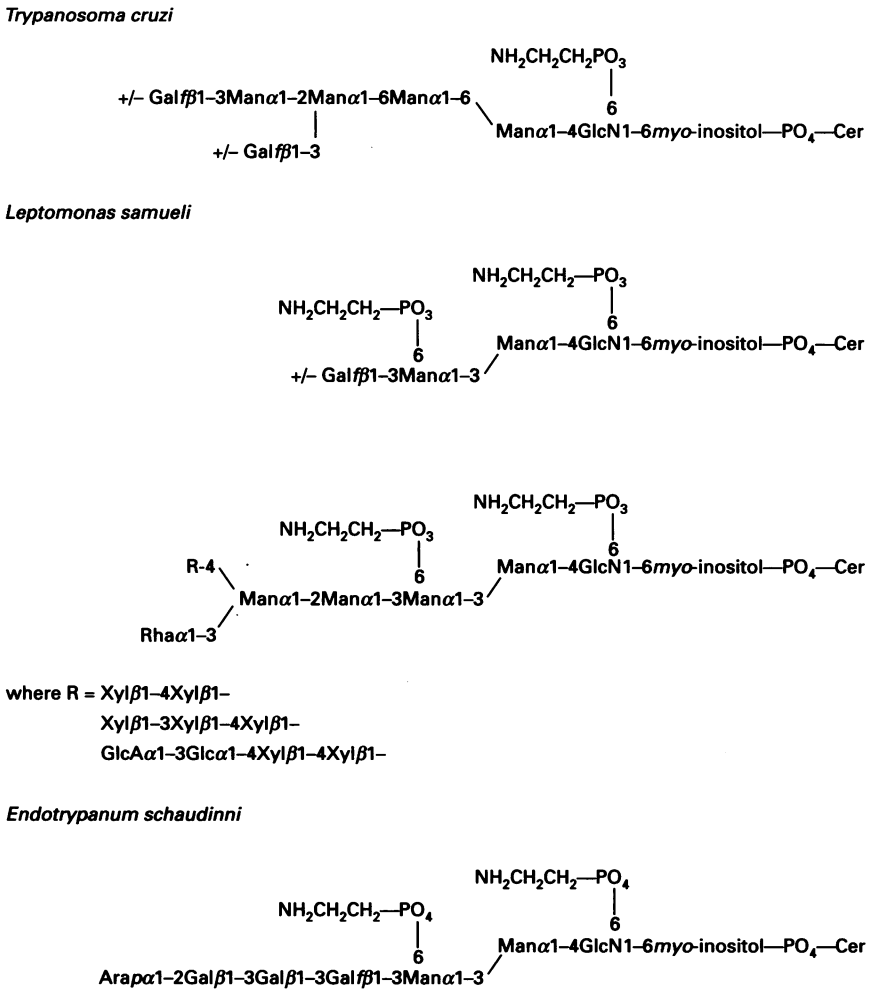


Figure 7 Structures of the protein-free GPIs of *Trypanosoma cruzi* (from Previato et al., 1990; Lederkremer et al., 1991), *Endotrypanum schaudinni* (Previato et al., 1993) and *Leptomonas samueli* (Previato et al., 1992; J. O. Previato, R. Wait, C. Jones and L. Mendonça-Previato, unpublished work)

(King et al., 1987), and the shed material retains the *lyso*alkyl-PI lipid moiety (Ilg et al., 1992). This is consistent with a non-enzymic mechanism of release whereby LPG partitions out of the plasma membrane as either monomers or micelles. This property is probably a reflection of the weak attachment of LPG to the outer leaflet of the plasma membrane via a single aliphatic chain and may be important in allowing the rapid expression of new LPG structures on the cell surface during parasite development. Phosphoglycan chains, which lack both a lipid and the core region of the LPG anchor, are also released into the culture medium (Greis et al., 1992). It is not known whether this material is derived from LPG due to the action of an endoglycosidase or phosphodiesterase or whether it is a distinct biosynthetic product. These shed/secreted antigens have been used to serotype different strains of *Leishmania* and are a valuable tool in diagnostic and demographic studies of the disease (Schnur, 1982; Greenblatt et al., 1983; Handman et al., 1987).

Protein-free GPIs in other kinetoplastid parasites

Protein-free GPI glycolipids are the major cellular glycoconjugates in the insect stage of several other kinetoplastid parasites. In the digenetic parasite *T. cruzi*, the major molecule on the surface of the insect dwelling epimastigote stage is a heterogeneous lipopeptidophosphoglycan (LPPG) (Lederkremer et al., 1976). The expression of this molecule appears to be developmentally regulated as it is either absent from, or present in very low levels in, the stages that infect the mammalian host (Zingales et al., 1982). Recently, the complete primary structure of LPPG has been reported (Figure 7; Previato et al., 1990; Lederkremer et al., 1991). It contains the same tetrasaccharide backbone sequence as the protein GPI anchors of this species (Güther et al., 1992), but differs from these anchors in containing (1) up to two additional βGal*f* residues, (2) a residue of 2-aminoethylphosphonic acid on the 6 position of glucosamine, and (3) a ceramide lipid moiety [containing sphinganine and N-linked lignoceric ($C_{24:0}$) acid], instead of alkylacylglycerol (Lederkremer et al., 1990; Previato et al., 1990). A small amount of peptide is frequently present in purified fractions, although it is not known if these residues are covalently linked to the glycan.

A structurally related family of GPI glycolipids have recently been characterized from the monogenetic parasite, *Leptomonas samueli* (Previato et al., 1992) and from the insect-dwelling stage of the digenetic parasite, *Endotrypanum schaudinni* (Previato et

al., 1993). These glycolipids contain features in common with both the *Leishmania* type-2 GIPLs (in the glycan core) and the *T. cruzi* LPPG (in the inositolphosphoceramide lipid) (Figure 7). All these glycolipids appear to be substituted with either 2-aminoethylphosphonate (in *L. samueli*) or ethanolamine phosphate (in *T. schaudinni*) residues which are linked to both the core glucosamine and to the second mannose distal to the glucosamine. These structures may be extended with oligosaccharide sequences of variable length. In the *E. schaudinni* GPI, the terminal sequences are identical to those found in the side chains of *L. major* LPG (Figures 4 and 7), while in *L. samueli* these GPI glycolipids may be substituted with complex oligosaccharide chains containing rhamnose, xylose and glucuronic acid residues (Previato et al., 1992; J. Previato, R. Wait, C. Jones and L. Mendonça-Previato, unpublished work).

Protein-free GPIs in non-kinetoplastid parasites

There is evidence that some non-kinetoplastid protozoa may also synthesize non-protein linked GPIs. In particular, early studies identified a complex lipophosphonoglycan as the major cell surface glycoconjugate in *Acanthamoeba castellanii* (Dearborn et al., 1976). This glycoconjugate contained inositolphosphoceramide, and the monosaccharides mannose, glucosamine, galactose, xylose and galactosamine, although the structure of the glycan and the presence of the sequence Manα1–4GlcNα1–6*myo*-inositol has still to be determined. Similarly, an LPG-like structure has been identified in the human pathogen, *Entamoeba histolytica* (Stanley et al., 1992; Bhattacharya et al., 1992). Antibodies against this surface glycoconjugate inhibit adhesion of *E. histolytica* trophozoites to mammalian cells, suggesting that it may be involved in mediating host–parasite interactions. A GPI species with the structure Manα1–2Manα1–3Manα1–4GlcN-PI containing undefined phosphate constituents has also been described in the ciliate *Tetrahymena mimbres* (Weinhart et al., 1991).

EVOLUTION OF PROTEIN GPI ANCHORS AND FREE GPIs

Giardia lamblia and the kinetoplastid parasites are considered to be amongst the earliest diverging lineages in the eukaryotic line of descent, based on ribosomal RNA sequence analysis and morphological criteria (i.e. the absence of mitochondria, normal endoplasmic reticulum and Golgi in *G. lamlia*) (Sogin et al., 1989). The presence of GPI anchors in these organisms suggests that this type of anchor was present or evolved at the beginning of eukaryotic evolution. Indeed, the recent characterization of a novel phosphoglycerolipid with the structure, GlcNα1–6*myo*-inositol-*P*-dialkylglycerol, in the archaebacteria (Nishihara et al., 1992) raises the possibility of a relationship between the eukaryote GPIs and some prokaryote glycolipids. The presence of free GPIs as the major class of cellular glycolipid is, so far, unique to the protozoa. It is tempting to speculate that the high levels of GPI anchor expression in the ancestors of the kinetoplastid protozoa pre-adapted them for expanding their GPI metabolism for functions that have complemented their evolution, firstly to monogenetic parasitism (of annelids and insects) and subsequently to digenetic parasitism (of insects and mammals). The degree of structural relatedness in the core regions of the free GPIs of *Leishmania* spp., *T. cruzi*, *E. schaudinni* and *L. samueli* suggests that these glycolipids may have been inherited from a common monogenetic ancestor (Lake et al., 1988). Moreover, their continued expression in parasites such as *Leptomonas*, which only infects insects, suggests that their primary, or ancestral, function was related to parasite survival in the insect gut. This is supported by structure–function studies on *Leishmania* LPG, which also indicate that some of the species-specific differences in LPG structure are adaptations to different sandfly vectors. It is surprising that free GPIs are apparently absent from the bloodstream or insect forms of *T. brucei* given that this species is thought to have evolved from the same ancestral organism as *Leishmania* and *T. cruzi* (Lake et al., 1988). In this case, it is possible that free GPI glycolipids have been down-regulated and replaced by the elaborate oligosaccharide side chains on the GPI anchors of the VSG and PARP surface proteins. Interestingly, the protein portion of PARP has similar physicochemical properties to *Leishmania* LPG, by virtue of containing an acidic $(Asp\text{-}Pro)_2$-$(Glu\text{-}Pro)_{22-29}$ repeat domain which is predicted to take up an extended conformation (Figure 3; Roditi et al., 1989). Thus LPG and PARP may represent examples of convergent evolution, giving rise to the expression of molecules of similar size, charge distribution and surface density on the insect stages of these two parasites.

CONCLUSIONS

The GPI anchors of plasma membrane proteins are ubiquitous throughout the eukaryotes. In higher eukaryotes, these anchors are only utilized by a minority of surface proteins, although there is evidence that GPI anchorage is frequently required for certain specific tasks related to multicellular existence. In the protozoa, GPI anchors appear to have been selected as the most useful form of surface protein anchorage. This is most pronounced in many parasitic protozoa, particularly the kinetoplastida, which have also expanded upon the conserved GPI biosynthetic pathway to produce a diverse array of unique structures. There is evidence that several of these parasite-specific structures are essential for survival of the parasite in the insect and mammalian hosts. We postulate that the evolution of these novel GPI metabolic pathways may have occurred in parallel with the evolution of these organisms to occupy new ecological niches as mono- and digenetic parasites. These studies predict that the expression of protein free GPIs may be quite common in other parasitic protozoa and that an improved understanding of their function will provide information on host–parasite interactions. Finally, some pathways of GPI biosynthesis are likely to be unique to these organisms and be potential targets for the development of antiparasite drugs.

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