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Parasite and mammalian GPI biosynthetic pathways can be distinguished using synthetic substrate analogues

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Glycosylphosphatidylinositol (GPI) structures are attached to many cell surface glycoproteins in lower and higher eukaryotes. GPI structures are particularly abundant in trypanosomatid parasites where they can be found attached to complex phosphosaccharides, as well as to glycoproteins, and as mature surface glycolipids. The high density of GPI structures at all life-cycle stages of African trypanosomes and *Leishmania* suggests that the GPI biosynthetic pathway might be a reasonable target for the development of anti-parasite drugs. In this paper we show that synthetic analogues of early GPI intermediates having the 2-hydroxyl group of the D-*myo*-inositol residue methylated are recognized and mannosylated by the GPI biosynthetic pathways of *Trypanosoma brucei* and *Leishmania major* but not by that of human (HeLa) cells. These findings suggest that the discovery and development of specific inhibitors of parasite GPI biosynthesis are attainable goals. Moreover, they demonstrate that inositol acylation is required for mannosylation in the HeLa cell GPI biosynthetic pathway, whereas it is required for ethanolamine phosphate addition in the *T.brucei* GPI biosynthetic pathway.

Keywords: glycosylphosphatidylinositol/HeLa/*Leishmania*/mannosyltransferase/Trypanosome

Introduction

The structure, function and biosynthesis of the GPI family of molecules has been extensively reviewed (McConville and Ferguson, 1993; Englund, 1993; Stevens, 1995; Takeda and Kinoshita, 1995; Udenfriend and Kokudula, 1995; Medof *et al.*, 1996). The smallest GPI structure found attached to glycoproteins is $NH_2CH_2CH_2PO_4$H-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol-1-PO_4H-lipid (EtNP-Man_3GlcN-PI), where the lipid may be diacylglycerol, alkylacylglycerol or ceramide (McConville and Ferguson, 1993). This minimal GPI-anchor structure may be embellished with additional ethanolamine phosphate groups and/or carbohydrate side chains in a species- and tissue-specific manner (McConville and Ferguson, 1993).

The tsetse fly transmitted African parasite *Trypanosoma brucei* expresses a dense cell-surface coat consisting of ~5×10^6 GPI-anchored variant surface glycoprotein (VSG) dimers (Cross, 1996). In addition to GPI-anchored glycoproteins, other trypanosomatid parasites, such as *Leishmania*, *Trypanosoma cruzi*, *Herpetomonas*, *Leptomonas* and *Phytomonas*, express a wide variety of GPI structures known as glycoinositol phospholipids (GIPLs) (McConville and Ferguson, 1993; Routier *et al.*, 1995; Redman *et al.*, 1995 and references therein). GIPLs are metabolic end-products expressed at the cell surface and are classified as type-1 if they contain the motif Manα1-6Manα1-4GlcNα1-6*myo*-inositol-1-PO_4H-lipid or as type-2 if they contain the motif Manα1-3Manα1-4GlcNα1-6*myo*-inositol-1-PO_4H-lipid or as hybrid if they contain both motifs (i.e. Manα1-6(Manα1-3)Manα1-4GlcNα1-6*myo*-inositol-1-PO_4H-lipid) (McConville and Ferguson, 1993). The largest characterized type-2 GIPL structures are the lipophosphoglycans (LPGs) of the *Leishmania* (McConville and Ferguson, 1993; McConville *et al.*, 1995), which contain phosphosaccharide-repeat domains. LPGs are known to be major virulence factors for these parasites (Turco and Descoteaux, 1992). GPI-anchored glycoproteins and/or GIPLs are also abundant on non-trypanosomatid protozoan parasites such as *Plasmodium falciparum* (Gerold *et al.*, 1996), *Toxoplasma gondii* (Tomavo *et al.*, 1989), *Trichomonas* (Singh *et al.*, 1994) and *Entamoeba* (Bhattacharya *et al.*, 1992). In contrast, higher eukaryotes express lower densities of GPI-anchored glycoproteins and do not express GIPL structures.

Proteins destined to be GPI anchored are attached to a preassembled GPI precursor in the endoplasmic reticulum in exchange for a hydrophobic COOH-terminal peptide (Udenfriend and Kokudula, 1995). The basic sequence of events in GPI precursor biosynthesis has been studied in *T.brucei* (Masterson *et al.*, 1989, 1990; Menon *et al.*, 1990; Güther and Ferguson, 1995), *T.cruzi* (Heise *et al.*, 1996), *T.gondii* (Tomavo *et al.*, 1992), *P.falciparum* (Gerold *et al.*, 1994), *Saccharomyces cerevisiae* (Sipos *et al.*, 1994) and mammalian cells (Hirose *et al.*, 1992; Puoti and Conzelmann, 1993; Mohney *et al.*, 1994 and references therein). Some features of the biosynthesis of GPI-like GIPLs and the GPI-like anchor of the LPG of *Leishmania major* have also been described (Proudfoot *et al.*, 1995; Smith *et al.*, 1997). In all cases, GPI biosynthesis involves the addition of GlcNAc to phosphatidylinositol (PI), to give GlcNAc-PI, which is de-*N*-acetylated to form GlcN-PI (Doering *et al.*, 1989; Hirose *et al.*, 1991; Stevens, 1993; Milne *et al.*, 1994). In *T.brucei*, *L.major* and human (HeLa) cells, de-*N*-acetylation has been shown to be a prerequisite for the mannosylation of GlcN-PI to form later GPI intermediates (Smith *et al.*, 1996, 1997; Sharma *et al.*, 1997). The GlcNAc-PI de-*N*-acetylases from these organisms show similar substrate specificities (Sharma *et al.*, 1997; Smith *et al.*, 1997).

From GlcN-PI onwards there are several significant

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differences among the *T.brucei*, *L.major* and HeLa GPI biosynthetic pathways. For example: (i) the *L.major* pathway leads predominantly to the formation of GIPL/LPG intermediates containing the type-2 GIPL sequence Manα1-3Manα1-4GlcNα1-6PI, whereas the *T.brucei* and HeLa pathways lead to the formation of GPI anchor intermediates containing the sequence Manα1-6Manα1-4GlcNα1-6PI. (ii) Acylation of the 2-hydroxyl group of D-*myo*-inositol [to form (acyl)PI-containing intermediates] occurs with *T.brucei* and HeLa GPI intermediates, but not with *L.major* GPI intermediates. (iii) Acylation of D-*myo*-inositol occurs only after the first mannosylation of early *T.brucei* GPI intermediates (Güther and Ferguson, 1995) but appears to occur before mannosylation in HeLa cells (Hirose *et al.*, 1992; Puoti and Conzelmann, 1993; Doerrler *et al.*, 1996). (iv) Acylation of D-*myo*-inositol can be inhibited by PMSF in *T.brucei* but not in HeLa cells (Güther *et al.*, 1994). (v) Additional ethanolamine phosphate groups are added to mammalian GPI intermediates during biosynthesis (Puoti and Conzelmann, 1993; Kamitani *et al.*, 1992), whereas no such modifications are found in *T.brucei* or *L.major*. (vi) Only *T.brucei* performs fatty acid remodelling, a process whereby the *sn*-2 and *sn*-1 fatty acids of EtNP-Man_3GlcN-PI are removed sequentially and replaced with myristic acid (Masterson *et al.*, 1990).

The apparent difference between the acceptor substrate specificities of the parasite Dol-P-Man:GlcN-PI α1-4 mannosytransferase and the mammalian equivalent (Dol-P-Man:GlcN-(acyl)PI α1-4 mannosytransferase) noted in (iii) above prompted us to synthesize two analogues of early GPI intermediates, namely D-GlcNAcα1-6(2-*O*-methyl)D-*myo*-inositol-1-PO_4H–3-*sn*-1,2-dipalmitoylglycerol [GlcNAc-(2-*O*-Me)PI] and D-GlcNα1-6(2-*O*-methyl)D-*myo*-inositol-1-PO_4H–3-*sn*-1,2-dipalmitoylglycerol [GlcN-(2-*O*-Me)PI], in which the 2-hydroxyl group of the inositol ring is blocked by methylation. Mass spectrometric studies of human CD52 (Treumann *et al.*, 1995), *T.brucei* glycolipid C (Güther *et al.*, 1996) and procyclic acidic repetitive protein (Treumann *et al.*, 1997) have revealed that inositol acylation occurs exclusively on the 2-hydroxyl group. Thus, the presence of the 2-*O*-methyl group in the substrate analogues should prevent inositol acylation and so allow the involvement of the inositol 2-hydroxyl group and the role of inositol-acylation in GPI biosynthesis to be assessed.

Results

GlcN-(2-O-Me)PI and GlcNAc-(2-O-Me)PI act as mannose acceptors in the trypanosome cell-free system

The trypanosome cell-free system (Masterson *et al.*, 1989) has been modified to probe the substrate specificity of Dol-P-Man:GlcN-PI α1-4 mannosyltransferase (Smith *et al.*, 1996). The modified assay included *N*-ethylmaleimide (NEM) to inhibit the UDP-GlcNAc:PI α1-6 GlcNAc-transferase (Milne *et al.*, 1992), thereby suppressing the production of endogenous mannose acceptors and allowing the [^{3}H]mannosylation of exogenous substrates to be examined.

As described previously (Smith *et al.*, 1996), the addition of GlcNAc-PI produced [^{3}H]Man-labelled Man_{1-3}GlcN-PI, Man_3GlcN-(acyl)PI and EtNP-Man_3GlcN-PI (glycolipid A′) (Figure 1, lane 2). By contrast, the addition of the substrate analogues GlcN-(2-*O*-Me)PI (Figure 1, lanes 3–6) and GlcNAc-(2-*O*-Me)PI (Figure 1, lanes 7–10) produced the novel [^{3}H]mannosylated glycolipid species T1–T5. The yield of T5 is higher with GlcN-(2-*O*-Me)PI and, in some experiments (e.g. see Figure 3), the yield of T4 is low and an additional glycolipid (T6) is observed when using this compound. The *N*-acetylated analogue GlcNAc-(2-*O*-Me)PI appeared to be more efficiently [^{3}H]mannosylated than its non-N-acetylated counterpart GlcN-(2-*O*-Me)PI, particularly at low (5–20 μM) substrate concentrations. Similar behaviour is observed with the natural substrates, where GlcNAc-PI is typically mannosylated six times more efficiently than GlcN-PI (Smith *et al.*, 1996). As with the natural substrates, it would appear that the substrate analogues are best presented to the α-mannosyltransferases via the GlcNAc-PI de-*N*-acetylase enzyme. Evidence for the de-*N*-acetylation of GlcNAc-(2-*O*-Me)PI prior to mannosylation is provided below.

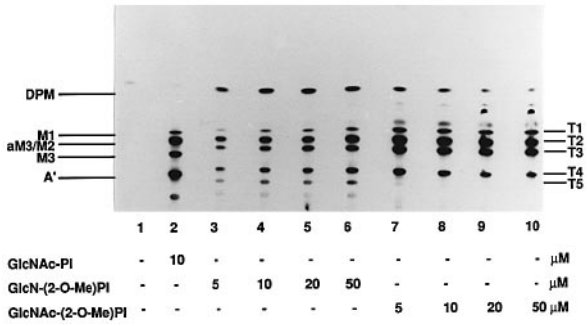

Fig. 1. Synthetic substrate and substrate analogues are mannosylated by the *Trypanosoma brucei* cell-free system. The trypanosome cell-free system was incubated with GDP-[^{3}H]Man either alone (lane 1) or in the presence of various concentrations of synthetic GlcNAc-PI (lane 2), GlcN-(2-*O*-Me)PI (lanes 3–6) and GlcNAc-(2-*O*-Me)PI (lanes 7–10). The labelled glycolipids were extracted and analysed by HPTLC and fluorography. DPM, dolichol-phosphate-mannose. The products of GlcNAc-PI, indicated on the left of the chromatogram, are Man_1GlcN-PI (M1), Man_2GlcN-PI (M2), Man_3GlcN-PI (M3), Man_3GlcN-(acyl)PI (aM3) and EtNP-Man_3GlcN-PI (A′). The products obtained from GlcN-(2-*O*-Me)PI and GlcNAc-(2-*O*-Me)PI are the novel glycolipids T1–T5.

Characterization of glycolipids T1–T4

The structures of glycolipids T1–T4 [generated from GlcNAc-(2-*O*-Me)PI] were investigated using chemical and enzymatic treatments (Figure 2). A conventional series of GPI intermediates (Figure 1, lane 2) were treated in parallel in order to provide positive controls for each treatment (data not shown). Glycolipids T1–T4 showed a uniform increase in R_f-values upon *N*-acetylation (Figure 2, lane 1) and were sensitive to nitrous acid deamination (Figure 2, lane 6); this is consistent with each glycolipid containing a single amino group in the form of glucosamine. The glycolipids were resistant to PI-PLC (Figure 2, lane 3), as would be expected from the presence of the 2-*O*-methyl group on the inositol ring, and were sensitive to the action of serum GPI-PLD (Figure 2, lane 4), confirming their identities as GPI structures. The sensitivity of the bands to JBAM indicated that ethanolamine phosphate had not been added to any of the glycolipids.

In order to define the structures of the glycan headgroups of T1–T4, each glycolipid was purified by preparative HPTLC and after deacylation by mild alkaline hydrolysis was split into two aliquots. One aliquot was subjected in turn to deamination, reduction, dephosphorylation and re-*N*-acetylation, whereas the other was subjected to dephosphorylation and *N*-acetylation. The former treatment converts GlcN into 2,5-anhydromannitol (AHM) with simultaneous cleavage of the glycosidic linkage to the 2-*O*-methyl-*myo*-inositol residue (Ferguson *et al.*, 1985), whereas the latter retains the 2-*O*-methyl-*myo*-inositol residue and converts GlcN into GlcNAc (Ralton *et al.*, 1993). The sizes of the glycans isolated from glycolipids T1–T4 by the treatments, as measured by Bio-Gel P4 gel-filtration, are shown in Table I. The sizes of the AHM-containing glycans were identical to those of authentic Manα1-4AHM (for T1 and T4), Manα1-6Manα1-4AHM (for T2) and Manα1-2Manα1-6Manα1-4AHM (for T3). The hydrodynamic volumes of the 2-*O*-methyl-inositol-containing glycans were also consistent with these assignments (Table I).

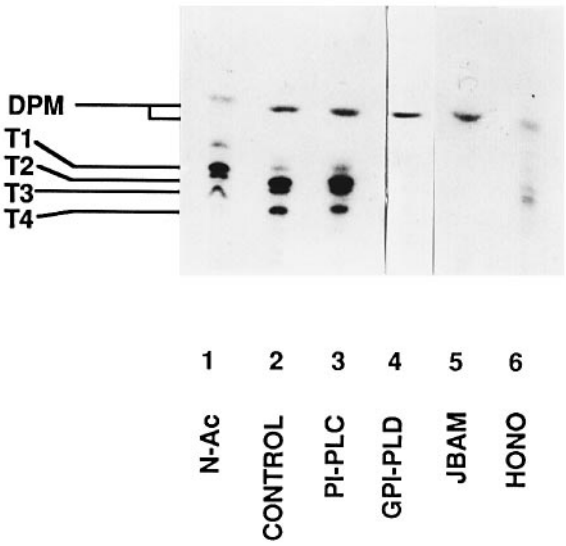

Fig. 2. Characterization of glycolipids T1–T4. The novel glycolipids produced from GlcNAc-(2-*O*-Me)PI by the trypanosome cell-free system (lane 2) were either *N*-acetylated (lane 1) or treated with bacterial PI-PLC (lane 3), human serum GPI-PLD (lane 4), jack bean α-mannosidase (lane 5) or nitrous acid (lane 6). The products were partitioned between water and butan-1-ol and the butan-1-ol phases were analysed by HPTLC and fluorography. DPM, dolichol-phosphate-mannose.

Since glycolipids T1 and T4 have the same glycan structure, differences between their R_f-values (Figure 1) must be attributed to differences between their lipid structures. The most likely explanation is that T4 is a *lyso*-form of T1. This was confirmed by phospholipase A_2 digestion, which converted glycolipids T1, T2 and T3 into glycolipids T4, T5 and T6 (Figure 3, compare lanes 2 and 3). Mild alkaline hydrolysis of glycolipids T1–T6, followed by butan-1-ol/water partitioning, confirmed that the lipid components of all of the glycolipids were alkali labile (Figure 3, lane 1), consistent with the presence of either diacylglycerol (T1–T3) or monoacylglycerol moieties (T4–T6).

Taken together, the data suggest that glycolipids T1–T6 have the structures indicated in Table I, namely, Man_{1-3}GlcN-(2-*O*-Me)PI (T1–T3) and Man_{1-3}GlcN-*lyso*(2-*O*-Me)PI (T4–T6).

The formation of these glycolipids from GlcNAc-(2-*O*-Me)PI shows that methylation of the 2-hydroxyl group of the D-*myo*-inositol residue of GlcNAc-PI has no effect on substrate recognition and turnover by the trypanosomal GlcNAc-PI de-*N*-acetylase and α-mannosyltransferases. On the other hand, methylation of this hydroxyl group appears to prevent the addition of ethanolamine phosphate to Man_3GlcN-(2-*O*-Me)PI. Finally, the Man_{1-3}GlcN-(2-*O*-Me)PI products can undergo significant deacylation at the *sn*-2 position of the diacylglycerol moiety, indicating that the presence of the 2-*O*-methyl group does not affect substrate recognition and turnover by a trypanosomal PLA_2-like activity.

GlcN-(2-O-Me)PI and GlcNAc-(2-O-Me)PI act as mannose acceptors in the Leishmania cell-free system

The *Leishmania* cell-free system (Brown *et al.*, 1996) has been modified to probe the substrate specificity of Dol-P-Man:GlcN-PI α1-4 mannosyltransferase (Smith *et al.*, 1997). The modified assay included dithiothreitol to stimulate the mannosylation of exogenous acceptor substrates.

As previously described (Smith *et al.*, 1997), both Dol-P-Man and endogenous Manα1-4GlcN-PI (glycolipid E) were labelled with [^{3}H]Man in the absence of an exogenous

Table I. Proposed structures of glycolipids T1–T6 and Bio-Gel P4 analyses of the headgroups derived from glycolipids T1–T4

Glycolipid	Proposed structure	Headgroup structure and size	
		Method 1[a]	Method 2[b]
T1	Manα1-4GlcNα1-6(2-*O*-Me)PI	Man_1AHM (2.2 ± 0.1 Gu)	Man_1GlcNAc-(2-*O*-Me)Ino (3.6 ± 0.1 Gu)
T2	Manα1-6Manα1-4GlcNα1-6(2-*O*-Me)PI	Man_2AHM (3.1 ± 0.1 Gu)	Man_2GlcNAc-(2-*O*-Me)Ino (4.4 ± 0.1 Gu)
T3	Manα1-2Manα1-6Manα1-4GlcNα1-6(2-*O*-Me)PI	Man_3AHM (4.2 ± 0.1 Gu)	Man_3GlcNAc-(2-*O*-Me)Ino (5.5 ± 0.1 Gu)
T4	Manα1-4GlcNα1-6*lyso*(2-*O*-Me)PI	Man_1AHM (2.2 ± 0.1 Gu)	Man_1GlcNAc-(2-*O*-Me)Ino (3.6 ± 0.1 Gu)
T5	Manα1-6Manα1-4GlcNα1-6*lyso*(2-*O*-Me)PI	n.d.	n.d.
T6	Manα1-2Manα1-6Manα1-4GlcNα1-6*lyso*(2-*O*-Me)PI	n.d.	n.d.

[a]The sizes determined for authentic GPI neutral glycans standards were: Manα1-4AHM, 2.3 Gu; Manα1-6Manα1-4AHM, 3.2 Gu and Manα1-2Manα1-6Manα1-4AHM, 4.2 Gu (Ferguson, 1992).
[b]The sizes of GlcNAcα1-6*myo*-inositol and GlcNAcα1-6(2-*O*-methyl)*myo*-inositol were determined as 2.2 and 2.8 Gu, respectively. The 2-*O*-methyl group appears to add ~0.6 Gu to the measured size. The sizes determined for authentic Manα1-6Manα1-4GlcNAc-Ino and Manα1-2Manα1-6Manα1-4GlcNAc-Ino were 4.0 ± 0.1 and 4.9 ± 0.1 Gu, respectively (Ralton *et al.*, 1993). The headgroup assignments shown in the table are therefore consistent with the measured sizes.
n.d., not determined.

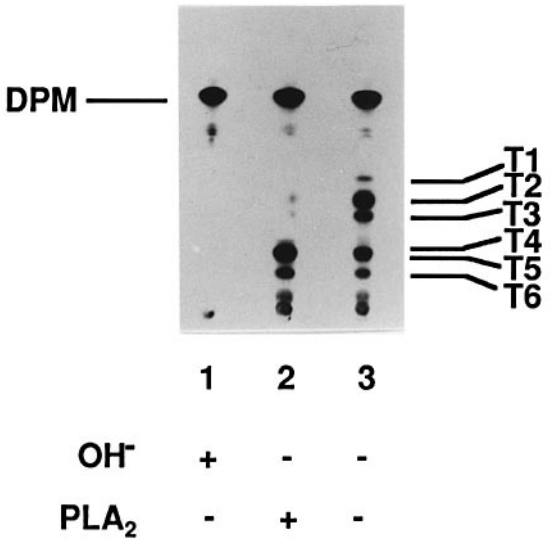

Fig. 3. Base treatment and phospholipase A_2 digestion of glycolipids T1–T6. The novel glycolipids produced from GlcN-(2-*O*-Me)PI by the trypanosome cell-free system (lane 3) were subjected to mild alkaline hydrolysis (lane 1) and phospholipase A2 digestion (lane 2). The products were partitioned between water and butan-1-ol and the butan-1-ol phases were analysed by HPTLC and fluorography. DPM, dolichol-phosphate-mannose.

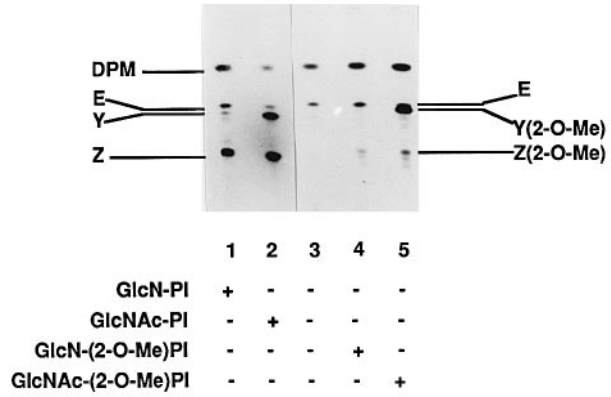

Fig. 4. Synthetic substrates and substrate analogues are mannosylated by the *Leishmania major* cell-free system. The *Leishmania* cell-free system was incubated with GDP-[^{3}H]Man either alone (lane 3) or in the presence of synthetic GlcN-PI (lane 1), GlcNAc-PI (lane 2), GlcN-(2-*O*-Me)PI (lane 4) and GlcNAc-(2-*O*-Me)PI (lane 5). The labelled glycolipids were extracted and analysed by HPTLC and fluorography. DPM, dolichol-phosphate-mannose; E, endogenous Man_1GlcN-PI (formed from endogenous GlcN-PI); Y, exogenous Man_1GlcN-PI; Z, exogenous Man_1GlcN-*lyso*PI; Y(2-*O*-Me), Man_1GlcN-(2-*O*-Me)PI and Z(2-*O*-Me), Man_1GlcN-*lyso*(2-*O*-Me)PI (see Table II).

acceptor (Figure 4, lane 3), whereas the presence of exogenous GlcN-PI or GlcNAc-PI produced [^{3}H]Man-labelled Manα1-4GlcN-PI (glycolipid Y) and Manα1-4GlcN-*lyso*PI (glycolipid Z) (Figure 4, lanes 1 and 2). The addition of GlcN-(2-*O*-Me)PI (Figure 4, lane 4) and GlcNAc-(2-*O*-Me)PI (Figure 4, lane 5) produced two novel glycolipids, Y(2-*O*-Me) and Z(2-*O*-Me) having R_f-values slightly higher than those of glycolipids Y and Z, repectively. Glycolipids Y(2-*O*-Me) and Z(2-*O*-Me) were characterized by chemical and enzymatic treatments (summarized in Table II) and were assigned the structures Manα1-4GlcN-(2-*O*-Me)PI and Manα1-4GlcN-*lyso*(2-*O*-Me)PI, respectively.

The formation of these glycolipids from GlcNAc-(2-*O*-Me)PI shows that methylation of the 2-hydroxyl group of the D-*myo*-inositol residue of GlcNAc-PI does not prevent substrate recognition and turnover by the *Leishmania* GlcNAc-PI de-*N*-acetylase and Dol-P-Man:GlcN-PI α1-4 mannosyltransferase. Furthermore, some deacylation at the *sn*-2 position of the diacylglycerol moiety of Man_1GlcN-(2-*O*-Me)PI is evident, suggesting that the presence of the 2-*O*-methyl group does not prevent substrate recognition and turnover by a *Leishmania* PLA_2-like activity. As previously observed with the natural GlcN-PI and GlcNAc-PI substrates (Smith *et al.*, 1997 and Figure 4, lanes 1 and 2), the presence of the *N*-acetyl group results in increased mannosylation of GlcNAc-(2–0-Me)PI compared with GlcN-(2-*O*-Me)PI (Figure 4, compare lanes 4 and 5), suggesting a degree of substrate channelling between the de-*N*-acetylase and the α1-4 mannosyltransferase.

GlcN-(2-O-Me)PI and GlcNAc-(2-O-Me)PI do not act as mannose acceptors in the HeLa cell-free system

The HeLa cell-free system (Hirose *et al.*, 1992; Güther *et al.*, 1994) has been modified to probe the substrate specificity of human Dol-P-Man:GlcN-(acyl)PI α1-4 mannosyltransferase (Sharma *et al.*, 1997). The modified assay included CoA to stimulate inositol acylation of GPI intermediates (Stevens and Zhang, 1994).

As reported previously (Sharma *et al.*, 1997), Dol-P-Man and endogenous EtNP-Manα1-4GlcN-(acyl)PI (glycolipid H5) were labelled with [^{3}H]Man in the absence of an exogenous acceptor (Figure 5, lane 1) whereas the addition of exogenous GlcN-PI resulted in the formation of exogenous H2 (Manα1-4GlcN-(acyl)PI and exogenous H5 that has a slightly lower R_f-value than that of endogenous H5 (Figure 5, lanes 2 and 7). In contrast, the addition of GlcN-(2-*O*-Me)PI or GlcNAc-(2-*O*-Me)PI did not result in the formation of any additional glycolipids (Figure 5, lanes 3 and 4). Furthermore, the presence of these substrate analogues (at an equimolar concentration to GlcN-PI) did not affect the processing of GlcN-PI to yield exogenous H2 and H5 (Figure 5, lanes 5 and 6), even when the cell-free system was preincubated with the substrate analogues prior to the addition of GlcN-PI (Figure 5, lanes 7–9).

These data show that methylation of the 2-hydroxyl group of the D-*myo*-inositol residue of GlcN-PI prevents substrate recognition and turnover by the HeLa cell Dol-P-Man:GlcN-(acyl)PI α1-4 mannosyltransferase. Furthermore they show that the 2-*O*-methyl group cannot substitute for the 2-*O*-acyl group in GlcN-(acyl)PI with respect to substrate recognition.

Discussion

The results presented in this paper are summarized in Figure 6. They support the following conclusions about GPI biosynthesis: (i) both the trypanosomal and *Leishmania* GlcNAc-PI de-*N*-acetylases can tolerate methylation of the 2-hydroxyl group of the D-*myo*-inositol residue. However, the failure of the HeLa cell-free system to mannosylate GlcN-(2-*O*-Me)PI (see below) prevented assessment of the ability of the HeLa cell de-N-acetylase to recognise GlcNAc-(2-*O*-Me)PI. (ii) Both the trypanosomal

Table II. Characterisation of the [^{3}H]mannose-labelled glycolipids synthesized in the *Leishmania* cell-free system

Glyoclipid	Treatment							Assignment
	JBAM	PI-PLC	GPI-PLD	HONO	APAM	BASE	PLA_2	
DPM	–	–	–	–	–	–	–	Dol-P-Man
E	+	+	+	+	–	+[a]	+	Man_1GlcN-PI[a]
Y	+	+	+	+	–	+	+	Man_1GlcN-PI
Z	+	+	+	+	–	+	–	Man_1GlcN-*lyso*PI
Y(2-*O*-Me)	+	–	+	+	–	+	+	Man_1GlcN-(2-*O*-Me)PI
Z(2-*O*-Me)	+	–	+	+	–	+	–	Man_1GlcN-*lyso*(2-*O*-Me)PI

Summary of the data used to assign the structures to the [^{3}H]mannose-labelled glycolipids; (+) indicates a positive digestion and (–) indicates resistance to the treatment. The treatments were with jack bean α-mannosidase (JBAM), *B.thuringiensis* PI-PLC (PI-PLC), human serum GPI-PLD (GPI-PLD), nitrous acid (HONO), *Aspergillus phoenicis* Manα1-2Man-specific α-mannosidase (APAM), mild alkali (BASE) and pig pancreas phospholipase-A_2 (PLA_2).

[a]Mild alkaline hydrolysis of this glycolipid produced a new glycolipid having an R_f-value slightly higher than glycolipid Z, commensurate with the presence of an alkylacylglycerol lipid group in the endogenous *L.major* GPI intermediates (Proudfoot *et al.*, 1995; Smith *et al.*, 1997).

and *Leishmania* Dol-P-Man:GlcN-PI α1-4 mannosyltransferases can tolerate methylation of the 2-hydroxyl group of the D-*myo*-inositol residue, whereas the comparable HeLa cell enzyme cannot recognise this 2-*O*-methylated substrate. (iii) The trypanosomal Dol-P-Man:Man_1GlcN-PI α1-6 mannosyltransferase and Dol-P-Man:Man_2GlcN-PI α1-2 mannosyltransferase can tolerate methylation of the 2-hydroxyl group of the D-*myo*-inositol residue, whereas the trypanosomal phosphatidylethanolamine:Man_3GlcN-PI ethanolamine phosphotransferase cannot recognise this 2-*O*-methylated substrate. (iv) Both the trypanosomal and *Leishmania* PLA_2-like enzymes can tolerate methylation of the 2-hydroxyl group of the D-*myo*-inositol residue.

The observations made in point (ii) are perhaps the most significant since they show that GlcN-(2-*O*-Me)PI and GlcNAc-(2-*O*-Me)PI are selective substrates for the parasite GPI pathways. This suggests that the discovery and development of inhibitors that are selective for the parasite GPI pathways are attainable goals.

The observations made in points (i), (ii) and (iii) also clarify several fundamental features of GPI biosynthesis. Firstly, the suggestion that the trypanosomal Dol-P-Man:GlcN-PI α1-4 mannosyltransferase requires a free hydroxyl group at the 2-position of the D-*myo*-inositol residue for substrate recognition (Güther and Ferguson, 1995; Smith *et al.*, 1996) must be revised. This notion arose from the observation that inositol-acylation of GlcN-PI does not occur until after the first αMan residue is added (Güther and Ferguson, 1995). However, in the light of the successful mannosylation of GlcN-(2-*O*-Me)PI, this could be reinterpreted in terms of the accessibility of substrates to the inositol-acyltransferase. This view would be consistent with the suggestion of substrate channelling between the de-*N*-acetylase and the first α-mannosyltransferase (Smith *et al.*, 1996) such that GPI intermediates might only be available to the inositol acyltransferase after emerging from a de-*N*-acetylase/α-mannosyltransferase complex.

Secondly, the prediction that inositol acylation is required in trypanosomes for efficient ethanolamine-phosphate addition (Güther and Ferguson, 1995) appears to be correct. The presence of the methyl group in GlcN-(2-*O*-Me)PI precludes 2-*O*-acylation of the inositol ring and appears to block completely ethanolamine-phosphate addition. This observation suggests that the trypanosomal inositol acyltransferase might be a potential target for the development of potent GPI-pathway inhibitors, particularly as the mammalian inositol acyltransferase appears to differ in its donor-substrate and inhibition characteristics (Güther *et al.*, 1994, 1996). However, since there is no evidence that *Leishmania* perform inositol acylation (Proudfoot *et al.*, 1995 and references therein), an inositol acyltransferase inhibitor would not function as a general anti-trypanosomatid agent.

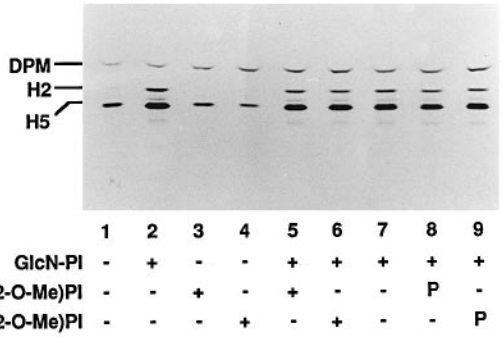

Fig. 5. Unlike synthetic GlcN-PI, the synthetic substrate analogues GlcN-(2-*O*-Me)PI and GlcNAc-(2-*O*-Me)PI are not mannosylated by the HeLa cell-free system. The HeLa cell-free system was incubated with GDP-[^{3}H]Man either alone (lane 1) or in the presence of GlcN-PI (lane 2), GlcN-(2-*O*-Me)PI (lane 3), GlcNAc-(2-*O*-Me)PI (lane 4), an equimolar mixture of GlcN-PI and GlcN-(2-*O*-Me)PI (lane 5) and an equimolar mixture of GlcN-PI and GlcNAc-(2-*O*-Me)PI (lane 6). In lanes 7–9 the cell-free system was preincubated (P) for 10 min either alone (lane 7) or with GlcN-(2-*O*-Me)PI (lane 8) or GlcNAc-(2-*O*-Me)PI (lane 9) prior to the addition of GlcN-PI. The labelled glycolipids were extracted and analysed by HPTLC and fluorography. DPM, dolichol-phosphate-mannose; H2, Man_1GlcN-(acyl)PI and H5, EtNP-Man_1GlcN-(acyl)PI.

Thirdly, the prediction that inositol acylation of GlcN-PI is a prerequisite for mannosylation in HeLa (and other mammalian) cells appears to be correct. This notion was originally supported by the observations that the majority of GPI intermediates in mammalian cells are inositol acylated (Hirose *et al.*, 1992; Puoti and Conzelmann, 1993) and that mannosylation is greatly stimulated by CoA (Stevens and Zhang, 1994; Sharma *et al.*, 1997) or acyl-CoA (Doerrler *et al.*, 1996). In the latter study, the use of synthetic GlcN-PI substrates provided strong evidence for this model but could not entirely rule out the

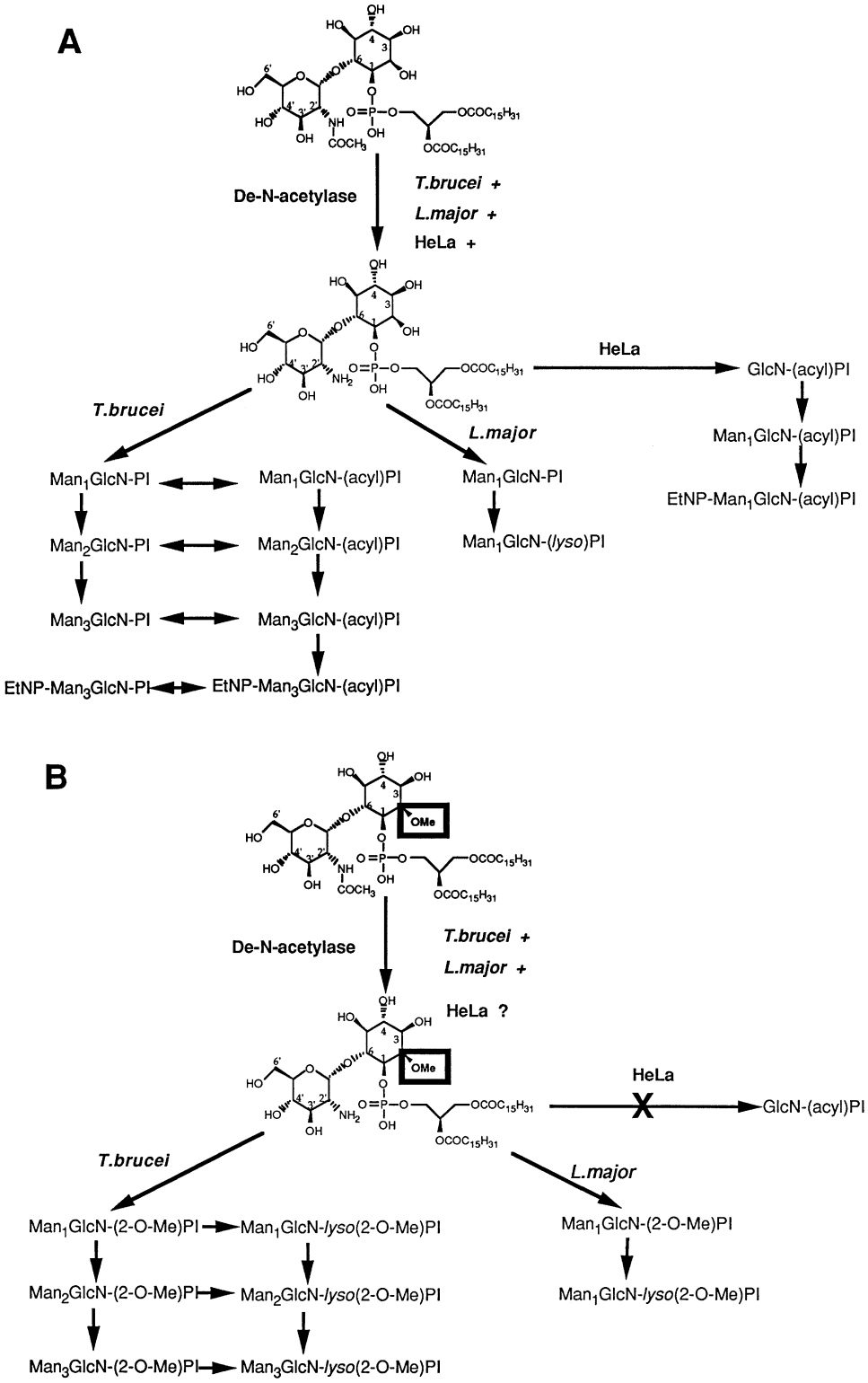

Fig. 6. Summary of the fates of the synthetic substrates. The *T.brucei* cell-free system converts GlcNAc-PI to GlcN-PI which is then mannosylated and acylated to produce Man_3GlcN-(acyl)PI which is the substrate for ethanolamine phosphate addition (**A**). All of the intermediates are in equilibrium between their inositol acylated and deacylated forms. The *T.brucei* cell-free system converts GlcNAc-(2-*O*-Me)PI to GlcN-(2-*O*-Me)PI but it can not acylate the 2-*O*-methylated substrates and, consequently, ethanolamine phosphate addition to Man_3GlcN-(2-*O*-Me)PI is prevented (**B**). The accumulated $Man_{1\text{-}3}GlcN$-(2-*O*-Me)PI intermediates undergo substantial conversion to their *lyso* forms by the action of a PLA_2-like activity. The *L.major* cell-free system does not utilize the inositol acylation reaction and processes the natural and 2-*O*-methylated substrates in a similar way (A and B). The HeLa cell-free system converts GlcNAc-PI to GlcN-PI which is acylated and subsequently mannosylated to form Man_1GlcN-(acyl)PI which is the substrate for the addition of an ethanolamine phosphate residue (A). In contrast, the HeLa cell-free system can not acylate GlcN-(2-*O*-Me)PI and does not recognize GlcN-(2-*O*-Me)PI (B).

possibility that mannosylation precedes inositol acylation. For example, acyl-CoA-dependent mannosylation would be observed if Dol-P-Man:GlcN-PI α1-4 mannosyltransferase underwent product inhibition such that mannosylation was undetectable until such inhibition was relieved by inositol acylation. In contrast, the inability of GlcN-(2-*O*-Me)PI to undergo mannosylation by the HeLa cell-free system provides more direct evidence that inositol 2-*O*-acylation is a prerequisite for mannosylation. It is worth noting that, unlike the trypanosomal and *Leishmania* cell-free systems, there is no evidence for substrate channelling between the de-*N*-acetylase and mannosyltransferases in the HeLa cell-free system (Sharma *et al.*, 1997). This is also consistent with the need for GlcN-PI to access the inositol acyltransferase prior to reaching the mannosytransferases.

The significance of point (iv) is less clear, because it is not known whether the PLA_2-like activities that act on the [^{3}H]mannosylated products derived from exogenous substrates are specific for GPI structures. However, it is possible that the PLA_2-like activities are those of GPI fatty acid remodelling (Masterson *et al.*, 1990) and/or myristate exchange (Buxbaum *et al.*, 1996; Werbovetz and Englund, 1997) for the trypanosome cell-free system and of LPG biosynthesis for the *Leishmania* cell-free system.

Materials and methods

Materials

GDP-[2-^{3}H]mannose (14.9–17.8 Ci/mmol) and En^{3}Hance^{TM} were purchased from Dupont NEN. Jack bean α-mannosidase (JBAM) and pig pancreas phospholipase A_2 (PLA_2) were purchased from Boehringer Mannheim and *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) and *Aspergillus phoenicis* α-mannosidase (APAM) from Oxford GlycoSystems. Whole human serum was used as a source of glycosylphosphatidylinositol specific phospholipase D (GPI-PLD). *n*-Octyl β-D-glucopyranoside was obtained from Calbiochem. Ion-exchange resins (AG–50X12 and AG–3X4) were obtained from Bio-Rad. All the other reagents were purchased from Merck-BDH or Sigma.

Substrates and substrate analogues

D-GlcNα1-6D-*myo*-inositol-1-HPO_4–3-*sn*-1,2-dipalmitoylglycerol (GlcN-PI) was synthesized according to Cottaz *et al.* (1993). D-GlcNα1-6D-(2-*O*-methyl)*myo*-inositol-1-HPO_4–3-*sn*-1,2-dipalmitoylglycerol [GlcN-(2-OMe)PI] was prepared according to Crossman *et al.* (1997). These compounds were *N*-acetylated as described below for the radiolabelled glycolipids. The purity of the synthetic substrates was checked by negative ion electrospray mass spectrometry prior to use and the concentrations of stock solutions of the synthetic substrates were measured by analysis of the *myo*-inositol content by GC-MS, as described in Smith *et al.* (1996).

Preparation of Trypanosomes membranes

Bloodstream forms of *T.brucei* (variant MITat.1.4) were isolated from infected rats and mice. Trypanosome membranes (trypanosome cell-free system) were prepared as previously described by Masterson *et al.* (1989), except that the cells were not pre-incubated with tunicamycin prior to lysis. Aliquots (5×10^8 cells/ml) were snap-frozen in liquid N_2 and stored at –70°C.

Preparation of Leishmania major membranes

Leishmania major (V121) promastigotes were grown to 1.25×10^7 cells/ml in Schneider's medium supplemented with 10% heat-inactivated fetal calf serum. The cells were pelleted, washed with ice-cold phosphate-buffered saline and suspended in 0.1 mM *N*-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) containing 1 μg/ml leupeptin to give a final density of 1×10^9 cells/ml. The cells were were disrupted twice in a nitrogen cavitation bomb at 2.8 MPa and an equal volume of 0.1 M HEPES (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 20% (v/v) glycerol, 0.1 mM TLCK, 1 μg/ml leupeptin was then added (Brown *et al.*, 1996). Aliquots (5×10^8 cell equivalents/ml) were snap-frozen in liquid N_2 and stored at –70°C.

Preparation of HeLa membranes

HeLa cells were grown at 37°C in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere. The HeLa cell-free system was prepared according to Güther *et al.* (1994) with the following modifications. Subconfluent HeLa cells were treated with 5 μg/ml tunicamycin for 2 h at 37°C and were harvested after incubation (10 min at 37°C) with phosphate buffered saline (PBS) containing 0.5 mM EDTA instead of trypsin. The cells were washed twice with 30 ml of PBS to remove EDTA and were then hypotonically lysed in water containing 0.1 mM TLCK and 0.1 μg/ml of leupeptin. An equal volume of 100 mM HEPES–NaOH buffer (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM TLCK, 0.1 μg/ml leupeptin and 20% (w/v) glycerol was added. Aliquots (1×10^7 cell equivalents/ml) were snap frozen in liquid N_2 and stored at –70°C.

Trypanosome cell-free system assay

Trypanosome membranes were washed twice in 0.1 M HEPES buffer (pH 7.4), containing 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM TLCK and 2 μg/ml leupeptin, and were then suspended at 5×10^8 cell equivalents/ml in 2× concentrated incorporation buffer: 0.1 M HEPES (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 20% (v/v) glycerol, 2.5 μg/ml tunicamycin, 0.2 mM TLCK and 2 μg/ml leupeptin (Masterson *et al.*, 1989). Unless stated otherwise, the 2× concentrated incorporation buffer was supplemented with freshly prepared 0.2 M *N*-ethylmalemide (NEM) and 10 mM (0.3% w/v) *n*-octyl β-D-glucopyranoside. The resuspended lysate was vortexed, briefly sonicated and added to a tube containing dry GDP-[^{3}H]Man (0.3 μCi per 10^7 cell equivalents). After sonication for 1 min, aliquots of 20 μl (1×10^7 cell equivalents) were withdrawn and added to the reaction tubes containing an equal volume of 10–100 μM solutions of the various GlcN-PI analogues in 10 mM *n*-octyl β-D-glucopyranoside. The reaction tubes were incubated at 30°C for 1 h whereafter the reactions were terminated by the addition of 270 μl of chloroform:methanol (1:1, v/v). The glycolipid products were recovered in the chloroform/methanol/water-soluble fraction, which was evaporated and partitioned between butan-1-ol and water, as previously described (Smith *et al.*, 1996). Aliquots of the butan-1-ol phase containing the glycolipid products were subjected to HPTLC analysis both before and after enzymatic and chemical treatments.

Leishmania major cell-free system assay

Leishmania major membranes were thawed and washed twice as described for the trypanosome assay. The pelleted membranes were suspended in 2× incorporation buffer, as described above, except that the the buffer was supplemented with 2 mM DTT instead of NEM and did not contain *n*-octyl β-D-glucopyranoside. The suspension of membranes was added to a tube containing dry GDP-[^{3}H]Man (0.5 μCi per 2×10^8 cell equivalents) and sonicated for 1 min. Aliquots of 50 μl (2×10^8 cell equivalents) were added to the reaction tubes containing an equal volume of 60 μM solutions of the various GlcN-PI analogues in water. The reaction tubes were incubated at 30°C for 1 h. After termination of the reactions by the addition of 666 μl of chloroform: methanol (1:1, v/v), the glycolipids were extracted and processed as described above.

HeLa cell-free system assay

HeLa cell lysate was thawed and supplemented with 0.5 mM DTT, 1 mM Coenzyme-A, 10 mM ATP, 5 mM $MnCl_2$, 2 μg/ml leupeptin, 0.1 mM TLCK and 1 μg/ml tunicamycin. Aliquots of 100 μl (1×10^6 cell equivalents) were added to tubes containing dry GDP-[^{3}H]Man (2.5 μCi) and the synthetic GlcN-PI analogues to give a final concentration of 100 μM. The reaction tubes were incubated at 35°C for 1.5 h whereafter and the glycolipids were extracted and processed as described above. Note: the results in Figure 5 and elsewhere (Sharma *et al.*, 1997) show that the HeLa cell-free system used in these studies only generates the mannosylated GPI intermediates H2 and H5, even if excess UDP-GlcNAc is added to the membranes instead of GlcN-PI (data not shown). This is different from the results of Hirose *et al.* (1992) and our own results (Güther *et al.*, 1994) where larger GPI intermediates were observed. This discrepancy indicates that the HeLa cell-free system is culture and/or membrane-preparation dependent with respect to the observed final products.

HPTLC

Samples and glycolipid standards were applied to 10 cm aluminium-backed silica gel 60 HPTLC plates (Merck) which were developed with chloroform/methanol/1 M ammonium acetate/13 M ammonium hydroxide/water (180:140:9:9:23, v/v), except for the HPTLC plate in Figure 1 that was developed using chloroform/methanol/water (10:10:3, v/v). Radiolabelled components were detected by fluorography at –70°C using Kodak XAR–5 film and an intensifying screen after spraying the plates with En^{3}Hance^{TM}.

Enzyme treatments of radiolabelled glycolipids

Digestions with APAM, JBAM, PI-PLC and GPI-PLD and processing of the products for analysis by HPTLC were performed as described previously (Güther *et al.*, 1994; Smith *et al.*, 1996). Pig pancreas PLA_2 digests were performed at 37°C in 40 µl of 25 mM Tris–HCl (pH 8.0), 2 mM $CaCl_2$, and 0.1% sodium deoxycholate with the addition of 8 U of enzyme at hourly intervals over 3 h, followed by further incubation at 37°C for 12 h.

Chemical treatments of radiolabelled glycolipids

Deamination of glycolipids was carried out in 20 µl of 0.1 M sodium acetate (pH 4.0), containing 0.01% Zwittergent 3–16. Aliquots (10 µl) of freshly prepared 0.5 M $NaNO_2$ were added at hourly intervals with incubation at 60°C for 4 h. Lipidic products were extracted into butan-1-ol for analysis by HPTLC.

Glycolipids were *N*-acetylated at 0°C in 100 µl of saturated $NaHCO_3$ by the addition of three aliquots (2.5 µl) of acetic anhydride over 20 min. The reaction mixture was warmed to room temperature and *N*-acetylated glycolipids were extracted into butan-1-ol. Residual salts were removed by washing the butan-1-ol phase with water.

Glycan analysis

Radiolabelled glycolipids from the trypanosome assay were purified by preparative HPTLC. They were eluted from the excised silica with chloroform/methanol/water (10:10:3, v/v), dried and delipidated by incubation (5 h, 50°C) with 300 µl of concentrated aqueous ammonia/50% propan-1-ol (1:1, v/v). The radiolabelled soluble glycan products were recovered in the aqueous phase of a butan-1-ol/water partition and were treated in one of two ways: (i) deamination, reduction, aqueous HF dephosphorylation, re-*N*-acetylation and desalting by passage through AG50X12(H^+) over AG3X4(OH^-) ion-exchange resins (Ferguson, 1992) to yield neutral glycans terminating in 2,5-anhydromannitol (AHM). (ii) Aqueous HF dephosphorylation, *N*-acetylation and desalting by passage through AG50X12(H^+) over AG3X4 (OH^-) ion-exchange resins (Ferguson, 1992) to yield neutral glycans containing GlcNAc and 2-*O*-methyl-*myo*-inositol.

The neutral glycans resulting from these procedures were dissolved in water containing glucose oligomer internal standards and the aqueous solution was filtered through a 0.2 µm membrane and analysed by Bio-Gel P4 gel filtration using an Oxford Glycosystems GlycoMap. Fractions (250 µl) were collected and counted for radioactivity.

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