Specificity of GlcNAc-PI de-*N*-acetylase of GPI biosynthesis and synthesis of parasite-specific suicide substrate inhibitors

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The substrate specificities of *Trypanosoma brucei* and human (HeLa) GlcNAc-PI de-*N*-acetylases were determined using 24 substrate analogues. The results show the following. (i) The de-*N*-acetylases show little specificity for the lipid moiety of GlcNAc-PI. (ii) The 3'-OH group of the GlcNAc residue is essential for substrate recognition whereas the 6'-OH group is dispensable and the 4'-OH, while not required for recognition, cannot be epimerized or substituted. (iii) The parasite enzyme can act on analogues containing β GlcNAc or aromatic *N*-acyl groups, whereas the human enzyme cannot. (iv) Three GlcNR-PI analogues are de-*N*-acetylase inhibitors, one of which is a suicide inhibitor. (v) The suicide inhibitor most likely forms a carbamate or thiocarbamate ester to an active site hydroxy-amino acid or Cys or residue such that inhibition is reversed by certain nucleophiles. These and previous results were used to design two potent (IC₅₀ = 8 nM) parasite-specific suicide substrate inhibitors. These are potential lead compounds for the development of anti-protozoan parasite drugs.

Keywords: de-*N*-acetylase/glycosylphosphatidylinositol/ mannosyltransferase/suicide inhibition/*Trypanosoma brucei*

Introduction

A significant proportion of eukaryotic cell-surface glycoproteins are attached to the plasma membrane by covalent linkage to a glycosylphosphatidylinositol (GPI) membrane anchor. The structure and biosynthesis of GPI membrane anchors and related molecules have recently been reviewed (Ferguson, 1999; Kinoshita and Inoue, 2000; McConville and Menon, 2000; Morita *et al.*, 2000a). The basic GPI core structure attached to protein comprises NH₂CH₂CH₂-PO₄H-6Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6D-*myo*-inositol-1-HPO₄-lipid (EtNP-Man₃GlcN-PI), where the lipid can be diacylglycerol, alkylacylglycerol or ceramide. This minimal GPI structure may be embellished with additional ethanolamine phosphate groups and/or carbohydrate side chains in a species- and tissue-specific manner (Ferguson *et al.*, 1999).

Protozoa tend to express significantly higher densities of cell-surface GPI-anchored proteins than do higher eukaryotes. For example, *Trypanosoma brucei*, the causative agent of African sleeping sickness, expresses a dense cell-surface coat consisting of $\sim 5 \times 10^6$ dimers of a GPI-anchored variant surface glycoprotein. This protects the parasite from the alternative complement pathway of the host and, through antigenic variation, from specific immune responses (Cross, 1996). A variety of GPI-related structures, such as lipophosphoglycans, glycoinositolphospholipids (GIPLs) and mucin-like structures, are expressed by other trypanosomatid parasites (Ferguson, 1999, and references therein). *Plasmodium* (Gerold *et al.*, 1996), *Toxoplasma* (Striepen *et al.*, 1997), *Trichomonas* (Singh *et al.*, 1994) and *Entamoeba* (Moody-Haupt *et al.*, 2000) also have abundant GPI-anchored glycoproteins and/or GIPLs. Inhibitors able to arrest the formation of GPI-anchored proteins and/or GPI-related molecules on the plasma membrane of parasitic protozoa should prove useful in the development of anti-parasitic agents. This notion has been validated, at least for *T.brucei*, where disruption of the *TbGPI10* gene encoding the third mannosyltransferase of GPI anchor biosynthesis has been shown to be lethal for the bloodstream form of the parasite (Ferguson, 2000; Nagamune *et al.*, 2000). Furthermore, GIPLs appear to be essential for the survival of *Leishmania* (Ilgoutz *et al.*, 1999) and *Trypanosoma cruzi* (Garg *et al.*, 1997).

The sequence of events underlying GPI biosynthesis has been studied in *T.brucei* (Masterson *et al.*, 1989, 1990; Menon *et al.*, 1990a,b; Güther and Ferguson, 1995; Morita *et al.*, 2000b), *T.cruzi* (Heise *et al.*, 1996), *Toxoplasma gondii* (Striepen *et al.*, 1999), *Plasmodium falciparum* (Gerold *et al.*, 1999), *Leishmania* (Smith *et al.*, 1997a; Ralton and McConville, 1998), *Saccharomyces cerevisiae* (Sütterlin *et al.*, 1998; Flury *et al.*, 2000) and mammalian cells (Hirose *et al.*, 1992; Puoti and Conzelmann, 1993; Chen *et al.*, 1998, and references therein). In all cases, GPI biosynthesis involves the addition of GlcNAc to phosphatidylinositol (PI) to give D-GlcNAc α 1-6D-*myo*-inositol-1-HPO₄-sn-1,2-diacylglycerol (GlcNAc-PI), which is then de-*N*-acetylated to form D-GlcN α 1-6D-*myo*-inositol-1-HPO₄-sn-1,2-diacylglycerol (GlcN-PI) (Doering *et al.*, 1989; Milne *et al.*, 1994; Watanabe *et al.*, 1999, 2000). De-*N*-acetylation is a prerequisite for the mannosylation of GlcN-PI to form later GPI intermediates (Nakamura *et al.*, 1997; Sharma *et al.*, 1997). The GlcNAc-PI de-*N*-acetylases from protozoan and mammalian sources are similar with regard to their specificities for the acyl (R) group removed from GlcNR-PI substrates (Sharma *et al.*, 1997), but differ with regard to their specificity for the *myo*-inositol residue. Thus, the trypanosomal enzyme can de-*N*-acetylate GlcNAc-PI containing either D- or L-*myo*-

inositol, whereas the human (HeLa) enzyme strictly requires D-*myo*-inositol (Sharma *et al.*, 1999).

Notable differences between the *T.brucei* and mammalian GPI biosynthetic pathways occur from GlcN-PI onwards, including the timing of inositol acylation and deacylation (Güther and Ferguson, 1995), the addition of extra ethanolamine phosphate groups to mammalian GPI anchors (Hirose *et al.*, 1992; Puoti and Conzelmann, 1993) and fatty acid remodelling of *T.brucei* GPI anchors (Masterson *et al.*, 1990). The processing of GlcN-PI is a key step in the GPI biosynthetic pathway; inositol acylation (the transfer of fatty acid to the 2-OH group of the D-*myo*-inositol residue) of GlcN-PI either precedes or follows a first mannosylation, as in mammalian cells and *T.brucei*, respectively (Güther and Ferguson, 1995; Doerrler *et al.*, 1996; Smith *et al.*, 1997b). This difference was exploited in the discovery of the first generation of specific inhibitors of the parasite GPI biosynthetic pathway *in vitro* (Smith *et al.*, 1999). Thus, D-GlcN α 1-6D-2-*O*-hexadecyl-*myo*-inositol-1-HPO₄-*sn*-1,2-dipalmitoylglycerol [GlcN-(2-*O*-hexadecyl)-PI] was shown to inhibit the first mannosyltransferase (MT-1), whereas D-GlcN α 1-6D-2-*O*-octyl-*myo*-inositol-1-HPO₄-*sn*-1,2-dipalmitoylglycerol [GlcN-(2-*O*-octyl)-PI] and its *N*-acetylated version inhibited inositol acylation of Man₁₋₃GlcN-PI and prevented the subsequent addition of an ethanolamine phosphate bridge (Smith *et al.*, 1999). Another series of parasite-specific GPI pathway inhibitors containing L-*myo*-inositol inhibited *T.brucei* MT-1 (Smith *et al.*, 2000), whereas a terpenoid natural product inhibited yeast and human, but not parasite, GPI biosynthesis (Sütterlin *et al.*, 1997).

In this paper we describe the first mechanism-based suicide inhibitor of GPI biosynthesis, and combine features of this molecule with others to produce two parasite-specific suicide substrate inhibitors.

Results and discussion

Optimization of the GlcN[³H]Ac-PI de-*N*-acetylase assay

The release of [³H]acetate from GlcN[³H]Ac-PI by the GlcNAc-PI de-*N*-acetylase of the trypanosome and HeLa cell-free systems was followed for up to 4 h under different conditions. The presence of GDP-Man (1 mM), which allows the conversion of the GlcN-PI product to downstream mannosylated intermediates, stimulated trypanosomal GlcNAc-PI de-*N*-acetylase activity by ~10%. The addition of CoA and an ATP-regenerating system, which allows the *in situ* formation of acyl-CoA and hence the formation of GlcN-(acyl)PI, stimulated the HeLa GlcNAc-PI de-*N*-acetylase activity by ~15%. The addition of 1 mM GDP-Man stimulated the HeLa activity by a further 10%. Neither enzyme was affected by the addition of a 10-fold excess of GlcN-PI over GlcN[³H]Ac-PI, indicating that there is no direct feedback product inhibition. Since substrate turnover was linear only within 60–90 min, all subsequent assays to determine initial rates were performed up to 120 min in the presence of either GDP-Man for the trypanosomal system or GDP-Man and CoA/ATP-regenerating system for the HeLa cell-free system. Under optimal conditions, the specific activity of the trypanosome cell-free system de-*N*-acetylase for

Table I. Release of [³H]acetate from GlcN[³H]Ac-PI substrate analogues by the trypanosome and HeLa cell-free systems

Analogue	Trypanosome		HeLa	
	Initial rate ^a	% ^b	Initial rate ^a	% ^b
GlcNAc-PI	1.35	100	0.75	100
3-dGlcNAc-PI	0.00	0	0.00	0
4-dGlcNAc-PI	1.40	103	0.69	92
6-dGlcNAc-PI	1.53	108	0.90	120
GlcNAc4Me-PI	0.30	22	0.15	20
ManNAc-PI	0.01	1	0.00	0
GalNAc-PI	0.01	1	0.00	0
GlcNAc- β -PI	1.42	104	0.00	0
GlcNAc-[L]-PI	0.08	6	0.00	0
GlcNAc-[L]-(2- <i>O</i> -methyl)-PI	0.09	7	0.01	1
GlcNAc-(2- <i>O</i> -methyl)-PI	1.05	77	0.00	0
GlcNAc-(2- <i>O</i> -octyl)-PI	0.99	73	0.00	0
GlcNAc-(2- <i>O</i> -hexadecyl)-PI	0.02	2	0.00	0
GlcNAc-I- <i>P</i> -C18	1.30	96	0.80	107
GlcNAc-I- <i>P</i> -C8	0.24	18	0.04	5
GlcNAc-PI(diC8)	1.37	101	0.65	86
GlcNAc-PI(diC18)	1.31	97	0.89	118
GlcNAc-PI(Me,C18)	1.41	104	0.66	88

^aThe initial reaction rates (pmol [³H]acetate released min⁻¹ mg⁻¹ protein) were estimated from the initial linear range of [³H]acetate release. Each time point was measured in triplicate and measurements were within $\pm 3\%$ of the mean value. The values given here are the mean of at least two estimates each of the initial rates; individual estimates were within $\pm 4\%$ of the mean value. Background levels of non-GPI-specific de-*N*-acetylation (4 and 1% for the trypanosomal and HeLa systems, respectively) were estimated from the initial rates of [³H]acetate released from GlcN[³H]Ac α 1-S-C₈ and GlcN[³H]Ac β 1-S-C₈, which do not compete for the GlcNAc-PI de-*N*-acetylase. The values of the initial rates recorded in the table have been adjusted accordingly.

^bInitial reaction rate relative to that for GlcNAc-PI (100%).

GlcN[³H]Ac-PI is almost double that of the HeLa system (Table I), which is consistent with a greater turnover rate for the protozoan GPI pathway.

Comparison of the specificities of the trypanosome and HeLa GlcNAc-PI de-*N*-acetylases

Various GlcN[³H]Ac-PI analogues (Figure 1) were tested as substrates (Table I). In both systems, the deoxy compounds 4-dGlcNAc-PI and 6-dGlcNAc-PI were de-*N*-acetylated at a similar rate to that of GlcNAc-PI, suggesting that the 4- and 6-OH groups of the GlcNAc residue are not involved in substrate recognition. However, the methylated analogue GlcNAc4Me-PI is de-*N*-acetylated at one-fifth of the rate of GlcNAc-PI, whereas GalNAc-PI (the 4'-epimer) is not a substrate in either system. This suggests that there are steric constraints in the active site that disfavor substitution or epimerization of the 4'-OH group. These results are consistent with a previous observation that substitution of the 4'-OH group with an α Man residue, as in Man α 1-4GlcNAc-PI, prevents substrate recognition by the trypanosomal de-*N*-acetylase (Sharma *et al.*, 1997).

The 3-dGlcNAc-PI analogue is not a substrate (nor an inhibitor) for the de-*N*-acetylase of either system, implying that the 3'-OH group is a crucial hydrogen-bond acceptor or donor involved in substrate recognition by the de-*N*-acetylases. Neither is ManNAc-PI (the 2'-epimer) a substrate; this is not surprising since the *N*-acetyl group

Fig. 1. Synthetic substrate analogues of GlcNAc-PI. Their abbreviated names and the m/z values of their $[M-H]^{-1}$ pseudomolecular ions are indicated.

now occupies an axial orientation on the pyranose ring compared with its equatorial orientation in the natural substrate.

Surprisingly, GlcNAc-β-PI is as good a substrate as the natural α-anomer (GlcNAc-PI) for the trypanosomal enzyme, although it is not a substrate for the HeLa enzyme. Modelling studies suggest that the geometric arrangement of the 2'-acetamido, 3'-OH and phosphodiester groups is remarkably similar for both anomers, although the relative position of the inositol ring differs significantly (Figure 2). This, in turn, suggests that there are no significant interactions between the 2-, 3-, 4- and 5-OH groups of *D-myo*-inositol and the trypanosomal de-*N*-acetylase. This contrasts with the recognition of GlcN-PI by trypanosomal MT-1, which appears to make essential hydrogen bonds to the 3- and 5-OH groups of the *D-myo*-inositol residue (Smith *et al.*, 2000). The absence of an interaction between the parasite de-*N*-acetylase and the 2-OH group of the *D-myo*-inositol residue can also be inferred from the ability of the trypanosomal enzyme to act on substrates alkylated at this position, notably GlcNAc-(2-*O*-methyl)-PI and GlcNAc-(2-*O*-octyl)-PI (Sharma *et al.*, 1999) (Table I). Furthermore, the absence of an interaction between the parasite de-*N*-acetylase and the 2- and 4-OH groups of the *D-myo*-inositol residue is supported by the slow turnover of GlcNAc-[L]-PI (Table I), which can adopt a conformation similar to GlcNAc-PI apart from the orientations of the 2- and 4-OH groups (Sharma *et al.*, 1999; Smith *et al.*, 2000). Clearly, the HeLa cell de-*N*-acetylase, which recognizes neither GlcNAc-β-PI nor GlcNAc-[L]-PI (Table I), is more stringent with regard to the configurations of the glycosidic linkage and the *myo*-inositol residue, and it is

Fig. 2. Molecular models of GlcNAcα1-6*D-myo*-inositol-1-HPO₄-lipid and GlcNAcβ1-6*D-myo*-inositol-1-HPO₄-lipid. The energy-minimized structures (using an unrestrained MOPAC molecular dynamics simulation at 300 K) show how similar the two isomers are with respect to the relative positions of the GlcNAc residue and the phosphodiester group. For simplicity, the lipid group in these models is represented as a simple alkyl chain.

tempting to speculate that the HeLa enzyme makes essential hydrogen bonds with the α1-6-linked *D-myo*-inositol residue of GlcNAc-PI.

All of the lipid variants of GlcNAc-PI (Figure 1) were good substrates for the trypanosomal and HeLa de-*N*-acetylases, whether presented as a glycerolipid or a single alkyl chain (Table I). However, the more hydrophobic (longer chain) analogues were better substrates in the HeLa cell-free system than in the trypanosomal cell-free system. The poorest substrate in both cell-free systems was the short-chain analogue GlcNAc-I-P-C8, presumably because it failed to interact sufficiently with the membrane

bilayer in order to bring it into intimate contact with the membrane-bound de-*N*-acetylases. Thus, the primary role of the lipid component of GlcNAc-PI in substrate recognition appears to be to insert the substrate into the membrane. This differs from the situation for the mammalian UDP-GlcNAc:PI α 1-6 GlcNAc-transferase complex, which shows specificity for the glycerolipid component of the PI acceptor substrate (Watanabe *et al.*, 1998).

Substrate specificity of the trypanosomal de-*N*-acetylase for the GlcNR-PI substrate analogues

We have previously defined the substrate specificities of the trypanosomal and HeLa de-*N*-acetylases with respect to the nature of the acyl group (R) released from GlcNR-PI analogues (Sharma *et al.*, 1997). Both enzymes were active when R was acetyl or propionyl, barely active when R was butyryl, isobutyryl or pentanoyl, and inactive when R was hexanoyl. The most sensitive way of assessing substrate turnover for GlcNR-PI analogues is to use the cell-free system as a coupled assay, exploiting the fact that GPI mannosylation reactions can proceed from GlcN-PI, but not from GlcNR-PI (Sharma *et al.*, 1997). In the trypanosomal cell-free system, this assay is performed in the presence of *N*-ethylmaleimide (NEM), which inhibits UDP-GlcNAc:PI α 1-6 GlcNAc-transferase without affecting the downstream enzymes (Milne *et al.*, 1992). This prevents the labelling of endogenous GPI intermediates (Figure 3A, lane 1), and simplifies the interpretation of the effects resulting from the addition of synthetic substrates and substrate analogues (Smith *et al.*, 1996).

The addition of 10 μ M GlcN-PI and GlcNAc-PI primed the production of GPI intermediates up to and including glycolipids A' and C' (Figure 3A, lanes 2 and 3). The products were identified by standard treatments, including digestions with jack bean α -mannosidase, phosphatidylinositol-specific phospholipase C and GPI-specific phospholipase D, and nitrous acid deamination and base hydrolysis (data not shown). Products identical to those obtained with GlcNAc-PI were formed when D-GlcN(benzoyl) α 1-6D-*myo*-inositol-1-HPO₄-sn-1,2-dipalmitoylglycerol (GlcNBz-PI) was added (Figure 3A, lanes 4 and 5). All of the mannosylated products were sensitive to nitrous acid deamination, demonstrating that they had been de-*N*-acylated (data not shown). The enhanced priming of the trypanosomal GPI biosynthetic pathway by GlcNAc-PI and GlcNBz-PI compared with GlcN-PI (Figure 3A, lane 2) is consistent with previous reports pointing to substrate channelling between the de-*N*-acetylase and MT-1 (Smith *et al.*, 1996). The data show that GlcNBz-PI is a good substrate for the trypanosomal GlcNAc-PI de-*N*-acetylase. This result was unexpected in view of the poor turnover of the *N*-butyryl, *N*-isobutyryl and *N*-pentanoyl derivatives of GlcN-PI (Sharma *et al.*, 1997), and suggests that the planar aromatic ring of the *N*-benzoyl group is beneficial for substrate recognition. The ability of the trypanosomal de-*N*-acetylase to turnover GlcNR-PI analogues with even larger aromatic groups [i.e. D-GlcN(2-carboxybenzoyl) α 1-6D-*myo*-inositol-1-HPO₄-sn-1,2-dipalmitoylglycerol (GlcNBzc-PI) and 2-deoxy-2-(3-phenylthioureido)-D-Glc α 1-6D-*myo*-inositol-1-HPO₄-sn-1,2-dipalmitoylglycerol (GlcPtU-PI); Figure 3B, lanes 5

Fig. 3. GlcNR-PI analogues as substrates for the trypanosomal GlcNAc-PI de-*N*-acetylase. (A) The trypanosomal cell-free system was incubated with GDP-[³H]Man and NEM alone (lane 1), or together with either GlcN-PI, GlcNAc-PI or GlcNBz-PI at the concentrations indicated (lanes 2–5). (B) The trypanosomal cell-free system was incubated with GDP-[³H]Man and NEM alone (lane 1), or together with the compounds indicated at a final concentration of 10 μ M (lanes 2–8). In both cases, the radiolabelled glycolipid products were analysed by HPTLC and fluorography. The products are: DPM, dolichol-phosphate-mannose; M1–3, Man_{1–3}GlcN-PI; aM2–3, Man_{2–3}GlcN-(acyl)PI; A', EtNP-Man₃GlcN-PI; C', EtNP-Man₃GlcN-(acyl)PI.

and 8] supports this view, although these analogues were turned over less rapidly than GlcNAc-PI (Figure 3B, lane 2).

None of the four other GlcNR-PI analogues, i.e. D-GlcNMe₂ α 1-6D-*myo*-inositol-1-HPO₄-sn-1,2-dipalmitoylglycerol (GlcNMe₂-PI), 2-deoxy-2-ureido-D-Glc α 1-6D-*myo*-inositol-1-HPO₄-sn-1,2-dipalmitoylglycerol (GlcNCONH₂-PI), D-GlcNmaleoyl α 1-6D-*myo*-inositol-1-HPO₄-sn-1,2-dipalmitoylglycerol (GlcNMal-PI) and D-GlcN(benzyl) α 1-6D-*myo*-inositol-1-HPO₄-sn-1,2-dipalmitoylglycerol (GlcNBn-PI), tested in the trypanosomal cell-free system was a substrate for the de-*N*-acetylase (Figure 3B, lanes 3, 4, 6 and 7). Of these, only GlcNCONH₂-PI and GlcNMal-PI might be expected to be potential substrates since the others do not possess an *N*-acyl group on which the de-*N*-acetylase might act (Figure 1).

Inhibition of trypanosomal de-*N*-acetylase by GlcNR-PI substrate analogues

The GlcNR-PI analogues that were not substrates for the trypanosomal GlcNAc-PI de-*N*-acetylase were tested as inhibitors.

Using the indirect de-*N*-acetylase assay (which relies on the de-*N*-acetylation of exogenous GlcNAc-PI and subsequent [³H]mannosylation of the GlcN-PI produced),

Fig. 4. Inhibition of GPI biosynthesis in the trypanosomal cell-free system by GlcNR-PI substrate analogues. (A) The trypanosomal cell-free system was incubated with GDP-[³H]Man and NEM either alone (lane 1) or together with GlcNAc-PI (lane 2), or with GlcNAc-PI after pre-incubation with the compounds indicated (lanes 3–7). (B) The trypanosomal cell-free system was incubated with GlcN[³H]Ac-PI alone (squares) or in the presence of equimolar GlcNAc-PI (diamonds), GlcNBz-PI (circles), GlcNMe₂-PI (triangles) or GlcNCONH₂-PI (crosses), and the release of [³H]acetate was measured against time. (C) The trypanosomal cell-free system was incubated with GDP-[³H]Man and NEM either alone (lane 1) or with GlcNAc-PI (lane 2) or GlcNCONH₂-PI (lane 3), or with GlcNAc-PI after pre-incubation with various concentrations of GlcNCONH₂-PI (lanes 4–8).

GlcNMal-PI and GlcNPtu-PI showed no significant inhibition when compared with the control (Figure 4A, lanes 2, 3 and 7), whereas GlcNBn-PI, GlcNMe₂-PI and GlcNCONH₂-PI inhibited the processing of exogenous GlcNAc-PI (Figure 4A, lanes 4–6, respectively). In this assay, inhibition could result from inhibition of the de-*N*-acetylase and/or MT-1. To throw light on which, if any, of these analogues inhibited the de-*N*-acetylase, they were pre-incubated with trypanosome membranes and the rate of [³H]acetate release from GlcN[³H]Ac-PI was measured (Figure 4B). Pre-incubation with an equimolar amount of unlabelled GlcNAc-PI or GlcNBz-PI reduced the rate of release of [³H]acetate to 50–60% of the control, as expected. By contrast, the release of [³H]acetate from GlcN[³H]Ac-PI was completely inhibited following pre-incubation with an equimolar amount of GlcNMe₂-PI or GlcNCONH₂-PI, demonstrating that both of these analogues inhibited the de-*N*-acetylase. The indirect assay was also used to assess the potency of the two inhibitors;

Fig. 5. GlcNR-PI inhibitors of trypanosome GPI biosynthesis inhibit MT-1 as well as GlcNAc-PI de-*N*-acetylase. The trypanosomal cell-free system was incubated with GDP-[³H]Man and NEM either alone (lane 2) or with GlcN-PI (lane 1), or with GlcN-PI after pre-incubation with the compounds indicated (lanes 3–8).

GlcNMe₂-PI gave complete inhibition at 1 μ M (data not shown), whereas GlcNCONH₂-PI gave complete inhibition at 80 nM with an IC₅₀ of $\sim$ 8 nM (Figure 4C). This is the most potent inhibitor of GPI biosynthesis reported thus far.

Inhibition of trypanosomal MT-1 by GlcNR-PI substrate analogues

Next to be assessed was the ability of GlcNR-PI analogues to inhibit MT-1 and/or later enzymes in the GPI pathway. This was achieved by pre-incubating the trypanosomal cell-free system with the analogues prior to incubation with GlcN-PI and GDP-[³H]Man. GlcNMal-PI and GlcPtu-PI showed little or no inhibition of the mannosylation of GlcN-PI when compared with the control (Figure 5, lanes 1, 3 and 7). GlcNBn-PI caused significant, but incomplete inhibition, whereas GlcNMe₂-PI and GlcNCONH₂-PI produced complete inhibition (Figure 5, lanes 4, 5 and 6). These inhibitory effects cannot be ascribed to non-specific effects, resulting from increased concentrations of synthetic lipid in the system, since pre-incubation with an equivalent concentration of GlcNAc-PI stimulated the biosynthetic pathway (Figure 5, lane 8).

These data suggest that GlcNCONH₂-PI, GlcNMe₂-PI and, to a lesser extent, GlcNBn-PI, inhibit MT-1 as well as the de-*N*-acetylase. Furthermore, in a titration experiment similar to that shown in Figure 4C, but using GlcN-PI as the substrate to specifically assay MT-1, the IC₅₀ for GlcNCONH₂-PI was also $\sim$ 8 nM (data not shown). Given the evidence for substrate channelling between the two enzymes (Smith *et al.*, 1996, 1997b), the most likely explanation for this joint inhibition is a physical overlap between the substrate/product and substrate binding sites of the de-*N*-acetylase and MT-1, respectively, such that occupation of the former blocks the latter.

The mechanism of inhibition of GPI biosynthesis by GlcNR-PI analogues

The cell-free system was pre-incubated with the known MT-1 inhibitor D-GlcNA α 1-6L-*myo*-inositol-1-HPO₄-*sn*-1,2-dipalmitoylglycerol (GlcN-[L]-PI) (Smith *et al.*, 2000) and each of the three de-*N*-acetylase/MT-1 inhibitors described herein, i.e. GlcNCONH₂-PI, GlcNBn-PI and GlcNMe₂-PI, prior to the addition of GlcN-PI

Fig. 6. The reversible and irreversible inhibition of trypanosome GPI biosynthesis by GlcNR-PI analogues. **(A)** The trypanosomal cell-free system was incubated with GDP-[³H]Man and NEM alone (lane 1) or with GlcN-PI (lane 2), or with GlcN-PI after pre-incubation with the compounds indicated (lanes 3–6). Alternatively, membranes were pre-incubated with GDP-[³H]Man and NEM alone (lanes 7 and 8), or with GlcN-[L]-PI (lane 9), GlcNCONH₂-PI (lane 10), GlcNBn-PI (lane 11) or GlcNMe₂-PI (lane 12) prior to membrane washing (+) and incubation with GlcN-PI (lanes 8–12). **(B)** The trypanosomal cell-free system was incubated with GDP-[³H]Man and NEM alone (lane 1) or with GlcN-PI (lane 2), or with GlcN-PI after pre-incubation for 5 min with GlcNCONH₂-PI (lane 3). The same membranes were also pre-incubated with (lanes 4–7) or without (lane 8) GlcNCONH₂-PI, and subsequently washed with HEPES incorporation buffer alone (lanes 4 and 8) or with buffer containing DTT, hydroxylamine (NH₂OH) or hydrazine (NH₂NH₂) (lanes 5–7), prior to incubation with GlcN-PI.

and GDP-[³H]Man. The negative and positive controls are shown in Figure 6A (lanes 1 and 2), and the expected inhibition by the aforementioned compounds is shown in Figure 6A (lanes 3–6). When this experiment was repeated with a washing step between the pre-incubation stage and the addition of GlcN-PI, the inhibition caused by GlcN-[L]-PI and GlcNBn-PI was abolished (Figure 6A, lanes 9 and 11), suggesting that they are competitive inhibitors. However, the inhibition caused by GlcNCONH₂-PI and GlcNMe₂-PI was irreversible (Figure 6A, lanes 10 and 12), presumably due to a strong (possibly covalent) interaction between the enzyme and the inhibitor. Further investigation revealed that the inhibition caused by GlcNMe₂-PI, but not that by GlcNCONH₂-PI, could be partly reversed by washing the membranes with 2 M NaCl (data not shown), suggesting that the interaction between GlcNMe₂-PI and the de-*N*-acetylase-MT-1 complex is non-covalent, and is likely therefore to involve charge-charge interaction(s).

The stability of the GlcNCONH₂-PI enzyme-inhibitor complex was also examined. The trypanosomal cell-free system was pre-incubated with GlcNCONH₂-PI so as to allow the enzyme-inhibitor complex to form, and it was then incubated with GlcN-PI before or after washing the

Fig. 7. Inhibition of HeLa cell GPI biosynthesis by GlcNR-PI analogues. **(A)** The HeLa cell-free system was incubated with GDP-[³H]Man alone (lane 1) or with GlcNAc-PI (lanes 2 and 6), or with GlcNAc-PI after pre-incubation with the compounds indicated (lanes 3–5). The products are: DPM, dolichol-phosphate-mannose; H2, Man₁GlcN-(acyl)PI; ENDO and EXOG H5, endogenous and exogenous EtNP-Man₁GlcN-(acyl)PI derived from endogenous GlcN-PI or exogenous synthetic GlcNAc-PI, respectively. **(B)** The HeLa cell-free system was incubated with GlcN[³H]Ac-PI alone (squares) or in the presence of equimolar GlcNAc-PI (diamonds), GlcNBn-PI (circles), GlcNMe₂-PI (triangles) or GlcNCONH₂-PI (crosses), and the release of [³H]acetate was measured against time.

membranes. In both cases, inhibition was complete (compare Figure 6B, lanes 3 and 4, with the positive controls, lanes 2 and 8), again demonstrating irreversible inhibition by GlcNCONH₂-PI. However, washing the GlcNCONH₂-PI-treated membranes with dithiothreitol (DTT), hydroxylamine and hydrazine reversed the inhibition (Figure 6B, lanes 5–7).

Substrate specificity and inhibition of the HeLa cell de-*N*-acetylase

A number of the foregoing GlcNR-PI analogues were tested in the human (HeLa) cell-free system. In this system, NEM cannot be used to suppress endogenous GPI biosynthesis; consequently, some endogenous GPI glycolipids (H2 and H5) are formed as well as Dol-P-[³H]Man (Figure 7A, lane 1). The addition of GlcNAc-PI leads to the formation of significantly more H2 and H5 (Figure 7A, lanes 2 and 6), as previously described (Sharma *et al.*, 1997; Smith *et al.*, 1997b). The *R_f* of H5 produced from exogenous GlcNAc-PI is lower than that of the endogenous H5 (Figure 7A, compare lanes 1 and 2) because the dipalmitoylglycerol lipid component of the synthetic substrate is less hydrophobic than the glycerolipid component of endogenous GPI intermediates. The addition of GlcNBz-PI, GlcNCONH₂-PI or GlcNMe₂-PI did not stimulate the synthesis of H2 and H5 (data not shown), indicating that these compounds are not substrates for the de-*N*-acetylase. The result with GlcNBz-PI is in contrast to that for the trypanosome cell-free system (Figure 3A,

Fig. 8. Selective inhibition of the trypanosome de-*N*-acetylase by GlcNCONH₂-β-PI and GlcNCONH₂-(2-*O*-octyl)-PI. The trypanosome (A, C and E) and HeLa cell (B, D and F) cell-free systems were incubated with GlcN[³H]Ac-PI after pre-incubation with various concentrations of GlcNCONH₂-PI (A and B), GlcNCONH₂-β-PI (C and D) or GlcNCONH₂-(2-*O*-octyl)-PI (E and F), and the release of [³H]acetate was measured against time.

lane 4), demonstrating that GlcNBz-PI is a parasite-specific substrate.

As with the trypanosomal system, pre-incubation of the HeLa cell-free system with GlcNBn-PI, GlcNMe₂-PI and GlcNCONH₂-PI inhibited the processing of exogenous GlcNAc-PI, the former the least (Figure 7A, lanes 3–5). However, none of them inhibited the formation of endogenous H5, suggesting that they inhibited the de-*N*-acetylase rather than MT-1. The inhibitory effects could not be attributed to the concentration of synthetic lipid in the system (totalling 200 μM) since the addition of 200 μM GlcNAc-PI showed greater stimulation of the biosynthetic pathway than did 100 μM GlcNAc-PI (Figure 7A, compare lanes 2 and 6).

Inhibition of GPI biosynthesis in the HeLa cell-free system by GlcNRPI analogues was investigated further using the direct de-*N*-acetylase assay. The membranes were pre-incubated with the GlcNR-PI analogues, and the rate of [³H]acetate released from GlcN[³H]Ac-PI was measured (Figure 7B). In the presence of an equimolar amount of unlabelled GlcNAc-PI, the rate of [³H]acetate released was halved, whereas pre-incubation with GlcNBn-PI had no effect, indicating that this analogue did not compete efficiently with GlcN[³H]Ac-PI for the HeLa de-*N*-acetylase. However, in agreement with the results with the trypanosomal enzyme, GlcNCONH₂-PI and GlcNMe₂-PI were potent inhibitors of the HeLa de-*N*-acetylase. This suggests that although the parasite and

Fig. 9. A proposed mechanism for the action of GlcNAc-PI de-*N*-acetylase. **(A)** A proposed mechanism for cleavage of the acetyl function from GlcNAc-PI via the formation of an *O*-acetyl enzyme intermediate. **(B)** A proposed mechanism for inhibition of the de-*N*-acetylase by GlcNCONH₂-PI. Note: the data do not exclude the possibility that the active site residue is a hydroxy-amino acid instead of Cys.

human enzymes show different substrate specificities, they operate by much the same reaction mechanism.

Design of parasite-specific suicide substrate inhibitors

The selective recognition by parasite GlcNAc-PI de-*N*-acetylase of GlcNAc-β-PI (this study) and GlcN-(2-*O*-octyl)-PI (Sharma *et al.*, 1999) was combined with the suicide substrate properties of GlcNCONH₂-PI to produce the compounds 2-deoxy-2-ureido-D-Glcβ1-6D-*myo*-inositol-1-HPO₄-*sn*-1,2-dipalmitoylglycerol (GlcNCONH₂-β-PI) and 2-deoxy-2-ureido-D-Glcα1-6D-(2-*O*-octyl)*myo*-inositol-1-HPO₄-*sn*-1,2-dipalmitoylglycerol [GlcNCONH₂-(2-*O*-octyl)-PI].

The non-selective inhibitor GlcNCONH₂-PI and the two novel compounds were tested as inhibitors of the trypanosomal and HeLa cell enzymes using the direct assay (measuring release of [³H]acetate from GlcN[³H]Ac-PI). In agreement with the indirect coupled assay (Figure 4C), the IC₅₀ for GlcNCONH₂-PI against the trypanosome enzyme was ~8 nM (Figure 8A). This compound was less active against the HeLa enzyme (IC₅₀ between 0.1 and 1 μM), but, nevertheless, inhibitory (Figure 8B). In contrast, while GlcNCONH₂-β-PI and GlcNCONH₂-(2-*O*-octyl)-PI were equally potent inhibitors as GlcNCONH₂-PI for the trypanosomal enzyme (Figure 8C and E), neither of these compounds inhibited the HeLa cell enzyme at concentrations up to 100 μM (Figure 8D and F).

Summary

Based on the work in this paper and by others (Milne *et al.*, 1994; Sharma *et al.*, 1997, 1999; Smith *et al.*, 1997b, 1999, 2000), certain structural features have been identified as being responsible for the interactions between the trypanosomal de-*N*-acetylase and GlcNAc-PI. The essential features are: (i) the phosphodiester group appears to be important for recognition and may interact with positively

charged residue(s) on the enzyme; (ii) the 3'-OH group of the GlcNAc residue appears to be essential for substrate recognition, suggesting that it acts as a hydrogen-bond acceptor or donor; (iii) the 4'-OH group of the GlcNAc residue is not essential for substrate recognition, but epimerization, methylation or mannosylation of this position reduces or abolishes substrate turnover; (iv) the 6'-OH group of the GlcNAc residue is not essential for substrate recognition; (v) the 2-, 3-, 4- and 5-OH groups of the D-*myo*-inositol residue do not appear to be involved in substrate recognition by the de-*N*-acetylase, hence the ability of the parasite enzyme to act on GlcNAc-[L]-PI, GlcNAc-β-PI and GlcNAc-(2-*O*-alkyl)PI. The latter point illustrates a fundamental difference between the parasite and human (HeLa) de-*N*-acetylases; namely, that the human enzyme is configurationally and anomerically specific for the GlcNAcα1-6D-*myo*-inositol component of GlcNAc-PI, whereas the parasite enzyme is not.

The irreversible inhibition of the trypanosomal GlcNAc-PI de-*N*-acetylase by GlcNCONH₂-PI, and reactivation by the nucleophiles hydroxylamine, hydrazine and DTT at neutral pH, can reasonably be explained by postulating a reaction mechanism that involves either a hydroxyl or sulfhydryl group at the active site. Attack on the carbonyl group of the *N*-acetyl function of the GlcNAc residue by a Ser/Thr group, for example, would liberate GlcN-PI and generate an *O*-acetyl-enzyme intermediate (Figure 9A), which is subsequently hydrolysed to liberate acetic acid and regenerate the Ser/Thr OH group. However, loss of ammonia from the tetrahedral intermediate formed with GlcNCONH₂-PI (Figure 9B) would still leave the enzyme covalently bound as a carbamate ester to the substrate analogue. This model would account for the reactivation of the enzyme on treatment with hydroxylamine, hydrazine and DTT, requiring nucleophilic attack on the carbonyl group of the carbamate ester to lead to C-O bond cleavage and restoration of the

Ser/Thr OH group at the active site. It would also apply should the initial attack on GlcNCONH₂-PI involve a Cys SH group, culminating in the formation of an enzyme-linked thiocarbamate ester. However, the insensitivity of the de-*N*-acetylase to sulfhydryl alkylating reagents such as iodoacetic acid, iodoacetamide and NEM suggests that a hydroxy-amino acid may be more likely. Site-directed mutagenesis studies will be performed to see which residues are essential for enzymatic activity.

Finally, we exploited the more fastidious nature of the human de-*N*-acetylase (Sharma *et al.*, 1999; Smith *et al.*, 1999; this study) by combining features that provide specificity for the parasite de-*N*-acetylase (i.e. β anomeric linkage or 2-*O*-alkylation of the D-*myo*-inositol residue) with the *N*-acyl function of the non-specific inhibitor GlcNCONH₂-PI to produce GlcNCONH₂- β -PI and GlcNCONH₂-(2-*O*-octyl)-PI. These compounds, which are active *in vitro*, are leads for the design and synthesis of parasite-specific inhibitors that may be active *in vivo*. Such compounds could impact significantly on the development of trypanocidal drugs, since the GPI biosynthetic pathway has been validated as a therapeutic target against African trypanosomes (Ferguson, 2000; Nagamune *et al.*, 2000).

Materials and methods

Substrates and substrate analogues

The compounds shown in Figure 1 were synthesized as previously described (Cottaz *et al.*, 1993; Crossman *et al.*, 1997, 1999; Borissov *et al.*, 2001; Dix *et al.*, 2001), except for D-GlcN α 1-6L-2-*O*-methyl-*myo*-inositol-1-HPO₄-*sn*-1,2-dipalmitoylglycerol (GlcN-[L]-(2-*O*-methyl)-PI), which was prepared in a similar manner to the corresponding D-*myo*-inositol analogue (Crossman *et al.*, 1997), and D-GalN α 1-6D-*myo*-inositol-1-HPO₄-*sn*-1,2-dipalmitoylglycerol (GalN-PI), D-ManN α 1-6D-*myo*-inositol-1-HPO₄-*sn*-1,2-dipalmitoylglycerol (ManN-PI), D-GlcN β 1-6D-*myo*-inositol-1-HPO₄-*sn*-1,2-dipalmitoylglycerol (GlcN- β -PI), D-GlcN α 1-6D-*myo*-inositol-1-HPO₄-octadecyl (GlcN-I-P-C18) and D-GlcN α 1-6D-*myo*-inositol-1-HPO₄-octyl (GlcN-I-P-C8) (A.Crossman, M.J.Paterson and J.S.Brimacombe, details to be published elsewhere). *N*-acetyl derivatives of the compounds were prepared by standard procedures (Smith *et al.*, 1996).

Preparation of GlcNR-PI substrate analogues

The GlcNR-PI substrate analogues GlcNBz-PI, GlcNBz-PI and GlcNMal-PI were prepared by dissolving GlcN-PI in tetrahydrofuran/methanol (1:1, v/v) containing 5% triethylamine, and then adding half a volume of tetrahydrofuran containing an excess of either benzoic, phthalic or maleic anhydrides, respectively. After 15 min at 0°C, the reaction was quenched with an equal volume of water. GlcPtu-PI was prepared in a similar manner using pyridine and phenyl isothiocyanate. GlcNMe₂-PI was prepared by reductive methylation: to a solution of GlcN-PI in propan-1-ol/0.5 M HEPES pH 7.4 (1:1, v/v) was added formaldehyde (5%, v/v) followed by sodium cyanoborohydride (20 mM) and incubated at room temperature (RT) for 1 h. GlcNBn-PI was prepared in a similar manner to GlcNMe₂-PI, except that benzaldehyde was used instead of formaldehyde. GlcNCONH₂-PI analogues were prepared by mixing a solution of the corresponding GlcN-PI analogue in propan-1-ol/0.5 M HEPES pH 7.4 (1:1, v/v) with an equal volume of 10 mM sodium cyanate dissolved in the same buffer and incubated at RT for 2 h. After removal of the solvent, the GlcNR-PI substrate analogues were purified using 100 mg Isolute cartridges, as described below for GlcN^{[3]H}Ac-PI. The identity and purity of each substrate analogue were assessed by negative-ion electrospray mass spectrometry (Figure 1), and the concentration of each stock solution was ascertained by measuring the inositol content by selected ion-monitoring gas chromatography-mass spectrometry (Ferguson, 1994).

N-^{[3]H}acetylation and purification of the substrate analogues

GlcN-PI and analogues thereof were *N*-acetylated with [³H]acetic anhydride as now described for GlcN-PI. GlcN-PI (10 nmol) in 150 μ l of dry tetrahydrofuran/methanol (2:1, v/v) containing 5 μ l of triethylamine was treated with 100 nmol [³H]acetic anhydride (50.0 Ci/mmol) for 30 min at RT, followed by 50 nmol of non-radioactive acetic anhydride for 30 min at RT. The reaction was quenched with 100 μ l of water, whereafter GlcN^{[3]H}Ac-PI was purified by diluting the reaction mixture with 5 ml of 5% propan-1-ol in 100 mM ammonium acetate and loading onto a pre-equilibrated 500 mg C8 Isolute cartridge. The cartridge was washed with the same buffer ($\geq$ 200 ml) until radioactivity in the eluate had reached background levels. GlcN^{[3]H}Ac-PI was eluted from the cartridge with 10 ml of 80% propan-1-ol in 100 mM ammonium acetate, which was evaporated to dryness. The residue was desalted by dissolution in butan-1-ol and washing the organic solution with water. [Analogues with short lipid chains (e.g. GlcN^{[3]H}Ac-I-P-C8) were freeze-dried three times and residual acetic acid was removed by co-evaporation with toluene.] Purified GlcN^{[3]H}Ac-PI was dissolved in 1 ml of butan-1-ol, and aliquots used to determine the inositol content and specific activity. The GlcN^{[3]H}Ac-PI analogues were diluted with the corresponding non-radioactive compound to give a final specific activity of 18.2 mCi/mmol. The GlcN^{[3]H}Ac-PI substrate analogues were analysed by HPTLC, to determine their radiochemical purity (data not shown). The deoxy-GlcN^{[3]H}Ac-PIs and GlcN^{[3]H}Ac4Me-PI have slightly higher *R_f* values than GlcN^{[3]H}Ac-PI, as have GlcN^{[3]H}Ac- β -PI, ManN^{[3]H}Ac-PI and GalN^{[3]H}Ac-PI. GlcN^{[3]H}Ac-[L]-PI has the same *R_f* value as GlcN^{[3]H}Ac-PI, while that of GlcN^{[3]H}Ac-[L]-(2-*O*-methyl)-PI is slightly higher. The lipid-modified GlcN^{[3]H}Ac-PI analogues have significantly different *R_f* values due to differences in the lipid component.

Preparation of trypanosomal and HeLa membranes

Trypanosoma brucei and HeLa cell membranes (cell-free systems) were prepared as described previously (Masterson *et al.*, 1989; Smith *et al.*, 1996, 1997b), except that HeLa aliquots were frozen at 1×10^7 and 2×10^7 cell equivalents/ml for GDP-[³H]Man labelling and GlcN^{[3]H}Ac-PI de-*N*-acetylase assays, respectively.

Direct de-*N*-acetylase assay

Washed trypanosome membranes were suspended in incorporation buffer (Smith *et al.*, 1996, 1997b) supplemented with NEM. The lysate was sonicated and 40 μ l aliquots (5×10^7 cell equivalents) were added to reaction tubes containing either 0.5 nmol (10 000 c.p.m.) of GlcN^{[3]H}Ac-PI or a substrate analogue in 10 μ l of incorporation buffer supplemented with *n*-octyl β -D-glucopyranoside (*n*-OG) (0.3% w/v) and GDP-Man (1 mM), unless stated otherwise. After brief sonication, the reaction mixtures were incubated at 30°C.

HeLa cell lysate was thawed and supplemented as previously described (Smith *et al.*, 1997b) with 100 μ M CoA, ATP-regenerating system (100 μ M ATP, 5 mM phosphocreatine and 5 U of creatine phosphokinase) and 1 mM GDP-Man, unless stated otherwise. Aliquots of 100 μ l (2×10^6 cell equivalents) were added to reaction tubes containing either 1.5 nmol (30 000 c.p.m.) of GlcN^{[3]H}Ac-PI or a substrate analogue, sonicated briefly and incubated at 35°C.

Reactions were terminated by the addition of 50 μ l of propan-1-ol, followed by vortexing and snap-freezing. Each GlcN^{[3]H}Ac-PI analogue was studied at least twice with triplicate samples at each time interval. Samples were thawed, adjusted to 1 ml with 100 μ l of 1 M ammonium acetate and water, and then applied to a pre-equilibrated 100 mg C8 Isolute cartridge. The cartridge was washed with 2 ml of 5% propan-1-ol in 100 mM ammonium acetate, and the eluate (containing the released [³H]acetate) was counted for radioactivity. The unreacted GlcN^{[3]H}Ac-PI analogue was then eluted with 2 ml of 80% propan-1-ol in 100 mM ammonium acetate, and the eluate was counted for radioactivity. All input radioactivity was accounted for.

Inhibition assays were conducted the same way, except that the membranes were pre-incubated with potential inhibitors for 5 min prior to being added to GlcN^{[3]H}Ac-PI.

Indirect de-*N*-acetylase assay

The indirect assay detects the transfer of [³H]Man to de-*N*-acylated products and has been described previously (Smith *et al.*, 1996, 1997b). Inhibition assays were conducted in a similar manner, except that the membranes were pre-incubated with potential inhibitors for 5 min prior to being added to GlcN-PI or GlcNAc-PI. Some inhibition assays (such as those in Figure 6A) were conducted by pre-incubation of the membranes (5×10^7 cell equivalents) with GDP-[³H]Man (0.5 μ Ci) and a potential

inhibitor for 10 min at RT, in order to allow Dol-P-[³H]Man to be formed and inhibition to take place. Thereafter, the membranes were pelleted (16 000 g for 2 min at 4°C), resuspended in 200 µl of fresh incorporation buffer supplemented with NEM, sonicated briefly, pelleted again and resuspended in 25 µl of fresh 2× incorporation buffer supplemented with both NEM and n-OG (0.3%, w/v). The suspensions were added to an equal volume of GlcN-PI (50 µM) in n-OG (0.3%, w/v), sonicated briefly and incubated at 30°C for 1 h.

Reactivation studies

The reversibility of the inhibition by GlcNCONH₂-PI was studied using an assay similar to that used to obtain the data for Figure 6B. Membranes (5 × 10⁷ cell equivalents) were incubated in incorporation buffer with GlcNCONH₂-PI (2.5 µM) for 5 min to allow inhibition of the de-N-acetylase to take place. Thereafter, the membranes were pelleted and the activity of the enzyme-inhibitor complex was analysed after brief sonication and incubation on ice for 15 min with either 100 µl of 25 mM DTT or 100 µl of 100 mM hydroxylamine or 100 µl 25 mM hydrazine (all in incorporation buffer). Following incubation, the membranes were pelleted, washed with 200 µl of fresh incorporation buffer, sonicated briefly, pelleted again and resuspended in 25 µl of fresh 2× incorporation buffer supplemented with both NEM and n-OG (0.3% w/v). The suspensions were added to an equal volume of GDP-[³H]Man (0.5 µCi) and GlcN-PI (50 µM) in n-OG (0.3% w/v), sonicated and incubated at 30°C for 1 h.

Enzymatic and chemical treatments of radiolabelled glycolipids and HPTLC

Digestions with jack bean α-mannosidase, glycosylphosphatidyl-inositol specific phospholipase D and phosphatidylinositol-specific phospholipase C, and base hydrolysis, deamination, N-acetylation and HPTLC were performed as previously described (Güther *et al.*, 1994; Smith *et al.*, 1996, 1997b).

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