Substrate specificity of the *N*-acetylglucosaminyl-phosphatidylinositol de-*N*-acetylase of glycosylphosphatidylinositol membrane anchor biosynthesis in African trypanosomes and human cells

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De-*N*-acetylation of *N*-acetylglucosaminyl-phosphatidylinositol (GlcNAc-PI) is the second step of glycosylphosphatidylinositol (GPI) membrane anchor biosynthesis in eukaryotes. This step is a prerequisite for the subsequent mannosylation of glucosaminyl-phosphatidylinositol (GlcN-PI) which leads to mature GPI membrane anchor precursors, which are transferred to certain proteins in the endoplasmic reticulum. The substrate specificities of the GlcNAc-PI de-*N*-acetylase activities of African trypanosomes and human (HeLa) cells were studied with respect to the *N*-acyl groups (R) that could be removed from a series of

GlcNR-PI substrates, where R = acetyl (Ac), propionyl (Pr), butyryl (Bu), isobutyryl (iBu), pentanoyl (Pen) or hexanoyl (Hex). The data show that the trypanosomal and HeLa enzymes had similar specificities and that the turnover of GlcNR-PIs by the trypanosomal enzyme was in the order GlcNAc-PI > GlcNPr-PI ≫ GlcNBu-PI ≈ GlcNiBu-PI ≈ GlcNPen-PI ≫ GlcNHex-PI. The trypanosome and HeLa de-*N*-acetylases were unable to de-*N*-acetylate mannosylated GlcNAc-PI intermediates, which explains why de-*N*-acetylation must precede mannosylation in the GPI biosynthetic pathway.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) membrane anchors are found on many eukaryotic cell-surface glycoproteins, and their structures, functions and biosynthesis have been extensively reviewed [1–6]. The minimum GPI-anchor structure is NH₂CH₂CH₂-HPO₄-6Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6-*myo*-inositol-1-HPO₄-lipid (EtNP-Man₃GlcN-PI), where the lipid may be diacylglycerol, alkylacylglycerol or ceramide [1].

Proteins destined to be GPI-anchored are attached to the amino group of the terminal ethanolamine phosphate (EtNP) residue by a transamidation reaction whereby a hydrophobic C-terminal peptide is exchanged for a preassembled GPI precursor in the endoplasmic reticulum [5]. The minimum GPI-anchor structure described above is highly conserved but it may be modified with additional ethanolamine phosphate groups and with carbohydrate side chains in a species- and tissue-specific manner [1].

The biosynthesis of GPI anchors involves the addition of GlcNAc to phosphatidylinositol (PI), to give GlcNAc-PI, which is de-*N*-acetylated to form GlcN-PI [6–9]. A Chinese hamster ovary cell mutant that is deficient in GlcNAc-PI de-*N*-acetylase activity has been described recently [10] but the defective gene has yet to be isolated. De-*N*-acetylation is a prerequisite for the mannosylation of GlcN-PI to form later GPI intermediates [11] and it has been suggested that this step is regulated in mammalian cells by GTP [9].

The GlcNAc-PI de-*N*-acetylase of *Trypanosoma brucei* has been partially purified and characterized [12] using a series of GlcNAc-PI analogues [13] to assess the importance of the fatty

acids and glycerophosphate components and the absolute configuration of the *myo*-inositol residue for substrate recognition. The study concluded that: (i) the fatty acids assist in the presentation of the substrate to the enzyme, but are not essential for substrate recognition; (ii) a phosphodiester linkage between the *myo*-inositol and glycerol residues is essential for substrate recognition; (iii) *D*-*myo*-inositol can be replaced by *L*-*myo*-inositol without loss of substrate recognition; (iv) product inhibition by GlcN-PI and acetate is negligible.

In the present study, we report the substrate specificity of *T. brucei* and human (HeLa cell) GlcNAc-PI de-*N*-acetylases with respect to the size of the *N*-acyl (R) group that can be cleaved from a series of GlcNR-PI substrates. We also assess the ability of these enzymes to de-*N*-acetylate mannosylated GlcNAc-PI intermediates.

MATERIALS AND METHODS

Materials

GDP-[1-³H]Man (15.0 Ci/mmol), [9,10-³H]myristic acid (11.2 Ci/mmol) and En³Hance[®] were purchased from Du Pont-NEN, and jack-bean α -mannosidase (JBAM) was from Boehringer-Mannheim. *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) was a gift from Dr. M. Low, Columbia University, New York, NY, U.S.A. Whole human serum was used as a source of GPI-specific phospholipase D (GPI-PLD). Ion-exchange resins (AG-50X12 and AG-3X4) were obtained from Bio-Rad, and all the other reagents were purchased from Sigma.

Abbreviations used: AHM, 2,5-anhydromannitol; GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; Pr, propionyl; Bu, butyryl; iBu, isobutyryl; Pen, pentanoyl; Hex, hexanoyl; JBAM, jack-bean α -mannosidase; GC, gas chromatography.

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Preparation of trypanosomes and the trypanosome cell-free system

Bloodstream forms of *T. brucei* (variant MITat1.4) were isolated from infected rats and mice. Trypanosome membranes (trypanosome cell-free system) were prepared as previously described by Masterson et al. [14], except that the cells were not preincubated with tunicamycin before lysis.

Preparation of HeLa cells and the HeLa cell-free system

HeLa cells were grown at 37 °C in Dulbecco's modified minimal essential medium supplemented with 10 % fetal calf serum in a 5 % CO₂ atmosphere. The HeLa cell-free system was prepared as described by Güther et al. [15], except that subconfluent HeLa cells were treated with 5 µg/ml tunicamycin for 2 h at 37 °C and then incubated for 10 min at 37 °C with 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 Tos-Lys-CH₂Cl and 0.1 µg/ml leupeptin. An equal volume of 100 mM Hepes/NaOH buffer, pH 7.4, containing 50 mM KCl, 10 mM MgCl₂, 0.1 mM Tos-Lys-CH₂Cl, 0.1 µg/ml leupeptin and 20 % (w/v) glycerol was added to the lysate. Aliquots containing either 10⁷ or 2 × 10⁷ cell equivalents/ml were stored at -70 °C.

Preparation of semisynthetic [³H]myristate-labelled GlcNR-[³H]PI and Man₃GlcNAc-[³H]PI

The procedure used was similar to that described by Milne et al. [8]. Trypanosomes (variant MITat 1.4) from the buffy coat of infected rat blood were washed and suspended at 5 × 10⁷ cells/ml in complete RPMI 1640 medium (Gibco) buffered with 25 mM Hepes/NaOH, pH 7.4, and supplemented with 2 mg/ml BSA (fatty acid-free). The cells were preincubated with 0.8 mM PMSF for 15 min at 37 °C. [³H]Myristic acid complexed to BSA (1 mol/mol) was added in RPMI 1640 medium to give a final concentration of 50 µCi/ml. After 30 min at 37 °C, the trypanosomes were pelleted and extracted with chloroform/methanol/water (10:10:3, by vol.) at 2.5 × 10⁸ cells/ml with vortex-mixing and sonication. After centrifugation, the supernatants were evaporated to dryness in a stream of nitrogen and the products were partitioned between 200 µl of butan-1-ol and 200 µl of water. After vortex-mixing and centrifugation the radiolabelled glycolipids were recovered in the upper butan-1-ol phase which was concentrated to dryness in a SpeedVac evaporator, and the residue was subjected to high-performance TLC (HPTLC). Man₃GlcN-PI labelled with [³H]myristate in the lipid moiety, which accumulates in the presence of PMSF [16], was located on the HPTLC plate using a Raytest RITA linear analyser (Lab-Logic, Sheffield, Yorkshire, U.K.). The excised silica was extracted with methanol/pyridine/water (2:1:1, by vol.) and the Man₃GlcN-[³H]PI intermediate was recovered by evaporation of the solvent under a stream of nitrogen. It was treated in two ways. (i) For the production of GlcN-[³H]PI, Man₃GlcN-[³H]PI was digested with 30 µl of 50 units/ml JBAM in 0.1 M sodium acetate buffer, pH 5.0, containing 0.1 % (w/v) taurodeoxycholate at 37 °C for 18 h. The product (GlcN-[³H]PI) was recovered in the organic phase after partitioning between butan-1-ol and water. (ii) For the production of Man₁GlcN-[³H]PI, Man₃GlcN-[³H]PI was digested with 25 µl of 4 units/ml JBAM in 0.1 M sodium acetate buffer, pH 5.0, containing 0.1 % (w/v) taurodeoxycholate at 37 °C for 5 h. The products of this partial digest (a mixture of Man₃GlcN-[³H]PI, Man₂GlcN-[³H]PI, Man₁GlcN-[³H]PI and GlcN-[³H]PI) were recovered in the organic phase after partitioning between butan-1-ol and water. Man₁GlcN-

[³H]PI was then obtained by preparative HPTLC, as described above for Man₃GlcN-[³H]PI.

Purified Man₁GlcN-[³H]PI and GlcN-[³H]PI were N-acetylated in 100 µl of saturated NaHCO₃ at 0 °C by the addition of three aliquots (2.5 µl) of acetic anhydride at 10 min intervals. After warming to room temperature, Man₁GlcNAc-[³H]PI and GlcNAc-[³H]PI were recovered by extraction into butan-1-ol. GlcN-[³H]PI was also N-acylated under the same conditions with propionic, butyric and isobutyric anhydrides to produce the corresponding GlcNR-[³H]PIs.

Synthetic GlcNR-PI substrates

D-GlcNAc1-6D-*myo*-inositol-1-HPO₄-(*sn*-1,2-dipalmitoylglycerol) (GlcN-PI) was synthesized as described by Cottaz et al. [17] and was N-acylated at 0 °C in 100 µl of saturated NaHCO₃ by the addition of three aliquots (2.5 µl) of the appropriate acid anhydride (acetic, propionic, butyric, isobutyric, pentanoic or hexanoic anhydride) at 10 min intervals. The reaction mixture was allowed to warm to room temperature and the N-acylated glycolipids were isolated and desalted by extraction into butan-1-ol. The butan-1-ol phases were evaporated to dryness, the residues were dissolved in 20 % propan-1-ol/10 mM ammonium acetate (solvent A) and the resulting solutions were applied to a reverse-phase HPLC column (Kromasil 5 µm C8; 25 cm × 0.46 mm) equilibrated with the same solvent. The column was eluted first with solvent A for 10 min, then with a linear gradient to 50 % propan-1-ol/10 mM ammonium acetate over 20 min and finally with a linear gradient to 90 % propan-1-ol/10 mM ammonium acetate over 120 min with a flow rate of 0.5 ml/min throughout. The GlcNR-PI products were eluted at about 70 % propan-1-ol.

The authenticity and purity of the synthetic N-acylated compounds was checked by negative-ion electrospray MS. The concentrations of solutions containing GlcNR-PIs were measured by analysing the *myo*-inositol content by gas chromatography (GC)-MS, as described below.

Trypanosome de-N-acetylase assays using [³H]myristate-labelled GlcNR-[³H]PIs and Man₁GlcNAc-[³H]PI

The trypanosome cell-free system was used as the source of GlcNAc-PI de-N-acetylase. Trypanosome membranes were washed twice in 0.1 M Hepes buffer, pH 7.4, containing 25 mM KCl, 5 mM MgCl₂, 0.1 mM Tos-Lys-CH₂Cl and 2 µg/ml leupeptin, and were then suspended at 5 × 10⁸ cell equivalents/ml in incorporation buffer: 50 mM Hepes, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 5 mM MnCl₂, 10 % (v/v) glycerol, 1.25 µg/ml tunicamycin, 0.1 mM Tos-Lys-CH₂Cl, 1 µg/ml leupeptin, 10 mM *N*-ethylmaleimide and 10 mM (0.3 %, w/v) *n*-octyl β-D-glucopyranoside. Aliquots (50 µl) of this lysate were added to tubes containing dry [³H]myristate-labelled GlcNR-[³H]PI (5000 c.p.m.), vortex-mixed and incubated at 35 °C for 16 h. The reactions were terminated by the addition of 333 µl of chloroform/methanol (1:1, v/v). The glycolipid products were recovered in the chloroform/methanol/water-soluble fraction, which was evaporated to dryness and the residue was partitioned between butan-1-ol and water, as described by Güther et al. [15]. The butan-1-ol phases containing the glycolipids were analysed by HPTLC.

HeLa cell de-N-acetylase assays on [³H]myristate-labelled GlcNR-[³H]PIs and Man₁GlcNAc-[³H]PI

HeLa cell lysate aliquots of 1 ml (2 × 10⁷ cell equivalents) were thawed and supplemented with 0.5 mM dithiothreitol, 0.2 µg/ml

tunicamycin, 0.1 mM Tos-Lys- CH_2Cl , 5 mM MnCl_2 and 2 $\mu\text{g}/\text{ml}$ leupeptin. Aliquots (100 μl) of the supplemented lysate were added to tubes containing dry [^3H]myristate-labelled GlcNR-[^3H]PI (5000 c.p.m.), vortex-mixed and incubated at 37 °C for 16 h. The reactions were terminated by the addition of 666 μl of chloroform/methanol (1:1, v/v). The glycolipid products were recovered as described above and analysed by HPTLC.

Trypanosome de-N-acylation/[^3H]mannosylation assay

Aliquots (50 μl) of washed trypanosome membranes suspended in incorporation buffer were added to tubes containing dry GDP-[^3H]Man (0.5 μCi) and 365 pmol of synthetic GlcNR-PI substrate and were then incubated at 30 °C for 1 h. The reactions were terminated by the addition of 333 μl of chloroform/methanol (1:1, v/v). The glycolipid products were recovered as described above and analysed by HPTLC both before and after enzymic and chemical treatments (see below).

HeLa cell de-N-acylation/[^3H]mannosylation assay

HeLa cell lysate was thawed and supplemented as described above and, unless otherwise indicated, 1 mM CoA and 10 mM ATP were added to the lysates. Aliquots (100 μl) of the lysate were added to tubes containing dry GDP-[^3H]Man (2.5 μCi) and 1400 pmol of synthetic GlcNR-PI substrate and were then incubated at 37 °C for 1.5 h. The reactions were terminated by the addition of 666 μl of chloroform/methanol (1:1, v/v). The glycolipid products were recovered as described above and analysed by HPTLC both before and after enzymic and chemical treatments. The major radiolabelled glycolipid formed in the assay was purified by preparative HPTLC and then subjected to chemical modification and glycan analysis as described below.

Enzymic and chemical treatments of radiolabelled glycolipids

Digestion of the glycolipid extracts with the enzymes JBAM, PI-PLC and GPI-PLD and subsequent product analysis by HPTLC was carried out as described previously [15]. HNO_2 deamination of the glycolipids was performed 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 during incubation at 60 °C for 4 h. The products of deamination were recovered for HPTLC analysis by extraction into butan-1-ol.

HPTLC

Samples and glycolipid standards were applied to 10 or 20 cm aluminium-backed silica gel 60 HPTLC plates (Merck) which were developed in chloroform/methanol/1 M ammonium acetate/13 M ammonium hydroxide/water (180:140:9:9:23, by vol.). Radiolabelled components were detected using a phosphorimager (see below) and/or, after spraying with $\text{En}^3\text{Hance}^{\text{TM}}$, by fluorography at -70 °C using Kodak XAR-5 film and an intensifying screen.

Phosphorimager quantification of [^3H]mannosylated glycolipids on HPTLC plates

To quantify the relative amounts of radioactivity in particular glycolipid bands, some of the HPTLC plates were exposed to tritium screens which were subsequently analysed in a Molecular Dynamics phosphorimager. The relative amounts of substrate de-N-acylation in the de-N-acylation/[^3H]mannosylation assays were estimated by integrating the phosphorimager signals cor-

responding to the exogenous H5 band (for the HeLa cell assay; Figure 6) or corresponding to the $\text{Man}_1\text{GlcN-PI}$ to glycolipid A region (for the trypanosome assay; Figure 2).

Inositol analysis

GC-MS (Hewlett Packard 5970-MSD) was used to measure the *myo*-inositol content of the synthetic GlcNR-PIs. Aliquots of the compounds were mixed with 50 pmol of the internal standard [1,2,3,4,5,6- ^3H]*myo*-inositol and treated with 50 μl of 6 M HCl at 110 °C for 16–18 h. After removal of the acid by evaporation, the products were converted into their trimethylsilyl derivatives for analysis by GC-MS, as described by Ferguson [18].

Electrospray MS

A Micromass Quattro spectrometer was used to acquire negative-ion electrospray mass spectra over the range m/z 150–1150. Samples (5–20 μl at approx. 20 pmol/ μl) were introduced into the ion source in aq. 50 % acetonitrile at a flow rate of 10 $\mu\text{l}/\text{min}$. Scans were averaged and processed using MassLynx software.

Glycan head-group analysis of the major radiolabelled glycolipid generated in the HeLa cell-free system

The major product labelled with GDP-[^3H]Man in the presence of synthetic GlcNAc-PI in the HeLa cell-free system (designated as H5 in Figure 5) was purified by HPTLC as described above. The glycolipid was delipidated by base hydrolysis [100 μl of conc. ammonia/methanol (1:1, v/v), 50 °C for 6 h], dried, dissolved in 50 μl of 0.1 M sodium acetate, pH 4.0, and treated with 50 μl of freshly prepared 0.5 M NaNO_2 for 2.5 h at room temperature. Subsequent NaB^3H_4 reduction was performed by the addition of 25 μl of 0.8 M boric acid and transfer of the mixture into 50 μl of 0.1 M NaOH containing 1 M NaB^3H_4 with incubation for 16 h at room temperature. The reaction mixture was acidified with acetic acid and desalted by passage through AG50X12(H^+), drying and removal of boric acid and acetic acid by co-evaporation with methanol and toluene respectively. The product so obtained was dephosphorylated with 50 μl of aq. 48 % HF (0 °C for 48 h); saturated LiOH was used to neutralize the acid, which precipitated as LiF [18]. The supernatant containing the deaminated, reduced and dephosphorylated neutral glycan fraction was desalted by passage through a tandem column of ion-exchange resins [AG50X12(H^+) over AG3X4(OH^-)].

Glycan analysis

The neutral glycan fraction obtained from the above procedure was filtered through a 0.2 μm membrane and analysed by Bio-Gel P4 gel filtration using an Oxford Glycosystems GlycoMap. Fractions (330 μl) were collected and counted for radioactivity.

RESULTS AND DISCUSSION

Specificity of the trypanosome GlcNAc-PI de-N-acetylase for the N-acyl moiety

GlcN-[^3H]PI, radiolabelled in the fatty acids of the PI moiety, was prepared by labelling trypanosomes with [^3H]myristic acid in the presence of PMSF. This leads to an accumulation of the GPI intermediate $\text{Man}_3\text{GlcN-PI}$ [16], arising from the inhibition of inositol acylation [15] and the subsequent addition of ethanolamine phosphate [19]. The $\text{Man}_3\text{GlcN-PI}$ intermediate that accumulates in the cells undergoes fatty acid remodelling with the introduction of [^3H]myristate into the PI moiety [15]. This intermediate was purified by HPTLC and then digested with

Figure 1 Turnover of GlcNR-[^3H]PI species by the trypanosome cell-free system

The GlcNR-[^3H]PI substrates were incubated with (+) and without (–) the trypanosome cell-free system (enzyme) as indicated. The extracted glycolipid fractions were analysed by HPTLC and fluorography. Standards of [^3H]myristate-labelled GlcN-[^3H]PI and GlcNAc-[^3H]PI are shown in lanes 1 and 2 respectively. Note: the labelled bands in lanes 2–10 migrating ahead of the major labelled GlcNR-[^3H]PI species are most likely GlcNR-[^3H]PI species that have undergone partial O-acylation during N-acylation.

JBAM to produce GlcN-[^3H]PI [8,12]. GlcN-[^3H]PI was N-acylated with acetyl (Ac), propionyl (Pr), butyryl (Bu) and isobutyryl (iBu) groups to produce radiolabelled GlcNR-[^3H]PI substrates (where R = Ac, Pr, Bu or iBu) for studies with the trypanosomal GlcNAc-PI de-N-acetylase. The substrates were presented to the trypanosome cell-free system in the presence of 0.3% *n*-octyl β -D-glucopyranoside, and the extent of de-N-acylation was assessed by extracting the glycolipid fraction and analysing it by HPTLC and fluorography (Figure 1). GlcNAc-[^3H]PI, the natural substrate, underwent ~36% turnover to GlcN-PI under these conditions (Figure 1, lanes 3 and 4), whereas the turnover of GlcNPr-[^3H]PI (Figure 1, lanes 5 and 6) was significantly less (17%) and those of GlcNBu-[^3H]PI (Figure 1, lanes 7 and 8) and GlcNiBu-[^3H]PI (Figure 1, lanes 9 and 10) were barely detectable.

The specificity of the GlcNAc-PI de-N-acetylase for the N-acyl group was also investigated using an indirect assay based on the ability of chemically synthesized (non-radiolabelled) GlcNR-PI substrates to undergo mannosylation by the trypanosome cell-free system. Previous studies showed that de-N-acylation of exogenous GlcNAc-PI precedes the addition of the three α Man residues in the GPI biosynthetic pathway [11] so that mannosylation of exogenous substrates can be used as an indirect (but highly sensitive) measure of GlcNR-PI de-N-acylation.

GlcN-PI, synthesized as described by Cottaz et al. [17], was N-acylated with the appropriate anhydride, and the N-acylated products were purified by reverse-phase HPLC. The purified N-acylated compounds (including the N-pentanoyl and N-hexanoyl derivatives GlcNPen-PI and GlcNHex-PI) were analysed by negative-ion ES-MS, which showed that all of the preparations were pure, and quantified by GC-MS measurement of the *myo*-inositol content.

The assay shown in Figure 2 reveals that, in the absence of an exogenous substrate, the cell-free system labelled with GDP-[^3H]Man produces Dol-P-[^3H]Man (the immediate donor for α -mannosyltransferases of the GPI pathway [20]) and low levels of labelled endogenous GPI intermediates (Figure 2, lane 2). The addition of synthetic GlcN-PI (the natural acceptor substrate for the first α -mannosyltransferase of the GPI pathway) leads to the formation of additional GPI intermediates (Figure 2, lane 3) which migrate fractionally more slowly than the endogenous

Figure 2 De-N-acylation and subsequent [^3H]mannosylation of synthetic GlcNR-PI substrates by the trypanosome cell-free system

The cell-free system was incubated with GDP-[^3H]Man in the absence (lane 2) and presence (lanes 3–9) of synthetic GlcNR-PI substrates, as indicated. The extracted glycolipid fractions were analysed by HPTLC and fluorography. A standard of [^3H]myristate-labelled $\text{Man}_3\text{GlcN-PI}$ is shown in lane 1. The assignments to the various bands are based on their sensitivities to JBAM HNO_2 and PI-PLC and R_f values (results not shown) [11,19]. DPM, Dol-P-[^3H]Man.

GPI species. This is presumably due to shorter acyl chains being attached to the glycerol backbone in synthetic GlcN-(*sn*-1,2-diacyl)PI compared with those of endogenous intermediates [21]. As noted previously, the addition of GlcNAc-PI produces substantially more mannosylated GPI intermediates than does the addition of GlcN-PI (compare Figure 2, lanes 3 and 4), suggesting that GlcN-PI is best presented to the α -mannosyltransferases via the de-N-acetylase [11]. The compounds GlcNPr-PI, GlcNBu-PI, GlcNiBu-PI and GlcNPen-PI were also able to undergo de-N-acylation and subsequent mannosylation (Figure 2, lanes 5–8), whereas GlcNHex-PI showed no detectable de-N-acylation/mannosylation activity (Figure 2, lane 9). The relative order of substrate processing was GlcNAc-PI > GlcNPr-PI $\gg$ GlcNiBu-PI $\approx$ GlcNBu-PI $\approx$ GlcNPen-PI. The mannosylated GPI intermediates portrayed in lanes 3–8 were sensitive to both PI-PLC [except for $\text{Man}_3\text{GlcN(acyl)PI}$ which is inherently PI-PLC resistant] and HNO_2 , confirming their identities as de-N-acylated GPI intermediates (results not shown).

Taken together, the two assays employed above show that the relative rate of de-N-acylation of GlcNR-PI decreases with increasing size of the N-acyl group. There is one slight discrepancy between the two assays; GlcNBu-[^3H]PI appears to be turned over more efficiently than GlcNiBu-[^3H]PI (Figure 1) whereas GlcNBu-PI and GlcNiBu-PI gave similar results in the de-N-acylation/[^3H]mannosylation assay (Figure 2). The reason for this discrepancy is not clear, but it may reflect the fact that the substrates are presented to the membranes at a much lower final concentration when radiolabelled GlcNR-[^3H]PI substrates are used. However, it can be concluded that both the N-butyryl and N-isobutyryl derivatives are relatively poor substrates whereas the N-propionyl derivative is a relatively good substrate when compared with the natural N-acetyl substrate.

The presence of mannose residues prevents de-N-acylation of GlcNAc-PI by the trypanosome cell-free system

Partial digestion of the GPI intermediate $\text{Man}_3\text{GlcN-[}^3\text{H]PI}$ with JBAM produced a mixture of $\text{Man}_3\text{GlcN-[}^3\text{H]PI}$, $\text{Man}_2\text{GlcN-[}^3\text{H]PI}$, $\text{Man}_1\text{GlcN-[}^3\text{H]PI}$ and GlcN-[$^3\text{H]PI}$ (results not shown).

Figure 3 Demonstration that Man₁GlcNAc-[³H]PI is not a substrate for the trypanosome de-N-acetylase

GlcNAc-[³H]PI and Man₁GlcNAc-[³H]PI were incubated with (+) (lanes 4 and 8) and without (—) (lanes 3 and 7) the cell-free system (enzyme), and the products were analysed by HPTLC and fluorography against standards of GlcNAc-[³H]PI (lane 1), GlcN-[³H]PI (lane 2), Man₁GlcNAc-[³H]PI (lane 5) and Man₁GlcN-[³H]PI (lane 6). Note: the minor bands migrating above and below the major Man₁GlcNAc-[³H]PI bands in lanes 5, 7 and 8 are indicative of the presence of traces of GlcNAc-[³H]PI and Man₂GlcNAc-[³H]PI respectively formed from GlcN-[³H]PI and Man₂GlcN-[³H]PI carried over from the HPTLC purification of Man₁GlcN-[³H]PI (see lane 6).

A fraction enriched in Man₁GlcN-[³H]PI was prepared by preparative HPTLC (Figure 3, lane 6) and was N-acetylated to produced Man₁GlcNAc-[³H]PI (Figure 3, lane 5). This material was tested as a substrate for the de-N-acetylase using the assay described for Figure 1. Under conditions where approx. 50% of the GlcNAc-[³H]PI control was converted into GlcN-[³H]PI (Figure 3, lanes 3 and 4), there was no detectable conversion of Man₁GlcNAc-[³H]PI into Man₁GlcN-[³H]PI (Figure 3, lanes 7 and 8). Identical experiments were performed using Man₂-GlcNAc-[³H]PI and Man₃-GlcNAc-[³H]PI as substrates. Neither of these compounds was turned over by the de-N-acetylase (results not shown).

These data suggest that substitution of the 4-hydroxy group of the GlcNAc residue with a bulky α Man residue prevents substrate recognition by the trypanosomal de-N-acetylase. Such a substrate specificity is consistent with the idea that de-N-acetylation must precede mannosylation in trypanosome GPI biosynthesis. It further suggests that de-N-acetylation is an essential step in GPI biosynthesis that cannot be bypassed.

Comparative studies on the substrate specificity of the HeLa cell GlcNAc-PI de-N-acetylase

In a similar experiment to that recorded in Figure 1, GlcNR-[³H]PI substrates were added to the HeLa cell-free system and the turnover of substrates was assessed by the formation of GlcN-[³H]PI. In this experiment, CoA was omitted from the reaction mixture because it did not stimulate de-N-acylation and reduced our ability to assess de-N-acetylation, since part of the GlcN-[³H]PI produced was converted into GlcN-(acyl)[³H]PI (results not shown).

The data in Figures 4(A) and 4(B) show that the HeLa cell-free system will de-N-acylate GlcNAc-[³H]PI (Figure 4B, lanes 2 and 3) and GlcNPr-[³H]PI (Figure 4A, lanes 3 and 4) and, whereas the turnover of GlcNiBu-[³H]PI (Figure 4A, lanes 7 and 8) is lower than that of GlcNBu-[³H]PI (Figure 4A, lanes 5 and 6) is barely

Figure 4 Turnover of GlcNR-[³H]PI and Man₁GlcNAc-[³H]PI species by the HeLa cell-free system

GlcNPr-[³H]PI, GlcNBu-[³H]PI and GlcNiBu-[³H]PI (**A**) and GlcNAc-[³H]PI and Man₁GlcNAc-[³H]PI (**B**) were incubated with (+) and without (—) the HeLa cell-free system (enzyme), as indicated. The extracted glycolipid fractions were analysed by HPTLC and fluorography. Standards of [³H]myristate-labelled GlcN-[³H]PI are shown in lanes 1 and 9 (**A**) and lane 1 (**B**); standards of GlcNAc-[³H]PI are shown in lane 2 of both panels.

detectable. These results are similar to those of the trypanosomal cell-free system, as was the inability of the HeLa cell-free system to de-N-acetylate Man₁GlcN-[³H]PI (Figure 4B, lanes 6 and 7).

The substrate specificity of the de-N-acetylase of the HeLa cell-free system was also assessed using an indirect de-N-acetylation/[³H]mannosylation assay similar to that described above. However, in this case mannosylation of exogenous GlcNAc-PI proved to be extremely variable for different batches of the HeLa cell-free system. This variability was reduced and the efficiency of mannosylation of the exogenous substrate was greatly increased when reduced CoA and ATP [22] were included in the reaction mixture (Figure 5A). On the basis of its R_p value, the major [³H]mannosylated product (Figure 5A) was tentatively assigned as the H5 glycolipid described by Hirose et al. [23], namely EtNP-Man₁GlcN-(acyl)PI. This structure was confirmed by chemical and enzymic treatments on a glycolipid extract similar to that shown (Figure 5A). Thus the H5 band (Figure 5B, lane 1) was shown to be resistant to JBAM (Figure 5B, lane 2) and *B. thurigiensis* PI-PLC (Figure 5B, lane 3), whereas the positive-control substrates (Man₃GlcN-[³H]PI and GlcN-[³H]PI respectively) were sensitive to these enzymes (Figure 5B, lanes 7 and 11). Like the positive-control Man₃GlcN-[³H]PI substrate (Figure 5B, lanes 8 and 9), the H5 band was sensitive to human

Figure 5 De-N-acetylation and [³H]mannosylation of exogenous GlcNAc-PI by the HeLa cell-free system

(A) The HeLa cell-free system was incubated with GDP-[³H]Man and synthetic GlcNAc-PI with (+) and without (–) reduced CoA, as indicated. The glycolipid products were extracted and analysed by HPTLC and fluorography. (B) [³H]Mannose-labelled H5 glycolipid (lane 1) was digested with JBAM, PI-PLC, GPI-PLD and HONO, as indicated (lanes 2–5). Positive controls for JBAM, HONO and GPI-PLD digestion, using Man₃GlcN-[³H]PI as a substrate, are shown in lanes 6–9 and a positive control for PI-PLC digestion, using GlcN-[³H]PI as a substrate, is shown in lanes 10 and 11. The products of these control digestions include GlcN-PI, PI, phosphatidic acid (PA), dimyristoylglycerol (DMG) respectively, as well as a trace of non-esterified fatty acid (FA) in the case of the PI-PLC digest. DPM, Dol-P-[³H]Man. (C) Bio-Gel P4 chromatogram showing the elution at about 2.3 glucose units (GU) of Man α 1-4AHM derived from the H5 glycolipid.

serum GPI-PLD and to HNO₂ deamination (Figure 5B, lanes 4 and 5). It is worth noting that the [³H]mannose-labelled GPI-PLD product of H5 [EtNP-Man₁GlcN-(acyl)inositol] runs as a heterogeneous band on HPTLC (Figure 5B, lane 4). This suggests that there may be some heterogeneity in the acyl chain attached to the inositol ring, as has been observed in human erythrocyte acetylcholinesterase [24], *T. brucei* glycolipid C [25] and *T. brucei* procyclic acid repetitive protein [26]. This conclusion would be broadly consistent with the ability of the mammalian inositol-acyltransferase to use different acyl-CoA donors [27] but it is at variance with the study of Seveler et al. [28] on the GlcN-(acyl)PI that accumulates in HeLa S3 cells, which suggests that, in that case, the acyl chain is exclusively palmitate.

Taken together, the properties described above are consistent with a GlcN- and Man-containing, inositol-acylated GPI structure in which the Man residue(s) are protected from the action of JBAM by an ethanolamine phosphate group. To determine how many Man residues were present, the putative H5 band was purified by preparative HPTLC and deacylated with alkali. The deacylated product was deaminated and reduced [to convert the GlcN residue into 2,5-anhydromannitol (AHM) and to release

Figure 6 De-N-acylation and subsequent [³H]mannosylation of synthetic GlcNR-PI substrates by the HeLa cell-free system

The cell-free system was incubated with GDP-[³H]Man without (lane 3) and with (lanes 2 and 4–9) various synthetic GlcNR-PI substrates, as indicated. The extracted glycolipid fractions were analysed by HPTLC and fluorography. A standard of [³H]myristate-labelled Man₃GlcN-PI is shown in lane 1. The H5 glycolipid band consists of an upper band of endogenous H5 glycolipid, formed by [³H]mannosylation and processing of endogenous GlcN-(acyl)PI, and a slightly lower H5 glycolipid band (lanes 2, 4, 5 and 7) resulting from de-N-acylation, inositol acylation, [³H]mannosylation and processing of exogenous GlcNR-PIs. DPM, Dol-P-[³H]Man.

the inositol residue] and then dephosphorylated to yield a neutral glycan fraction. Analysis of this fraction by Bio-Gel P4 gel filtration revealed a single glycan with a hydrodynamic volume identical with that of authentic Man α 1-4AHM and equal to 2.3 glucose units (Figure 5C) [18,29]. Taken together, these data identify the H5 band as EtNP-Man₁GlcN-(acyl)PI and validate the assay for measuring the first α -mannosyltransferase of the HeLa cell GPI biosynthetic pathway and therefore, indirectly, the de-N-acetylase. A similar assay has recently been described using a Chinese hamster ovary cell-free system and synthetic GlcN-(sn-1,2-dioctoyl)PI [27].

In the absence of an exogenous substrate, the HeLa cell-free system labelled with GDP-[³H]Man produces radiolabelled Dol-P-Man and endogenous H5 glycolipid (Figure 6, lane 3). When synthetic GlcN-PI, GlcNAc-PI and GlcNPr-PI are added a slightly less polar H5 band is produced, running just below the endogenous H5 band (Figure 6, lanes 2, 4 and 5). This is consistent with de-N-acylation of the substrates and subsequent mannosylation and addition of ethanolamine phosphate. However, this band was barely produced with GlcNiBu-PI (Figure 6, lane 7) and could not be detected with GlcNBu-PI, GlcNPen-PI and GlcNHex-PI as substrates (Figure 6, lanes 6, 8 and 9). Quantification of the [³H]mannosylated products generated in the trypanosome and HeLa cell-free systems (as measured by phosphorimager densitometry of the Man₁GlcN-PI to glycolipid A bands in Figure 2 and of the exogenous glycolipid H5 band in Figure 6) showed that GlcNPr-PI generated 30 and 34 % respectively of the products generated with GlcNAc-PI. Thus it seems that the de-N-acetylases of the HeLa and trypanosome cell-free systems have similar substrate specificities.

It is worth noting that the trypanosome cell-free system processes GlcNAc-PI roughly 6 times more efficiently than GlcN-PI ([11] and Figure 2, compare lanes 3 and 4) whereas the HeLa cell-free system processes GlcN-PI roughly 1.5 times more efficiently than GlcNAc-PI (Figure 6, compare lanes 2 and 4). This observation may be significant since it has been suggested that the enhanced processing of GlcNAc-PI over GlcN-PI indicates a degree of substrate channelling between the trypanosome de-N-acetylase and the first α -mannosyltransferase [11]. Such substrate channelling between these two enzymes is not evident in the HeLa cell-free system, possibly because acylation of the inositol ring of the GlcN-PI intermediate is a prerequisite for mannosylation in mammalian cell-free systems [27].

Conclusions

The trypanosome and HeLa cell-free systems de-N-acylate and process GlcNAc-PI and, with lower efficiency, GlcNPr-PI. De-N-acylation of GlcNBu-PI with the HeLa cell-free system is just detectable by the de-N-acylation/[³H]mannosylation assay, whereas de-N-acylation of this derivative, as well as of GlcNBu-PI and GlcNPen-PI, is more evident in the equivalent trypanosome assay. However, this apparent difference may be due to differences in the limits of detection of de-N-acylation in the HeLa and trypanosome cell-free systems. The similar relative reduction in substrate turnover by HeLa and trypanosome membranes on exchanging the N-acetyl group for an N-propionyl group in GlcNR-PI suggests that the substrate specificities of the parasite and human GlcNAc-PI de-N-acetylase enzymes are quite similar. Furthermore, the substantial reduction in substrate turnover on increasing the size of the N-acyl group beyond N-propionyl suggests that there is relatively little scope for change in the N-acyl structure that can be tolerated by the enzymes. These two factors imply that suicide substrate inhibitors, based on reactive N-acyl groups, are unlikely to be selective for and effective solely against the parasite enzyme. Finally, the inability of the trypanosome and HeLa cell de-N-acetylases to de-N-acetylate mannosylated GlcNAc-PI intermediates explains why de-N-acetylation must precede mannosylation in the GPI biosynthetic pathway.

Note added in proof (received 9 October 1997)

The cloning of a candidate rat GlcNAc-PI de-N-acetylase has recently been reported [30].

We thank Angela Mehler and Lucia Güther for advice and assistance and Robert Field for helpful discussions. This work was supported by a programme grant from the Wellcome Trust and a project grant from the MRC. M.A.J.F. is a Howard Hughes International Research Scholar. D.K.S. thanks the MRC for a Ph.D. studentship.

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