Substrate Specificity of the Dolichol Phosphate Mannose: Glucosaminyl Phosphatidylinositol α1-4-Mannosyltransferase of the Glycosylphosphatidylinositol Biosynthetic Pathway of African Trypanosomes*

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The biosynthesis of glycosylphosphatidylinositol (GPI) precursors in Trypanosoma brucei involves the α-mannosylation of α-D-GlcN1-6-o-myo-inositol-1-PO4-sn-1,2-diacylglycerol (GlcN-PI). An assay for the first mannosyltransferase of the pathway, Dol-P-Man:GlcN-PI α1-4-mannosyltransferase, is described. Analysis of the acceptor specificity revealed that the enzyme requires the myo-inositol residue of the GlcN-PI substrate, and by the presence of the NH2 group of the D-GlcN residue; (b) that the enzyme requires the presence of the NH2 group of the α-GlcN residue; (c) that GlcNAc-PI is more efficiently presented to the enzyme than GlcN-PI, suggesting a degree of substrate channeling via the preceding GlcNAc-PI-de-N-acetylace transferase; and (d) that the fatty acid and phosphoglycerol components of the phosphatidyl moiety are important for enhancing substrate presentation and substrate recognition, respectively; and (e) that α-D-GlcN1-6-o-myo-inositol is the minimum structure that can support detectable acceptor activity. Analysis of the donor specificity revealed that short chain (C5 and C15) analogues of dolichol phosphate can act as substrates for the trypanosomal dolichol-phosphomannose synthetase, whereas the corresponding mannyropyranosides cannot act as donors for the Dol-P-Man:GlcN-PI α1-4-mannosyltransferase.

Glycosylphosphatidylinositol (GPI) membrane anchors are widely distributed among the eukaryotes. They anchor proteins to the outer leaflet of the plasma membrane and may be associated with other functions, such as signal transduction and protein targeting. The structure, biosynthesis, and function of GPI anchors have been reviewed, most recently by Englund (1993), McConville and Ferguson (1993), Stevens (1995), Udenfriend and Kodukula (1995), and Takeda and Kinoshita (1995). The tsetse fly-transmitted African trypanosomes, which cause human sleeping sickness and a variety of livestock diseases, are able to survive in the mammalian bloodstream by virtue of their dense cell-surface coat. This coat consists of 10 million copies of a 55-kDa GPI-anchored glycoprotein called the variant surface glycoprotein (VSG) (Cross, 1990). The relative abundance of the VSG protein in Trypanosoma brucei renders this organism extremely useful for the study of GPI anchor biosynthesis. The structure of the VSG GPI anchor is known (Ferguson et al., 1987), and the principal features of the GPI biosynthetic pathway in trypanosomes were elucidated using a cell-free system based on washed trypanosome membranes (Masterson et al., 1989, 1990). The first step in the pathway involves the transfer of GlcNAc from UDP-GlcNAc to endogenous phosphatidylinositol (PI), via a sulfodryl-dependent GlcNAc transferase (Halley et al., 1992) to form GlcNAc-PI, which is rapidly de-N-acetylated (Boer et al., 1989) to give glucosaminyl PI (GlcN-PI). Three α-mannosyl residues are sequentially transferred onto GlcN-PI from dolichol phosphate mannosyl (Dol-P-Man) (Sugiyama et al., 1990b) to produce the intermediate Manα1-2Manα1-6Manα1-4GlcN-PI (Man-GlcN-PI). All of the mannosylated intermediates can be found in both acylated and nonacylated inositol forms, and a Manα1,GlcN-acyl)PI species appears to be the preferred substrate for ethanolamine phosphate (EtNP) adduction (Güther and Ferguson, 1995), the donor for which is phosphatidylethanolamine (Menon and Stevens, 1992; Menon et al., 1993). The EtNP-Manα1,GlcN-acyl)PI (glycolipid C+) species is then deacylated to form glycolipid A+, which undergoes a series of fatty acid remodeling reactions (Masterson et al., 1990) in which the fatty acids of the PI moiety are removed and replaced with myristate to yield the mature GPI precursor glycolipid A. This mature precursor is transferred to the VSG polypeptide in the endoplasmic reticulum in exchange for a carboxyl-terminal GPI signal peptide, reviewed by Udenfriend and Kodukula (1995).

The GPI biosynthetic pathways in mammalian cells (Sugiyama et al., 1991; Lemansky et al., 1991; Hirose et al., 1991, 1992a, 1992b; Kamitani et al., 1992; Puoti and Brimacombe, 1999; Mooney et al., 1994) and yeast (Costello and Orlean, 1992; Sipos et al., 1994), as well as in other protozoa, such as Toxoplasma (Tomavo et al., 1992a, 1992b) and Plasmodium falciparum (Gerdol et al., 1994), appear to be broadly similar to that described above for the bloodstream form of T. brucei. Some notable differences among the trypanosomal, mammalian, and yeast GPI pathways include the almost quantitative acylation of all mammalian and
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yeast GPI intermediates from GlcN-PI onwards and the addition of extra ethanolamine phosphate groups to the mammalian intermediates. The fatty acid remodeling reactions, as described above, appear to be unique to the bloodstream form of African trypanosomes.

In this paper, we show that exogenously added GlcN-PI and analogues thereof can prime the GPI pathway in a trypanosome cell-free system, thereby providing a convenient means for probing the substrate specificity of Dol-P-Man:GlcN-PI α1-4-mannosyltransferase, the first mannosyltransferase of the GPI biosynthetic pathway.

EXPERIMENTAL PROCEDURES

Materials—GDP-[1-3H]mannose (15 mCi/mmol) and En3Hance20 were purchased from DuPont NEN, and jack bean α-mannosidase was from Boehringer Mannheim. Bacillus thuringiensis phosphatidylinisitol-specific phospholipase C was a gift from Dr. M. Low, Columbia University, New York, NY, and whole human serum was used as a source of glycosylphosphatidylinositol-specific phospholipase D. Ion exchange resins (AG-50X12 and AG-3X4) were obtained from Bio-Rad. Zwittergent 3–16, o-nitro-β-galactosyanide, and n-octyl-1-thio-β-glucopyranoside were obtained from Calbiochem, Nonidet P-40 was obtained from Pierce, and Triton X-100 was from Aldrich. The novel detergent β-o-glucopyranosyl octyl sulfone was prepared by five additions of peracetic acid (10 μl) at 5-min intervals to solid n-octyl-1-thio-β-glucopyranoside (25 mg) while on ice. The excess of acid was removed by evaporation, and the product was recovered from the organic phase of a butan-1-ol/water partition. All of the other reagents were purchased from Sigma.

Substrates and Substrate Analogues—GlcN-PI, d-GlcNα1-6-o-myo-inositol-1-PO4-sn-1,2-dipalmitoylglycerol (GlcN-PI-[1,2]), d-GlcNα1-6-o-myo-inositol-1-PO4-glycerol (GlcN-Ino-P-glycerol), d-GlcNα1-6-o-myo-inositol-1-PO4 (GlcN-Ino-P), and d-GlcNα1-6-o-myo-inositol (GlcN-Ino) were synthesized according to Cottaz et al. (1993, 1995a, 1995b). d-GlcNα1-6-o-myo-inositol-1,2-cyclic phosphate (GlcN-Ino-1,2-P) was a gift from Professor J. van Boom, Leiden University, The Netherlands. These compounds were N-acetylated as described below.

d-GlcNα1-6-o-myo-inositol-1-PO4-sn-1,2-dipalmitoylglycerol (GlcN-PI) and d-deoxy-GlcNα1-6-o-myo-inositol-1-PO4-sn-1,2-dipalmitoylglycerol (d-deoxy-GlcN-PI) were synthesized according to Cottaz et al. (1995b). The authenticity and purity of the synthetic compounds was checked by electrospray mass spectrometry prior to use (see Fig. 1 for the recorded pseudomolecular ions), and the concentrations of stock solutions were measured by analyzing the myo-inositol content by gas chromatography/mass spectrometry, as described below. Isoamyl phosphate (CH3–CH2–CH2–PO4H) were synthesized.2 Briefly, isoamyl alcohol and toluene were used at a final concentration of 0.5 mM, and the assays were terminated as described above. The products were recovered in the aqueous phase of a butan-1-ol/water partition and analyzed for glycan as described below.

Preparation of Trypanosomes—Bloodstream forms of T. brucei (varient MITat1.4) were isolated from infected rats and mice. Trypanosome membranes (trypanosome cell-free system) were prepared as described previously by Masterson et al. (1989), except that the cells were not preincubated with tunicamycin prior to lysis.

Dol-P-Man:GlcN-PI α1-4 Manneyltransferase Assays—The trypanosome cell-free system was used as the source of the enzyme. Trypanosome membranes were washed twice in 0.1 m Hepes buffer, pH 7.4, containing 25 mM KCl, 5 mM MgCl2, 0.1 mM N\'-tosyl-L-lysine chloromethyl ketone and 2 μg/ml leupeptin, and resuspended at 5 × 108 cell equivalents/ml in 2 × concentrated incorporation buffer (0.1 m Hepes, pH 7.4, 50 mM KCl, 10 mM MgCl2, 10 mM MnCl2, 20% (v/v) glycerol, 2.5 μg/ml tunicamycin, 0.2 μg/ml TLCCK, 2 μg/ml leupeptin in Hepes buffer, pH 7.4, 50 mM KCl, 10 mM MgCl2, 10 mM MnCl2, 20% (v/v) glycerol) (Masterson et al., 1989)). Unless stated otherwise, the 2 × concentrated incorporation buffer was supplemented with freshly prepared 0.2 m N-ethylmaleimide and 10 mM (0.3% (w/v)) n-octyl-β-D-glucopyranoside. The resuspended lysate was vortexed, sonicated briefly, and then added to a tube containing dry GDP-[3H]Man (0.3 μCi/107 cell equivalents)

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Table I. Mannosyltransferase activity in the presence of detergents

| Detergent                  | CMC  | Concentration | Activity* |
|----------------------------|------|---------------|-----------|
| None (control)             | -    | -             | 100       |
| Zwittergent 3–16 (Z)       | 0.06 | 1.00          | 10        |
| CHAPS (Z)                  | 10.00| 15.00         | 10        |
| Sodium deoxycholate (I)    | 6.00 | 10.00         | 0         |
| Triton X-100 (N)           | 0.90 | 2.00          | 10        |
| NP-40 (N)                  | 0.30 | 1.00          | 10        |
| n-Octyl β-D-glucopyranoside (N) | 25.00 | 50.00         | 0         |
| n-Octyl 1-thio-β-D-glucopyranoside (N) | 9.00  | 20.00         | 0         |
| β-D-glucopyranosyl octyl sulphone (N) | 20.00 | 40.00         | 0         |
| n-Octyl β-o-glucopyranosylamine (N) | 80.00 | 200.00        | 0         |

* Activity is expressed as the percent of [3H]Man incorporated into GPI intermediates compared with the control.
Z, zwitterionic; I, ionic; N, nonionic.

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

N-Acetylation of glycans was performed at 0 °C in 100 μl of saturated NaHCO3 by the addition of three aliquots (2.5 μl) of acetic anhydride at 10-min intervals. The reaction mixture was allowed to come to room temperature, and the solution of N-acetylated glycan was desalted by passage through AG50X12(H+) ion-exchange resin and evaporated, and residual acetic acid was removed by coevaporation with toluene (2 × 50 μl).

Dephosphorylation was performed with 50 μl of 48% aqueous H2SO4 (0 °C for 48–60 h). Saturated LiOH was used to neutralize H2SO4, which precipitated as Li2SO4 (Ferguson, 1992).

Glycan Analysis—Radiolabeled products were mixed with 2,500 cpm of an internal standard, Manα1–2-Manα1–6-Galα1–6-Galα1–3-Manα1–4-Iβ1–H2,5-anhydromannitol (GalαManα1–4-H+) (Ferguson et al., 1988). In the analysis of glycolipid products, the internal standard was added prior to delipidation, and the soluble glycan products were recovered in the aqueous phase of a butan-1-d/water partition. The samples were treated in two ways:
(a) Dephosphorylated/N-acetylated glycans were generated by N-acetylation, passage through AG50X12(H+) ion-exchange resin, aqueous HF dephosphorylation, and re-N-acetylation (Ferguson, 1992).
(b) Dephosphorylated/deaminated glycans were generated by deamination and subsequent NaBH4 reduction followed by aqueous HF dephosphorylation and re-N-acetylation (Ferguson, 1992). The neutral glycans from these procedures were dissolved in water and mixed with oligomeric glucose internal standards, filtered through a 0.2-μm membrane, and analyzed by Bio-Gel P4 gel filtration using an Oxford Glycosystems GlycoMap. Fractions (250 μl) were collected and counted for radioactivity. The yields of [3H]Man-containing glycan products for each substrate were normalized using the internal standard GalαManα1–4-H+ (AHM).

Insoluble Analysis—Gas chromatography/mass spectroscopy (Hewlett Packard MSD 5970 series) was used to measure the myo-inositol content of synthetic substrates and analogues. Aliquots of samples were mixed with the internal standard myo-[2,3,4,5,6-3H]inositol (100 pmoles) and hydrolyzed with 50 μl of 6 N HCl at 110 °C for 16–18 h. After enzymatic, the products were converted into their trimethylsilyl derivatives and analyzed by gas chromatography/mass spectroscopy, as described by Ferguson (1992).

Electrospray Mass Spectrometry—A VG Quattro (Fisons Instruments) spectrometer was used to acquire positive and negative-ion electrospray mass spectra over the mass range m/z 150-1150. Samples (5–20 μl at approximately 20 pmol/μl) were introduced in 50% aqueous acetonitrile at a flow rate of 10 μl/min, and several scans were averaged using MassLynx software. The m/z values of the pseudomolecular ions for each of the synthetic compounds are given in Fig. 1.

RESULTS

Optimization of Assay Conditions—The standard incubation conditions of the trypanosome cell-free system can be used to monitor the activity of the enzymes of the GPI biosynthetic pathway using endogenous acceptor substrates present in the membranes (Masterson et al., 1989, 1990; Menon et al., 1990a, 1990b). This system has been adapted to allow the use of exogenous substrates and substrate analogues to probe the substrate specificity of the first mannosyltransferase of the pathway. The formation of GPI intermediates from endogenous PI was suppressed by omitting UDP-GlcNAc and including N-ethylmaleimide, a potent inhibitor of the UDP-GlcNAcPI α1–6 GlcNAc-transferase (Milne et al., 1992). The addition of N-ethylmaleimide also inhibits the endogenous GPI-specific phosphopilase C (Herold et al., 1986) and prevents the degradation of GPI intermediates when detergents are included. Attempts to solubilize the Dol-P-Man:GlcN-PI α1–4-mannosyltransferase were unsuccessful because all of the detergents investigated largely destroyed the enzyme activity when employed above the critical micellar concentration (CMC), see Table I. Significantly, all active preparations primed with exogenous GlcN-PI produced essentially the same spectrum of products (see Fig. 2, lanes 1 and 3, and Table II). In view of this, the activity of Dol-P-Man:GlcN-PI α1–4-mannosyltransferase was measured by summation of the radioactivity found in all mannosylated GPI intermediates. The incorporation of [3H]Man into these intermediates was linear over 90 min, and an incubation time of 60 min was chosen for the assay.

In the system without added detergent, enzymatic activity was observed between pH 6.0 and 9.0, with a maximum at about pH 7.4 (data not shown). All subsequent assays were performed at pH 7.4.

The assay is dependent on endogenous Dol-P-Man synthetase, which converts GDP-[3H]Man to Dol-P-[3H]Man in situ.
The enzyme is thermally unstable (Prado-Figueroa et al., 1994) so that the temperature of the assay was limited to 30 °C. The loss in GPI pathway activity using detergents above their CMCs was not associated with the loss of Dol-P-Man synthetase activity, since the formation of Dol-P-[3H]Man is usually stimulated under these conditions (data not shown).

The data in Table I show that several detergents can support GPI biosynthesis below their CMCs, but only CHAPS and n-octyl β-D-glucopyranoside (and certain derivatives thereof) were able to stimulate the pathway. The significant (3.5-fold) stimulation of the pathway with 10 mM (0.3% (w/v)) n-octyl β-D-glucopyranoside was exploited in all subsequent assays. Stimulation of the GPI pathway was not due to stimulation of Dol-P-Man synthetase, since Triton X-100 was at least as effective as n-octyl β-D-glucopyranoside in stimulating Dol-P-Man synthesis, whereas it did not stimulate GPI biosynthesis (data not shown). The sensitivity of the pathway to the detergent environment is illustrated by the inhibitory effect of n-octyl 1-thio-β-D-glucopyranoside (Fig. 2, lane 2), which can be reversed by oxidation of the sulfur atom to the more polar sulfone (Fig. 2, lanes 3 and 4).

Acceptor Substrate Specificity—The cell-free system was incubated with GlcNAc-PI (the natural acceptor substrate) and various GlcNAc-PI analogues in the presence of 10 mM n-octyl β-D-glucopyranoside and GDP-[3H]Man (Fig. 3). In all cases, the membranes produced labeled Dol-P-[3H]Man and a low level of endogenous GPI intermediates, ranging from Manα1-4GlcNAc-PI to EtNP-Manα2–GlcNAc-PI (see lane 3). The only substrates producing an increased amount of labeled intermediates were GlcNAc-PI (lane 1) and GlcNAc-PI (lane 4). Of these, GlcNAc-PI was superior in priming the pathway, resulting in at least a 6-fold increase in product formation compared with GlcNAc-PI.3 The identities of the individual bands were assigned on the basis to their Rf values and sensitivities to jack bean α-mannosidase, PI-specific phospholipase C, GPI-specific phospholipase D, and nitrous acid deamination (Table II). The GlcNAc-PI analogues Glc-PI, 2-deoxy-Glc-PI, and GlcNAc-PI showed no detectable acceptor activity (lanes 2, 5, and 6).

In order to compare the abilities of glycolipid and water-soluble substrates to prime the GPI pathway (see Fig. 4), they were incubated with the cell-free system in the presence of n-octyl β-D-glucopyranoside (10 mM) and GDP-[3H]Man, as described above. The glycan components of the products were recovered following (a) N-acetylation, aqueous HF dephosphorylation and re-N-acetylation or (b) deamination/reduction and aqueous HF dephosphorylation. The radiolabeled neutral glycans so produced were fractionated on a Bio-Gel P4 column. In

3 The enhanced acceptor activity of GlcNAc-PI over GlcNAc-PI was not due to differences in the solubilities of these substrates in the assay buffer, which contains 10 mM n-octyl β-D-glucopyranoside. This was assessed by ultra-centrifugation of solutions of GlcNAc-PI and GlcNAc-PI, containing respective [3H]myristate-labeled tracers, which showed that both substrates were fully soluble.
In the normal cell-free system assay, GDP-[\textsuperscript{3}H]Man is converted into Dol-P-[\textsuperscript{3}H]Man by the action of endogenous Dol-P-Man synthetase on endogenous (C50 and C55) dolichol phosphate (Fig. 5, lane 1). The addition of exogenous (C40-C65) dolichol phosphate to the cell-free system stimulated the production of additional Dol-P-[\textsuperscript{3}H]Man (lane 2) but did not result in a significant increase in the labeling of the GPI intermediates. The addition of the dolichol phosphate analogues didehydrofarnesol phosphate and isoamyl phosphate to the cell-free system inhibited the formation of endogenous Dol-P-[\textsuperscript{3}H]Man (lanes 3 and 4, respectively), and both analogues were mannosylated by Dol-P-Man synthetase to form didehydrofarnesol and isoamyl phospho-[\textsuperscript{3}H]mannose, respectively. Neither of the latter compounds was able to donate [\textsuperscript{3}H]mannose, via Dol-P-Man:GlcN α-1-4-mannosyltransferase, to the awaiting exogenous GlcN-PI acceptor. Didehydrofarnesol phospho-[\textsuperscript{3}H]mannose was recovered in the butan-1-ol phase, and the HPTLC profile revealed two bands, corresponding to the cis- and trans-isomers (lane 3). Isoamyl phospho-[\textsuperscript{3}H]mannose was recovered in the aqueous phase of a water/butan-1-ol partition, whereas there was no endogenous Dol-P-[\textsuperscript{3}H]Man present in the butan-1-ol phase (lane 4). The identities of the [\textsuperscript{3}H]mannosylated analogues were established by their sensitivities to mild acid hydrolysis.

**DISCUSSION**

Protein-linked GPI anchors and GPI-related glycolipids, such as the lipophosphoglycans and glycoinositol-phospholipids of the Leishmania, are particularly abundant in protozoa (McConville and Ferguson, 1993). All of these molecules, several of which are essential for parasite infectivity, share the structural motif Man\textsubscript{a}1–4GlcN\textsubscript{a}1–6-myo-inositol-1-PO\textsubscript{4}-lipid. Thus the enzymes responsible for the synthesis of this conserved unit represent attractive targets for the development of anti-parasite agents. In this paper we disclose the substrate specificity of one of these enzymes, the trypanosomal Dol-P-Man:GlcN-PI α-1-4-mannosyltransferase.

A previous report by DeLuca et al. (1994) describes an assay for a comparable mammalian Dol-P-Man:GlcN-(acyl)PI α-1-4-mannosyltransferase, based on the microsomes of mutant CHO-K1 cells that were unable to synthesize or utilize endogenous Dol-P-Man and which therefore accumulated endogenous GlcN-(acyl)PI. Using this assay, they were able to study the donor substrate specificity of the enzyme, which exhibited strict specificity for the β-anomer of Dol-P-Man, although the dolichol moiety could be replaced by a similar polyisoprenyl moiety without abolishing enzymatic activity. The assay described here for the trypanosomal Dol-P-Man:GlcN-PI α-1-4-mannosyltransferase differs from that of DeLuca et al. (1994).
in that it relies on the addition of exogenous (synthetic) acceptors and can be used to determine both donor and acceptor specificities.

The trypanosomal dolichols are unusually short \( \alpha \)-unsaturated polyisoprenes \((C_{40-50})\) (Low et al., 1991). We have investigated the possibility that the dolichyl phosphate moiety of the Dol-P-Man donor might be replaced by even shorter analogues, namely didehydrofarnesol phosphate (an \( \alpha \)-unsaturated \( C_{15} \) tri-isoprene) or isomayl phosphate (a saturated \( C_{9} \) isoprene). Both of these analogues were mannosylated by the trypanosomal Dol-P-Man synthetase, but the products (didehydrofarnesol-P-[\( ^{3}H \)]Man and isomayl-P-[\( ^{3}H \)]Man) were unable to act as donors for the trypanosomal Dol-P-Man:GlcN-PI [\( \alpha \)-1-4-mannosyltransferase. Although the addition of exogenous dolichyl phosphate increased the amount of Dol-P-[\( ^{3}H \)]Man, the synthesis of [\( ^{3}H \)]Man-labeled GPI intermediates was not affected. Thus, under the conditions of the assay, the levels of Dol-P-[\( ^{3}H \)]Man generated in the cell-free system appear to exceed the requirements of the GPI biosynthetic pathway, and the addition of exogenous dolichyl phosphate is not required.

Several synthetic compounds were tested for their ability to act as acceptors for the Dol-P-Man:GlcN-PI [\( \alpha \)-1-4-mannosyltransferase. As expected, synthetic GlcN-PI (i.e. the natural acceptor) was a good acceptor, and the enzyme showed selectivity for the \( \alpha \)-myo-inositol component (Figs. 3 and 4). The selectivity of the Dol-P-Man:GlcN-PI [\( \alpha \)-1-4-mannosyltransferase toward the \( \alpha \)-myo-inositol component suggests that the orientation of one or more hydroxyl groups and/or the spatial orientation of the phosphatidyl moiety (relative to \( \alpha \)-GlcN) are crucial for acceptor substrate recognition. Such substrate specificity suggests that the Dol-P-Man:GlcN-PI [\( \alpha \)-1-4-mannosyltransferase activity is truly specific for the GPI pathway.

The 6-fold increase in acceptor activity of GlcNAc-PI over GlcN-PI was unexpected, since GlcNAc-PI is thought to require prior de-N-acetylation to GlcN-PI in order to function as a substrate for the first mannosyltransferase (Englund, 1993; Stevens, 1995; Takeda and Kinoshita, 1995). The fact that all of the GPI products from Man-GlcN-PI were sensitive to nitrous acid deamination (a reaction that requires a free NH\(_2\) group on the GlcN residue) indicates that GlcNAc-PI was either de-N-acetylated before mannosylation or quantitatively de-N-acetylated immediately afterwards. The latter course seems unlikely since exogenous GlcNAc-PI does not require prior mannosylation to be a substrate for the de-N-acetylase (Doering et al., 1989), and the in situ generation of Dol-P-Man does not stimulate the de-N-acetylation of exogenous GlcNAc-PI (Sharma and Ferguson, unpublished data). Assuming that de-N-acetylation of GlcNAc-PI normally precedes mannosylation, the enhanced acceptor activity of GlcNAc-PI over GlcN-PI suggests that the GlcN-PI de-N-acetylase and the Dol-P-Man:GlcN-PI [\( \alpha \)-1-4-mannosyltransferase are associated in a complex that allows a degree of substrate channelling from the de-N-acetylase to the acceptor site of the mannosyltransferase. The fact that the product (Man\(_\alpha\)-1-GlcN-PI) of Dol-P-Man:GlcN-PI [\( \alpha \)-1-4-mannosyltransferase activity is always further processed to later GPI intermediates (see Fig. 3 and Table II) suggests that other enzymes of the pathway are also physically associated in the endoplasmic reticulum membrane with these enzymes. The existence of such a complex could explain the liability of the pathway to detergents above their CMC values. The possibility that the first step of the GPI pathway (i.e. the transfer of GlcN-acetoPI) in mammalian (Takeda and Kinoshita, 1995) and yeast (Leidich et al., 1995) cells also involves a complex is supported by the fact that at least three separate gene products are required for this step.

The inability of Glc-P-1 and 2-deoxy-Glc-P-1 to act as acceptors suggests that the NH\(_2\) group of the GlcN residue is necessary for acceptor activity. Neither of these compounds inhibited the mannosylation of GlcN-PI, suggesting that they do not bind significantly to the active site of Dol-P-Man:GlcN-PI [\( \alpha \)-1-4-mannosyltransferase. It is possible that the NH\(_2\) group of GlcN-PI is required to form either a salt bridge to a negatively charged residue on the enzyme or a hydrogen bond to a residue in or near the active site. Such a model would explain the requirement for de-N-acetylation prior to mannosylation and also suggests that de-N-acetylation of GlcNAC-PI may be an important control point in GPI biosynthesis. Such control might be exercised in the mammalian pathway, where stimulation of a comparably enzyme by GTP has been demonstrated (Stevens, 1993). We find no such GTP effect for the trypanosomal de-N-acetylase (Milne et al., 1994). However, these parasites synthesize a significant excess of GTP precursors over that required for protein anchorage (Masterson and Ferguson, 1991; Talton et al., 1993) and may regulate the GPI pathway via catabolism (Güther et al., 1994).

GlcN-Ino-P-glycerol, which lacks the two fatty-acid components of the natural substrate, was a much less efficient acceptor than GlcN-PI (Fig. 4). This suggests that the lipid moieties have a role either in substrate recognition or, more likely, in presenting the substrate to the membrane-bound enzyme. The removal of the glycolal component (as in GlcN-Ino-P) or the glycerol-phosphate component (as in GlcN-Ino) further reduced the acceptor activity, whereas GlcN-Ino-1,2-P had hardly any detectable acceptor activity. Thus, a phospho group at C-1 of the \( \alpha \)-myo-inositol appears to play a role in substrate recognition, unless it involves the C-2 hydroxyl group in the formation of a cyclic phosphate, as is the case with GlcN-Ino-1,2-P. The notion that the C-2 hydroxyl group of myo-inositol is involved in substrate recognition is supported by the observation that trypanosomes do not acylate this position until after the first Man residue has been added (Güther and Ferguson, 1995). This contrasts with observations made with mammalian and yeast cells where acylation of the C-2 hydroxyl group of myo-inositol appears to precede all mannosylations (Stevens, 1995). Thus, the parasite Dol-P-Man:GlcN-PI [\( \alpha \)-1-4-mannosyltransferase

\[ \text{FIG. 5. Analysis of donor substrate specificity of dolichol-P-Man:GlcN-1-4-mannosyltransferase.} \]

A: Dol-P-Man; B: Dol-P-Man:GlcN-1-4-mannosyltransferase; C: Control. The trypanosome cell-free system (1 \( \times \) 10\(^7\) cell equivalents/sample) was incubated at 30°C for 1 h with GDP-[\( ^{3}H \)]Man and 10 mm \( \beta \)-o-glucopyranoside in the presence of 35 \( \mu \)M GlcN-PI without further additions (lane 1) or with added 25 \( \mu \)M dolichol phosphate (lane 2), 50 \( \mu \)M didehydrofarnesol phosphate (lane 3), and 50 \( \mu \)M isomayl phosphate (lane 4). Glycolipids were extracted and analyzed by HPTLC with fluorographic detection.

4. Sharma, T. K. Smith, and M. A. J. Ferguson, unpublished results.
may have a different, and potentially exploitable, acceptor substrate specificity to those of the comparable mammalian and yeast enzymes.

The ability of GlcN-Ino and GlcNAC-Ino (but not GlcN or GlcNAc) to act as weak acceptors for the Dol-P-Man:GlcN-PI α1-4-mannosyltransferase suggests that the minimum structural features for acceptor substrate recognition are present within the structure GlcN-Ino. Furthermore, the products generated from these acceptors by the cell-free system included substantial quantities of Man3-GlcN-Ino, suggesting that Man-GlcN-Ino and Man2-GlcN-Ino contain sufficient structural information for recognition by the second and third α-mannosyltransferases, respectively, of the GPI pathway. The salient features recognized within GlcN-Ino will be investigated using synthetic analogues having hydroxyl group deletions or substitutions on the α-GlcN and α-myoinositol residues. The 5-fold decrease in acceptor activity of GlcN-Ino-P-glycerol, compared with GlcN-PI, suggests that the presence of a hydrophobic group in a similar location to the diacylglycerol group of GlcN-PI might usefully increase the presentation of potential inhibitors to the enzyme. The inclusion of a phosphodiester group at C-1 of the α-GlcN and α-myoinositol ring might also be advantageous with regard to binding potential inhibitors to the enzyme, although it might be difficult for such compounds to cross the cell membrane.

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Substrate Specificity of the Dolichol Phosphate Mannose: Glucosaminyl Phosphatidylinositol 14-Mannosyltransferase of the Glycosylphosphatidylinositol Biosynthetic Pathway of African Trypanosomes

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