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Substrate Specificity of the *Plasmodium falciparum* Glycosylphosphatidylinositol Biosynthetic Pathway and Inhibition by Species-Specific Suicide Substrates†

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ABSTRACT: The substrate specificities of the early glycosylphosphatidylinositol biosynthetic enzymes of *Plasmodium* were determined using substrate analogues of D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol (GlcN-PI). Similarities between the *Plasmodium* and mammalian (HeLa) enzymes were observed. These are as follows: (i) The presence and orientation of the 2′-acetamido/amino and 3′-OH groups are essential for substrate recognition for the de-*N*-acetylase, inositol acyltransferase, and first mannosyltransferase enzymes. (ii) The 6′-OH group of the GlcN is dispensable for the de-*N*-acetylase, inositol acyltransferase, all four of the mannosyltransferases, and the ethanolamine phosphate transferase. (iii) The 4′-OH group of GlcNAc is not required for recognition, but substitution interferes with binding to the de-*N*-acetylase. The 4′-OH group of GlcN is essential for the inositol acyltransferase and first mannosyltransferase. (iv) The carbonyl group of the natural 2-*O*-hexadecanyl ester of GlcN-(acyl)PI is essential for substrate recognition by the first mannosyltransferase. However, several differences were also discovered: (i) *Plasmodium*-specific inhibition of the inositol acyltransferase was detected with GlcN-[L]-PI, while GlcN-(2-*O*-alkyl)PI weakly inhibited the first mannosyltransferase in a competitive manner. (ii) The *Plasmodium* de-*N*-acetylase can act on analogues containing *N*-benzoyl, GalNAc, or βGlcNAc whereas the human enzyme cannot. Using the parasite specificity of the later two analogues with the known nonspecific de-*N*-acetylase suicide inhibitor [Smith, T. K., et al. (2001) *EMBO J. 20*, 3322−3332], Gal$NCONH_2$-PI and Glc$NCONH_2$-β-PI were designed and found to be potent (IC_{50} ∼0.2 μM), *Plasmodium*-specific suicide substrate inhibitors. These inhibitors could be potential lead compounds for the development of antimalaria drugs.

Plasmodium falciparum is a virulent human parasite, causing malaria in approximately 400 million people, resulting in over 2 million deaths worldwide annually, mainly in tropical and subtropical countries. These protozoa have been shown to synthesize glycosylphosphatidylinositol (GPI)[1] membrane anchors (*1*), forming their major glycoconjugates at the intraerythrocytic stage of their life cycle (*2*, *3*).

The structure and biosynthesis of GPI membrane anchors, which are ubiquitous to all eukaryotes, have been recently reviewed (*4*−*7*).

The conserved GPI core comprises $NH_2CH_2CH_2PO_4H$-6Manα1−2Manα1−6Manα1−4GlcNα1−6-D-*myo*-inositol-1-HPO_4-lipid (EtNP-Man_3GlcN-PI), where the lipid can be diacylglycerol, alkylacylglycerol, or ceramide. This minimal structure may be embellished with additional ethanolamine phosphate groups and/or carbohydrate side chains in a species- and tissue-specific manner (*8*).

Biosynthesis of GPIs in *Plasmodium* has been established using metabolic labeling (*1*) and detailed characterization of isolated and purified GPIs (*9*). The mature GPI anchor has been identified as a Man_4 core with one ethanolamine phosphate attached to the third mannose allowing linkage to protein. The diacylglycerol moiety of PI is slightly

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[1] Abbreviations: GPI, glycosylphosphatidylinositol; GlcN-PI, D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcN-β-PI, D-GlcNβ1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; 3-dGlcN-PI, 3-deoxy-D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; 4-dGlcN-PI, 4-deoxy-D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; 6-dGlcN-PI, 6-deoxy-D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcN4-Me-PI, 4-*O*-methyl-D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GalN-PI, D-GalNα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; ManN-PI, D-ManNα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcN-(2-*O*-methyl)-PI, D-GlcNα1−6-D-2-*O*-methyl-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcN-(2-*O*-octyl)-PI, D-GlcNα1−6-D-2-*O*-octyl-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcN-(2-*O*-hexadecyl)-PI, D-GlcNα1−6-D-2-*O*-hexadecyl-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcN-[L]-PI, D-GlcNα1−6-L-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcN-[L]-(2-*O*-methyl)-PI, D-GlcNα1−6-L-2-*O*-methyl-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; Glc$NCONH_2$-PI, 2-deoxy-2-ureido-D-Glcα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; Glc$NCONH_2$-β-PI, 2-deoxy-2-ureido-D-Glcβ1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; Gal$NCONH_2$-PI, 2-deoxy-2-ureido-D-Galα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcNBn-PI, D-GlcN(benzyl)α1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol.

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heterogeneous in the acyl chains but contains mainly C18:0 and C18:1 in the S_N1 and S_N2 positions, respectively. The third fatty acid is attached to the inositol at the 2-position and is specific, being either palmitate (90%) or myristate (10%) (*9*).

P. falciparum GPIs have been identified as pathogenic factors, able to activate host cell macrophages and induce inflammatory cyctokines (*10*−*13*).

Major cell surface proteins of *P. falciparum* merozoites MSP-1, MSP-2, and MSP-4 as well as functionally important proteins, i.e., transferrin receptor, serine protease, and heat shock proteins, are all GPI anchored (*14*−*18*). These observations suggest that GPI biosynthesis is vital for the parasite. This is supported by recent evidence that shows GPIs are synthesized in a stage-specific manner and are crucial for the development and survival of the parasite (*19*).

A *Plasmodium* cell-free system has been established and used previously to highlight differences of the parasite and host biosynthetic pathways (*20*, *21*). Inhibitors able to arrest the formation of GPI-anchored proteins on the plasma membrane of parasitic protozoa should prove useful in the development of antiparasitic agents. This notion has been validated, at least for *Trypanosoma 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 (*22*, *23*). Furthermore, glycosylinositolphospholipids (GIPLs) appear to be essential for the survival of *Leishmania* (*24*) and *Trypanosoma cruzi* (*25*).

The sequence of events underlying GPI biosynthesis has been studied in *T. brucei* (*26*−*34*), *T. cruzi* (*35*), *Toxoplasma gondii* (*36*), *P. falciparum* (*20*), *Leishmania* (*37*, *38*), *Saccharomyces cerevisiae* (*39*, *40*), and mammalian cells (*41*−*43* and references cited therein). In all cases, GPI biosynthesis involves the addition of GlcNAc to phosphatidylinositol (PI) to give GlcNAc-PI, which is then de-N-acetylated to form GlcN-PI (*44*−*47*). De-N-acetylation is a prerequisite for the formation of later GPI intermediates (*48*, *49*). Inositol acylation (the transfer of fatty acid to the 2-OH group of the D-*myo*-inositol residue) of GlcN-PI precedes mannosylation, for both mammalian and *Plasmodium* pathways (*20*, *32*, *50*). The donor for this acylation in both cases is acyl-CoA; however, there is some confusion as to the acyl donor in some mammalian cell lines (*50*, *51*). Notable differences between the pathways occur during the dolichol phosphate-mannose-dependent chain elongation of the mannose chain (*30*, *52*). For example, the addition of extra ethanolamine phosphate groups to mammalian GPI anchors (*41*, *42*) and the addition of a fourth mannose in the case of the *Plasmodium* pathway (*1*, *20*) must occur so that a mature GPI anchor can be attached to protein via the transamidase complex (*53*).

Differences in the *T. brucei* and HeLa GPI biosynthetic pathways have previously been exploited, leading to the discovery of the first generation of specific inhibitors of the parasite GPI biosynthetic pathway *in vitro* (*32*, *33*). Another series of parasite-specific GPI pathway inhibitors containing L-*myo*-inositol inhibited *T. brucei* MT-1 (*54*), whereas a terpenoid natural product inhibited yeast and human, but not parasite, GPI biosynthesis (*55*). More recently, the first mechanism-based suicide inhibitor of GPI biosynthesis was described (*56*).

In order to discover inhibitors able to discriminate between host and parasite GPI biosynthetic pathways, it is vital to probe the specificity of these enzymes. In this paper we have used the *Plasmodium* cell-free system with an army of GlcNAc-PI and GlcN-PI analogues to elucidate the substrate specificities of the early biosynthetic enzymes. By exploiting differences between the *Plasmodium* and mammalian enzymes, we were able to produce *Plasmodium*-specific suicide substrate inhibitors.

MATERIALS AND METHODS

Materials. GDP-[2-^{3}H]mannose (14.9−17.8 Ci/mmol), UDP-[6-^{3}H]GlcNAc (38.5 Ci/mmol), and [^{3}H]acetic anhydride (50.0 Ci/mmol) were purchased from NEN, jack bean α-mannosidase, *Aspergillus pheonicus* α-mannosidase, and glycosylphosphatidylinositol-specific phospholipase D were from Roche, and *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C was from Glyko. EA-Wax was obtained from EABiotech Ltd. All other reagents were purchased from Merck-BDH or Sigma.

Substrates and Substrate Analogues. D-GlcNα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcN-PI) and D-GlcNα1−6-L-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcN-[L]-PI) were synthesized as previously described (*57*). D-GlcNα1−6-D-2-*O*-methyl-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcN-(2-*O*-methyl)-PI), D-GlcNα1−6-D-2-*O*-octyl-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcN-(2-*O*-octyl)-PI), and D-GlcNα1−6-D-2-*O*-hexadecyl-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcN-(2-*O*-hexadecyl)-PI) were obtained as previously described (*58*, *59*). D-GlcNα1−6-L-2-*O*-methyl-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcN-[L]-(2-*O*-methyl)-PI) was prepared in a manner similar to that of the corresponding D-*myo*-inositol analogue (*58*). 3-Deoxy-D-GlcNα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (3-dGlcN-PI), 4-deoxy-D-GlcNα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoyl-glycerol (4-dGlcN-PI), 6-deoxy-D-GlcNα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (6-dGlcN-PI), and 4-*O*-methyl-D-GlcNα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcN4Me-PI) were synthesized as described recently (*60*, *61*). The preparation of D-GalNα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GalN-PI), D-ManNα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (ManN-PI), and D-GlcNβ1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcN-β-PI) will be published elsewhere (Crossman and Brimacombe, unpublished data). *N*-Acetyl derivatives of all of the above compounds were prepared by standard procedures (*48*).

Preparation of GlcNR-PI Substrate Analogues. The GlcNR-PI substrate analogues D-GlcNBzα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcNBz-PI), D-GlcNBnα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcNBn-PI), 2-deoxy-2-ureido-D-Glcα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcNCONH$_2$-PI), 2-deoxy-2-ureido-D-Glcβ1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcNCONH$_2$-β-PI), and 2-deoxy-2-ureido-D-Glcα1−6-D-2-*O*-octyl-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GlcNCONH$_2$-(2-*O*-octyl)-PI) were prepared and purified as described previously (*56*). 2-Deoxy-2-ureido-D-Galα1−6-D-*myo*-inositol-1-HPO$_4$-*sn*-1,2-dipalmitoylglycerol (GalNCONH$_2$-PI) was prepared in a manner

similar to that of GlcNCONH$_2$-PI (*56*) but using GalN-PI instead of GlcN-PI.

The identity and purity of each of the synthetic substrate analogues were assessed by negative ion electrospray mass spectrometry, and the concentration of each stock solution was ascertained by measuring the inositol content by selected ion monitoring gas chromatography−mass spectrometry (*48*, *62*).

N-[^{3}H]Acetylation and Purification of the Substrate Analogues. Synthetic GlcN-PI and analogues thereof were N-acetylated with [^{3}H]acetic anhydride as described previously (*60*). The GlcN[^{3}H]Ac-PI analogues were diluted with the corresponding nonradioactive compound to give a final specific activity of 18.2 μCi/mmol.

Preparation of P. falciparum Membranes. Plasmodium falciparum strain FCBR was maintained as described previously (*63*). The development and multiplication of *Plasmodium* cultures were followed by microscopic evaluation of Giemsa-stained smears. Parasite cultures were routinely checked for contamination with *Mycoplasma*.

Parasites (30−40 h after invasion) were washed and harvested by lysis with saponin (*1*) and lysates prepared as previously described (*26*). The membranes were collected by centrifugation (15000*g*, 10 min, 4 °C), washed in buffer containing 100 mM NaHepes (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM TLCK, and 1 μg/mL leupeptin, and stored in the same buffer containing 20% (v/v) glycerol at −80 °C and used within 3 months.

De-N-acetylase Assay. Membranes were washed and suspended in wash buffer supplemented with 50 mM $MnCl_2$, 1 mM DTT, 100 μM CoA, and the ATP regenerating system (100 μM ATP, 5 mM phosphocreatine, and 5 units of creatine phosphokinase), unless stated otherwise. Aliquots of 100 μL (2×10^8 cell equivalents) were added to reaction tubes containing 1.5 nmol (30000 cpm) of GlcN[^{3}H]Ac-PI or a substrate analogue, sonicated briefly, and incubated at 35 °C. The reactions were terminated at various time intervals (0, 15, 30, and 60 min for initial rate determination and 0, 15, 30, 60, 90, and 120 min for inhibition studies) by the addition of 50 μL of propan-1-ol, followed by vortexing and snap-freezing. Each GlcN[^{3}H]Ac-PI substrate analogue was studied in triplicate at each time interval. Samples were processed and counted for radioactivity as described previously (*56*).

Mannosylation Assay. Membranes were washed and suspended in wash buffer supplemented with 50 mM $MnCl_2$, 1 mM DTT, 100 μM CoA, and the ATP regenerating system, unless stated otherwise, sonicated briefly, and added to dry GDP-[^{3}H]Man (1 μCi per 1×10^8 cell equivalents). After brief sonication, aliquots of 100 μL (1×10^8 cell equivalents; 2.73 mg of total membrane protein, 2.41 mg of phospholipid phosphorus) were added to reaction tubes containing 5 μL of the GlcN-PI analogue in 10 mM *n*-octyl β-D-glucopyranoside or 1 mM UDP-GlcNAc, sonicated, and thereafter incubated at 35 °C for 90 min. The glycolipid products were extracted twice in chloroform/methanol/water (10/10/3, v/v), recovered from a butan-1-ol partitioning, and analyzed by HPTLC using solvent system A, both before and after enzymatic or chemical treatments.

Endogenous Priming Assay. Membranes were washed and suspended in wash buffer supplemented with 50 mM $MnCl_2$, 1 mM DTT, and 100 μM Mys-CoA, unless stated otherwise, sonicated briefly, and added to dry UDP-[^{3}H]GlcNAc (2 μCi per 1×10^8 cell equivalents). After brief sonication, aliquots of 100 μL (1×10^8 cell equivalents) were incubated at 35 °C for 90 min. The glycolipid products were extracted as above and analyzed by HPTLC using solvent system B.

Inhibition Studies. Inhibition assays were conducted as described in the above assays, except that the membranes were preincubated with potential inhibitors at various concentrations for 5 min prior to being added to GlcN-PI, GlcNAc-PI, GlcN[^{3}H]Ac-PI, or UDP-[^{3}H]GlcNAc. Inhibition of the early steps of the pathway involved 20 mM *N*-ethylmaleimide (NEM), 20 mM *N*-phenylmaleimide (NPM) dissolved in MeOH, 20 mM iodoacetamide (IA), 1 mM phenylmethanesulfonyl fluoride (PMSF) dissolved in dry propan-2-ol, and 1 mM diisopropyl fluorophosphate (DFP).

Preparation of HeLa Membranes. HeLa cell membranes (cell-free system) were prepared as described previously (*32*, *48*).

HeLa Cell-Free System Assays. HeLa cell lysate was thawed and supplemented as previously described (*56*), in brief, with 2.5 mM $MnCl_2$, 2 mg/mL leupeptin, 0.1 mM TLCK, 1 μg/mL tunicamycin, 1 μM DTT, 100 mM CoA, and the ATP regenerating system (100 μM ATP, 5 mM phosphocreatine, and 5 units of creatine phosphokinase), and added to dry GDP-[^{3}H]Man (1 μCi per 1×10^8 cell equivalents). After brief sonication, aliquots of 100 μL were added to reaction tubes containing the GlcN-PI analogue and incubated at 35 °C for 1.5 h. The glycolipid products were recovered and analyzed by HPTLC as described above. Inhibition studies were performed in exactly the same manner except that the membranes were preincubated with the potential inhibitors for 5 min prior to being added to GlcN-PI or GlcNAc-PI.

HPTLC. Samples and glycolipid standards were applied to 10 cm aluminum-backed silica gel 60 HPTLC plates which were developed using either solvent system A, chloroform/methanol/water (4/4/1 v/v), or solvent system B, chloroform/methanol/water (10/10/3 v/v). Radiolabeled components were detected by fluorography at −70 °C after being soaked in EA-Wax and using Kodak XAR-5 film with an intensifying screen.

Enzymatic and Chemical Treatments of Radiolabeled Glycolipids. Digestions with jack bean α-mannosidase (JBαM), *A. pheonicus* α-mannosidase (APαM), glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD), and phosphatidylinositol-specific phospholipase C (PI-PLC), base hydrolysis, deamination, and N-acetylation were performed as previously described (*32*, *48*).

RESULTS

Priming the Plasmodium GPI Biosynthetic Pathway with Exogenous Substrates. Washed *P. falciparum* membranes (cell-free system) were incubated with GDP-[^{3}H]Man in the presence or absence of various compounds. With only GDP-[^{3}H]Man present (Figure 1A, lane 1), endogenous dolichol phosphate-[^{3}H]mannose (DPM) is formed, because of the endogenous dolichol phosphate-mannose synthetase, together with two endogenous GPI glycolipids (aM4 and E-aM4). The addition of UDP-GlcNAc (Figure 1A, lane 4) results in the formation of additional [^{3}H]mannosylated glycolipids (aM1-4 and E-aM3-4), previously identified as Pf η, ϵ, δ, γ, α, and β, respectively (*1*, *20*). These have been formed by utilizing

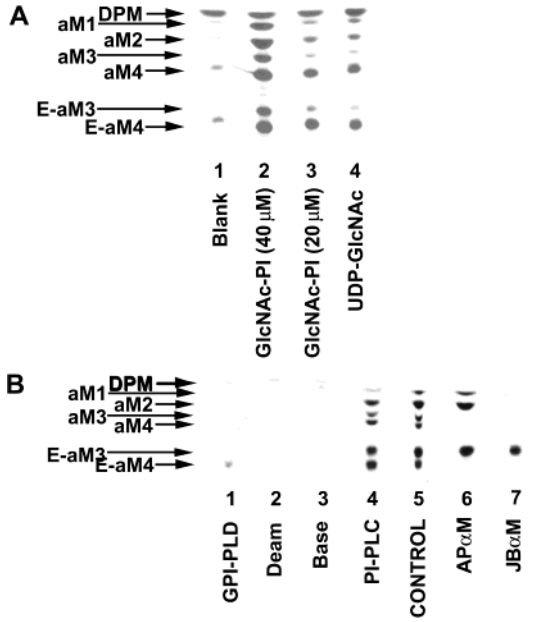

FIGURE 1: Priming of the *Plasmodium* cell-free system with endogenous and exogenous acceptors. (A) Washed parasite membranes were incubated with GDP-[^{3}H]Man alone (lane 1), with GlcNAc-PI (lanes 2 and 3) at the indicated concentrations, or with UDP-GlcNAc (lane 4). [^{3}H]Man-labeled glycolipids were extracted and analyzed by HPTLC using solvent system A and fluorography. (B) Characterization of the [^{3}H]Man-labeled glycolipids formed from exogenous acceptor GlcNAc-PI. The [^{3}H]mannosylated glycolipids were subjected to GPI-PLD, PI-PLC, APαM, and JBαM digestions, base hydrolysis, and deamination. Products that partitioned into butan-1-ol were analyzed by HPTLC using solvent system A and fluorography. The identities of the bands are Dol-P-Man (DPM), Man_{1-4}-GlcN-(acyl)PI (aM1−aM4), EtN-P-Man_3-GlcN-(acyl)PI (E-aM3), and EtN-P-Man_4-GlcN-(acyl)PI (E-aM4).

the action of UDP-GlcNAc:PIα1−6GlcNAc transferase and endogenous PI to form GlcNAc-PI which is subsequently processed further. The conditions were optimized, for the addition of the exogenous acceptor GlcNAc-PI (Figure 1A, lanes 2 and 3) resulted in the formation of additional [^{3}H]-mannosylated glycolipids, compared to the blank (Figure 1A, lane 1), whose R_f values are similar to those formed with UDP-GlcNAc. The level of the exogenously formed [^{3}H]-mannosylated glycolipids increased with the doubling of the concentration of exogenously added GlcNAc-PI.

Preincubation of the cell-free system with amphomycin and $CaCl_2$ prevented the formation of Dol-P-[^{3}H]Man from GDP-[^{3}H]Man and prevented the formation of the [^{3}H]-mannosylated intermediates (data not shown). This suggests that at least the first mannosyltransferase is Dol-P-Man dependent, consistent with other GPI pathways where all of the mannosyltransferases have DPM as their Man donor (*30*, *52*).

Analysis of the [^{3}H]mannosylated glycolipids formed from the exogenous acceptor GlcNAc-PI (Figure 1A, lanes 2 and 3) revealed them all to be sensitive to GPI-PLD (Figure 1B, lane 1), deamination (Figure 1B, lane 2), and base treatment (Figure 1B, lane 3) but resistant to PI-PLC (Figure 1B, lane 4). This indicates that the [^{3}H]glycolipids are inositol-acylated GPI anchors that have a diacylglycerol PI anchor and contain a non-N-acetylated glucosamine component. All of the [^{3}H]-glycolipids except E-aM3 were sensitive to JBαM (Figure 1B, lane 7), indicating that they have at least one terminal αMan group. Of these, only glycolipids aM3, aM4, and

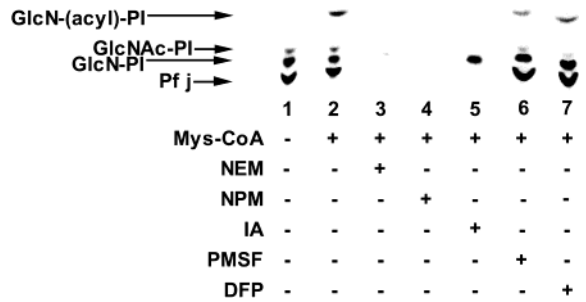

FIGURE 2: Inhibition of the early plasmodial GPI biosynthetic enzymes: UDP-GlcNAc transferase and inositol acyltransferase. Washed *Plasmodium* membranes were incubated with UDP-[^{3}H]-GlcNAc, either alone (lane 1) or in the presence of Mys-CoA (lanes 2−7) after preincubation with either 20 mM *N*-ethylmaleimide (NEM) (lanes 3), 20 mM *N*-phenylmaleimide (NPM) (lane 4), 20 mM iodoacetamide (IA) (lane 5), 1 mM phenylmethanesulfonyl fluoride (PMSF), (lane 6), or 1 mM diisopropyl fluorophosphate (DFP) (lane 7). [^{3}H]GlcN-labeled glycolipids were extracted and analyzed by HPTLC using solvent system B and fluorography. The identities of the bands are given on the left of the chromatogram as GlcN-PI, GlcNAc-PI, GlcN-(acyl)PI, and Pf j.

E-aM4 were sensitive to APaM (Figure 1B, lane 6), suggesting that they have at least one terminal Manα1−2Man group. The [^{3}H]mannosylated glycolipids were analyzed further by headgroup analysis. Neutral glycan headgroups were obtained from the HPTLC purified glycolipids, deacylated, deaminated, reduced, dephosphorylated, and desalted by passage through AG50 × 12 (H^+) and AG3 × 4 (OH^-) before being analyzed by Bio-Gel P4 gel filtration. The neutral [^{3}H]glycans obtained from aM1, aM2, aM3, aM4, E-aM3, and E-aM4 eluted from the column at 2.2, 3.1, 4.1, 5.2, 4.1, and 5.1 GU, respectively, consistent with previous assignments of the endogenous intermediates (*62*). The R_f values of the [^{3}H]mannosylated glycolipids from the exogenous acceptor (Figure 1A, lanes 2 and 3) coincided with those of the endogenously primed glycolipids (Figure 1A, lane 4), suggesting not only that they have the same mannosylated glycan headgroup but also that the total lipid hydrophobicity of the endogenous glycolipids must be similar to those formed from the exogenous synthetic D-GlcNAcα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol (C16:0).

Priming the endogenous pathway with UDP-[^{3}H]GlcNAc in the presence of DTT (Figure 2A, lane 1), resulted in the formation of three [^{3}H]GlcN glycolipids. [^{3}H]GlcNAc-PI and [^{3}H]GlcN-PI were formed from the UDP-GlcNAc:PI GlcNAc transferase and the de-*N*-acetylase while the third [^{3}H]GlcN glycolipid, Pf j, has recently been identified as a glycosphingolipid (*64*). The addition of myristoyl-CoA (Mys-CoA) (Figure 2A, lane 2) causes the formation of a new [^{3}H]GlcN glycolipid, GlcN-(acyl)PI, by virtue of the inositol acyltransferase which, like that of the mammalian pathway, is acyl-CoA dependent and acts prior to mannosylation (*41*, *51*).

The substitution of DTT with sulfhydryl alkylating agents such as *N*-ethylmaleimide (NEM) or *N*-phenylmaleimide (NPM) in the presence of UDP-[^{3}H]GlcNAc and Mys-CoA (Figure 2, lanes 3 and 4, respectively) prevented the formation of any [^{3}H]GlcN glycolipids compared with controls (Figure 2, lanes 1 and 2), suggesting that, like other GPI biosynthetic pathways, the UDP-GlcNAc:PI GlcNAc transferase complex is inhibited by sulfhydryl alkylating agents due to modification of an active site Cys residue (*45*).

Surprisingly, the alkylating agent iodoacetamide did not inhibit the formation of [^{3}H]GlcNAc-PI or its subsequent de-N-acetylation to form [^{3}H]GlcN-PI (Figure 2, lane 5). However, inositol acylation of [^{3}H]GlcN-PI is completely inhibited by iodoacetamide compared to that of the control (Figure 2, lane 2). Thus, whereas iodoacetamide is unable to inhibit the GlcNAc transferase, it does inhibit the inositol acyltransferase, suggesting that this enzyme also contains an active site Cys residue(s). Supporting evidence for this is that, in the presence of NEM, exogenous GlcN-PI with either acyl-CoA or CoA with an ATP regenerating system and GDP-[^{3}H]Man resulted in no exogenously [^{3}H]mannosylated glycolipids (data not shown). All further experiments were preformed in the presence of DTT to allow inositol acylation of GlcN-PI substrate analogues to take place prior to mannosylation.

No inhibition was observed of the GlcNAc transferase, de-*N*-acetylase, or the inositol acyltransferase in this system in the presence of the serine protease inhibitors PMSF and DFP (Figure 2, lanes 6 and 7).

Interestingly, the formation of Pf j from UDP-[^{3}H]GlcNAc, identified as a glycosphingolipid (*64*), is also inhibited by sulfhydryl alkylating agents but not serine protease inhibitors (Figure 2, lanes 3−7).

Acceptor Substrate Specificity of the Plasmodium Inositol Acyltransferase and Mannosyltransferase. The cell-free system was incubated with GlcN-−PI and various GlcN-PI analogues (Figure 3). In all cases, the membranes produced labeled DPM and low levels of endogenous GPI intermediates, i.e., aM4 and E-aM4 (Figure 3A, lane 1; Figure 3B, lane 1; Figure 3C, lane 1). The only acceptors that produced additional [^{3}H]mannosylated products were GlcN-PI (Figure 3A, lane 2; Figure 3B, lane 3; Figure 3C, lane 2), GlcNAc-PI (Figure 3B, lanes 4 and 7; Figure 3C, lane 3), and 6-dGlcN-PI and 6-dGlcNAc-PI (Figure 3B, lanes 8 and 9, respectively). This suggests that these analogues are substrates for the de-*N*-acetylase, inositol acyltransferase, all four mannosyltransferases, and, ultimately, the ethanolamine phosphate transferase.

The substrate channeling between the de-*N*-acetylase and the first mannosyltransferase observed in *T. brucei* and *Leishmania major* (*37*, *48*) is not observed in the *Plasmodium* cell-free system. There is little or no difference in the level of priming of either GlcN-PI or GlcNAc-PI (Figure 3B, lanes 3 and 4, respectively) or 6-dGlcN-PI and 6-dGlcNAc-PI (Figure 3B, lanes 8 and 9, respectively). This is similar to the HeLa pathway whereby GlcN-PI must be inositol-acylated prior to mannosylation, preventing substrate channeling between the de-*N*-acetylase and first mannosyltransferase.

The following GlcN-PI analogues showed no additional [^{3}H]mannosylated glycolipids to the endogenous DPM, aM4, and E-aM4 (Figure 3A, lane 1) and are thus not substrates for either or both of the inositol acyltransferase and first mannosyltransferase: Glc-PI, 2-dGlc-PI, 3-dGlcN-PI, 4-dGlcN-PI, GlcN4Me-PI, GlcN(Ac)-(2-*O*-methyl)-PI, GlcN(Ac)-(2-*O*-octyl)-PI, GlcN(Ac)-(2-*O*-hexadecyl)-PI (Figure 3A, lanes 3−13, respectively), GlcN-β-PI, GlcN-[L]-PI (Figure 3B, lanes 5 and 6), GalN(Ac)-PI, and ManN(Ac)-PI (Figure 3C, lanes 4−7, respectively). Delipidated fragments of GlcN-PI, GlcN-Ino-*P*-gly and GlcN-Ino, were also tested as acceptors, but neither were [^{3}H]mannosylated (data not shown). Lipid variants of GlcN-PI were also tested in the *Plasmodium* cell-free system but were found to be very poorly processed compared with the synthetic dipalmitoylated GlcN-PI analogues used in this study (data not shown). This phenomenon may be associated to the different properties of *Plasmodium* membranes, with respect to insertion of exogenous phospholipids. However, it is a reasonable assumption that the lipid phosphodiester is an essential structural feature of substrates for the *Plasmodium* GPI pathway, as it is for other systems (*48*, *56*).

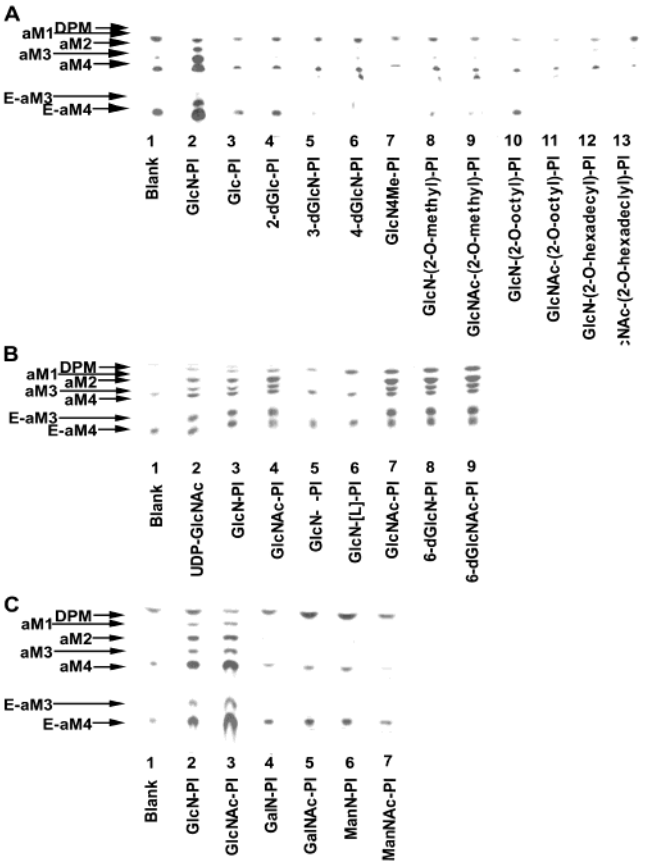

FIGURE 3: Substrate specificity of the *Plasmodium* mannosyltransferase. (A) Washed parasite membranes were incubated with GDP-[^{3}H]Man alone (lane 1) or with 30 μM GlcN-PI (lane 2) or analogues thereof (lanes 3−13). (B) Washed parasite membranes were incubated with GDP-[^{3}H]Man either alone (lane 1) or with 500 μM UDP-GlcNAc (lane 2) or 30 μM GlcN-PI (lane 3), GlcNAc-PI (lanes 4 and 7), GlcN-β-PI (lane 5), GlcN-[L]-PI (lane 6), 6-dGlcN-PI (lane 8), or 6-dGlcNAc-PI (lane 9). (C) Washed parasite membranes were incubated with GDP-[^{3}H]Man either alone (lane 1) or with 30 μM GlcN-PI (lane 2), GlcNAc-PI (lane 3), GalN-PI (lane 4), GalNAc-PI (lane 5), ManN-PI (lane 6), or ManNAc-PI (lane 7). All [^{3}H]Man-labeled glycolipids were extracted and analyzed by HPTLC using solvent system A and fluorography. The identities of the bands indicated on the left of the chromatogram are given in Figure 1.

Inhibition of the Plasmodium Inositol Acyltransferase and Mannosyltransferase by GlcN-PI Substrate Analogues. Those GlcN-PI analogues that were not substrates for the *Plasmodium* GPI pathway were tested as inhibitors against the [^{3}H]-mannosylation of exogenously added GlcN-PI. No significant inhibition was observed by preincubation with Glc-PI, 3-dGlcN-PI, GlcN4Me-PI, GalN-PI, ManN-PI, and GlcN-β-PI (Figure 4A, lanes 6, 7, and 9−12, respectively) when compared with the control (Figure 4A, lane 3). The processing of exogenously added GlcN-PI was inhibited by

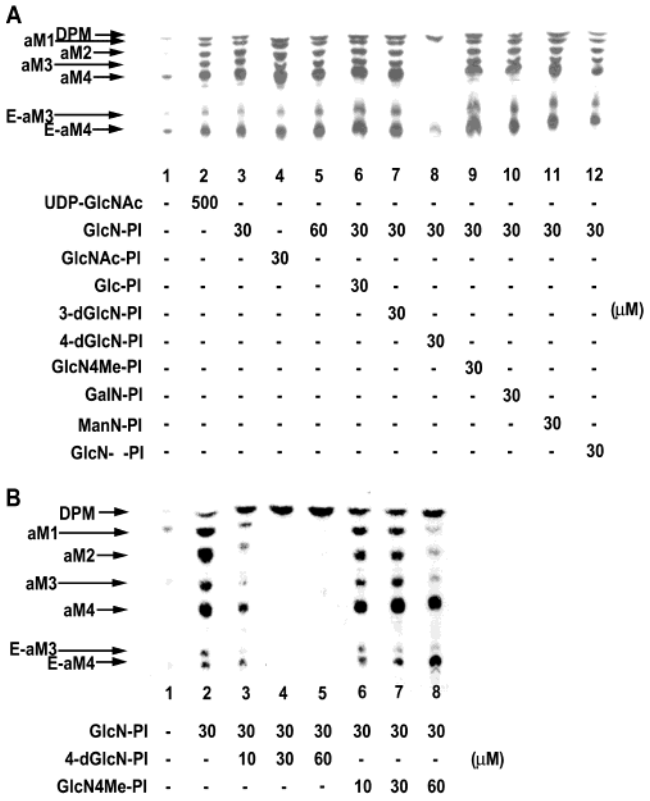

FIGURE 4: Inhibition of the plasmodial cell-free system by GlcN-PI analogues. (A) Washed membranes were incubated with GDP-[^{3}H]Man alone (lane 1) or with 30 μM GlcN-PI after preincubation with GlcN-PI analogues (lanes 5−12). (B) Washed membranes were incubated with GDP-[^{3}H]Man alone (lane 1) or with GlcN-PI before (lane 2) or after preincubation with 4-dGlcN-PI (lanes 3−5) or GlcN4Me-PI (lanes 6−8) followed at the indicated concentrations. [^{3}H]Man-labeled glycolipids were extracted and analyzed by HPTLC using solvent system A and fluorography. The identities of the bands indicated on the left of the chromatogram are given in Figure 1.

4-dGlcN-PI (Figure 4A, lane 8). This inhibitory effect cannot be ascribed to nonspecific effects resulting from increased concentration of synthetic lipid in the system, since GlcN-PI at 60 μM (Figure 4A, lane 4) still resulted in good priming of the pathway.

The observed inhibition with 4-dGlcN-PI was repeated at various concentrations (Figure 4B, lanes 3−5), and an IC_{50} of ~10 μM was estimated. The closely related GlcN4Me-PI analogue was also titrated against GlcN-PI (Figure 4B, lanes 6−8), with an estimated IC_{50} of ~60 μM, showing it to be a weaker inhibitor. This implies that the additional methoxy group at the 4′-position of GlcNAc interferes with the binding to the active site. This inhibition may be caused by the inhibitor analogues binding to and blocking the inositol acyltransferase, Alternatively, these analogues may be inositol-acylated, and these products may bind and inhibit the mannosyltransferase.

Inhibition of the Plasmodium Mannosyltransferase by GlcN-(2-O-alkyl)PI and GlcN-[L]-PI Substrate Analogues. As the GlcN-(2-*O*-alkyl)PI analogues are not substrates for the *Plasmodium* GPI pathway (Figure 3), they were assessed as to whether they might be inhibitors of *Plasmodium* GPI biosynthesis, as they are not substrates (Figure 3A); these analogues were preincubated with the cell-free system prior to addition of exogenous GlcN-PI (Figure 5A). All three analogues (GlcN-(2-*O*-methyl)PI, GlcN-(2-*O*-octyl)PI, and

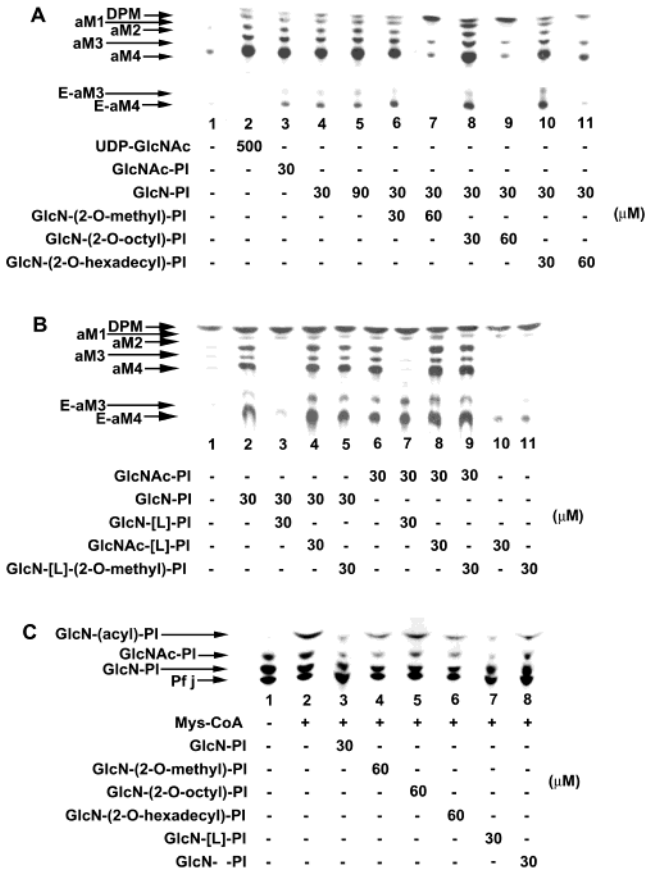

FIGURE 5: Inhibition of the plasmodial cell-free system by GlcN-PI analogues with modified *myo*-inositol. (A) Washed membranes were incubated with GDP-[^{3}H]Man alone (lane 1) or with UDP-GlcNAc-PI, GlcNAc-PI (lane 3), GlcN-PI (lanes 4 and 5), or GlcN-PI after preincubation with GlcN-(2-*O*-alkyl)-PI analogues (lanes 6−11) at the indicated concentration. (B) Washed membranes were incubated with GDP-[^{3}H]Man alone (lane 1) or with GlcN-PI (lane 2), GlcNAc-PI (lane 6), GlcN-[L]-PI (lane 10), GlcN-[L]-(2-*O*-methyl)-PI (lane 12), or GlcN-PI or GlcNAC-PI after preincubation of GlcN-[L]-PI analogues (lanes 3−5 and 7−9) at the indicated concentration. [^{3}H]Man-labeled glycolipids were extracted and analyzed by HPTLC using solvent system A and fluorography. The identities of the bands indicated on the left of the chromatogram are given in Figure 1. (C) Washed membranes were incubated with UDP-[^{3}H]GlcNAc alone (lane 1) or in the presence of Mys-CoA (lanes 2−8) with preincubation with either GlcN-PI (lane 3) or GlcN-(2-*O*-alkyl)-PI analogues (lanes 4−6), GlcN-[L]-PI (lane 7), or GlcN-β-PI (lane 8) at the indicated concentrations. [^{3}H]GlcN-labeled glycolipids were extracted and analyzed by HPTLC using solvent system B and fluorography. The identities of the bands are given on the left of the chromatogram as GlcN-PI, GlcNAc-PI, GlcN-(acyl)PI, and Pf j.

GlcN-(2-*O*-hexadecyl)PI) showed the same effect. At an equal molar ratio to GlcN-PI (30 μM), they showed little or no inhibition (Figure 5A, lanes 6, 8, and 10). However, at twice the molar ratio (60 μM) significant inhibition was observed (Figure 5A, lanes 7, 9, and 11). This inhibition is not due to some effect of increased synthetic lipid concentration, as GlcN-PI at 90 μM (Figure 5A, lane 5) results in priming of the pathway comparable with that at 30 μM (Figure 5A, lane 4). Thus, inhibition may be either at the level of inositol acylation or mannosylation. To elucidate this, inositol acylation in the absence and presence of GlcN-(2-*O*-alkyl)PI analogues was investigated (Figure 5C). This was achieved by preincubation of the cell-free system with 60 μM GlcN-(2-*O*-alkyl)PI analogues prior to incubation with

UDP-[^{3}H]GlcNAc and Mys-CoA (Figure 5C, lanes 4−6). The level of [^{3}H]GlcN-(acyl)PI formed was comparable with the control (lane 2) and clearly visible above the negative control without Mys-CoA (Figure 5C, lane 1). These data suggest that GlcN-(2-*O*-alkyl)PI analogues do not interfere with inositol acylation of GlcN-PI and are therefore probably weakly inhibiting the first mannosyltransferase, probably in a competitive manner, by interacting with the GlcN-(acyl)-PI binding site.

The nonacceptor activity of GlcN-[L]-PI prompted the possibility that it may act as a competitive inhibitor, as in *T. brucei* (*54*). Various GlcN-[L]-PI analogues were preincubated with the membranes prior to priming with either exogenous GlcN-PI (Figure 5B, lanes 3−6) or GlcNAc-PI (Figure 5B, lanes 7−9). GlcNAc-[L]-PI and GlcN-[L-(2-*O*-methyl)-PI were not substrates (Figure 5B, lanes 10 and 11, respectively) and showed no inhibition of the de-N-acetylation, inositol acylation, or mannosylation of either GlcN-PI or GlcNAc-PI (Figure 5B, lanes 4, 5 and 8, 9, respectively) when compared with the controls (Figure 5B, lanes 2 and 6). GlcN-[L]-PI caused significant, but incomplete, inhibition of the processing of GlcNAc-PI (Figure 5B, lane 7) but complete inhibition of the processing of GlcN-PI (lane 3). The approximate IC_{50} for the inhibition of GlcN-PI processing by GlcN-[L]-PI is 10 μM (data not shown). Thus, the addition of either an *N*-acetyl group or methyl group on the 2-OH of the *myo*-inositol renders GlcN-[L]-PI incapable of inhibition. Inhibition was not observed with various moieties of GlcN-[L]-PI; these included [L]-PI, GlcN-[L]-Ino-*P*-gly, and GlcN-[L]-Ino (data not shown). This suggests that the mode of inhibition in this system is different from that observed previously for the *T. brucei* GPI pathway (*54*). In this *Plasmodium* system there must be specific recognition of the whole GlcN-[L]-PI molecule, at either the inositol acylation and/or mannosylation step, for inhibition to take place. This was investigated by assessing inositol acylation in the absence and presence of GlcN-[L]-PI (Figure 5C). This was achieved by preincubation of the cell-free system with GlcN-[L]-PI prior to incubation with UDP-[^{3}H]GlcNAc and Mys-CoA (Figure 5C, lane 7). The level of [^{3}H]GlcN-(acyl)-PI formed was significantly reduced compared with the control (Figure 5C, lane 2), similar to that observed in the presence of a competing acceptor, e.g., GlcN-PI (Figure 5C, lane 3). These data suggest that GlcN-[L]-PI is either competitively inhibiting the inositol acyltransferase, preventing the acylation of GlcN-PI, or being inositol-acylated itself and is subsequently interacting with the first mannosyltransferase, probably in a competitive manner in the GlcN-(acyl)-PI active site.

Substrate Specificity of the Plasmodium De-N-acetylase Using GlcNR-PI Analogues. The substrate specificity of the de-*N*-acetylase with respect to the nature of the acyl group (R) released from GlcNR-PI analogues has been studied previously in the *T. brucei* system by means of a coupled assay (*49*, *56*). This study employs a similar assay, such that their subsequent inositol acylation and [^{3}H]mannosylation (Figure 6) measure the de-N-acetylation of GlcNR-PI analogues. The substrate specificity of the *Plasmodium* de-*N*-acetylase is similar to those previously determined for the trypanosomal and HeLa de-*N*-acetylases. They are active when R is acetyl or propionyl, barely active when R is butyryl, and inactive as a substrate when R is pentanoyl.

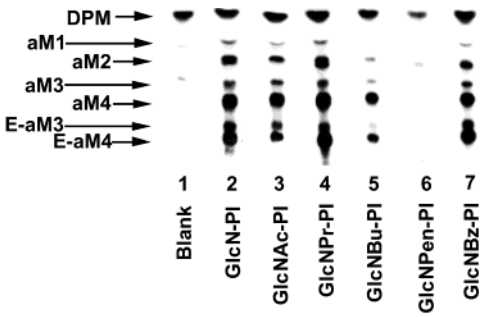

FIGURE 6: Substrate specificity of the *Plasmodium* de-*N*-acetylase using GlcNR-PI analogues. Washed parasite membranes were incubated with GDP-[^{3}H]Man either alone (lane 1) or with 30 μM GlcN-PI (lane 2), GlcNAc-PI (lane 3), GlcNPr-PI (lane 4), GlcNBu-PI (lane 5), GlcNPen-PI (lane 6), or GlcNBz-PI (lane 7). [^{3}H]Man-labeled glycolipids were extracted and analyzed by HPTLC using solvent system A and fluorography. The identities of the bands indicated on the left of the chromatogram are given in Figure 1.

Table 1: Release of [^{3}H]Acetate from GlcN[^{3}H]Ac-PI Substrate Analogues by the *Plasmodium* and HeLa Cell-Free Systems

	Plasmodium		HeLa[c]	
analogue	initial rate[a]	%[b]	initial rate[a]	%[b]
GlcNAc-PI	0.89	100	0.75	100
3-dGlcNAc-PI	0.00	0	0.00	0
4-dGlcNAc-PI	0.85	95	0.69	92
6-dGlcNAc-PI	0.93	105	0.90	120
GlcNAc4Me-PI	0.33	37	0.15	20
ManNAc-PI	0.00	0	0.00	0
GalNAc-PI	0.61	68	0.00	0
GlcNAc-β-PI	0.30	34	0.00	0
GlcNAc-[L]-PI	0.04	4	0.00	0
GlcNAc-[L]-(2-*O*-methyl)-PI	0.03	3	0.01	1
GlcNAc-(2-*O*-methyl)-PI	0.04	4	0.00	0
GlcNAc-(2-*O*-octyl)-PI	0.04	4	0.00	0
GlcNAc-(2-*O*-hexadecyl)-PI	0.01	1	0.00	0

[a] The initial reaction rates [pmol of [^{3}H]acetate released min^{-1} (mg of protein)$^{-1}$] were estimated from the initial linear range of [^{3}H]acetate release. Each time point was measured in triplicate, and measurements were within ±4% of the mean value. Background levels of non-GPI-specific de-N-acetylation (3% and 1% for the *Plasmodium* and HeLa systems, respectively) were estimated from the initial rates of [^{3}H]acetate released from GlcN[^{3}H]Acα1-S-C_8, 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. [b] Initial reaction rate relative to that for GlcNAc-PI (100%). [c] HeLa data set previously determined (*56*).

Surprisingly, the *Plasmodium* de-*N*-acetylase, like that of the *T. brucei*, can remove a benzoyl group as in GlcNBz-PI (Figure 6, lane 7) whereas the HeLa enzyme cannot (*56*).

Comparison of the Specificity of the Plasmodium and HeLa GlcNAc-PI De-N-acetylases. Optimization of the conditions for the de-N-acetylation of GlcNAc-PI was found to include CoA and ATP, allowing inositol acylation of GlcN-PI. Various GlcNAc-PI analogues were tested as substrates (Table 1) for the *Plasmodium* de-*N*-acetylase, and initial rates were compared with those previously determined for the HeLa enzyme (*56*). The relative rates of de-N-acetylation of the natural substrate GlcNAc-PI are comparable, with the *Plasmodium* enzyme slightly more active.

Similar to the HeLa enzyme, the deoxy compounds 4d- and 6d-GlcNAc-PI were de-N-acetylated at rates comparable to those of the natural substrate GlcNAc-PI, suggesting that the 4′- and 6′-OH groups of the GlcNAc moiety are not involved in substrate recognition. The 3d-GlcNAc-PI and

the 2′-epimer (ManNAc-PI) analogues are not substrates in either system, implying the importance of the 2′- and 3′-positions in substrate binding. The GlcN4Me-PI analogue is a fair substrate in both systems but better in the *Plasmodium* than the HeLa system. This difference at the 4′-position is reflected further by the surprisingly good substrate properties of the 4′ epimer (GalNAc-PI) for the *Plasmodium* enzyme. This analogue is totally unrecognized by the HeLa enzyme. Another *Plasmodium*-specific substrate, GlcNAc-β-PI, suggests that the de-*N*-acetylase has little or no interaction with the hydroxyl groups on the *myo*-inositol. This is supported by the finding that GlcNAc-[L]-PI and the GlcNAc-(2-*O*-alkyl)PI analogues whose *myo*-inositol hydroxyl groups are modified are substrates (albeit poor ones) whereas they are not substrates for the HeLa enzyme.

Plasmodium Specific Inhibition of the De-N-acetylase. The selective recognition of GalNAc-PI and GlcNAc-β-PI by the *Plasmodium* GlcNAc-PI de-*N*-acetylase was combined with the properties of $GlcNCONH_2$-PI, which has previously been shown to be a potent suicide substrate inhibitor for both *T. brucei* and HeLa de-*N*-acetylases, to produce $GalNCONH_2$-PI and $GlcNCONH_2$-β-PI. These two novel compounds and $GlcNCONH_2$-PI were tested as inhibitors of the *Plasmodium* and HeLa cell enzymes using an indirect assay, relying on de-N-acetylation, inositol acylation, and mannosylation of exogenous GlcNAc-PI. The priming of the plasmodial pathway was inhibited totally by 0.2 mM $GlcNCONH_2$-PI (Figure 7A, lane 3) when compared to the control (Figure 7A, lane 2). Inhibition was also observed with $GlcNCONH_2$-β-PI and $GalNCONH_2$-PI (Figure 7A, lanes 5 and 6), where the former shows weaker inhibition at 0.2 μM as there are still some [^{3}H]mannosylated intermediates being formed. The inhibition observed with $GalNCONH_2$-PI is not due to the novel analogue being a competitive substrate for the de-*N*-acetylase as GalNAc-PI is (Table 1) or that the product of the de-N-acylation i.e., GalN-PI, is neither a substrate nor an inhibitor of inositol acyltransferase or mannosyltransferase (Figure 3C, lane 4; Figure 4A, lane 10, Figure 7A, lane 4). Inhibition of only the de-*N*-acetylase by $GlcNCONH_2$-β-PI or $GalNCONH_2$-PI is demonstrated by the priming of the *Plasmodium* pathway with GlcN-PI, which proceeds downstream of the inhibited de-*N*-acetylase even in the presence of 30 μM $GlcNCONH_2$-β-PI or $GalNCONH_2$-PI (Figure 7A, lanes 7−9). By contrast, neither 100 μM GalNAc-PI nor $GalNCONH_2$-PI had any effect on the processing of exogenous GlcNAc-PI to H2 and H5 in the HeLa cell-free system (Figure 7B, lanes 3 and 4) compared to the control (Figure 7B, lane 2). These data indicated that $GalNCONH_2$-PI is a selective inhibitor of the *Plasmodium* cell-free system, presumably via the inhibition of the de-*N*-acetylase. The latter assumption was confirmed by assaying the de-*N*-acetylase directly by measuring the rate of [^{3}H]acetate released from GlcN[^{3}H]Ac-PI or GalN[^{3}H]Ac-PI (Figure 8). First, it was deemed necessary to show that both GlcNAc-PI and GalNAc-PI were being de-N-acetylated by the same enzyme; thus competition assays were conducted. Preincubation with an equimolar amount of unlabeled GlcNAc-PI followed by GlcN[^{3}H]Ac-PI reduced the rate of released [^{3}H]acetate by 50% as expected. Addition of an equimolar amount of unlabeled GalNAc-PI gave ~75% of the control, as might be expected for a weaker competitive substrate. When the rate of [^{3}H]acetate released from GalN[^{3}H]Ac-PI was measured, the addition of unlabeled GalNAc-PI gave 50% of the control and the addition of an equimolar amount of GlcNAc-PI gave 25% of the control, as expected for a better competing substrate for the de-*N*-acetylase. This suggests that GalNAc-PI and GlcNAc-PI are both competing for the same enzyme, GlcNAc-PI de-*N*-acetylase.

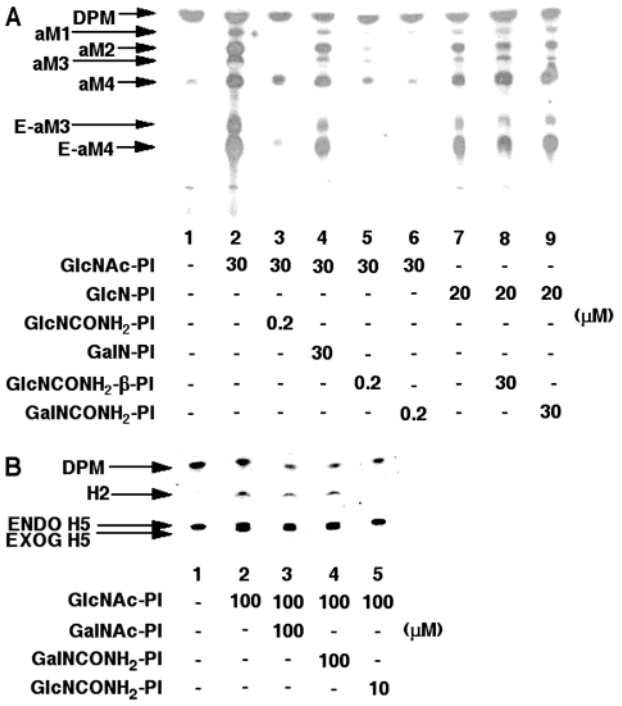

FIGURE 7: Specific inhibition of plasmodial GPI biosynthesis. (A) Washed membranes were incubated with GDP-[^{3}H]Man alone (lane 1) or with GlcNAc-PI (lane 2) or GlcN-PI (lane 7). Inhibition studies were carried out by preincubation with either $GlcNCONH_2$-PI (lane 3), GalN-PI (lane 4), $GlcNCONH_2$-β-PI (lanes 5 and 8), or $GalNCONH_2$-PI (lanes 6 and 9) followed by GlcNAc-PI or GlcN-PI at the indicated concentrations. [^{3}H]Man-labeled glycolipids were extracted and analyzed by HPTLC using solvent system A and fluorography. The identities of the bands indicated on the left of the chromatogram are given in Figure 1. (B) The HeLa cell-free system was incubated with GDP-[^{3}H]Man alone (lane 1) or with GlcNAc-PI before (lane 2) or after preincubation with GalNAc-PI (lane 3), $GalNCONH_2$-PI (lane 4), or $GlcNCONH_2$-β-PI (lane 5) at the indicated concentrations. [^{3}H]Man-labeled glycolipids were extracted and analyzed by HPTLC using solvent system A and fluorography. The identities of the bands indicated on the left of the chromatogram are DPM, Man_1-GlcN-(acyl)PI (H2), and EtNP-Man_1-GlcN-(acyl)PI (H5). Note: H5 produced by the processing of synthetic GlcNAc-PI has a lower R_f than endogenous H5 due to differences in the PI glycolipid moieties (*49*).

The known nonselective inhibitor $GlcNCONH_2$-PI (*56*) and the two novel compounds were also tested as inhibitors of the *Plasmodium* de-*N*-acetylase using the direct assay (measuring release of [^{3}H]acetate from GlcN[^{3}H]Ac-PI). In agreement with the indirect coupled assay (Figure 7A), all three analogues reduced the rate of de-N-acetylation of GlcN-[^{3}H]Ac-PI (Figure 8C), the most potent being $GlcNCONH_2$-PI followed by $GlcNCONH_2$-β-PI and $GalNCONH_2$-PI (Figure 8C, structures shown in Figure 8D), reflecting the order of specificity previously observed (Table 1 and Figure 7A). The $GlcNCONH_2$-PI analogue has previously been shown to inhibit the HeLa de-*N*-acetylase, while neither $GlcNCONH_2$-β-PI nor $GalNCONH_2$-PI inhibited the HeLa enzyme at concentrations up to 100 μM (Figure 7B).

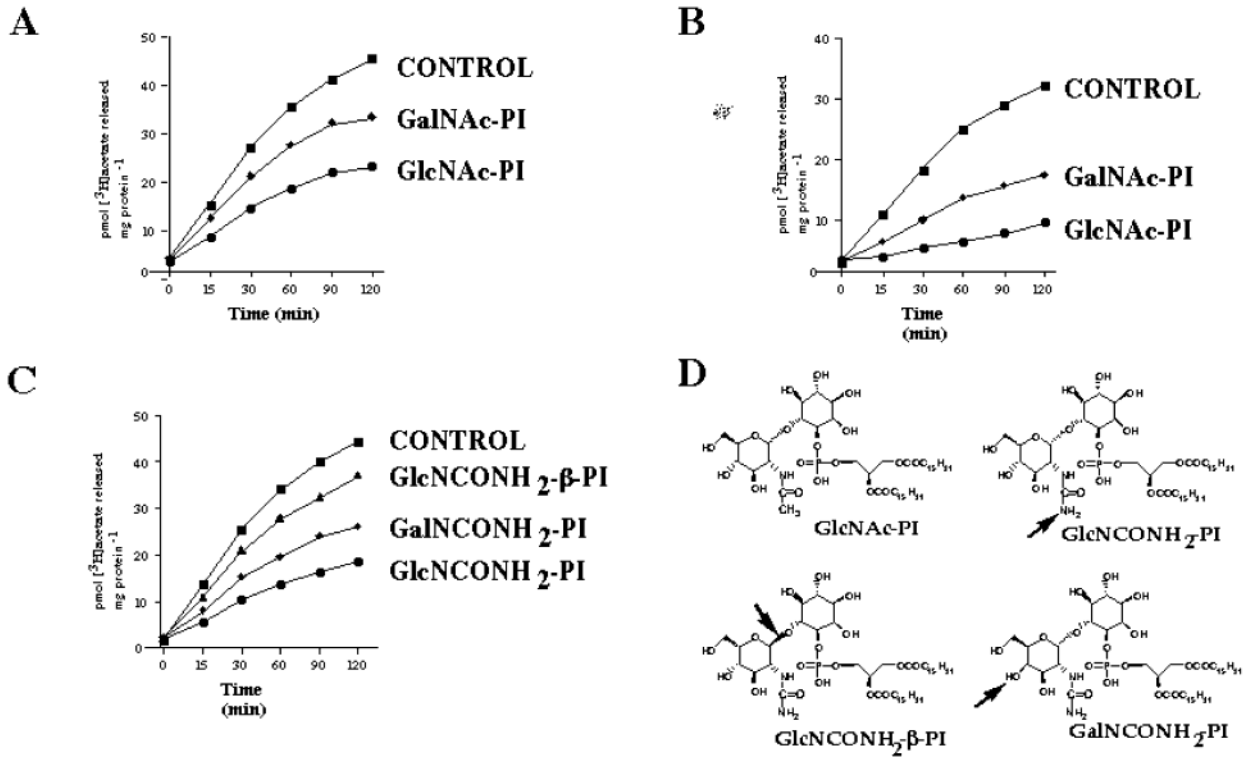

FIGURE 8: Inhibition of the *Plasmodium* de-*N*-acetylase by $GlcNCONH_2$-PI and $GalNCONH_2$-PI analogues. (A) The *Plasmodium* cell-free system was incubated with GlcN[^{3}H]Ac-PI alone (squares) or in the presence of equimolar GlcNAc-PI (circles) or GalNAc-PI (diamonds), and the release of [^{3}H]acetate was measured against time. (B) The *Plasmodium* cell-free system was incubated with GalN[^{3}H]Ac-PI alone (squares) or in the presence of equimolar GlcNAc-PI (circles) or GalNAc-PI (diamonds), and the release of [^{3}H]acetate was measured against time. (C) The *Plasmodium* cell-free system was incubated with GlcN[^{3}H]Ac-PI alone (squares) or in the presence of 0.15 μM $GlcNCONH_2$-PI (circles), $GlcNCONH_2$-β-PI (triangles), or $GalNCONH_2$-PI (diamonds), and the release of [^{3}H]acetate was measured against time. Each time point was measured in triplicate, and measurements were within $\pm$4% of the mean value. (D) Structures of GlcNAc-PI and analogues thereof which act as suicide substrate inhibitors of the de-*N*-acetylase.

DISCUSSION

Eukaryotes all have a very similar GPI biosynthetic pathway leading to a conserved core structure. Nevertheless, significant differences in the timing of certain biosynthetic steps as well as more subtle differences in the substrate specificities of the biosynthetic enzymes have been described (*30*−*33*, *37*, *49*−*50*, *54*). GPI biosynthesis in *Plasmodium* has been studied using a cell-free system prepared from the asexual intraerythrocytic stage to identify the intermediates of the pathway (*20*). In this study we have used such a cell-free system with an array of synthetic substrate analogues to probe the substrate specificities of the early biosynthetic enzymes in the pathway, identifying differences between parasite and host (*P. falciparum* and man) enzymes, which could be therapeutic targets against malaria. This methodology has successfully highlighted differences between trypanosomal and HeLa GPI biosynthetic enzymes (*31*−*33*, *49*, *54*, *56*).

The addition of exogenous GlcN(Ac)-PI substrate to the *Plasmodium* cell-free system produces GPI intermediates similar to those formed by adding UDP-GlcNAc. The mature GPI anchor of *P. falciparum* contains *sn*-1-stearoyl-2-oleoylglycerol whereas the exogenous synthetic GlcN(Ac)-PI contains *sn*-1,2-dipalmitoylglycerol. However, the difference in polarity of the natural C36:1 and the synthetic C32:0 barely alters the R_f values of the GPI intermediates.

Lipid variants of GlcN(Ac)-PI were also tested in the *Plasmodium* cell-free system but were found to be very poorly processed compared with the synthetic dipalmitoyl GlcN-PI analogues used in this study. This phenomenon may be associated to the different nature of *Plasmodium* membranes compared with other cells. Alternatively, it may hint toward some specificity for the PI moiety by the biosynthetic enzymes, as has been suggested for the mammalian UDP-GlcNAc:PI GlcNAc transferase. In any case it is a reasonable assumption that the lipid phosphodiester is an essential structural feature for substrate recognition by *Plasmodium* GPI biosynthetic enzymes, as it is for other systems (*48*, *56*).

Unlike the trypanosomal cell-free system, the addition of NEM cannot be used to suppress the formation of early endogenous GPI intermediates because sulfhdryl alkylating agents inhibit the inositol acyltransferase as well as the UDP-GlcNAc:PI GlcNAc transferase, and the former is essential for acylation of exogenous GlcN-PI and subsequent [^{3}H]-mannosylation. This is similar to the mammalian GPI pathway. An alternative way to suppress the labeling of endogenous GPI intermediates is to preincubate the membranes with unlabeled GDP-Man before the addition of GDP-[^{3}H]Man. However, very low concentrations of GDP-Man have been shown to have an adverse effect on the formation of mannosylated GPI intermediates in the *Plasmodium* cell-free system (*1*), so this is not viable.

As a consequence, some endogenous GPI intermediates are [^{3}H]mannosylated and observed, even when an inhibitor of the de-*N*-acetylase, inositol acyltransferase, or first mannosyltransferase is present in the assay.

Inositol acylation in the *Plasmodium* cell-free system has been shown to be mediated by acyl-CoA, with a preference for C14 and C16 chain length (*1*) and consistent with a recent detailed structural analysis of a mature GPI anchor showing 10% C14:0 and 90% C16:0 acyl chains linked to inositol (*9*). These observations, taken together with the noninhibitory effect of serine protease inhibitors, support the theory that those GPI pathways with inositol acylation/deacylation steps which are mediated by acyl-CoA have a Cys residue prone to alkylation, while those which require no cofactor in washed membranes, e.g., *T. brucei*, are unaffected by sulfhydryl alkylating agents but are inhibited by specific serine protease inhibitors. This may not be surprising in light of the differences in the timing and function of inositol acylation in the trypanosomal and other GPI pathways such as *Plasmodium*, yeast, and mammalian (*1*, *20*, *30*, *39*−*43*).

The substrate specificity of the *Plasmodium* inositol acyltransferase and the first mannosyltransferase was investigated and appears similar to that of the mammalian GPI pathway (*65*). The essential features are the following: (a) The presence and orientation of the amino group of the GlcN-PI play a key role in acceptor substrate recognition, possibly by allowing an interaction with a negatively charged group on the enzyme. (b) The 3′-OH group of the GlcN residue appears to be essential for substrate recognition, suggesting that it acts as a hydrogen bond acceptor or donor. (c) The 4′-OH group of the GlcN residue is essential, as this is the position of mannosylation, but it is not essential for substrate recognition, as shown by the competitive inhibitory effect of the 4′-deoxy analogue. Methylation or epimerization of this 4′-OH group reduces or abolishes this competitive inhibitory effect. (d) The 6′-OH group of the GlcN residue is not essential for substrate recognition. (e) The orientation of the 2-OH group of the D-*myo*-inositol residue, the site of inositol acylation, is essential for acylation and subsequent mannosylation. Consequently, the *Plasmodium* system is unable to utilize GlcN-[L]-PI, GlcN-*β*-PI, or GlcN-(2-*O*-alkyl)PI analogues as a substrate. Like the mammalian system (*33*), GlcN-(2-*O*-hexadecyl)PI is not a substrate for the first mannosyltransferase, despite its similarity to the natural substrate GlcN-(2-*O*-hexadecanoyl)PI. This suggests that substrate recognition by the first mannosyltransferase is dependent on the carbonyl group of the natural 2-*O*-hexadecanoyl ester, which is absent in the 2-*O*-hexadecyl ether. However, the weak competitive inhibition by GlcN-(2-*O*-alkyl)PI analogues in the *Plasmodium* system suggests that these analogues may bind sufficiently to the active sites of the inositol acyltransferase and/or the first mannosyltransferase to block them. Binding to the latter enzyme is more likely since GlcN-(2-*O*-alkyl)PI analogues do not prevent the formation of GlcN-(acyl)PI (Figure 5C). Such inhibition is not observed in the mammalian system but may be similar to the inhibition previously observed with GlcN-(2-*O*-hexadecyl)PI and the trypanosomal first mannosyltransferase (*33*), even though inositol acylation in trypanosomes only occurs after the formation of Man_1GlcN-PI.

The competitive inhibition observed with GlcN-[L]-PI in the *Plasmodium*, but not HeLa, system is similar to that previously described for the trypanosomal system (*54*), such that methylation of the 2-OH of the [L]-*myo*-inositol abolishes inhibition. However, unlike the trypanosomal inhibition, where modification of the GlcN moiety or even its removal has no effect on inhibition, N-acetylation of GlcN-[L]-PI abolishes inhibition in the *Plasmodium* cell-free system. As previously observed, GlcNAc-PI is unable to interact with the inositol acyltransferase directly; a free amino group must be present; therefore, GlcNAc-(acyl)PI is never formed (de-N-acetylation is a prerequisite for acylation and mannosylation). The same order of biosynthetic steps would apply to GlcNAc-[L]-PI, and as this is only de-N-acetylated at a insignificant rate, no GlcN-[L]-PI would ever be formed from GlcNAc-[L]-PI. Thus GlcN-[L]-PI must inhibit by binding to the active sites of the inositol acyltransferase or the first mannosyltransferase as the formation of GlcN-(acyl)PI is drastically reduced in the presence of GlcN-[L]-PI (Figure 5C). The 4-OH group on the [L]-*myo*-inositol which occupies the acylation site has the wrong orientation; thus an acyl chain cannot be added, or if acylation does take place, the first mannosyltransferase specificity allows partial binding of this unnatural GlcN-(acyl)[L]-PI but does not allow mannosylation. In either situation, methylation of GlcN-[L]-PI (GlcN-[L]-(2-*O*-methyl)PI) may cause a steric clash, preventing binding to an active site.

The substrate channeling previously observed between the GlcNAc-PI de-*N*-acetylase and first mannosyltransferase in the trypanosomal cell-free system (*48*) is not observed in the *Plasmodium* system. Unlike the trypanosomal pathway, the *Plasmodium* and mammalian pathways require immediate inositol acylation after de-N-acetylation of GlcNAc-PI (prior to mannosylation). Therefore, a direct interaction between the de-*N*-acetylase and the first mannosyltransferase is not expected.

The substrate specificity of the *Plasmodium* GlcNAc-PI de-*N*-acetylase was also investigated. The requirement for the free amino group of GlcN-PI prior to mannosylation as observed above, and as described for other organisms (*37*, *48*, *56*), was utilized in the indirect assay. Elongation of the acyl chain beyond *N*-propyl had a detrimental effect on the rate of de-N-acylation, as previously described for the trypanosomal and HeLa enzymes (*49*). However, the de-N-acylation of the *N*-benzoyl derivative by the *Plasmodium* enzyme (this study) and the trypanosomal, but not HeLa, de-*N*-acetylase (*56*) shows a fundamental difference between protozoan parasite and human de-*N*-acetylases.

Further studies of the *Plasmodium* de-*N*-acetylase revealed the structural features required for the interactions between the enzyme and GlcNAc-PI (Table 1). Like the trypanosomal and HeLa enzymes (*56*), the following apply: (a) The orientation of the *N*-acetamido group is essential (the epimer ManNAc-PI is not a substrate. (b) The 3-OH group of the GlcNAc residue is essential. (c) The 6′-OH and 4′-OH groups are not essential, but methylation of the latter reduces turnover significantly. Like the trypanosomal (but not the HeLa) enzyme, the *Plasmodium* enzyme can de-N-acetylate GlcNAc-*β*-PI, suggesting that the precise orientation of the 2-, 3-, 4-, and 5-OH groups of the D-*myo*-inositol residue is not essential and substrate recognition is principally driven by the juxtaposition of the phosphodiester group, the 2′-acetamido and 3′-OH groups of the GlcNAc-PI. Finally, the enzyme has the unique property of recognizing GalNAc-PI, the 4′-epimer of GlcNAc-PI; this lack of specificity at the 4-OH of the amino sugar may be a result that *Plasmodium* does not possess a UDP-GlcNAc 4′-epimerase and thus never utilizes GalNAc (*66*).

The fundamental differences between the substrate specificities of the *Plasmodium* and human (HeLa) de-*N*-acetylases were exploited by combining features that provide *Plasmodium* specificity (i.e., β-anomeric linkage or 4′-OH epimer) with the *N*-acyl function of the known general de-*N*-acetylase suicide substrate inhibitor $GlcNCONH_2$-PI (*56*) to produce $GlcNCONH_2$-β-PI and $GalNCONH_2$-PI. Both of these analogues (see Figure 8D for structures) were found to specifically inhibit the *Plasmodium* de-*N*-acetylases at the submicromolar level, without affecting the mammalian pathway at 100 μM. It is reasonable to assume that the mechanism of inhibition by these compounds is similar to that described for the inhibition of the trypanosomal enzyme by $GlcNCONH_2$-PI; i.e., covalent modification of the enzyme through the formation of a carbamate ester between the enzyme and the substrate analogue (*56*). Inhibition of the trypanosomal, *Plasmodium*, and Hela de-*N*-acetylases by $GlcNCONH_2$-PI suggests that all GlcNAc-PI de-*N*-acetylases operate by the same mechanism.

The *Plasmodium*-specific inhibitors discovered in this report, even though they are only active in vitro, are potential leads for the design and synthesis of antimalarial drugs that may be active in vivo.

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