11-6-1996

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## Recommended Citation

Doerrler, W., Ye, J., Falck, J., & Lehrman, M. (1996). Acylation of glucosaminyl phosphatidylinositol revisited. Palmitoyl-CoA dependent palmitoylation of the inositol residue of a synthetic dioctanoyl glucosaminyl phosphatidylinositol by hamster membranes permits efficient mannosylation of the glucosamine residue. *Journal of Biological Chemistry, 271* (43), 27031-27038. https://doi.org/10.1074/jbc.271.43.27031

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 43, Issue of October 25, pp. 27031–27038, 1996

*Printed in U.S.A.*

# Acylation of Glucosaminyl Phosphatidylinositol Revisited

## PALMITOYL-CoA DEPENDENT PALMITOYLATION OF THE INOSITOL RESIDUE OF A SYNTHETIC DIOCTANOYL GLUCOSAMINYL PHOSPHATIDYLINOSITOL BY HAMSTER MEMBRANES PERMITS EFFICIENT MANNOSYLATION OF THE GLUCOSAMINE RESIDUE*

(Received for publication, April 15, 1996, and in revised form, August 5, 1996)

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**Two critical steps in the assembly of yeast and mammalian glycosylphosphatidylinositol (GPI) anchor precursors are palmitoylation of the inositol residue and mannosylation of the glucosamine residue of the glucosaminyl phosphatidylinositol (GlcNα-PI) intermediate. Palmitoylation has been reported to be acyl-CoA dependent in yeast membranes (Costello, L. C., and Orlean, P. (1992) *J. Biol. Chem.* 267, 8599–8603) but strictly acyl-CoA independent in rodent membranes (Stevens, V. L., and Zhang, H. (1994) *J. Biol. Chem.* 269, 31397–31403), and thus poorly conserved. In addition, it was suggested that acylation must precede mannosylation in both yeast (Costello, L. C., and Orlean, P. (1992) *J. Biol. Chem.* 276, 8599-8603) and rodent (Urakaze, M., Kamitani, T., DeGasperi, R., Sugiyama, E., Chang, H.-M., Warren, C. D., and Yeh, E. T. H. (1992) *J. Biol. Chem.* 267, 6459–6462) cells because GlcNα-acyl-PI accumulates *in vivo* when mannosylation is blocked. However, GlcNα-acyl-PI accumulation would also be expected if mannosylation and acylation were independent of each other.**

**These issues were addressed by the use of a synthetic dioctanoyl GlcNα-PI analogue (GlcNα-PI(C8)) as an *in vitro* substrate for GPI-synthesizing enzymes in Chinese hamster ovary cell membranes. GlcNα-PI(C8) was acylated in an manner requiring acyl-CoA. Thus, the process involving acyl-CoA reported for yeast has been conserved in mammals. Furthermore, both GlcNα-PI(C8) and GlcNα-acyl-PI(C8) could be mannosylated *in vitro*, but mannosylation of the latter was significantly more efficient. This provides direct support for the earlier suggestion that acylation precedes mannosylation in rodents cells. A similar result was also observed with the *Saccharomyces cerevisiae* mannosyltransferase.**

**In contrast, it has been reported that mannosylation of endogenous GlcNα-PI by *Trypansoma brucei* membranes occurs without prior acylation. The same result was obtained with GlcNα-PI(C8), confirming that the mannosyltransferase of trypanosomes is divergent from those in yeasts and rodents.**

Glycosylphosphatidylinositol (GPI)[1] anchored proteins have been observed throughout the eukaryotes including parasites, the yeast *Saccharomyces cerevisiae*, and mammals (1). GPI anchor addition to proteins consists of the transfer of a preassembled glycolipid, termed the anchor precursor, by a transamidase which removes a hydrophobic C-terminal peptide and attaches the anchor precursor to the newly exposed carboxyl group. In all species, the anchor precursor is synthesized by transfer of GlcNAc from UDP-GlcNAc to the inositol residue of phosphatidylinositol to form GlcNAc-PI; deacetylation of the GlcNAc to generate GlcNα-PI; transfer of three residues of mannose from mannose-P-dolichol (MPD) to yield $Man_3$-GlcNα-PI; and addition of ethanolamine-P to the third mannose to yield EthN-P-$Man_3$-GlcNα-PI, the GPI anchor precursor (1). The amino group of the ethanolamine residue serves as the eventual attachment point for the protein.

Additional modifications of the anchor precursor are possible depending on the species. One such modification has been observed in *S. cerevisiae* and several mammalian cell lines, the palmitoylation of the 2-position of the inositol on the GlcNα-PI intermediate. This modification renders the intermediate and the resulting GPI anchor precursor resistant to bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) digestion. Although some GPI anchored proteins remain resistant to PI-PLC, most are PI-PLC sensitive indicating that the palmitate is removed at some point.

Several aspects of GPI palmitoylation remain controversial. The function of the palmitate remains unclear. In addition, it has been suggested in both *S. cerevisiae* (2) and murine cells (3) that inositol palmitoylation must precede mannosylation of the GlcN residue. These suggestions were based primarily on the observations that mutants defective in the synthesis of the mannose donor, MPD, accumulate GlcNα-acyl-PI. However, while this type of result clearly shows that acylation can occur without prior mannosylation, it does not address whether mannosylation requires prior acylation. In other words, GlcNα-acyl-PI would be expected to accumulate in a mutant lacking MPD even if the mannosyltransferase did not require prior palmitoylation (Fig. 1). Indeed, there is no other precedent for such a lipid modification on the glycan being required for a glycosyltransferase reaction. Furthermore, the *Trypanosoma brucei* mannosyltransferase clearly does not require prior palmitoylation of inositol (4, 5), yet these enzymes are generally considered to be highly conserved among the eukaryotes.

Another difference deals with the identity of the source of the palmitoyl group on inositol. In experiments with *S. cerevisiae* membranes it was shown that the acyl group could originate from acyl-CoA (2). However, for murine membranes the acyl

* This work was supported by National Institutes of Health Grants GM38545 (to M. A. L.) and GM31278 (to J. R. F.) and Robert Welch Foundation Grants I-1168 (to M. A. L.) and I-782 (to J. R. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; CHO, Chinese hamster ovary; GlcN-PI, glucosaminyl phosphatidylinositol; MPD, mannose-P-dolichol, PI, phosphatidylinositol; PLC, phospholipase C; HF, hydrogen fluoride; PLD, phospholipase D.

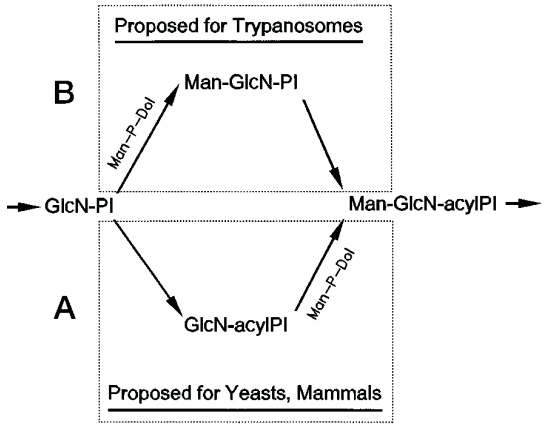


FIG. 1. **Pathways for conversion of GlcNα-PI to Man-GlcNα-acylPI.** As discussed in the text, opposite routes have been suggested for yeasts and mammals (route A) and trypanosomes (route B). In cells that fail to synthesize mannose-P-dolichol (Man-P-Dol), GlcN-acyl-PI would be expected to accumulate if both routes A and B were permissible or if route A was the only permissible pathway.

group was reported to originate from an endogenous membrane component other than palmitoyl-CoA, although the transfer of palmitate required the presence of free CoA (6). The latter study concluded that acyl-CoA was not a potential donor of the acyl group on inositol. In intact *T. brucei* (7) and with *T. brucei* membranes (8) it has also been suggested that the acyl donor is not acyl-CoA.

A central limitation of these earlier studies was the difficulty controlling the concentration and form of the GlcNα-PI acceptor, which was generated from endogenous lipid. In an effort to overcome this limitation, a recent report from Ferguson's group (9) described synthetic dipalmitoyl GPIs as efficient acceptors for the trypanosomal mannosyltransferase *in vitro*. In this paper, we describe the use of dioctanoyl GlcNα-PI (GlcNα-PI(C8)), a relatively water-soluble analogue of the GlcNα-PI intermediate, in which the *sn*-1,2 positions of the glycerol backbone contain octanoyl groups in ester linkage. In *in vitro* assays with Chinese hamster ovary (CHO) cell, *S. cerevisiae*, or *T. brucei* membranes, in the presence of GDP-[$^3$H]mannose to generate the [$^3$H]MPD donor, GlcNα-PI(C8) was found to be an efficient acceptor for GPI biosynthetic enzymes. We report on the use of GlcNα-PI(C8) with rodent membranes to re-examine both the reported requirement of the mannosyltransferase for prior palmitoylation, and the source of the palmitoyl group.

## EXPERIMENTAL PROCEDURES

*Materials*—GlcNα-PI(C8), GlcNα-PI(C16), and GlcNβ-PI(C8) were prepared as described (10). GDP-[$^3$H]mannose (15 Ci/mmol) was from American Radiolabeled Chemicals. [$^3$H]Palmitate (43 Ci/mmol) was from DuPont. All acyl-CoA derivatives were from Sigma. Cell culture supplies were from Life Technologies, Inc./BRL, except serum (Atlanta Biologicals). All other reagents were from reliable sources.

*Cell Lines and Membrane Preparations*—CHO-K1 cells (11) were maintained at 37 °C in F-12 medium buffered at pH 7.4 with 15 mM Na-HEPES and supplemented with 2% fetal calf serum and 8% calf serum, in the absence of antibiotics. Microsomal membranes were prepared from these cells as described (11) and stored in aliquots in Tris-buffered saline (TBS; 20 mM Tris-Cl, pH 7.4, 150 mM NaCl) containing 20% glycerol at −80 °C. *S. cerevisiae* membranes were prepared from a common wild-type laboratory strain (MMY011, gift of Dr. Joel Goodman) as described (2) and stored at −80 °C.

*T. brucei brucei* (12) membranes were prepared from organisms from infected rats after lysis and centrifugation (13, 14) and were a gift of Teddy Scott and Dr. Margaret Phillips of this institution. Membranes were stored in aliquots at −80 °C in 50 mM Na-HEPES (pH 7.4) containing 25 mM KCl, 5 mM $MgCl_2$, 0.05 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone, 0.5 μg/ml leupeptin, and 10% glycerol as described (4). Protein was measured using a dye-binding assay from Bio-Rad.

*In Vitro Assay for Acylation and Mannosylation of GlcNα-PI(C8)*—The zwitterionic form of GlcNα-PI(C8) ($C_{31}H_{58}NO_{17}P$, FW 747.6) was found in the butanol phase after butanol/water partitioning (data not shown). Thus, for *in vitro* assays the dry compound was dispersed in 0.03% (w/v) Triton X-100 to ensure reproducible dissolution, although pure water also appeared to dissolve the dry compound (data not shown). The dissolved compound was subsequently diluted 4-fold into a 0.1-ml reaction volume containing (final concentrations) 50 mM Tris-Cl (pH 7.4), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM 5′-AMP, and 0.26 μM GDP-[$^3$H]mannose. Some reactions also contained 2 or 10 μM acyl-CoA, 2 μM CoA, and/or 1 mM $Na_2ATP$. Reactions were initiated by addition of 50 μg of CHO-K1 or *S. cerevisiae* membrane protein. Trypanosomal membranes were assayed similarly, except that assays lacked Tris buffer and included 50 mM Na-HEPES (pH 7.4) and 5 mM KCl. Reactions were incubated at 37 °C for 20 min (CHO-K1, trypanosomes) or at 30 °C for 40 min (*S. cerevisiae)* at which time they were chilled on ice and extracted twice with 0.2 ml of water-saturated butanol. Pooled butanol extracts were backwashed once with 0.1 ml of butanol-saturated water and then dried under a stream of nitrogen. Lipids were dissolved in 30 μl of chloroform/methanol (2:1) and applied to a preactivated Whatman Silica Gel 60 TLC plate. TLC plates were developed in chloroform, methanol, 0.25% KCl (55:45:10), sprayed with fluor (15), and exposed to x-ray film (Kodak) for 2 to 4 days. In all figures, solvent flow is from bottom to top.

A small number of experiments were performed with [$^3$H]palmitoyl-CoA in place of GDP-[$^3$H]mannose. [$^3$H]Palmitoyl-CoA was synthesized by ligation of [$^3$H]palmitate to free CoA as described (16). Reactions with microsomes were performed essentially as described above with 0.2 μM [$^3$H]palmitoyl-CoA (43 Ci/mmol) in the absence or presence of 1 mM unlabeled GDP-mannose and/or 1 μg of GlcNα-PI(C8) per assay.

*Chemical and Enzymatic Treatments of Labeled Lipids*—Radiolabeled lipids were recovered by scraping the appropriate areas of TLC plates and extracting the silica with 1 ml of chloroform/methanol/water (10:10:3). Following a brief centrifugation to remove the silica the solvent was dried under a stream of nitrogen and partitioned with butanol and water.

For nitrous acid degradation (4), lipid was dissolved in 0.1 ml of 0.25 M sodium acetate (pH 3.25), 0.1% SDS, and 0.25 M sodium nitrite (or 0.25 M sodium chloride for controls) and incubated for 4 h at 37 °C. The oligosaccharides generated by nitrous acid degradation were subsequently reduced by the addition of 0.2 ml of 0.4 M sodium borate (pH 9.8) and 0.1 ml of 0.03 N NaOH containing 8 mg of sodium borohydride to the above reaction (pH of reaction = 10), with an additional incubation of 30 min at 30 °C. The reaction was stopped by the addition of 50 μl of glacial acetic acid.

For hydrogen fluoride treatment (17), lipids were dissolved by brief sonication in 0.1 ml of ice-cold 48% HF and incubated on ice for 60 h. The HF reaction was neutralized to a pH value below 4 by addition to 0.58 ml of frozen saturated lithium hydroxide, centrifuged briefly to remove precipitate, and adjusted to a pH value between 5 and 7 by addition of aliquots of saturated sodium bicarbonate. The supernatant was dried and partitioned between butanol and water. Acylated species were recovered predominantly in the butanol phase.

For GPI-PLD treatment (18), lipids were dissolved in 0.1 ml of 50 mM Tris-Cl (pH 7.4), 2.6 mM $CaCl_2$, 10 mM NaCl, 0.1% Triton X-100, and 0.01% Nonidet P-40 and incubated for 2 h at 37 °C in the presence of 10% fetal bovine serum, a rich source of GPI-PLD. The ability of serum to cleave GPIs in this study was fully inactivated by 1,10-phenanthroline, as expected for GPI-PLD, and in no case was any degradation of the GPI detected other than the expected cleavage of the phosphodiester linking the inositol residue to the glycerol moiety. Whole serum was also used as the source of GPI-PLD in a recent study on trypanosomal GPIs by Ferguson's group (9). For PI-PLC treatment, lipids were dissolved in 0.1 ml of 50 mM Tris-Cl (pH 7.4) plus 0.16% (w/v) Triton X-100 and incubated for 1 h in the presence of 100 milliunits of *Bacillus cereus* PI-PLC (Sigma). All phospholipase reactions mixtures were partitioned between butanol and water, with recovery of lipids in butanol.

For treatment with Jack bean α-mannosidase (Boehringer Mannheim), lipids were dissolved in 40 μl of a solution containing 50 mM sodium citrate (pH 4.5), 1 mM $ZnCl_2$, and 0.1% Triton X-100. Incubations were carried in the presence or absence of 0.1 unit of enzyme for 24 h at 37 °C at which time an additional aliquot of enzyme was added and incubation was continued for an additional 24 h.

*Bio-Gel P-2 Chromatography*—A 1 × 47-cm column of Bio-Gel P-2 (extra fine, Bio-Rad) was prepared in 50 mM acetic acid and run at a flow rate of 0.1 ml/min with a LKB peristaltic pump. 0.5-ml fractions were collected and subjected to liquid scintillation counting.

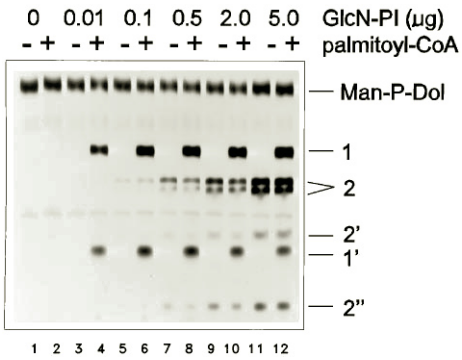


FIG. 2. **Effects of GlcNα-PI(C8) concentration and palmitoyl-CoA addition upon the formation of [$^3$H]mannosylated products by Chinese hamster ovary cell membranes.** Incubations of CHO membranes with GDP-[$^3$H]mannose were performed as described under "Experimental Procedures." Butanol-soluble products were analyzed by TLC. The amount of GlcNα-PI(C8) added and the presence or absence of 2 μM palmitoyl-CoA are indicated. The identification of mannose-P-dolichol (Man-P-Dol) is based on earlier work (29). The $R_F$ values of various lipids were: Man-P-Dol, 0.82; lipid 1, 0.65; lipid 2, 0.55/0.57; lipid 2′, 0.42; lipid 1′, 0.38; lipid 2″, 0.24.

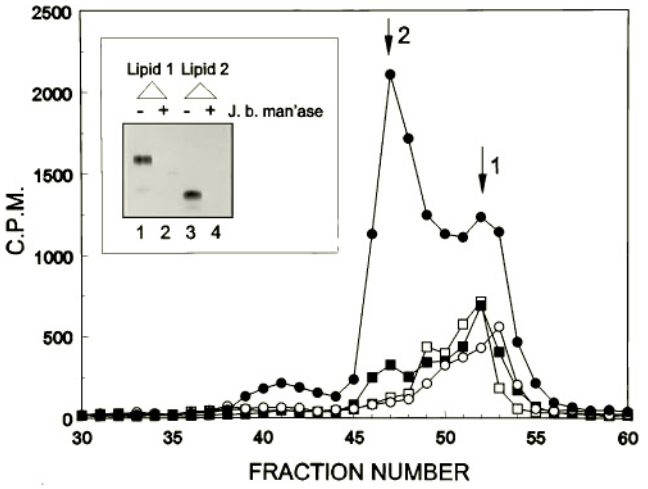


FIG. 3. **Bio-Gel P-2 chromatography.** Total [$^3$H]mannosylated products formed in the presence of 0.1 μg of GlcNα-PI(C8), with (*circles*) or without (*squares*) palmitoyl-CoA, and treated with (*closed symbols*) or without (*open symbols*) nitrous acid, were reduced and characterized by Bio-Gel P-2 chromatography as described under "Experimental Procedures." Standards are: *1*, free mannose; *2*, mannose-anhydromannitol, formed by nitrous acid treatment and reduction of Man-L (30). Each sample appeared to have substantial amounts of free mannitol that originated from GDP-[$^3$H]mannose. The small peak (*closed squares*) at fraction 47 may result from a trace amount of lipid 2 that formed with 0.1 μg of GlcNα-PI(C8) (see Fig. 2, *lane 5*). The nature of the minor peak (*closed circles*) at fraction 41 is unclear. *Inset*: lipids 1 and 2 were recovered from TLC plates and treated without (*lanes 1* and *3*) or with (*lanes 2* and *4*) Jack bean α-mannosidase. Butanol-soluble products were analyzed by TLC.

## RESULTS

*Choices of Isotopic Donor and Synthetic Acceptor*—GDP-[$^3$H]mannose was preferred over [$^3$H]palmitoyl-CoA for most *in vitro* experiments because the latter would fail to detect acyl-CoA independent mannosylation. In contrast to results reported for trypanosomal membranes (9), in initial experiments we found that synthetic GlcNα-PI with two C16 acyl (palmitoyl) chains linked to the glycerol moiety, to approximate the aliphatic chains found in natural GPIs, failed to yield mannosylated or acylated products with CHO membranes (data not shown). However, GlcNα-PI with two C8 acyl (octanoyl) chains ("GlcNα-PI(C8)") gave encouraging results (see below). The synthesis and structure of GlcNα-PI(C8), starting from the known (19) chiral D-*myo*-inositol derivative, was reported elsewhere (10).

*Characterization of [$^3$H]Mannosylated Products of GlcNα-PI(C8) Formed in the Absence and Presence of Palmitoyl-CoA*—GlcNα-PI(C8) was dissolved in buffer containing 0.03% (w/v) Triton X-100 and mixed with microsomal membranes from normal CHO-K1 cells. Incubations with GDP-[$^3$H]mannose (which formed [$^3$H]MPD) were complete after 20 min at 37 °C (data not shown). Lipids were recovered by organic extraction and analyzed by TLC and fluorography. The effects of GlcNα-PI(C8) concentration and addition of palmitoyl-CoA are shown in Fig. 2. Compared with control incubations of membranes with GDP-[$^3$H]mannose but no palmitoyl-CoA or GlcNα-PI(C8) (*lane 1*), in which [$^3$H]MPD was formed, incubations supplemented with palmitoyl-CoA (2 μM, *lane 2*) or low concentrations of GlcNα-PI(C8) (0.01 μg, *lane 3*) failed to yield additional labeled products. However, new products (*lanes 4, 6, 8, 10*, and *12*) termed lipids 1 and 1′ were observed upon incubation of palmitoyl-CoA together with GlcNα-PI(C8). The amounts of lipids 1 and 1′ correlated with the amount of GlcNα-PI(C8) up to approximately 0.01 μg/assay (data not shown), but lipid 1 and 1′ formation did not increase significantly with more GlcNα-PI(C8) (*lanes 6, 8, 10,* and *12*). This was explained by separate experiments (not shown) which demonstrated that 2 μM palmitoyl-CoA was limiting. In addition to lipids 1 and 1′, appreciable amounts of other products termed lipids 2 (a doublet), 2′, and 2″ were formed at concentrations of GlcNα-PI(C8) of 0.5 μg and above (*lanes 7–12*). Lipids 2, 2′, and 2″ were independent of addition of palmitoyl-CoA.

After recovery from TLC plates, lipids 1 and 2 were found to be sensitive to treatments with Jack bean α-mannosidase (Fig. 3, *inset*), nitrous acid, hydrogen fluoride, and GPI-PLD (see below). Bio-Gel P-2 column analyses of the nitrous acid fragments of lipid 1 (Fig. 3) and lipid 2 (data not shown) were consistent with a single mannose residue linked to glucosamine. Although the $Man_1$GlcN-acyl-PI intermediate is capable of modification with ethanolamine-P (1, 20), this modification appeared absent from lipids 1 and 2 because they were sensitive to Jack bean α-mannosidase. In addition, the nitrous acid fragments of lipids 1 and 2 failed to adhere to a quarternary aminoethyl ion exchange column under conditions (20) designed to bind GPIs with ethanolamine-phosphate modified mannose residues (data not shown). Thus, lipids 1 and 2 had the properties expected for GPIs with single unmodified α-linked mannose residues. Although lipids 1′, 2′, and 2″ remain to be characterized, it is feasible that they may result from further mannosylation of lipids 1 and 2. It remains unclear why lipid 2 is a doublet. It is possible that some form of acyl migration, as suggested (2), or remodeling may be involved.

The effects of palmitoyl-CoA addition suggested that lipid 1, but not lipid 2, contained acylated inositol. Initially, both lipids were found to be resistant to PI-PLC treatment, although a positive-control PI-PLC substrate added to the reaction mixtures was readily cleaved (data not shown). PI-PLC requires the 2-position of inositol to be unmodified, and resistance of GPIs to PI-PLC is usually a hallmark of inositol acylation (17). However, further analysis (Table I) indicated that lipid 1, but not lipid 2, contained acyl-inositol, suggesting that PI-PLC could not be used reliably with dioctanoyl GPIs.

As indicated in Table I, lipid 1 and the $^3$H-fragment obtained by a 60-h 0 °C HF treatment (to cleave the phosphodiester between the inositol and diacylglycerol groups) were soluble in butanol. However, the $^3$H-fragment of lipid 1 obtained by nitrous acid treatment (to cleave between inositol and glucosamine) was water-soluble (Fig. 3 and data not shown). This indicated that an apolar group, presumably an acyl chain, was

TABLE I

*Lipids 1 and 2 differ by the presence or absence of an apolar group on the inositol residue*

Intact [$^3$H]mannose-labeled lipids generated with CHO or *S. cerevisiae* membrane reactions, as well as lipid fragments created by various chemical and enzymatic treatments as described under "Experimental Procedures" and in Ref. 30, were partitioned between equal volumes of water-saturated butanol and butanol-saturated water. The percentage of radioactive material in the water phase is presented. In separate experiments, treatment 1, in which neutralization was performed immediately after adding HF, gave the same results as untreated lipids 1 and 2. In addition, it was found that approximately 90% of the radioactivity associated with the nitrous acid fragments of lipids 1 and 2 partitioned into the water phase (data not shown).

| Treatment | Percentage of radioactivity in water phase | |
|---|---|---|
| | Lipid 1 | Lipid 2 |
| CHO membrane reactions | | |
| 1. 0 h HF | 6.8 | 7.5 |
| 2. 60 h HF | 29.6[a] | 89.5 |
| 3. GPI-PLD | 6.1 | 77.7 |
| 4. $NH_4OH$ | 95.0 | 83.5 |
| 5. 60 h HF, then $NH_2$ | 91.6 | 95.1 |
| Yeast membrane reactions | | |
| 1. 0 h HF | 6.0 | 5.3 |
| 2. 60 h HF | 23.6[a] | 94.3 |

[a] Over several determinations, 20–30% of lipid 1 radioactivity consistently partitioned into water after 60 h of HF treatment, similar to what has been observed with trypanosomal $Man_3$-GlcN$\alpha$-acyl-PI (8). The results of treatment 1 ruled out excess alkalinity during neutralization as a cause, and treatment 3 indicated that lipid 1 was not contaminated with appreciable amounts of lipid 2 or a similar lipid. Re-partitioning of the water- and butanol-soluble materials of treatment 2 resulted in almost quantitative recovery in the original phases (data not shown), indicating that two distinct compounds were present. Thus, these observations suggest that the 60-h HF treatment cleaved a fraction of the ester-linked palmitate on lipid 1.

linked to the inositol. The apolar HF-fragment of lipid 1 became water-soluble upon treatment with weak base, consistent with an apolar goup attached by an ester linkage. In contrast, lipid 2 was butanol-soluble, but the $^3$H-fragment obtained by the 60-h 0 °C HF treatment treatment was water-soluble. In both cases, results similar to those with HF were obtained with GPI-PLD which also cleaves between the inositol and diacylglycerol groups.

This was a strong indication that lipid 1, but not lipid 2, contained acyl-inositol, and was consistent with the slower migration (and hence greater polarity) of lipid 2 by TLC (Fig. 2). Based on these data, lipid 1 most likely has the structure [$^3$H]Man-GlcN$\alpha$-acylPI(C8), while lipid 2 appears to be [$^3$H]Man-GlcN$\alpha$-PI(C8). At low concentrations of GlcN$\alpha$-PI(C8) only lipid 1 is observed, and formation of lipid 1 requires the presence of palmitoyl-CoA.

*Further Demonstration That Mannosylation at Low Concentrations of GlcN$\alpha$-PI(C8) Requires Inositol-acylation*—Repeated TLC analyses of radiolabeled lipids synthesized with 0.01 $\mu$g of GlcN$\alpha$-PI(C8), such as shown in Fig. 2 (*lanes 3* and *4*), gave no evidence of [$^3$H]mannose-labeled GlcN$\alpha$-PI(C8) in the absence of palmitoyl-CoA. However, we were concerned that possible mannosylated products could have been missed if they had migrated diffusely on the TLC plate or if they were obscured in some way. We reasoned that if the glycan cores were cleaved from the lipids of total reaction products, such artifacts could be ruled out by analysis of the cores by gel filtration. Total butanol-soluble reaction products were therefore treated with nitrous acid to cleave between GlcN$\alpha$ and inositol, with conversion of the GlcN$\alpha$ residue to anhydromannose. The products were then reduced with sodium borohydride to convert the anhydromannose to anhydromannitol. Gel filtration with a Bio-Gel P-2 column revealed a major peak that

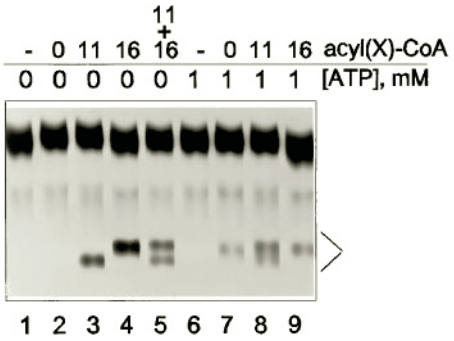


FIG. 4. **The source of the acyl chain donated to GlcN$\alpha$-PI(C8) is acyl-CoA.** Assays were performed with CHO membranes, GDP-[$^3$H]-mannose, and 0.1 $\mu$g of GlcN$\alpha$-PI(C8) as for Fig. 2. The nature of various CoAs (−, none added; *0*, 2 $\mu$M free CoA; *11*, 2 $\mu$M acyl($C_{11}$)-CoA; *16*, 2 $\mu$M acyl($C_{16}$)-CoA) and the concentration of ATP are indicated. In *lane 5,* the products of the acyl($C_{11}$)-CoA and acyl($C_{16}$)-CoA reactions were mixed. The *bracket* on the *right* indicates [$^3$H]Man-GlcN$\alpha$-acylPI(C8).

required both inclusion of palmitoyl-CoA in the reaction and nitrous acid treatment after the reaction, and that co-eluted with a mannose-anhydromannitol standard (Fig. 3). This provided a direct demonstration of palmitoylation-dependent mannosylation. However, this enhancement could not be quantified (see "Discussion").

*Inositol Acylation of GlcN$\alpha$-PI(C8) in CHO Membranes Is Acyl-CoA Dependent*—It has been reported that palmitoylation of GlcN$\alpha$-PI in rodent membranes is due to an acyl-CoA independent mechanism in which free CoA (which can be derived from hydrolysis of acyl-CoA) is a critical co-factor, and in which palmitate is acquired from an endogenous membrane component (6). Thus, it appeared likely that the results obtained in Figs. 2 and 3 were not due to an acyl-CoA dependent process. However, as shown in Fig. 4, substitution of palmitoyl($C_{16}$)-CoA (*lane 4*) with $C_{11}$-CoA (*lane 3*), as well as mixing the two (*lane 5*) yielded products with mobilities corresponding to the polarities of the respective acyl chain donors. Similar results were obtained if $C_{11}$-CoA was compared with $C_{16}$ palmitoyl-CoA generated by addition of free CoA and ATP to membranes (*lanes 7–9*). This result demonstrated that acyl-CoA was the true source of the acyl group transferred to lipid 1 by CHO membranes, in direct contrast to the results reported earlier (6). However, a concern remained that the altered mobilities of the products formed by addition of $C_{11/16}$-CoA could be explained by remodeling of one of the $C_8$ chains of GlcN$\alpha$-PI(C8) with $C_{11/16}$. In other words, it was necessary to demonstrate that the $C_{11/16}$ chains were transferred to the inositol residue. As shown in Fig. 5, the [$^3$H]GPI-PLD fragments, but not the [$^3$H]nitrous acid fragments of the lipids formed with $C_{11}$-CoA and $C_{16}$-CoA had altered mobilities corresponding to the polarities of the respective acyl donors. HF treatments gave results similar to those obtained with GPI-PLD (data not shown). This result showed conclusively that the acyl chains originating from acyl-CoA were transferred to the inositol residue, most likely to the 2-OH position.

The specificity for the acyl chain was tested with acyl-CoAs of different structure (Fig. 6). Formation of [$^3$H]Man-GlcN$\alpha$-acyl-PI(C8) was optimal with $C_{16}$-CoA, but appreciable [$^3$H]Man-GlcN$\alpha$-acyl-PI(C8) was also formed with acyl-CoAs containing $C_{10}$ to $C_{17}$ chains. In addition, substitution of $C_{16}$-CoA with $C_{16:1(9\text{-cis})}$-CoA was more detrimental than substitution with $C_{16:1(9\text{-trans})}$-CoA. Although lack of activity in this assay could be due to effects on the acyltransferase and/or the mannosyltransferase, it is clear that neither enzyme has a strict requirement for palmitate.

*Transfer of [$^3$H]Palmitate from [$^3$H]Palmitoyl-CoA to GlcN$\alpha$-*

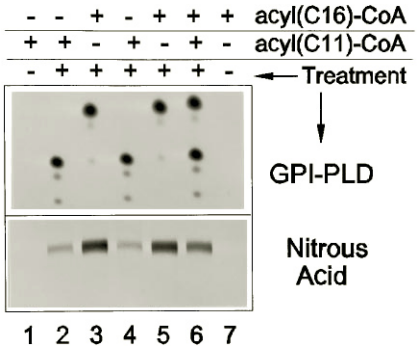


FIG. 5. **The acyl chain of acyl-CoA is transferred to the inositol residue of GlcNα-PI(C8).** [$^3$H]Man-GlcNα-acylPI(C8) was formed with acyl($C_{11}$)-CoA (*lanes 1, 2,* and *4*), acyl($C_{16}$)-CoA (*lanes 3, 5,* and *7*), or mixed (*lane 6*), and treated (*lanes 2–6*) with either GPI-PLD (*upper panel*) or nitrous acid (*lower panel*). After butanol/water partitioning the aqueous phases were desalted (with Dowex 50W-X8($H^+$) followed by Amberlite MB-3) and then analyzed in the case of nitrous acid, and the butanol phases were analyzed without desalting in the case of GPI-PLD. TLC was performed as described under "Experimental Procedures," except that the plate was developed 3 times with chloroform/methanol/water (10:10:3) and dried between runs.

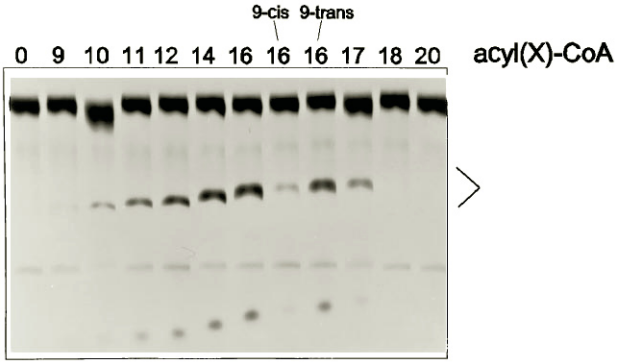


FIG. 6. **Dependence of the formation of Man-GlcNα-acyl-PI(C8) upon the nature of the acyl chain on the acyl-CoA donor.** Reactions were performed with 2 μM CoA ligated to acyl groups of the indicated chain length as for Fig. 4. $C_{16}$ acyl-CoAs with 9-cis or 9-trans double bonds were included. The *bracket* on the *right* indicates [$^3$H]Man-GlcNα-acyl-PI(C8).

*PI(C8)*—To provide independent confirmation that acyl-CoA served as a source of the palmitoyl group attached to the inositol residue, GlcNα-PI(C8) was tested as an acceptor in reactions containing [$^3$H]palmitoyl-CoA in place of GDP-[$^3$H] mannose. As shown in Fig. 7, a $^3$H-lipid designated lipid a was formed when GlcN-PI(C8) was incubated with membranes in the presence of [$^3$H]palmitoyl-CoA (compare *lanes 1* and *2* with *lane 3*), but not free [$^3$H]palmitate (data not shown). Due to its greater mobility, lipid a appeared less polar than lipid 1, and a portion of lipid a was converted to a species co-migrating with lipid 1 upon addition of non-radioactive GDP-mannose (*lane 4*). Both lipid a and its mannosylated form were sensitive to GPI-PLD (*lanes 5* and *6*), and appeared to yield lipid b and lipid c, respectively, as products. Lipids b and c were not formed if GlcN-PI(C8) was omitted (data not shown). By its migration lipid c was judged to be more polar than lipid b, and both were more polar than lipid 1. From these data, it is likely that lipid a is GlcN-[$^3$H]acylPI(C8), the species co-migrating with lipid 1 is Man-GlcN-[$^3$H]acyl-PI(C8), lipid b is GlcN-[$^3$H]acylinositol, and lipid c is Man-GlcN-[$^3$H]acylinositol. Therefore, these results provide independent evidence for the transfer of palmitate from palmitoyl-CoA to GlcN-PI(C8).

*Properties of GlcNβ-PI*—An analogue of GlcNα-PI(C8) with the non-physiological GlcNβ linkage was found to be a mannosyl acceptor with CHO membranes (10), and in conjunction

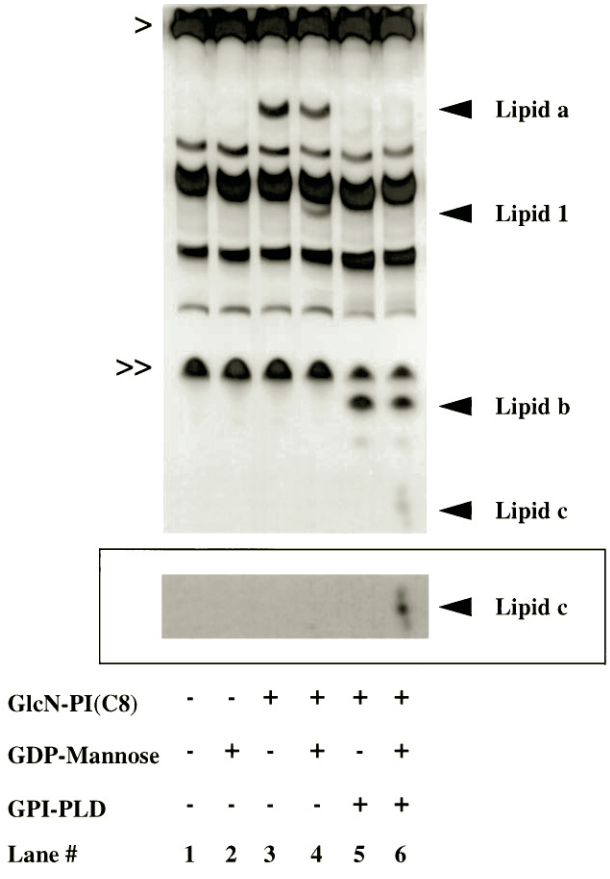


FIG. 7. **Transfer of [$^3$H]palmitate from [$^3$H]palmitoyl-CoA to GlcNα-PI(C8).** Reactions were performed and butanol-soluble products were examined by thin-layer chromatography as described under "Experimental Procedures," except that the plates were run twice with chloroform/methanol/water (10:10:3), with air-drying in between, to enhance separation between lipid 1 and the prominent $^3$H-material (not characterized) just above it. All reactions contained 0.2 μM [$^3$H]palmitoyl-CoA. 1 μg of GlcNα-PI(C8) was added to the reactions in *lanes 3–6*, non-radioactive GDP-mannose (1 mM) was added to the reactions in *lanes 2, 4*, and *6*, and the reactions in *lanes 5* and *6* were treated with GPI-PLD. The *single* and *double carets* indicate [$^3$H]palmitate ($R_F$ = 0.97), which formed from breakdown of [$^3$H]palmitoyl-CoA during the reaction, and [$^3$H]palmitoyl-CoA ($R_F$ = 0.47), respectively. The position of a lipid 1 standard ($R_F$ = 0.70; not shown) is indicated, as are the positions of lipids a ($R_F$ = 0.85), b ($R_F$ = 0.42), and c ($R_F$ = 0.27) discussed in the text. This figure (but no others in this article) was generated electronically from an autoradiogram with Adobe Photoshop software. To facilitate visualization of lipid c, a digitally enhanced image of the relevant portion of the autoradiogram (*box*) is shown.

with the current studies this was found to be due to the concerted actions of the palmitoyl-CoA dependent acyltransfer activity and the mannosyltransferase. The concentration dependence was similar to that of GlcNα-PI(C8) (data not shown). However, GlcNβ-PI(C8) was not mannosylated in the absence of palmitoyl-CoA. This suggests that the specificity for assuring the α-linkage of glucosamine in GPIs resides with the UDP-GlcNAc:PI αGlcNAc transferase rather than the acyltransferase or mannosyltransferase, and that the orientation of the GlcN residue may influence the requirement of the mannosyltransferase for prior acylation.

*Mannosylation of GlcNα-PI(C8) by S. cerevisiae and T. brucei Membranes*—To determine directly whether the yeast and trypanosomal mannosyltransferases had preferences for acylated acceptors, assays were performed with *S. cerevisiae* and *T. brucei* membranes in a manner similar to that of Fig. 2. As shown in Fig. 8, the total *S. cerevisiae* mannosyltransferase activity was enhanced at low acceptor concentrations by the addition of palmitoyl-CoA. The lipids designated 1 and 2 are

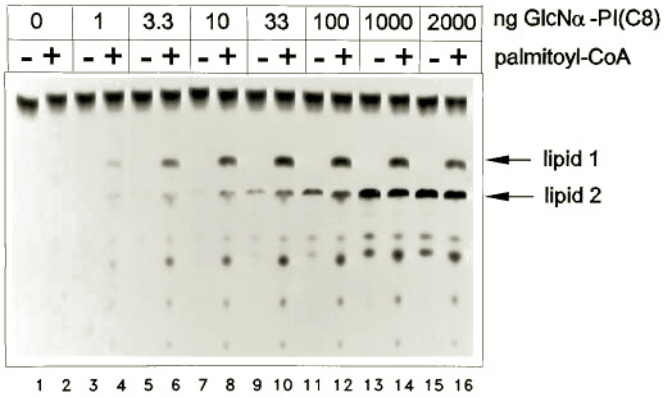


FIG. 8. **Mannosylation of GlcNα-PI(C8) by yeast membranes.** Reactions were performed as described in the text in the presence of 0–2000 ng/assay GlcNα-PI(C8) and 0 or 10 μM palmitoyl-CoA as indicated. The positions of lipids 1 and 2, tentatively identified as [$^3$H]Man-GlcNα-acylPI(C8) and [$^3$H]Man-GlcNα-PI(C8), respectively, are shown.

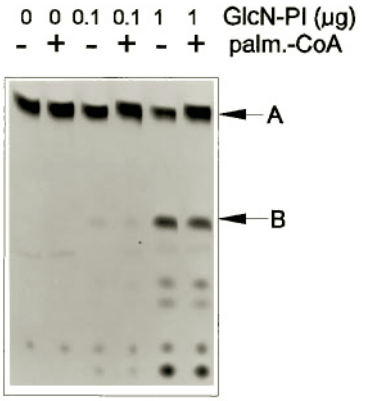


FIG. 9. **Mannosylation of GlcNα-PI(C8) by trypanosomal membranes.** Reactions were performed as described in the text in the presence of 0, 0.1, or 1.0 μg of GlcNα-PI(C8) and 0 or 2 μM palmitoyl-CoA. "*A*" is presumed to be MPD. "*B*" was found to have properties consistent with the structure [$^3$H]Man-GlcNα-PI(C8). The lipids migrating below "B" were not characterized.

presumed to be [$^3$H]Man-GlcNα-acyl-PI(C8) and [$^3$H]Man-GlcNα-PI(C8), respectively, based on co-migration with corresponding products from CHO membrane reactions and the results of HF treatments (Table I) used to determine the presence of apolar groups on the glycan moiety. Fig. 8 (*S. cerevisiae*) also revealed several subtle differences compared with results of Fig. 2 (CHO membranes): (i) lipid 2 formed with *S. cerevisiae* membranes was never observed to be a doublet. Hence, doublet formation appears to depend upon the source of membranes as opposed to an impurity in the acceptor preparation. (ii) With concentrations of GlcNα-PI(C8) of 33 ng/assay and below (*lanes 3–10*) palmitoyl-CoA clearly enhanced the synthesis of lipid 2, which lacks acyl-inositol (Table I), while there was little or no enhancement at the highest concentrations of GlcNα-PI(C8). (iii) Although the total yeast mannosyltransferase activity with low acceptor concentrations was enhanced by addition of palmitoyl-CoA, under no condition was lipid 1 made without appreciable synthesis of lipid 2. In contrast, we routinely observed synthesis of lipid 1 without appreciable lipid 2 when low concentrations of GlcNα-PI(C8) were incubated with CHO membranes (for example, *lane 4* of Fig. 2; Fig. 3). Observations ii and iii could be reconciled with a preference of the yeast mannosyltransferase for GlcNα-acyl-PI(C8) if the membranes contained an activity which was able to convert Man-GlcNα-acyl-PI(C8) into Man-GlcNα-PI(C8). The presence of such an activity is currently being investigated.

The outcomes of these studies contrasted sharply with those reported earlier for trypanosomes, *i.e.* that mannosylation occurs without acylation. As shown in Fig. 9, incubation of GlcNα-PI(C8) with *T. brucei* membranes gave results consistent with the earlier trypanosomal studies. Lipid "A" was formed in all reactions and is probably MPD synthesized from trypanosomal dolichol-P. Lipid "B" was strictly dependent upon addition of GlcNα-PI(C8), but independent of palmitoyl-CoA. Lipid "B" co-migrated with lipid 2 (Man-GlcNα-PI(C8)) and behaved the same as lipid 2 when tested in the manner indicated by Table I (data not shown). Additional species were noted that migrated more slowly than lipid "B" and that were also GlcNα-PI(C8) dependent/palmitoyl-CoA independent. These were not characterized further, although they likely represent further modification of "B."

With CHO membranes GlcNα-PI(C8) was a good acceptor while GlcNα-PI(C16) was completely ineffective (10). Similarly, with trypanosomal membranes GlcNα-PI(C8) was the better acceptor of the two, although there was clearly some activity with GlcNα-PI(C16) as reported (9). GlcNβ-PI(C8) also behaved similarly in the CHO and trypanosomal systems. The latter system appears capable of generating lipid 2, but not lipid 1, and there was no synthesis of lipid 2 in either case with GlcNβ-PI(C8) as acceptor.

## DISCUSSION

Most reactions involved in assembly of GPI anchor precursors are considered to be similar in all eukaryotic species examined (reviewed in Ref. 1). However, there are many aspects that are species-dependent. At the *sn*-1 position, GPI precursor intermediates typically have alkyl groups in mammals compared with acyl groups in yeasts (2) and trypanosomes (21). In insect-stage *T. brucei* the GPI can be in the *lyso* form (7). All GPI precursors synthesized by yeasts and mammals receive palmitic acid on inositol, while only a fraction of trypanosomal anchor precursors do so (4). Mammalian anchors usually receive an additional EthN-P unit on the first mannose, and sometimes on the second (20). After transfer to protein, anchors can receive a broad assortment of carbohydrate modifications which are species and probably tissue-dependent (reviewed in Ref. 22), and lipid remodeling can occur with myristate in trypanosomes (21) and with ceramide in yeast (23).

This paper deals with two enzymatic reactions, the acylation of inositol residue and the mannosylation of glucosamine residue of GlcNα-PI, which have been suggested to have very different mechanisms depending on the species. Both the sources of the acyl chains and the relative order of acylation and mannosylation have been reported as species-dependent. Prior analyses indicated that palmitoyl-CoA was the source of acyl chains in yeast (2), while some other palmitoyl source was used in trypanosomes (7, 8) and mouse (6). Furthermore, it has been shown that the first mannosylation event occurs before acylation in trypanosomes (5), while in yeast and mouse it had been reported that acylation must precede the first mannosylation (2, 3).

However, upon re-examination of these data it is clear that some other interpretations are possible. For example, regarding the acyl-CoA dependent activity reported with yeast membranes and the acyl-CoA independent activities reported for mouse and trypanosomal membranes, the possibility cannot be excluded that there are two different acyltransferase pathways in each species, but that only one was detected under the *in vitro* conditions used in each case (2, 6, 8). Similarly, it is feasible that the yeast and mammalian mannosyltransferases are independent of acylation, as proposed for trypanosomes, but that the failure to detect Man-GlcNα-PI in a number of studies could be due to rapid acylation. GlcNα-acyl-PI was

reported as an obligatory intermediate because it was identified in mouse (3) and yeast (2) mutants which failed to synthesize MPD, the mannose donor, but such results are equally consistent with a system of mutually independent acyltransferases and mannosyltransferases (Fig. 1). *In vitro* systems (2, 6) have been described which permit the synthesis of GlcN$\alpha$-PI from PI with little or no formation of GlcN$\alpha$-acyl-PI, but MPD was not supplied under these conditions so as to determine whether GlcN-acyl-PI was preferred for mannosylation.

It was important to re-examine some of these issues because if the acyltransferases and/or mannosyltransferases were truly different in yeasts and trypanosomes compared with mammals, it might be possible to target these enzymes for drug therapy. In addition, such results would suggest novel biochemical mechanisms. The limitation of the earlier experiments discussed above is that all GPI intermediates originated from endogenous lipids. Hence, it has been difficult to control the concentrations of potential acceptors such as GlcN$\alpha$-PI and GlcN$\alpha$-acyl-PI. As described in the Introduction, to solve this problem, we took advantage of chemically defined dioctanoyl GlcN$\alpha$-PI derivatives since the dioctanoyl modification was expected to enhance water solubility (24–26). Since no activity was observed in CHO membranes with equivalent synthetic di-C16 (palmitoyl) compounds (in contrast to results with trypanosomal membranes (9)), the use of dioctanoyl compounds with mammalian *in vitro* systems may represent a general technical advantage.

We identified a palmitoyl-CoA dependent acyltransferase activity, analogous to the one previously reported in yeast (2) but not detected in mouse (6) membranes, in our hamster membrane system when dioctanoyl compounds were used. Unlike the mouse system, free CoA was ineffective in the hamster system unless it was coupled to palmitate. This result demonstrated conservation of the mechanism of acylation between yeasts and rodents, and enabled us to control the acylation of GlcN$\alpha$-PI(C8). We observed no mannosylation of low concentrations of unacylated GlcN$\alpha$-PI(C8) by hamster membranes, by either TLC analysis of total butanol-soluble lipids or gel filtration chromatography of $HNO_2$-released glycans. This provides direct support for the earlier suggestions that GlcN$\alpha$-acylPI is an obligatory intermediate (2) and a "missing" biosynthetic "link" (3).

The CHO (Fig. 2) and yeast (Fig. 8) membrane data demonstrate that, at low acceptor concentrations, there is a consistently higher level of net mannose transfer in the presence of palmitoyl-CoA. Unlike the CHO data, interpretation of the yeast data was complicated by the possibility of a deacylase. In either case it was not feasible to reliably quantify the enhancement because the functional concentration of GlcN$\alpha$-acyl-PI(C8) in the assay and the maximum amount of lipid 1 that could be synthesized were dependent upon the amount of palmitoyl-CoA added and the efficiency of acyltransfer. Moreover, it was difficult to estimate the fraction of acceptor that became acylated. Such factors would be expected to have direct effects on the determination of apparent enzymatic parameters, and reliable measurements will therefore require a homogenous preparation of GlcN$\alpha$-acyl-PI(C8).

The mechanism by which acylation enhances mannosylation is unclear. Acylation may stabilize the insertion of the mannosyl acceptor into the membrane; promote partitioning of the acceptor into specific membrane microdomains, activate the mannosyltransferase directly, promote flip-flop (27), or facilitate the ability of the acceptor to achieve an optimal structural conformation or "stereochemical constraint" (8, 27, 28). This last idea appears consistent with the observation that it was possible to mannosylate $\alpha$GlcN-containing acceptor with or without inositol palmitoylation, but $\beta$GlcN-containing acceptor could be mannosylated only after palmitoylation. In other words, the necessity for palmitate may be governed by the stereochemistry of the acceptor.

From these studies it cannot be determined if acyl-CoA is the direct donor of the acyltransferase, as opposed to an endogenous membrane component serving as an intermediary to accept an acyl group from acyl-CoA and then donate it to GlcN-PI. Not surprisingly, many $^3$H-products were formed upon incubation of membranes with [$^3$H]palmitoyl-CoA (Fig. 7) in the absence or presence of GlcN-PI(C8). One of these could be a hypothetical intermediary. However, the steady-state amount of such a molecule must be low because no Man-GlcN-acyl-PI(C8) was formed in the absence of acyl-CoA.

These results have two important implications. First, the trypanosomal pathways for acyltransfer (which does not appear to use acyl-CoA) and mannosyltransfer (which does not require prior acylation) appear fundamentally different from those in yeasts and mammals. Hence, these enzymes may be useful targets for anti-trypanosomal drug design. Second, there is no precedent for a glycosyltransferase which requires the attachment of a lipid onto any part of a glycan other than the reducing or "non-growing" end. Thus, the yeast/mammalian mannosyltransferases may represent a new class of glycosyltransferase. Several properties of the CHO mannosyltransferase, including its donor substrate specificity, have been reported (29).

It is not clear why the acyl-CoA dependent activity was not detected in earlier studies with mouse membrane preparations (6), or why the acyl-CoA independent activity was undetected with our CHO membrane preparation, but there are several explanations. In our system it was not possible to add GTP, a potent stimulator of the acyl-CoA independent activity, as done earlier (6) since GTP interfered with the synthesis of [$^3$H]MPD from GDP-[$^3$H]mannose. It is also possible that the acyl-CoA independent activity does not recognize dioctanoyl acceptors, similar to our observations with PI-PLC, or that it must have a 1-alkyl-2-acyl acceptor as is typically the case in mammalian membranes. The failure to detect either enzyme in the respective systems could be due to some aspect of the membrane preparation or enzyme assay that may have inactivated one of the activities. Along these lines, we noted that 2,3-dimercaptopropanol, which was used to inhibit breakdown of UDP-[$^3$H]GlcNAc in mouse membranes assays (6), inhibited the CHO acyl-CoA dependent acyltransferase activity but not mannosylation of GlcN$\alpha$-acyl-PI.[2]

In summary, these results support the suggestion that GlcN$\alpha$-acylPI is an obligatory intermediate *in vivo* in yeast and rodent cells, and show that the ability of acyl-CoA to serve as an acyl source for GlcN$\alpha$-PI is conserved in yeasts and rodents. This indicates that the acyltransferase and mannosyltransferase may have significant differences, compared with those in trypanosomes, which could be exploited for drug therapy.

*Acknowledgments*—We thank Monica Sosa of the Summer Minority High School Research Apprentice Program (supported by National Institutes of Health Grant R25-RR10128) for assisting with initial studies, Margaret Phillips and Teddy Scott for help with trypanosomal membranes, members of the Joel Goodman lab for advice about *S. cerevisiae*, Sandy Hofmann's group for help with synthesis of [$^3$H]palmitoyl-CoA, and Biswanath Pramanik for assistance with cell culture.

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