Coenzyme A Dependence of Glycosylphosphatidylinositol Biosynthesis in a Mammalian Cell-free System*

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The biosynthesis of glycosylphosphatidylinositol (GPI) in mammals and yeast involves a step not observed in trypanosomes. This reaction, which is the inositol acylation of glucosamine phosphatidylinositol (GlcN-PI), occurs as the third step in the biosynthetic pathway. In this study, conditions were developed to stimulate this reaction in vitro. The synthesis of the GlcN-PI(acy1) from either UDP-[6-3H]GlcNAc or [6-3H]GlcNAc·PI by murine lymphoma cell microsomes was greatly enhanced by the addition of either CoA or palmitoyl-CoA. Stimulation of this reaction was optimal with 1 μM of either compound and required that the precursor, GlcN-PI, be synthesized in the presence of GTP, a specific effector of the formation of this glycolipid. That GlcN-PI(acy1) was generated from GlcN-PI was established by pulse-chase analysis. Because no acyl-chain specificity for acyl-CoA stimulation of GlcN-PI(acy1) synthesis was found and attempts to demonstrate direct transfer of [3H]palmitate from [3H]palmitoyl-CoA to the third intermediate in GPI biosynthesis were unsuccessful, the possibility that free CoA was the activator of this reaction was considered. CoA-stimulated GlcN-PI acylation occurred in the absence of ATP, an essential cofactor for acyl-CoA synthesis, indicating that free CoA is the endogenous effector of the third step in mammalian GPI biosynthesis. This finding is consistent with this inositol acylation being catalyzed by a CoA-dependent transacylase. Mannose-containing GPI intermediates were synthesized in vitro when GDP-mannose was added in the presence of GTP and CoA. Therefore, when effectors of the initial reactions in GPI biosynthesis are included, later steps in this pathway can be studied in mammalian cell-free systems.

Glycosylphosphatidylinositol (GPI) is a novel glycolipid which is utilized as a membrane anchor by proteins in a variety of eukaryotic organisms, including parasites, drosophilas, yeast and mammals (1, 2). GPI is biosynthesized in the endoplasmic reticulum and then transferred en bloc to a newly synthesized protein (3, 4). Studies using trypanosome membranes established that the GPI core is assembled by addition of the predicted carbohydrates to phosphatidylinositol (PI) (5–7). N-Acetylglucosamine (GlcNAc) is transferred from UDP-GlcNAc in the first reaction to form GlcNAc·PI. This glycolipid is then deacetylated to glucosamine-PI (GlcN-PI) and three mannoses are transferred from dolichol phosphomannose to form Man3GlcN-PI. Ethanolamine phosphate is then added to form the mature GPI core (8).

Studies of GPI biosynthesis using a set of murine lymphoma Thy-1-deficient mutants defective in GPI anchor biosynthesis (9, 10) have provided additional information about this pathway. Three complementary mutants (class A, C, and H) are unable to synthesize GlcN-PI, indicating that these gene products are required for the first step in the pathway (11, 12). Two of these genes, designated PIG-A (13) and PIG-H (14), have recently been expression cloned. Analysis of class A mRNA-derived cDNAs of a number of patients suffering from paroxysmal nocturnal hemoglobinuria indicates that defects in this gene are the most common cause of this acquired hemolytic anemia (15). Two other Thy-1-deficient mutants (class B and F) were found to be unable to add the third mannose to the core and the terminal phosphoethanolamine, respectively (12). These genes were recently expression cloned using the lymphoma cell mutants (16, 17).

Analysis of the glycolipid that accumulates in both the Thy-1-deficient mutant (class E) (18) and a yeast mutant (dpm1) (19) unable to add mannoses to the core because of a defect in dolichol phosphomannose synthesis (20) demonstrated that it is GlcN-PI modified by an acyl group on the inositol ring. The addition of an acyl chain to the inositol ring of GlcN-PI has been demonstrated in a cell-free system using yeast membranes (21). This modification renders GlcN-PI(acy1) resistant to PI-specific phospholipase C (PI-PLC) cleavage (22, 23). This evidence, coupled with the finding that all mannose-containing GPI intermediates in mammals are PI-PLC-resistant, has led to the conclusion that the inositol acylation of GlcN-PI must occur before mannose additions in mammalian and yeast GPI biosynthesis. Therefore, the third step in this pathway differs in these species from that in trypanosomes.

In this study, inositol acylation of GlcN-PI was investigated in microsomes prepared from EL4 murine lymphoma cells. This reaction was found to be stimulated by either CoA or palmitoyl-CoA. CoA rather than the palmitoyl-CoA was found to be the endogenous activator of GlcN-PI(acy1) synthesis, indicating that a fatty acyl-CoA is not the acyl donor for this reaction. Finally, in vitro synthesis of mannose-containing GPI precursors was demonstrated when GTP (a stimulator of the second step in GPI biosynthesis) (24) and CoA were included in the reaction to facilitate the earlier reactions in the pathway.

EXPERIMENTAL PROCEDURES

Materials—Horse serum and Dulbecco’s modified Eagle’s medium were purchased from Life Technologies, Inc. UDP-[6-3H]GlcNAc (25 Ci/mmol), GDP-[2-3H]mannose (15 Ci/mmol), [9,10-3H]palmitoyl-CoA (60 Ci/mmol), and [9,10-3H]palmitate (60 Ci/mmol) were obtained from.

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1 The abbreviations used are: GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; PLC, phospholipase C; PI-PLC, PI-specific phospholipase C; DMP, dimercapto propanol; GlcN, glucosamine.
American Radiolabeled Chemicals. PI-PLC from Bacillus cereus was from Boehringer Mannheim. Silica gel 60 TLC plates (E. Merck) were purchased from VWR Scientific. Dowex AG50X-8 mixed bed resin and AG50W-X12(10") cation exchange resin were from Bio-Rad. Aqueous HF, Amberlite DP-1 resin, CoA, acyl-CoAs, appryase, dimercaprotoanol, GTP, ATP, UDP-GlcNac, GDP-mannose, and all other chemicals were obtained from Sigma.

Cell Culture—The wild-type lymphoma cell line EL4 was purchased from the American Type Tissue Collection. The Thy-1-deficient cell line BW5147.3(Thy-1-/-) was the generous gift of Dr. Robert Hyman (Salk Institute). All cells were routinely grown in suspension culture as described previously (25).

Preparation of Microsomes from Lymphoma Cells—The microsomal fraction from the indicated lymphoma cell line was isolated using density centrifugation as described previously (24). The microsomes were frozen in a solution of 50 mM Hepes (pH 7.5), 0.5 mM dithiothreitol, 1 mM TLCK, 1 mg/ml leupeptin, and 10% glycerol and stored at -80°C. The activity of microsomes prepared in this manner was found to be stable for at least 6 months. Protein was quantitated using the bicinchoninic acid assay of Smith et al. (25).

In Vitro Biosynthesis of GPI Precursors—The intermediates in GPI biosynthesis were synthesized using a reaction mixture consisting of 50 mM Hepes (pH 7.5), 1 mM MgCl2, 0.5 mM dithiothreitol, 1 mM TLCK, 1 mg/ml leupeptin, and 0.2 mM GDP-mannose as described previously (24). ATP (1 mM) and dimercaprotoanol (DMP, 1 mM) were also included in the reaction mixture when UDP-[6-3H]GlcNac was the substrate. GTP (1 mM), CoA, palmitoyl-CoA, and GDP-mannose (200 mM) were added to the incubation as indicated. In experiments where GPI precursors were radiolabeled with GDP[14C]mannose (1 Ci), 100 mM GDP[14C]mannose was used in place of UDP[6-3H]GlcNac. Where only GlcNAc-Pi, GlcN-Pi, and GlcN-Pi(acyl) were being synthesized, the reaction was stopped by the addition of 0.5 ml of water and 3 ml of chloroform/methanol (1:2, v/v) containing 0.1 M HCl. The radiolabeled lipids were then extracted using the method of Bligh and Dyer (26) as described previously. In experiments in which GPI precursors beyond the third intermediate were synthesized, the reaction was stopped by the addition of 2 ml of chloroform/methanol (1:1, v/v). The sample was then centrifuged at 2000 rpm for 5 min and the lipid-containing supernatant collected. The pellet was washed once with 2 ml of chloroform/methanol/water (1:1:0.3, v/v) by centrifugation, after which the supernatant was combined with the first and dried in a SpeedVac concentrator. Lipids were resuspended in 400 ml of water-saturated butanol and extracted with 400 ml of water. After separation of the phases by centrifugation, the butanol phase was washed once with 200 ml of water and dried.

Analysis of GPI Precursors—The dried lipids were either treated with PI-PLC as described previously (11) or spotted directly onto Silica Gel G TLC plates. GPI precursors were analyzed using a solvent system of chloroform/methanol/1 M ammonium hydroxide (10:10:3, v/v, v/v). The radioactive lipids were visualized using a Bioscan System 200 Imaging Scanner or by spraying the plate with ENHANCE and exposing it to Kodak XAR-5 film. The various radiolabeled GPI precursors were quantitated using the two-dimensional analysis software of the Bioscan.

Measurement of Palmitoyl-CoA Synthesis—EL4 microsomes were incubated with reaction mixture supplemented with 1 mM CoA and 30 mM [9,10-3H]palmitate (final specific activity = 0.22 Ci/mmol) for the indicated time. The reaction was stopped by adding 1.5 ml of isopropyl alcohol/heptane/1 N H2SO4 (40:10:1, v/v) and the fatty acyl-CoAs separated from fatty acids as described by Dele (27). Briefly, the solution was partitioned into two phases by the addition of 0.6 ml of heptane and 0.9 ml of water. An aliquot (50 ml) of the upper phase was counted with a Wallac 1409 liquid scintillation counter. In each experiment, control reactions were done in which no enzyme source was added to determine the amount of the radioactive substrate which nonspecifically partitioned into the upper phase. This number was then subtracted from the total radioactivity found in the upper phase of each reaction to determine the amount of [9,10-3H]palmitoyl-CoA synthesized.

Structural Analysis of Mannose-containing GPI Intermediates—The mannose-containing intermediates M-A and M-B were biosynthesized from [3H]UDP-GlcNac by EL4 microsomes and extracted as described above. The glycolipids were digested with PI-PLC to eliminate unacylated GPI precursors with similar mobility, re-extracted, and separated by TLC. The spots corresponding to M-A and M-B were located by scanning the TLC plate with the Bioscan System 200 Imager and scraped. The glycolipids were extracted from the silica with 4 ml of chloroform/methanol/H2O (10/10/3, v/v) and dried. [3H]Mannose-labeled GPI intermediates synthesized from GDP[14C]mannose by trypanosome membranes as described previously (7) were the generous gift of Dr. Anant Menon (University of Wisconsin, Madison).

Neutral glycans were prepared from M-A, M-B, and the trypanosome intermediates by first treating the glycolipids with aqueous HF as described by Mayor and Menon (26). After neutralization with LiOH, an equal volume of water-saturated butanol was added to each sample and the butanol and water phases. Each phase was washed once and the washes combined with appropriate phase. The butanol extracts were then dried under vacuum and subjected to base hydrolysis in 0.1 N methanolic KOH at 37°C for 1 h. The reaction was neutralized with 10 ml of 1 N HCl, the products dried, resuspended in 100 ml water and combined with the water phases. The glycans were desalted by passage over AG50X-8 mixed bed resin and neutralized by N-acetylation with acetic anhydride (28). The neutral products were then desalted again by tandem ion exchange chromatography over a column consisting of 0.2 ml of Amberlite DE-1(5Na+) cation exchange resin over 0.4 ml of AG50W-X12(10") cation exchange resin. The final products were analyzed by TLC (three times) on Silica Gel 60 plates in a solvent system of butanol/ethanol/H2O (4:3:3, v/v) as described by Puoti and Conzelmann (29).

RESULTS

Effect of CoA or Palmitoyl-CoA on GlcN-Pi(acyl) Synthesis—The third step in the biosynthesis of GPI in yeast (21) and mammalian cells (18) is the acylation of GlcN-Pi to form GlcN-Pi(acyl). The endogenous donor of the acyl group has not been identified in mammalian cells. Therefore, to test if an acyl-CoA is the acyl donor, the effect of CoA or palmitoyl-CoA on GPI biosynthesis by EL4 lymphoma cell microsomes was assessed. The GPI precursors isolated after a 1-h incubation in the presence of the indicated effectors are shown in lane 1 of Fig. 1. As previously reported (24), in the presence of GTP (lane 2), GlcN-Pi was the predominant product and a third, more hydrophobic product appeared. Consistent with this being GlcN-Pi(acyl), this third product was nitrous acid sensitive, modified by chemical acetylation, resistant to hydrolysis by PI-PLC, and had identical mobility on TLC as the inositol-labeled glycolipid accumulated in the class E Thy-1-deficient mutant (data not shown). In the
synthesis. GPI precursors were synthesized from UDP-[6-3H]GlcNAc by EL4 microsomes for the indicated times in the presence of CoA alone (1 μM, Panel A) or CoA and GTP (1 μM, Panel B). GlcNAc-PI (open circles), GlcN-PI (filled circles), and GlcN-PI(acyl) (squares) were separated and quantitated as described under “Experimental Procedures.” The results shown are the average of triplicate determinations (± S.E.).

The total dependence of the stimulation of the third reaction by CoA or an acyl-CoA on GTP is further illustrated in the time course of the reaction shown in Fig. 2. In Panel A, in which only CoA was included in the reaction, the first product remained the predominant product synthesized during the course of the experiment. A small, constant proportion (~20%) of the GlcN-PI produced was acylated to GlcN-PI(acyl) (similar results were observed with palmitoyl-CoA) (data not shown). In contrast, when both CoA and GTP were added to the reaction, the desacylation of GlcNAc-PI was increased significantly and majority of the GlcN-PI was rapidly acylated. Within 20 min, the third GPI precursor was the predominant glycolipid produced by the microsomes.

GlcN-PI(acyl) Is Synthesized from GlcN-PI—To establish that GlcN-PI(acyl) was generated by the acylation of GlcN-PI, a pulse-chase experiment was done to establish a precursor-product relationship between these two glycolipids. These results are shown in Fig. 3. The pulse reaction was done with GTP present to generate GlcN-PI as the predominant reaction product. Upon addition of CoA (1 μM), the level of this glycolipid rapidly decreased to approximately 50% of the starting amount. Concurrently, the level of GlcN-PI(acyl) increased proportionately. The first intermediate in the pathway, GlcNAc-PI, remained fairly constant throughout the chase period. Therefore, GlcN-PI(acyl) clearly appears to be derived from GlcN-PI.

Acylation of CoA Is Not Required for Stimulation of GlcN-PI(acyl) Synthesis—To further investigate the role of CoA and/or acyl-CoA in the acylation of GlcN-PI, and to determine if acyl-CoA is the endogenous donor of the acyl group, several experiments were done. First, the optimal concentration of CoA and palmitoyl-CoA for stimulation of GlcN-PI acylation was determined. These results are shown in Fig. 4. The synthesis of GlcN-PI(acyl) was maximally stimulated by 1 μM CoA (Panel A) or palmitoyl-CoA (Panel B). Concentrations greater than 1 μM were inhibitory to both GlcN-PI(acyl) synthesis and the earlier steps in the pathway. The reason for this is unknown. Although very little GlcN-PI(acyl) was synthesized in the absence of GTP, the same concentration of CoA or palmitoyl-CoA (1 μM) stimulated the acylation reaction maximally when this nucleotide was left out of the reaction (data not shown).

To investigate the acyl-chain requirements for the third reaction in GPI biosynthesis, the ability of various chain length acyl-CoAs to stimulate GlcN-PI acylation was assessed. As shown in Fig. 5, all acyl-CoAs tested, from n-butyryl-CoA to oleoyl-CoA, stimulated GlcN-PI(acyl) synthesis to the same extent and in the same concentration range. Therefore, varying the length of the acyl chain of the acyl-CoA had no effect on GlcN-PI acylation. Furthermore, the GlcN-PI(acyl) synthesized in each of these reactions had the same mobility on TLC, suggesting that the exogenously added acyl group was not transferred to GlcN-PI.

Finally, to determine directly if the acyl group was donated by the acyl-CoA, the biosynthetic reaction was carried out in...
CoA Dependence of GlcN-PI Acylation

A. CoA

CoA (µM)  
0.01  0.1  1  10  100

GlcN-PI

GlcN-PI(acyl)

GlcNAc-PI

B. Pal-CoA

Pal-CoA (µM)  
0.01  0.1  1  10  100

GlcN-PI

GlcN-PI(acyl)

GlcNAc-PI

Fig. 4. Concentration dependence of the CoA stimulation of GlcN-PI(acyl) synthesis. GPI precursors were synthesized from UDP-[6-3H]GlcNAc by EL4 microsomes in the presence of GTP (1 mM) and the indicated concentration of CoA or palmitoyl-CoA. After 1 h, GlcNAc-PI (open circles), GlcN-PI (filled circles), and GlcN-PI(acyl) were separated by TLC and quantitated as described under "Experimental Procedures." The results shown are the average of triplicate determinations (± S.E.).

The presence of [3H]palmitoyl-CoA. Despite carrying out this reaction in the presence of GTP (1 mM), excess UDP-GlcNAc (100 µM), and levels of this compound expected to result in significant radioactivity incorporation into GlcN-PI(acyl), no evidence of transfer of [3H]palmitate to GlcN-PI(acyl) could be found (data not shown). Therefore, we were unable to demonstrate directly that an exogenously supplied acyl-CoA stimulated GlcN-PI(acyl) by acting as the acyl donor.

Taken together, the findings of a low optimal concentration of palmitoyl-CoA and CoA, no acyl chain specificity for the acyl-CoA-stimulated reaction, and no direct evidence of transfer of the acyl group from palmitoyl-CoA led us to consider that CoA rather than the acyl-CoA was stimulating GlcN-PI(acyl) synthesis. Therefore, the ability of CoA to stimulate GlcN-PI acylation under conditions in which CoA acylation could not occur was determined. This was accomplished by measuring GlcN-PI acylation in the absence of ATP or other nucleotide triphosphates (ATP is an essential requirement for acyl-CoA synthesis). A pulse-chase experiment was done in which the microsomes were incubated with UDP-[6-3H]GlcNAc in the presence of ATP and GTP to allow synthesis of GlcN-PI as the predominant GPI precursor. The nucleotide triphosphates were then removed using two different procedures. In the first, referred to as a the wash procedure, the microsomes were pelleted by centrifugation, resuspended in incubation buffer alone and incubated at 37 °C for 5 min. The microsomes were then repelleted and resuspended in the same volume of incubation buffer. The second procedure, referred to as the wash + apyrase procedure, was the same except that 1 unit of apyrase was included in the 5 min, 37 °C incubation. The chase was then initiated by the addition of excess unlabeled UDP-GlcNAc (100 µM) and CoA (1 µM). Parallel incubations were done in which the synthesis of [3H]palmitoyl-CoA from exogenously supplied [3H]palmitate was measured during the CoA chase to determine if the wash conditions were sufficient to block acyl-CoA synthesis.

The results of this experiment are shown in Fig. 6. In the case of the normal pulse-chase in which nothing is done to remove the ATP and GTP, the level of GlcN-PI decreased during the CoA chase while GlcN-PI(acyl) levels increased proportionately (Panel A). At intermediate times during the chase (15 and 45 min), significant amounts of [3H]palmitoyl-CoA were synthesized (Panel B). With either the wash or the wash + apyrase procedures, the total amount of radioactivity recovered was decreased by approximately 60% (Panels C and E). However, with both procedures, significant synthesis of GlcN-PI(acyl) occurred during the chase. Measurement of [3H]palmitoyl-CoA synthesis confirmed that no acyl-CoA was made under these conditions (Panels D and F), indicating that free CoA is responsible for the stimulation of GlcN-PI acylation.

In Vitro Synthesis of Mannose-containing GPI Intermediates—With conditions to increase synthesis of GlcN-PI(acyl) established, we then tested whether later GPI intermediates could be formed in vitro. Incubations were carried out in the presence of GTP, CoA, and GDP-mannose. The products formed are shown in Fig. 7. As observed previously (Fig. 1), in the absence of a mannose donor, GlcNAc-PI, GlcN-PI, and GlcN-PI(acyl) were synthesized (Fig. 7, lane 1). PI-PLC treatment of these products cleaved all but the acylated product, GlcN-PI(acyl) (lane 2). Upon addition of GDP-mannose to the
FIG. 6. ATP independence of GlcN-PI acylation. EL4 microsomes were incubated with UDP-[6-3H]GlcNAc in the presence of 1 mM GTP for 1 h to biosynthesize GlcN-PI. The microsomes were then subjected to either no treatment (Panel A, No Wash), the wash procedure described under "Results" (Panel C, Wash), or the wash procedure and an incubation with apyrase (Panel E). Excess UDP-GlcNAc (100 μM) and CoA (1 μM) was then added and the incubation continued for an additional hour. The amount of GlcN-PI, GlcN-PI, and GlcN-PI(acyl) synthesized before (solid bars, Panels A, C, and E) and after the CoA chase (hatched bars, Panels A, C, and E) was quantitated as described under "Experimental Procedures." In parallel incubations, EL4 microsomes were incubated for 1 h in reaction buffer without UDP-[6-3H]GlcNAc and then subjected to no treatment (Panel B), the wash procedure (Panel D), or the wash and apyrase incubation (Panel F). CoA (1 μM) and [9,10-3H]palmitate (30 μM) was then added to the reaction and the incubation was continued at 37°C for 15 (open bars, Panels B, D, and F) or 45 min (hatched bars, Panels B, D, and F). The amount of [9,10-3H]palmitoyl-CoA was quantitated as described under "Experimental Procedures." Triplicate determinations were made for each group and the results show the average ± S.E.

incubation, two additional products were detected (lane 3), both resistant to cleavage by PI-PLC (lane 4). These two products have been designated M-A and M-B because their precise structure has not yet been determined. To confirm that M-A and M-B were mannose-containing GPI intermediates, identical incubations were done using GDP-[2-3H]mannose as the source of the radiolabel rather than UDP-[6-3H]GlcNAc (lanes 5–8). In lanes 5 and 6, GTP, CoA, and GDP-[2-3H]mannose but no UDP-GlcNAc was included in the incubation. Several mannose-containing products were synthesized and all were resistant to PI-PLC-catalyzed hydrolysis. One of these products, shown with the top open arrowhead, had the same migration as M-A. With UDP-GlcNAc included in the incubation (lanes 7 and 8), PI-PLC-insensitive mannose-containing products with identical migration as both M-A and M-B were synthesized. Therefore, the two new UDP-[6-3H]GlcNAc-labeled products synthesized when GDP-mannose was added to the incubation appear to contain mannose.

To demonstrate that M-A and M-B were GPI intermediates, microsomes isolated from the class E Thy-1-deficient lymphoma mutant were tested for their ability to synthesize these glycolipids. These microsomes were incubated with UDP-[6-3H]mannose and excess cold GDP-[2-3H]mannose (lanes 9–12). As with the EL4 microsomes, BW6147.3(Thy-1–) microsomes synthesized GlcN-PI, GlcN-PI, and GlcN-PI(acyl) when incubated with GTP and CoA (lanes 9 and 10). However, no additional products were made when GDP-mannose was included in the incubation (lanes 11 and 12). The finding that M-A and M-B are not synthesized by microsomes unable to make mannose additions is consistent with these glycolipids being mannose-containing GPI precursors.

To establish that M-A and M-B are derived from the third GPI intermediate, a pulse-chase experiment was done (Fig. 8). The pulse incubation was done in the presence of GTP and CoA to maximize synthesis of GlcN-PI(acyl). The chase was initiated after 1 h by the addition of GDP-mannose and excess cold UDP-GlcNAc. As shown in Fig. 8, GlcN-PI(acyl) (open circles) and GlcN-PI (closed circles) levels remained fairly stable during the time course of the chase, indicating that these GPI intermediates were not undergoing further metabolism. The level of GlcN-PI(acyl) (open triangles) fell rapidly during the chase as the levels of M-A (open squares) and M-B (closed squares) increased. Therefore, the mannose-containing GPI intermediates appear to be synthesized from GlcN-PI(acyl).

Partial Structural Analysis of M-A and M-B—Neutral glycolipids from M-A and M-B were compared to trypanosome standards to determine the number of mannose residues each contained. Shown in Fig. 9 is the mobility of the decylated, dephosphorylated, and chemically acetylated glycolipids derived from M-A (top panel) and M-B (bottom panel). At the top of the M-A profile is the mobility of the trypanosome standards generated by the same procedure having 3, 2, or 1 mannose residue. The major component in the M-A sample migrated to the same position as the 1-mannose-containing standard (top panel). A smaller, more hydrophobic peak which does not correspond to any of the standards was also observed in the M-A sample. This probably is derived from GlcN-PI(acyl) that contaminated the original TLC-purified preparation of M-A (incomplete digestion with PI-PLC would leave residual GlcN-PI). The major species found in M-B (bottom panel) migrated with the standard containing three mannoses. There was minor contamination of this sample with a compound that appeared to be M-A. These analyses indicate that M-A contains one mannose and M-B has three mannoses.

DISCUSSION

The addition of CoA or palmitoyl-CoA was found to greatly increase the synthesis of GlcN-PI(acyl) by microsomes prepared from EL4 murine lymphoma cells. Because these two compounds can be readily interconverted by enzymes in the microsomes, it was not clear which was active in GlcN-PI acylation. The stimulation of this reaction appeared identical with CoA and palmitoyl-CoA and was optimal with 1 μM of either. If conversion of one species to the other is required (i.e. acyl-CoA to CoA or vice versa), then the finding that both compounds stimulated the same optimal concentration suggests that the conversion reaction occurred faster than the acylation reaction. The optimal concentration for palmitoyl-CoA is significantly lower than expected based on those found for other acyl-CoA-utilizing enzymes. For instance, serine palmitoyltransferase uses palmitoyl-CoA as the acyl donor in initial steps of sphingolipid biosynthesis and is maximally active in a microsomal preparation with at least 120 μM palmitoyl-CoA (31). Coupled with the lack of acyl chain specificity for the acyl-CoA
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**Fig. 7. Synthesis of later GPI intermediates.** Microsomes prepared from EL4 cells (lanes 1–8) or BW5147.3(Thy-1'e) cells (lanes 9–12) were incubated with UDP-[6-3H]GlcNAc, ATP, DMP, GTP, and CoA; lanes 3, 4, 11, and 12, ATP, DMP, GTP, CoA, and GDP-mannose; lanes 5 and 6, ATP, DMP, GTP, and CoA; and lanes 7 and 8, ATP, DMP, GTP, CoA, and UDP-GlcNAc. The radioactive glycolipids were extracted and analyzed by TLC as described under "Experimental Procedures." The samples in lanes 2, 4, 6, 8, 10, and 12 were treated with PI-PLC prior to separation by TLC. The open arrowheads to the left of lanes 9–12 indicate the position on the TLC plate where M-A and M-B are expected to run.

**Fig. 8. Later GPI intermediates M-A and M-B are derived from GlcN-PI(acyl).** A pulse-chase experiment was carried out in which EL4 microsomes were incubated in the presence of UDP-[6-3H]GlcNAc, ATP, DMP, GTP, and CoA for 1 h. Excess unlabeled UDP-GlcNAc (100 μM) and GDP-mannose (100 μM) was then added to the incubation to initiate the chase. The reaction was stopped at the indicated time points and the amount of GlcN-PI-acyl (open circles), GlcN-PI (closed circles), GlcN-PI(acyl) (open triangles), M-A (open squares), and M-B (closed squares) quantitated as described under "Experimental Procedures." Triplicate determinations were done for each group and the points shown are represent the average ± S.E.

stimulation and the inability to demonstrate the direct transfer of [3H]palmitate from [3H]palmitoyl-CoA to GlcN-PI(acyl), this finding led us to suspect that CoA may be a direct activator of this reaction. Therefore, the dependence of the CoA stimulation of GlcN-PI acylation on ATP was tested to determine if this effect was seen when CoA could not be acylated. Significant GlcN-PI(acyl) was synthesized in the absence of acyl-CoA formation, indicating that free CoA was capable of stimulating the third reaction in GPI biosynthesis. These findings rule out acyl-CoA as the donor of the inositol-linked acyl group on GlcN-PI(acyl) in mammalian cells. While the cellular source of this group is not established in this study, it seems likely that it comes from a membrane phospholipid and is transferred to GlcN-PI by a transacylase. The dependence of this reaction on CoA suggests that the enzyme uses this compound in the reaction. CoA-dependent, ATP-independent transacylases that mediate the transfer of fatty acids between phospholipids and lysophospholipids have been characterized in a number of tissues (32–35). These reactions involve transfer the sn-2-acyl chain from phospholipids to either the sn-1 position of lysophosphatidylinositol (35) or the sn-2 position of lyso-platelet-activating factor (32, 36), lysophosphatidylethanolamine (34, 37), lysophosphatidylserine (38), or lysophosphatidylcholine (33). In some cases (35, 37), the acyl transfer is suggested to be catalyzed by the reverse action of an acyl-CoA:lysophosphatide acyltransferase. Whether this is the case for the acylation of GlcN-PI can be determined from the data presented here. For all of these reactions, an acyl-CoA intermediate is formed during the transfer reaction. Because this intermediate is enzyme-associated, free fatty acids do not affect its rate of formation. We have found that the addition of 30 μM palmitate did not alter CoA-stimulated GlcN-PI(acyl) synthesis (data not shown). Therefore, the characteristics of the GlcN-PI acylation reaction are consistent with it being catalyzed by a CoA-dependent transacylase. Whether the acyl group comes from a specific phospholipid and the identity of this donor will be addressed in future studies.

Costello and Orlean (21) reported that acyl-CoA is the donor of the inositol acyl group of GlcN-PI(acyl) in yeast and showed direct transfer of [14C]palmitate from [14C]palmitoyl-CoA to the
third GPI intermediate. In this system, GlcN-PI acylation was stimulated by palmitoyl-CoA or CoA plus ATP but not with CoA alone. In trypanosomes, in which inositol acylation of GPI precursors occurs after addition of mannose residues, this reaction is not stimulated by either CoA or an acyl-CoA (7). Therefore, the acylation reaction appears to be different in each of the three species. While it occurs at the same step in yeast and mammals, the fatty acid transfer appears to be acyl-CoA-mediated in the former and CoA-dependent transacylase-mediated in the latter. Although less information is available about inositol acylation in trypanosomes, it appears that this reaction involves a CoA-independent transacylase (42). An additional difference between the three systems is that synthesis of GlcN-PI and latter GPI intermediates is dependent upon GTP in the mammalian system while these reactions do not appear to require this nucleotide triphosphate in the either the yeast or trypanosome systems. The consequences of this difference on GlcN-PI acylation is not clear.

When GlcN-PI(acyl) is synthesized in a coupled reaction from either UDP-[6-3H]GlcNAc or [6-3H]GlcN-PI, the stimulation by CoA is completely dependent on GTP. While we cannot rule out the possibility that this nucleotide has a direct effect on GlcN-PI acylation, the fact that this glycolipid is synthesized in its absence (if GlcN-PI is made in its presence as in the pulse-chase experiment in Fig. 6) suggests that it does not. One possible mechanism by which the third reaction could be dependent on GTP without being affected directly is if this nucleotide is involved in making GlcN-PI available for acylation. If this were the case, then GTP-mediated removal of GlcN-PI for acylation would have to relieve some end product inhibition for stimulation of GlcN-PI synthesis to be observed. Possible mechanisms by which GTP could affect the availability of GlcN-PI for acylation include physical relocation of this glycolipid within the microsome or transfer to the active site of the transacylase. While it is known that GlcN-PI and GlcN-CAc-PI are synthesized on the cytoplasmic face of the endoplasmic reticulum (39) and transfer of the mature GPI precursor occurs in the lumen (40), the topology of the other GPI sorors occurs in the lumen (40), the topology of the other GPI intermediates, and Drs. Chuck Rock, Dennis Voelker, Ten-Ching Lee, and David Lambeth for helpful discussions during the course of this work.

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