Stimulation of glycosylphosphatidylinositol biosynthesis in mammalian cell-free systems by GTP hydrolysis: evidence for the involvement of membrane fusion

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The second step in glycosylphosphatidylinositol (GPI) biosynthesis, the deacetylation of GlcNAc-phosphatidylinositol (GlcNAc-PI), has been shown to be stimulated by GTP hydrolysis [Stevens (1993) *J. Biol. Chem.* **268**, 9718–9724]. We have now developed a system to study this regulation that uses microsomes from cells defective in the first step in GPI biosynthesis (class A, C and H lymphoma mutants) and the second reaction in the pathway (G9PLAP.85). With this mixed-microsome system, the deacetylation of GlcNAc-PI was almost completely dependent on GTP hydrolysis. Because GlcNAc-PI synthesized by the G9PLAP.85 microsomes cannot readily move to the first-step-mutant microsomes to be deacetylated, this result indicated that the role of GTP was to facilitate the ‘apparent’ transfer of this

substrate between membrane vesicles. The microsomes could be stably preactivated by pretreatment with GTP before GPI biosynthesis was initiated, indicating that fusion was the most likely mechanism for this regulation. GlcNAc-PI deacetylation could also be stably preactivated in EL4 microsomes, suggesting that fusion also occurred in wild-type membranes. Some differential localization of the GlcNAc-PI synthetic and deacetylation activities with the endoplasmic reticulum was found. Therefore fusion seems to stimulate GPI biosynthesis in mammalian microsomes by bringing together the first two enzymes in the pathway in the same membrane vesicle.

Key words: deacetylation, homotypic fusion, regulation.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) membrane anchors are pre-assembled in the endoplasmic reticulum (ER) and then added *en bloc* to newly synthesized proteins containing the proper targeting information [1,2]. The biosynthesis of GPI occurs by the sequential assembly of the components of the glycan core on phosphatidylinositol (PI) [3–5]. In the first reaction, GlcNAc is transferred from UDP-GlcNAc to PI to make GlcNAc-PI. This intermediate is then deacetylated in the second step to form GlcN-PI. Assembly of the glycan core continues with the sequential transfer of three mannose residues from dolichol-phosphomannose to GlcN-PI. Finally, a phosphoethanolamine residue is added to the third mannose residue to complete the core structure.

In addition to the elucidation of the sequence of reactions, the study of GPI biosynthesis so far has resulted in the identification of the donors for each of the core components [3,6–9] and the cloning of several cDNA species that encode proteins involved in the pathway [10–15]. However, investigations into the enzymology and regulation of the pathway have been limited primarily to the second step, the deacetylation of GlcNAc-PI. The enzyme that catalyses this reaction, the GlcNAc-PI deacetylase, has been partly purified from trypanosomes by Milne et al. [16]. The substrate specificities of the enzyme from trypanosomes [16,17] and from humans [17] have also been examined. Characterization of this reaction in microsomes prepared from cultured mammalian cells revealed that GlcNAc-PI deacetylation

was stimulated by GTP hydrolysis [18]. This regulation of the second step of GPI biosynthesis seems species-specific because GTP did not stimulate this reaction in either yeast or trypanosome (V. L. Stevens, unpublished work) membranes. Although the stimulation of GlcNAc-PI deacetylation occurs with physiologically relevant concentrations of GTP, the mechanism of this regulation and its consequences for GPI biosynthesis *in vivo* are not known.

The isolation of a new mutant defective in GlcNAc-PI deacetylation [19], assigned to the class L complementation group, has provided an invaluable tool for studying this reaction and its regulation in mammalian cells. In principle, the phenotype of this mutant could arise from a defect in either the deacetylase itself or the GTP-regulatory component. Nakamura et al. [15] have recently cloned a cDNA that restores the GlcNAc-PI deacetylation in class L mutant. The protein encoded by this PIG-L gene does not seem to be the GTP regulatory component because it lacks the main sequence elements of GTP-binding motifs [20]. Although formal proof that the PIG-L protein is the deacetylase itself awaits demonstration of this activity in the expressed protein, it seems likely that the gene encodes this enzyme. If this is so, the apparent inability of PIG-L to bind nucleotides indicates that the enzyme and GTP-regulatory component are separate proteins.

In the present study we have used the class L mutant to establish a new assay for the regulation by GTP of GlcNAc-PI deacetylation. When class L microsomes were mixed with those isolated from a mutant cell line defective in the first step of GPI

Abbreviations used: ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; GTP[S], guanosine 5′-[γ-thio]triphosphate; PI, phosphatidylinositol; Tos-Lys-CH₂Cl (‘TLCK’), tosyl-lysylchloromethane.

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biosynthesis (classes A, C or H) [21], GlcNAc-PI deacetylation was found to be almost completely dependent on GTP. Characterization of this regulation suggested that the role of nucleotide hydrolysis was to facilitate substrate delivery to the deacetylase enzyme by stimulating the fusion of microsomal membranes. The relevance of this activity to GPI biosynthesis in wild-type cells was also investigated.

MATERIALS AND METHODS

Materials

Horse serum, Ham's F-12 medium, Dulbecco's modified Eagle's medium and G418 were purchased from Gibco-BRL. Fetal bovine serum was purchased from Atlanta Biologicals. UDP-[6-³H]GlcNAc (25 Ci/mmol) was obtained from American Radio-labelled Chemicals. Silica-gel 60 TLC plates (E. Merck) were purchased from VWR Scientific. En³Hance was from NEN-Dupont. CoA, GTP, ATP, UDP-GlcNAc, activated charcoal and all other chemicals were obtained from Sigma.

Cell culture

G9PLAP.85 cells were grown in monolayer cultures in Ham's F-12 medium containing 10% (v/v) fetal bovine serum, 100 i.u./ml penicillin, 100 µg/ml streptomycin and 300 µg/ml G418 (Gibco) in air/CO₂ (19:1) at 37 °C. Cells were harvested and passaged by removing the medium, washing with PBS and detaching from the tissue culture dish with 0.5 mM EDTA in PBS.

EL4, S49(Thy-1^a), TIMI(Thy-1^c) and S49(Thy-1^h) murine lymphoma cells were grown in suspension cultures in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) horse serum, 100 i.u./ml penicillin and 100 µg/ml streptomycin. The cells were passaged every 3 or 4 days and seeded at a density of 10⁵ cells/ml. Cell numbers were determined by counting with a haemocytometer.

Preparation of subcellular fractions

Microsomes were prepared from G9PLAP.85, EL4 and S49(Thy-1^a) cells by using differential centrifugation as described previously [18], then resuspended in microsome buffer [10 mM Hepes (pH 7.5)/0.5 mM EDTA/0.1 mM dithiothreitol/1 µg/ml leupeptin/0.1 mM tosyl-lysylchloromethane (Tos-Lys-CH₂Cl, 'TLCK')/10% (v/v) glycerol] by homogenization. Microsomes were stored at -80 °C for up to 6 months without loss of activity.

EL4 cells were fractionated by density-gradient centrifugation with the procedure described by Vidugiriene and Menon [22], with minor modifications. In brief, washed EL4 cells were resuspended in 0.25 M sucrose/10 mM Hepes (pH 7.5)/1 mM dithiothreitol/1 µg/ml leupeptin/0.1 mM Tos-Lys-CH₂Cl at a density of 5 × 10⁷ cells/ml. The cells were then lysed by nitrogen cavitation and a post-nuclear supernatant was prepared by centrifugation at 10000 g for 15 min at 4 °C. The post-nuclear supernatant (4.06 ml) was layered on a preformed sucrose gradient consisting of 2.52 ml of 38% (w/v) sucrose, 1.26 ml of 30% (w/v) sucrose and 1.26 ml of 20% (w/v) sucrose. This gradient was then centrifuged for 2 h at 10000 g (42000 rev./min) in a Sorvall TH-641 rotor. Four fractions of 1.96 ml (fraction 1), 2.1 ml (fraction 2), 2.38 ml (fraction 3) and 2.66 ml (fraction 4) were collected from the top of the tube. The pellet was resuspended in 1 ml of resuspension buffer by homogenization to make fraction 5. The fractions were assayed for organelle-specific activities to localize the plasma membrane (alkaline phosphodiesterase I [23]), Golgi (α-mannosidase II, [23]) and ER (dolichyl-

phosphate-mannoside synthase [24]) by methods described previously.

In some cases, microsomes or subcellular fractions were washed with activated charcoal by the method of Peveri et al. [25] before use, to deplete endogenous nucleotides. In brief, activated charcoal was prepared by preincubating 1% charcoal (w/v, in PBS) with 1% BSA (w/v, in PBS) for 2 h at 25 °C. The charcoal was then collected by centrifugation (10000 g for 5 min) and washed four times with 10 mM Hepes (pH 7.5)/100 mM KCl/3 mM NaCl/3.5 mM MgCl₂ by centrifugation. The activated charcoal was added directly to microsomes or subcellular fractions at a concentration of 5 mg/ml in 50 mM Hepes (pH 7.5)/2 mM EDTA/0.5 mM dithiothreitol/1 µg/ml leupeptin/0.1 mM Tos-Lys-CH₂Cl and incubated for 30 min at 4 °C. Finally, the charcoal was removed by centrifugation at 10000 g for 10 min.

Biosynthesis of GPI precursors *in vitro* and their analysis

The cell-free synthesis of GPI precursors [GlcNAc-PI, GlcN-PI and GlcN-PI(acyl)] from UDP-[6-³H]GlcNAc by microsomes or a subcellular fraction from a single cell line was performed as described previously [19]. In cases in which microsomes from two cell lines were mixed (referred to as the mixed-microsome system), conditions were the same except that approximately twice the amount of protein was added to the incubation mixture. GlcNAc-PI deacetylation was assayed directly with the use of radiochemically pure, enzymically prepared [³H]GlcNAc-PI [18] under the same conditions except that ATP and EDTA were omitted from the reaction mixture. Various concentrations of GTP were included, as indicated.

Preactivation of GlcNAc-PI deacetylation was accomplished by incubating the indicated microsomes or subcellular fraction in GTP (100 µM or 1 mM, as indicated)/1 mM ATP/10 mM Hepes (pH 7.5)/5 mM MgCl₂/0.5 mM dithiothreitol/1 µg/ml leupeptin/1 mM Tos-Lys-CH₂Cl at 30 °C for 20 min. The microsomes or fraction were then reisolated by centrifugation (436000 g for 15 min) with a TLA-100 rotor, resuspended in microsome buffer (without glycerol) and used in the biosynthetic incubation with UDP-[6-³H]GlcNAc, as described [19].

GPI precursors were extracted from the incubation mixture by the method of Bligh and Dyer [26] and fractionated by TLC on silica-gel 60 plates (E. Merck) with chloroform/methanol/1 M NH₄OH (10:10:3, v/v). Radiolabelled lipids were detected by fluorography by spraying the plate with En³Hance and exposing it to Kodak XAR-5 film. The GPI precursors were quantified with a Bioscan System 200 imaging scanner by using the two-dimensional analysis software.

In some cases the radiolabelled GPI precursors were separated by charge before TLC analysis. This was accomplished by chromatography on DEAE-cellulose, as described previously [18].

RESULTS

GTP dependence of GlcNAc-PI deacetylation in a mixed-microsome system

Previous studies have established that GTP stimulates the rate of synthesis and permits the acylation of the second intermediate in GPI biosynthesis in mammalian cell-free systems (i.e. the second and third reactions in the pathway) [18,27]. However, neither the mechanism by which GTP affects GlcNAc-PI deacetylation or the physiological relevance of this regulation is clear. To study this problem, a cell-free system was established in which microsomes prepared from a cell line defective in GlcNAc-PI bio-

Figure 1 The mixed-microsome system for the analysis of the GTP regulation of GlcNAc-PI deacetylation

G9PLAP.85 microsomes are able to synthesize GlcNAc-PI from UDP-GlcNAc (left panel) but not to deacetylate it (right panel). Microsomes from any of the mutant cell lines defective in GlcNAc-PI synthesis (class A, C, or H) cannot make this first intermediate but can deacetylate it if supplied with GlcNAc-PI from another source. In this mixed system, microsomes from the two mutants are mixed and the effect of GTP on the deacetylation of GlcNAc-PI is measured. The depiction of the GlcNAc-PI synthetase in the G9PLAP.85 microsomes as a heterotrimeric enzyme made up of the A, C and H proteins might or might not represent the true nature of this protein.

Figure 2 GlcNAc-PI deacetylation in the mixed-microsome system

Microsomes from G9PLAP.85 cells (lanes 1–3), S49(Thy-1^{-a}) cells (lane 4) or G9PLAP.85 cells and S49(Thy-1^{-a}) cells mixed together in approximately equal proportions (by protein, lanes 5–8) were incubated with UDP-[6-³H]GlcNAc in the presence of ATP alone (lanes 1, 4, 5 and 7) or with GTP (lanes 2 and 6), CoA (lane 3), or GTP and CoA (lane 8). After 1 h the GPI intermediates were extracted and analysed by TLC as described in the Materials and methods section.

synthesis [S49(Thy-1^{-a})] were mixed with microsomes from a cell line unable to catalyse the deacetylation of GlcNAc-PI (G9PLAP.85). This system is shown diagrammatically in Figure 1. The GPI intermediates synthesized from UDP-[6-³H]GlcNAc by this mixed system are shown in Figure 2. As expected, only GlcNAc-PI was made by G9PLAP.85 microsomes regardless of the addition of activators (Figure 2, lanes 1–3; the faint lower band is not GPI-related) and no products were produced by the S49(Thy-1^{-a}) microsomes defective in the first reaction (Figure 2, lane 4). When mixed together in the absence of GTP, the G9PLAP.85 and S49(Thy-1^{-a}) microsomes produced mostly GlcNAc-PI and a small amount of the second product, GlcN-PI

(Figure 2, lanes 5 and 7). However, large amounts of GlcN-PI were generated when GTP (100 μ M) was included in the incubation (Figure 2, lanes 6 and 8). Similar results were found when G9PLAP.85 microsomes were mixed with those prepared from either of the other two complementation groups defective in the first step of GPI biosynthesis [TIMI(Thy-1^{-c}) and S49(Thy-1^{-h})] (results not shown). Thus, in the mixed-microsome system, the deacetylation of GlcNAc-PI is almost completely dependent on GTP.

To determine whether endogenous GTP was responsible for the small amount of GlcNAc-PI deacetylation observed in the absence of added nucleotide triphosphate (Figure 2, lanes 5 and 7), the microsome preparations were subjected to extraction with charcoal. A comparison of the products synthesized before and after the depletion of endogenous GTP is shown in Figure 3. Both the G9PLAP.85 and S49(Thy-1^{-a}) microsomes were either not treated (Figure 3, lanes 1–3 and 7–9) or washed with charcoal to remove endogenous GTP (Figure 3, lanes 4–6 and 10–12) before incubation together in the presence of UDP-[6-³H]GlcNAc. Because the dolichol-linked product produced by the G9PLAP.85 microsomes in some reactions (GlcNAc-containing dolichol-phosphates, the faint lower band in lanes 1–3 of Figure 2) has a mobility on TLC similar to that of GlcN-PI, the radiolabelled products were fractionated on DEAE cellulose before chromatographic separation to allow the unambiguous identification of the second intermediate in GPI biosynthesis. Without extraction with charcoal, approx. 7% of the radiolabelled product made in the absence of added GTP was GlcN-PI (Figure 3, lane 2). After the depletion of endogenous GTP by extraction with charcoal, the amount of GlcN-PI made was decreased by approx. 70% to barely detectable levels (Figure 3, lane 5). Extraction with charcoal did not affect the ability of exogenous GTP to stimulate the deacetylation of GlcNAc-PI (Figure 3, lanes 7–12), indicating that this treatment did not perturb the cellular components involved either in this reaction or its regulation. These results demonstrate that most of the deacetylation of GlcNAc-PI in the mixed system, which is apparently GTP-independent, is actually supported by endogenous nucleotide triphosphate in the microsome preparations.

The stimulation by GTP of GlcNAc-PI deacetylation that we had previously characterized in wild-type microsomes was blocked by guanosine 5'-[γ -thio]triphosphate (GTP[S]), indicating that hydrolysis of the nucleotide triphosphate was required [18]. To determine whether this was also true of the GTP-dependent deacetylation of GlcNAc-PI in the mixed-microsome system, various concentrations of this hydrolysis-resistant GTP analogue were added to the reaction mixture. As shown in Figure 4, the presence of 50 μ M GTP[S] completely blocked the deacetylation of GlcNAc-PI supported by 100 μ M GTP. Therefore GTP hydrolysis is also required for the regulation of the second step of GPI biosynthesis in the mixed-microsome system.

Membrane fusion is implicated in the regulation of GlcNAc-PI deacetylation by GTP

In the mixed-microsome system, GlcNAc-PI is synthesized in one membrane but must be deacetylated in another. The hydrophobic nature of this glycolipid prohibits it from moving freely between lipid bilayers. Therefore some mechanism is required to mediate this 'apparent' transfer of GlcNAc-PI. We have previously shown that exogenous [³H]GlcNAc-PI added directly to microsomes derived from cells defective in GlcNAc-PI synthesis [S49(Thy-1^{-a}), S49(Thy-1^{-h}) and TIMI(Thy-1^{-c})] can be deacetylated without GTP hydrolysis (see Figure 8 in [18]), suggesting that the role of GTP in the mixed system is to stimulate

Figure 3 Effect of extraction of endogenous GTP with charcoal on GlcNAc-PI deacetylation in the mixed-microsome system

Mixtures of G9PLAP.85 and S49(Thy-1⁻a) microsomes were treated with buffer alone (lanes 1–3 and 7–9) or charcoal (lanes 4–6 and 10–12) before being incubated with UDP-[6-³H]GlcNAc in the absence (lanes 1–6) or presence (lanes 7–12) of GTP. GPI precursors were extracted and analysed either before (lanes 1, 4, 7 and 10) or after DEAE-cellulose chromatography to separate the neutral (lanes 2, 5, 8 and 11) and acidic (lanes 3, 6, 9 and 12) lipids.

Figure 4 Inhibition by GTP[S] of the GTP-induced deacetylation of GlcNAc-PI

Mixtures of microsomes isolated from G9PLAP.85 and S49(Thy-1⁻a) cells were pretreated with various concentrations of GTP[S] (GTP γ S) for 5 min before incubation with UDP-[6-³H]GlcNAc for 1 h in the presence or absence of 100 μ M GTP. Radiolabelled products were extracted and analysed by TLC. The percentage deacetylation indicates the percentage of the total product that was deacetylated; results are means $\pm$ S.E.M. for triplicate determinations.

the delivery of the GPI precursor to the membranes where deacetylation occurs. There are several possible mechanisms by which this 'apparent' transfer of GlcNAc-PI could occur. The three most likely are: (1) GTP stimulates the vesicle-mediated transfer of the GlcNAc-PI between the microsomes, (2) GTP stimulates protein-mediated transfer of the GlcNAc-PI between the microsomes, and (3) GTP brings the GlcNAc-PI to the S49(Thy-1⁻a) microsomes by stimulating the fusion of the two microsome populations. The first two possibilities require that GlcNAc-PI be synthesized before the GTP-mediated event. Therefore the stimulated rate of GlcNAc-PI deacetylation (referred to hereafter as the 'activated state') should be seen only in the presence of the nucleotide triphosphate. In the third mech-

anism, fusion would locate both the first-step and second-step enzymes in the same membrane and the subsequent removal of GTP would not affect deacetylation.

To determine whether GTP was mediating either a transport process or membrane fusion in the mixed-microsome system, the stability of the activated state was measured. This was accomplished in a two-step incubation experiment. In the first step, referred to as the preactivation incubation, the G9PLAP.85 and S49(Thy-1⁻a) microsomes were incubated together either with or without GTP for 30 min at 30 °C. The mixture was then centrifuged to pellet the membranes; the microsomes were resuspended in reaction buffer without GTP. UDP-[6-³H]GlcNAc was then added and a second, biosynthetic incubation was done at 37 °C for 1 h. The products formed in this experiment are shown in Figure 5. When preactivated in the presence of ATP only, the microsomes were unable to deacetylate GlcNAc-PI without the addition of further GTP to the biosynthetic incubation (Figure 5, lanes 1 and 2). Preactivation with both ATP and GTP resulted in the microsomes' being able to catalyse the second reaction even when no GTP was added during the second incubation (Figure 5, lane 3), indicating that GlcNAc-PI deacetylation was stimulated by GTP in the first incubation. The addition of GTP to the biosynthetic incubation after preactivation with GTP increased the amount of GlcNAc-PI deacetylation (Figure 5, lane 4), suggesting that further activation beyond that in the first incubation was possible. The level of deacetylation in the two-step experiment is comparable to that observed in the absence of preactivation (Figure 5, lane 7).

The possibility that residual GTP from the preactivation incubation that was carried through the centrifugation step stimulated GlcNAc-PI deacetylation in the second incubation was ruled out by the use of parallel incubations in which the amount of nucleotide triphosphate was quantified by the inclusion of [α -³²P]GTP. Less than 1% of the GTP in the preactivation incubation was found to remain after the centrifugation step (results not shown). This concentration of GTP (less than 1 μ M) does not stimulate GlcNAc-PI deacetylation beyond the background level seen before extraction of the

Figure 5 Effect of preactivation with GTP on GlcNAc-PI deacetylation in the mixed-microsome system

Mixtures of G9PLAP.85 and S49(Thy-1⁻) microsomes (lanes 1–4, 6 and 7) were either pretreated with ATP (lanes 1 and 2) or ATP plus GTP (lanes 3 and 4), or not pretreated (lanes 6 and 7) during the preactivation incubation. The microsomes were then reisolated by centrifugation and incubated with UDP-[6-³H]GlcNAc with either ATP (lanes 1, 3 and 6) or ATP plus GTP (lanes 2, 4 and 7) during the biosynthetic incubation. Shown in lane 5 is the product (GlcNAc-PI) made by G9PLAP.85 microsomes alone. The GPI precursors were then extracted and resolved by TLC.

Figure 6 Effects of GTP[S] on the preactivation of GlcNAc-PI deacetylation in the mixed-microsome system

Mixtures of G9PLAP.85 and S49(Thy-1⁻) microsomes were pretreated with either ATP alone (first and second pairs of bars) or ATP plus GTP (third and fourth pairs of bars) during the preactivation incubation. The membranes were then reisolated by centrifugation and incubated with UDP-[6-³H]GlcNAc in the presence of ATP only (wide-hatched bars), ATP plus GTP (black bars), ATP plus GTP[S] (grey bars) or ATP plus GTP plus GTP[S] (close-hatched bars). Products were extracted and analysed by TLC; the percentage deacetylation was calculated as the percentage of the total product that was deacetylated. Results are means $\pm$ S.E.M. for triplicate determinations.

microsomes with charcoal (see Figure 3, lane 2). Therefore the finding that the second step of GPI biosynthesis can be stably preactivated in the mixed-microsome system is consistent with

Figure 7 Preactivation of GlcNAc-PI deacetylation in wild-type microsomes

Microsomes isolated from EL4 cells were either not preactivated (first pair of bars) or preactivated by ATP alone (second pair of bars) or ATP plus GTP (third pair of bars). The microsomes were then reisolated by centrifugation and incubated with UDP-[6-³H]GlcNAc in the presence of ATP only (hatched bars) or ATP plus 100 μ M GTP (black bars). Products were extracted and analysed by TLC; the percentage deacetylation was calculated as the percentage of the total product that was deacetylated. Results are means $\pm$ S.E.M. for triplicate determinations.

GTP's mediating the delivery of GlcNAc-PI to the site of deacetylation by inducing membrane fusion.

Further evidence that fusion mediates the stimulation by GTP of GlcNAc-PI deacetylation in the mixed-microsome system was obtained by assessing the effect of GTP[S] in the preactivation experiment. These results are shown in Figure 6. As demonstrated previously, the stimulation of GlcNAc-PI deacetylation by 100 μ M GTP in the biosynthetic reaction (Figure 6, first pair of bars, black versus wide-hatched) was inhibited when 50 μ M GTP[S] was present in the same incubation (Figure 6, close-hatched bar of second pair). The deacetylation activated by preincubation with ATP and GTP (Figure 6, striped bar of third pair) was not affected by GTP[S] in the biosynthetic incubation (Figure 6, grey bar of fourth pair). However, the additional activation that occurred when GTP was added to the biosynthetic reaction after preactivation with GTP and ATP (Figure 6, black bar of third pair) was blocked by the hydrolysis-resistant analogue (Figure 6, close-hatched bar of fourth pair). Inclusion of GTP[S] in the first-step incubation completely inhibited the ability of 100 μ M GTP to preactivate the deacetylation reaction stably (results not shown). These findings indicate that the preactivation by GTP and ATP is separate from any stimulation that occurs in the biosynthetic reaction and are consistent with fusion's being the mechanism by which the problem of transferring GlcNAc-PI between different membranes is solved.

Comparison of the mixed-microsome system with wild-type microsomes

Characterization of the mixed-microsome system suggests that membrane fusion is the GTP-regulated process that, when stimulated, resulted in the 'apparent' transfer of GlcNAc-PI between microsomes and its subsequent deacetylation. To determine whether fusion was also responsible for the stimulation by GTP seen in membranes capable of both the first and second steps of GPI biosynthesis, the ability of GTP to preactivate GlcNAc-PI deacetylation stably was assessed in microsomes from wild-type EL4 cells; these results are shown in Figure 7.

Figure 8 Subcellular localization of the activities synthesizing and de-acetylating GlcNAc-PI

A post-nuclear supernatant from EL4 cells was fractionated by density-gradient centrifugation as described in the Materials and methods section. Five fractions from the top (fraction 1) to the bottom (fraction 5) of the gradient were isolated. (A) The activities of marker enzymes for plasma membrane (black bars), Golgi apparatus (hatched bars) and ER (grey bars) were measured. (B) The ability of each fraction to synthesize (first step; hatched bars) and deacetylate (second step; horizontally striped bars) GlcNAc-PI was quantified. In both panels the activities are expressed as a percentage of the total activity of that enzyme found in all the fractions. Results are means $\pm$ S.E.M. for triplicate determinations.

The typical effect of GTP on GlcNAc-PI deacetylation in wild-type microsomes is shown in the first pair of bars (Figure 7, black versus hatched). Preincubation with ATP alone did not change the amount of deacetylation that occurred in the 1 h biosynthetic incubation (Figure 7, hatched bar of second pair). Preincubation with GTP and ATP resulted in microsomes capable of the stimulated level of GlcNAc-PI deacetylation in the absence of additional GTP in the second incubation (Figure 7, hatched bar of third pair). Therefore the stimulated state can be stably preactivated in wild-type microsomes in the same way as in the mutant cell microsomes. That fusion can affect GlcNAc-PI deacetylation in wild-type membranes suggests that up to three different populations of microsomal vesicles could exist. Microsomes containing both the first-step and second-step enzymes would be capable of GlcNAc-PI without fusion and would account for the basal level of activity seen in the absence of GTP (Figure 7, hatched bar of first pair). The other populations would contain only the first-step enzymic machinery or the second-step activity. GTP-induced fusion between these populations and with the first type of vesicles would result in an increased level of GlcNAc-PI deacetylation in the presence of this nucleotide triphosphate.

Localization of GPI biosynthetic activities in ER fractions

If microsomes differ in their abilities to catalyse the first and second reactions of GPI biosynthesis, these activities might be differentially localized within the ER. To investigate this possibility, an EL4 cell-derived post-nuclear supernatant was fractionated on a sucrose gradient. The distribution of plasma membrane, Golgi and ER marker enzyme activities in the five fractions recovered from the gradient is shown in Figure 8(A). The ability of these fractions to catalyse the first reaction (measured as the synthesis of GlcNAc-PI from UDP-[6- 3 H]GlcNAc in the absence of GTP) and the second reaction (measured as the deacetylation of exogenously supplied [6- 3 H]GlcNAc-PI) of GPI biosynthesis is shown in Figure 8(B). Based on the marker enzymes, most of the ER is in fraction 4 but little is in fractions 3 and 5. Most of both GPI biosynthetic activities was found in fraction 4; small amounts of both were also measured in fraction 5. In fraction 3 there was negligible GlcNAc-PI biosynthetic activity (2% of total) but significant deacetylase activity (15% of total). Although modest, this difference in the localization of the first-step and second-step activities in fraction 3 was observed in three separate experiments. This observation is consistent with the proposal that some microsomes might contain only one of the two GPI biosynthetic activities being measured.

DISCUSSION

The deacetylation of GlcNAc-PI was found to be almost completely dependent on GTP hydrolysis when the initial reactions in GPI biosynthesis were reconstituted by mixing microsomes isolated from mutant cells defective in the first step (class A, C or H) and the second step (class L) in the pathway. Deacetylation was found to require the apparent transfer of GlcNAc-PI to the class L microsomes (where it is synthesized) to the class A microsomes (where it is deacetylated). This result suggests that the role of GTP was to facilitate the delivery of substrate to the deacetylase rather than to affect the deacetylase activity directly. Consistent with this is the observation that GlcNAc-PI added directly to microsomes containing the deacetylase can be metabolized in the absence of GTP [18]. Investigation into the mechanism by which this apparent transfer of GlcNAc-PI to the deacetylase was accomplished revealed that the mixed microsomes could be stably preactivated by incubation with GTP. After re-isolation and removal of the nucleotide triphosphate, the preactivated microsomes were competent in both GlcNAc-PI synthesis and deacetylation. This finding suggests that membrane fusion rather than some transport process was the mechanism by which the deacetylase gained access to GlcNAc-PI. The synthesis and deacetylation of GlcNAc-PI have been shown to take place on the cytoplasmic face of the ER in mammalian cells [24]. Therefore these two enzymic activities are expected to be oriented outwards in vesicles formed during the preparation of microsomes. If the two populations of vesicles come in contact with each other, the deacetylase in the G9PLAP.85 vesicle might be able to act on GlcNAc-PI in the outer leaflet of the S49(Thy-1 α) vesicle. In this case there would be no need to transfer the substrate from the membrane where it was synthesized to the deacetylase-containing microsomes. Because this does not occur in the absence of GTP, the role of hydrolysis of the nucleotide triphosphate would have to be either in allowing the deacetylase access to the GlcNAc-PI or making the substrate available to the enzyme. It seems unlikely that either of these mechanisms could be preactivated stably, as observed with the mixed-microsome system. Furthermore, Milne et al. [16] have shown that the fatty

acids are very important for the binding of GlcNAc-PI to the enzyme. Therefore GlcNAc-PI in a different membrane vesicle, in which its hydrophobic domains are not available, should be a very poor substrate for the deacetylase.

Although the preactivation experiment excluded mechanisms that require that GlcNAc-PI be synthesized before stimulation by GTP of the 'apparent' transport process (e.g. vesicular-mediated or protein-mediated transport), it did not eliminate the possibility that one of the GPI biosynthetic enzymes was exchanged to bring both activities into the same membrane. Transfer of the first step activity from the class L microsomes to the class A microsomes would require that the transmembrane PIG-A protein [11] exchange between membranes and associate with at least two other proteins (PIG-C and GPI-H) that participate in GlcNAc-PI synthesis [21,28]. Similarly, movement of the putative GlcNAc-PI deacetylase to the class L microsomes would require the extraction and re-insertion of a transmembrane protein into the lipid bilayer. Because these events are highly unlikely, the possibility that GTP stimulated the transfer of either the PIG-A or PIG-L proteins to the other microsome population seems remote.

To relate our findings in the mixed system with microsomes isolated from mutant cells to what is likely to occur in a wild-type cell, the ability of GlcNAc-PI deacetylation to be preactivated stably in EL4 microsomes was assessed and found to be identical with that in the mutant system. In both cases, inhibition of the GTP hydrolysis with GTP[S] blocked the preactivation (fusion) but not the subsequent preactivated deacetylation reaction. Therefore GTP seems to regulate the second step in GPI biosynthesis by bringing GlcNAc-PI to the deacetylase through intra-ER or homotypic fusion. These findings suggest that there is compartmentation of at least the first two steps in GPI biosynthesis within the ER. It is consistent with this idea that an analysis of the ability of different ER fractions to synthesize and deacetylate GlcNAc-PI revealed some differential localization of these two activities within this organelle. Therefore a perturbation of the ER, either by preparing microsomes or by isolating this organelle, would be expected to create one population of vesicles containing the deacetylase but not the GlcNAc-PI synthetase (corresponding to fraction 3) as well as a population of vesicles containing both enzymes. The latter would not require GTP for production of both the first and second intermediates in GPI biosynthesis and would be responsible for the basal level of deacetylation seen in the absence of GTP (see Figure 7). Fusion of the vesicles containing the deacetylase only with those having both GPI biosynthetic activities induced by GTP would then account for the increase in GlcNAc-PI deacetylation seen in the presence of this nucleotide triphosphate.

The existence of subcompartments or domains within the ER has previously been suggested [29,30]. The physiological relevance of this in GPI biosynthesis is not known. One possibility is that it gives the cell a mechanism for regulating GPI biosynthesis during periods when it is not efficient or beneficial for the cell to expend either energy or the initial substrates on this pathway. An example of such a period might be during cell division, when the ER is fragmented and split between two daughter cells before being reformed through fusion. By separating the biosynthetic enzymes into different ER vesicles, GPI synthesis could be down-regulated without directly affecting the activity of the enzymes. Although this remains speculation, this type of regulation would be expected to affect other metabolic pathways that use enzymes within the ER membrane. Consistent with this is the observation that the incorporation of GlcNAc into lipid acceptors as part of the assembly of carbohydrates on dolichol in the ER has been shown to be stimulated by GTP

hydrolysis [31]. Whether other pathways are also affected by ER fusion is not known but an effect would be predicted if this mechanism were to act as suggested.

In the mixed-microsome system, the activation of GlcNAc-PI deacetylation required both ATP and GTP but no cytosolic components. Therefore all the machinery for the apparent membrane fusion seems to be present in the microsomal membranes. These requirements are similar to those found to be needed for GTP-dependent fusion of rat liver microsomes revealed by electron microscopy [32–34]. With the rat liver microsomes, fusion was dependent on the membranes' being stripped of ribosomes and stimulated by the presence of various cytidine-containing nucleotide phosphates [33]. We have found that neither of these factors affects the GTP-dependent deacetylation of GlcNAc-PI in the mixed-microsome systems, suggesting that there are some important differences between the fusion that occurs in these two systems. Orsel et al. [35] recently characterized homotypic membrane fusion between ER vesicles isolated from Chinese hamster ovary cells with the use of an assay based on the concentration-dependent formation of pyrene excimers. This process was stimulated by ATP, GTP and cytosol. Treatment of the membranes with either GTP[S] or *N*-ethylmaleimide resulted in partial inhibition of the fusion. Although the need for GTP hydrolysis is similar to the requirement found in the present study, preliminary experiments indicate that the *N*-ethylmaleimide-sensitive fusion protein NSF has no role in the mixed-microsome system because the rate of deacetylation of GlcNAc-PI is unaffected by this thiol-acylating compound (E. S. Kristyenne, H. Zhang and V. L. Stevens, unpublished work). Therefore at least a portion of the Chinese hamster ovary ER vesicle fusion seems to be different from the process that we have measured in the mixed-microsome system. The lack of sensitivity to *N*-ethylmaleimide also indicates that our system is somewhat different from that measured in some other assays [36,37], including those used to demonstrate a role for a Rab GTPase in homotypic fusion [37]. Therefore the mixed-microsome system described here could provide a novel assay for the study of fusion that might be useful for the identification of the membrane-associated components required for this process. However, the use of this system for such purposes will require a direct demonstration that membrane fusion is the event mediating the apparent transfer of GlcNAc-PI between microsome populations. Such evidence can be obtained through immunoelectron microscopy studies, which will be possible once antibodies against the various proteins involved in the first two steps of GPI biosynthesis (the class A, C and H proteins) have been developed.

We thank Dr. Toon Stegmann, Dr. David Lambeth, Dr. Anant Menon and Dr. William Dowhan for suggestions and discussions on this research. This research was supported by National Institutes of Health Grant GM51419 (to V. L. S.).

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Received 18 December 1998/13 April 1999; accepted 17 May 1999