

The first step of glycosylphosphatidylinositol biosynthesis is mediated by a complex of PIG-A, PIG-H, PIG-C and GPI1

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Biosynthesis of glycosylphosphatidylinositol (GPI) is initiated by transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI). This chemically simple step is genetically complex because three genes are required in both mammals and yeast. Mammalian PIG-A and PIG-C are homologous to yeast GPI3 and GPI2, respectively; however, mammalian PIG-H is not homologous to yeast GPI1. Here, we report cloning of a human homolog of GPI1 (*hGPI1*) and demonstrate that four mammalian gene products form a protein complex in the endoplasmic reticulum membrane. PIG-L, which is involved in the second step in GPI synthesis, GlcNAc-PI de-*N*-acetylation, did not associate with the isolated complex. The protein complex had GPI-GlcNAc transferase (GPI-GnT) activity *in vitro*, but did not mediate the second reaction. Bovine PI was utilized ~100-fold more efficiently than soybean PI as a substrate, and lyso PI was a very inefficient substrate. These results suggest that GPI-GnT recognizes the fatty acyl chains of PI. The unusually complex organization of GPI-GnT may be relevant to selective usage of PI and/or regulation.

Keywords: *N*-acetylglucosaminyl transferase/ER/GPI anchor/GPI1

Introduction

Glycosylphosphatidylinositol (GPI) is used to anchor various eukaryotic proteins to membrane (reviewed by Ferguson and Williams, 1988; Kinoshita *et al.*, 1995). Post-translational attachment of the GPI anchor is essential for the surface expression of such proteins. A defect in GPI anchor synthesis affects various functions of cells, tissues and organs. Indeed, GPI knock-out mice generated by gene targeting were embryonic lethal, indicating the importance of GPI-anchored proteins in embryogenesis (Kawagoe *et al.*, 1996). An acquired deficiency in GPI anchoring in hematopoietic stem cells causes paroxysmal nocturnal hemoglobinuria (Takeda *et al.*, 1993). In the yeast *Saccharomyces cerevisiae*, the transport of a number of cell surface proteins is dependent on GPI anchoring, and an anchoring deficiency results in defective cell wall

synthesis and cell death (Conzelmann *et al.*, 1990; Leidich *et al.*, 1994, 1995; Lu *et al.*, 1995; Schönbacher *et al.*, 1995; Vossen *et al.*, 1995; Leidich and Orlean, 1996). Protozoan parasites, such as blood stage *Trypanosoma brucei* and sporozoites of *Plasmodium falciparum*, are coated, respectively, by GPI-anchored variant surface glycoproteins and by the circumsporozoite proteins. Therefore, a lack of surface expression of these proteins may compromise the viability of these parasites (reviewed by Thomas *et al.*, 1990; McConville and Ferguson, 1993; Ferguson, 1994).

The GPI anchors are synthesized and added to proteins in the endoplasmic reticulum (ER). GPI biosynthesis involves sequential additions of sugar and other components to phosphatidylinositol (PI) in six or more reaction steps (Englund, 1993; McConville and Ferguson, 1993). The pathway has been clarified in trypanosome (Masterson *et al.*, 1989, 1990), mammalian and yeast cells, and a number of genes involved in the pathway have been cloned, but little is known about the characteristics of the biosynthetic enzymes (reviewed by Kinoshita *et al.*, 1995).

The first step in GPI synthesis is the generation of *N*-acetylglucosaminyl phosphatidylinositol (GlcNAc-PI) from UDP-GlcNAc and PI, catalyzed by a GlcNAc transferase termed GPI-GlcNAc transferase (GPI-GnT) (Doering *et al.*, 1989). This step is mediated by more than one gene. In the mammalian system, three complementation groups of mutants, class A, H and C, are defective in this step (Stevens and Raetz, 1991; Sugiyama *et al.*, 1991). The corresponding genes, PIG-A (Miyata *et al.*, 1993), PIG-H (Kamitani *et al.*, 1993) and PIG-C (Inoue *et al.*, 1996) have all been cloned. Three *S.cerevisiae* mutants (*gpi1*, *gpi2* and *gpi3/spt14/cwh6*) are also defective in the first step (Leidich *et al.*, 1994, 1995; Schönbacher *et al.*, 1995; Vossen *et al.*, 1995). The yeast Gpi3 and Gpi2 proteins are homologous to the mammalian PIG-A and PIG-C proteins, respectively, but Gpi1p shows no similarity to the PIG-H protein. Furthermore, there is no open reading frame in the *S.cerevisiae* genome encoding an obvious counterpart of the PIG-H protein.

The PIG-A/Gpi3 protein has homology to the bacterial GlcNAc transferase RfaK, suggesting that it bears a catalytic site for GlcNAc transfer (Bessler *et al.*, 1994; Kawagoe *et al.*, 1994; Vossen *et al.*, 1995). Since the PIG-H and PIG-C/Gpi2 proteins show no significant homology to other proteins of known functions, their functions in turn cannot be predicted from their primary structures. PIG-A and PIG-H form a protein complex in the ER membrane (Watanabe *et al.*, 1996). PIG-C is also an ER membrane protein (Inoue *et al.*, 1996). These results suggest that three proteins are closely involved in GlcNAc transfer to PI. Consistent with this possible complexity, the yeast *gpi1*, *gpi2* and *gpi3* mutants show strong genetic interactions: no pairwise combination of those mutants is viable (Leidich *et al.*, 1995).

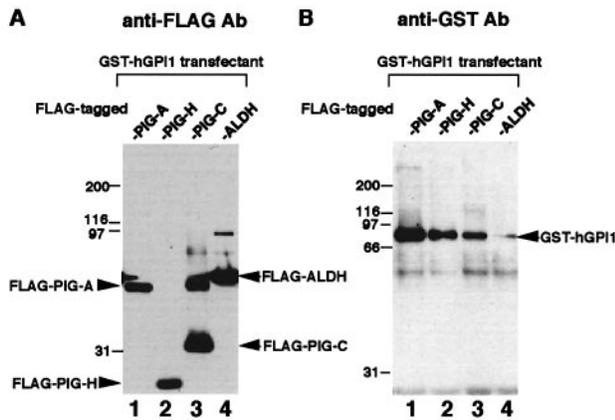


Fig. 3. Western blot analysis of the association of hGPII with PIG-A, PIG-H and PIG-C. JY25 cells were transfected with GST-tagged hGPII (tagged at the carboxy-terminus) together with FLAG-tagged PIG-A (lanes 1), PIG-H (lane 2), PIG-C (lane 3) or ALDH (lane 4). (A) FLAG-tagged proteins affinity-precipitated with anti-FLAG antibodies from each of the transfectants were separated by SDS-PAGE and Western blotted against anti-FLAG antibodies. Oligomeric FLAG-PIG-C is seen in lane 3 (Inoue *et al.*, 1996). (B) Western blotting of each of the precipitates against anti-GST antibodies. Molecular size markers (kDa) are on the left. Identities of bands are also indicated.

associates with PIG-A, PIG-H or PIG-C. We expressed glutathione-*S*-transferase (GST)-tagged hGPII in JY25 cells together with one of the three other proteins, which had each been tagged with the FLAG epitope. As a control, FLAG-tagged microsomal aldehyde dehydrogenase (msALDH), a non-relevant ER protein, was co-expressed with GST-tagged hGPII. After solubilization of the membrane fractions with 1% (w/v) digitonin, we precipitated FLAG-tagged proteins with anti-FLAG beads, and detected the proteins by Western blotting with anti-FLAG and anti-GST antibodies. As shown in Figure 3, FLAG-tagged PIG-A, PIG-H, PIG-C and msALDH were precipitated efficiently with anti-FLAG beads (Figure 3A, lanes 1–4). There were only trace amounts of these proteins remaining in the supernatants (data not shown). Western blotting of each of the FLAG-tagged precipitates against anti-GST antibodies (Figure 3B) demonstrated that GST-tagged hGPII was co-precipitated with FLAG-tagged PIG-A (lane 1), PIG-H (lane 2) or PIG-C (lane 3), but not significantly with FLAG-tagged msALDH (lane 4). We concluded that hGPII had associated specifically with PIG-A, PIG-H and PIG-C.

Associations of PIG-C with PIG-A and with PIG-H

We next tested if PIG-C associates with PIG-A or PIG-H. GST-tagged PIG-C was co-expressed in JY25 cells with FLAG-tagged PIG-A, PIG-H or msALDH. When FLAG-tagged PIG-A, PIG-H and msALDH were affinity precipitated, GST-tagged PIG-C was not co-precipitated significantly. (data not shown). Therefore, direct association of PIG-C with PIG-A or PIG-H was not demonstrated in this experiment in which only two components were overexpressed by co-transfection.

Formation of a complex consisting of PIG-A, PIG-H, PIG-C and hGPII

Since PIG-C associated with hGPII, and hGPII associated with PIG-A and PIG-H, we next examined whether a

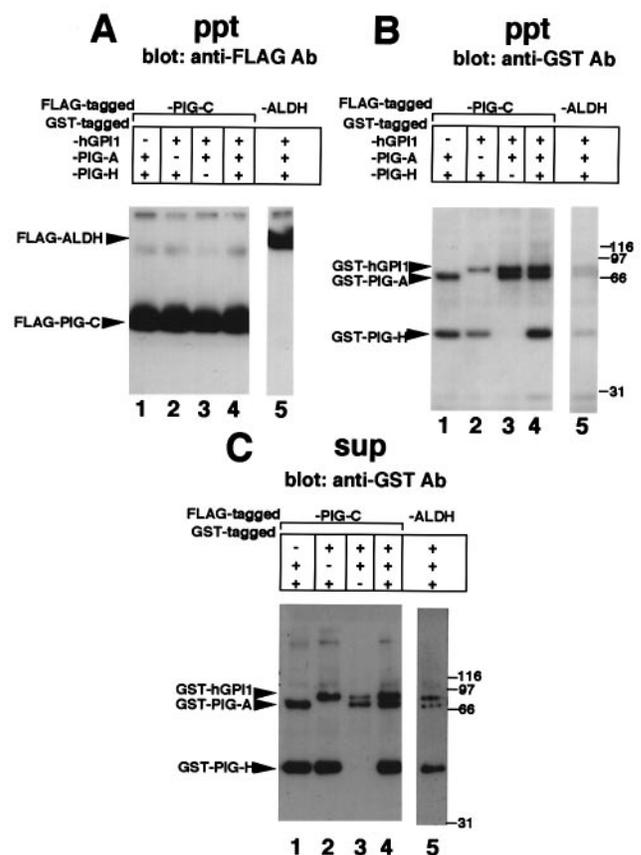


Fig. 4. Western blot analysis of the formation of a complex consisting of PIG-A, PIG-H, PIG-C and hGPII. JY25 cells were transfected with FLAG-tagged PIG-C together with GST-tagged PIG-A and PIG-H (lanes 1), with GST-tagged hGPII and PIG-H (lanes 2), with GST-tagged hGPII and PIG-A (lanes 3), or with GST-tagged hGPII, PIG-A and PIG-H (lanes 4). As a control, FLAG-tagged-ALDH was co-transfected with GST-tagged hGPII, PIG-A and PIG-H (lanes 5). In this experiment, hGPII was tagged at the carboxy-terminus. (A) FLAG-tagged proteins affinity-precipitated with anti-FLAG antibodies from each transfectant were separated by SDS-PAGE and Western blotted against anti-FLAG antibodies. (B) Western blotting of each precipitate against anti-GST antibodies. (C) GST-tagged proteins remaining in the supernatants after precipitation with anti-FLAG beads were collected with glutathione beads and analyzed by SDS-PAGE and Western blotting. Molecular size markers (kDa) are on the right.

complex of four proteins can be formed. JY25 cells were transfected with FLAG-tagged PIG-C together with pairwise combinations, or with all three of the GST-tagged PIG-A, PIG-H and hGPII proteins. As shown in Figure 4, FLAG-tagged PIG-C was precipitated efficiently with anti-FLAG beads in four combinations with other proteins (Figure 4A, lanes 1–4). Western blotting of each of the precipitates against anti-GST antibody (Figure 4B) demonstrated that co-transfected hGPII and PIG-A (lane 3), and hGPII and PIG-H (lane 2) were co-precipitated with PIG-C. Moreover, when all four proteins were co-transfected, the three other proteins were co-precipitated with PIG-C (lane 4). These results strongly suggest that all four proteins form a complex. When FLAG-tagged msALDH was used instead of FLAG-tagged PIG-C (Figure 4A and B, lanes 5), GST-tagged hGPII, PIG-A and PIG-H were not precipitated with anti-FLAG beads.

Interestingly, when PIG-A and PIG-H were co-transfected with PIG-C, both PIG-A and PIG-H were co-

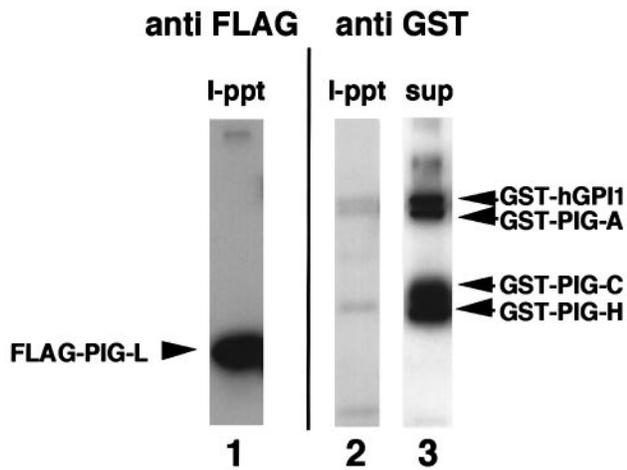


Fig. 5. Lack of association of PIG-L with the complex consisting of PIG-A, PIG-H, PIG-C and hGPII. JY25 cells were transfected with FLAG-tagged PIG-L together with GST-tagged hGPII, PIG-A, PIG-H and PIG-C. Proteins immunoprecipitated from a digitonin extract of the transfectant with anti-FLAG antibodies were separated by SDS-PAGE and Western blotted against anti-FLAG antibodies (lane 1) or anti-GST antibodies (lane 2). GST-tagged proteins remaining in the supernatant after immunoprecipitation were collected by glutathione beads, separated by SDS-PAGE and Western blotted against anti-GST antibodies (lane 3). hGPII was tagged at the carboxy-terminus.

precipitated with PIG-C (Figure 4B, lane 1). As described above, when PIG-C was co-expressed with PIG-A or with PIG-H alone, pairwise associations were not demonstrated. These results indicate that affinities between PIG-C and PIG-A, and PIG-C and PIG-H were so weak that their pairwise interactions were not detected, but that co-expression of three proteins together resulted in formation of a stable protein complex.

To determine ratios of GST-tagged proteins co-precipitated with FLAG-PIG-C, the intensities of bands of co-precipitated GST-tagged proteins (Figure 4B, lanes 1–4) were quantitated by means of image scanning. Their ratios were ~1:1 in all combinations (PIG-A:PIG-H in lane 1 = 1:1, hGPII:PIG-H in lane 2 = 1:1.3, hGPII:PIG-A in lane 3 = 1:0.8 and hGPII:PIG-A:PIG-H in lane 4 = 1:0.9:1.1). In contrast, ratios of GST-tagged proteins remaining in the supernatants varied, as seen in Figure 4C (PIG-A:PIG-H in lane 1 = 1:1.8, hGPII:PIG-H in lane 2 = 1:1.9, hGPII:PIG-A in lane 3 = 1:1.5 and hGPII:PIG-A:PIG-H in lane 4 = 1:0.6:1.5). The fact that three co-precipitated GST-tagged proteins had equimolar ratios strongly suggests that they represented complexes of four proteins rather than combinations of complexes of three proteins.

A lack of association of PIG-L with a complex consisting of PIG-A, PIG-H, PIG-C and hGPII

We further tested whether PIG-L, which is involved in the second step in GPI synthesis, the de-*N*-acetylation of GlcNAc-PI (Nakamura *et al.*, 1997), also associates with this protein complex. FLAG-tagged PIG-L was co-expressed with GST-tagged hGPII, PIG-A, PIG-C and PIG-H. The four proteins involved in the first step were not detected in precipitates of FLAG-tagged PIG-L (Figure 5, lanes 1 and 2), but remained in the supernatant (lane 3), indicating that PIG-L did not associate with the protein complex.

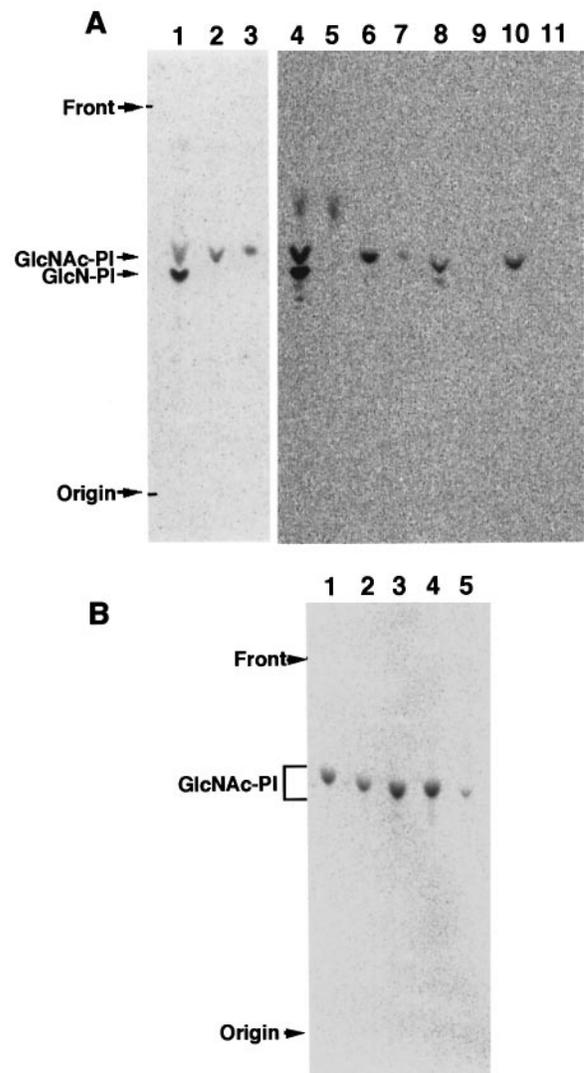


Fig. 6. GPI-GnT activity of the protein complex *in vitro*. (A) UDP-[6-³H]GlcNAc was incubated with lysates of JY5 cells transfected with GST-tagged PIG-A (lane 1), with a digitonin extract of the lysates (lane 2) or with the protein complexes collected by glutathione beads (lane 3). To characterize the glycolipids, products generated with cell lysates (lanes 4 and 5) or with the protein complexes (lanes 6 and 7) were treated with buffer (lanes 4 and 6) or PI-PLC (lanes 5 and 7). The products generated upon incubation with the protein complexes were incubated with wild-type (lanes 8 and 9) and deacetylase mutant (lanes 10 and 11) CHO cell lysates, and then with buffer (lanes 8 and 10) or PI-PLC (lanes 9 and 11). (B) Protein complexes were collected by glutathione beads from digitonin extracts of JY25 cells transfected with GST-tagged PIG-A (lane 1), PIG-H (lane 2), PIG-C (lane 3), hGPII (tagged at the amino-terminus) (lane 4) or ALDH (lane 5). The glutathione beads were incubated with UDP-[6-³H]GlcNAc and 100 μM bovine PI for 60 min at 37°C.

GPI-GnT activity of the complex consisting of PIG-A, PIG-H, PIG-C and hGPII

To test whether the protein complex consisting of PIG-A, PIG-H, PIG-C and hGPII has GlcNAc transferase activity *in vitro*, we transfected GST-tagged PIG-A into JY5 cells, a mutant defective in PIG-A, in order to restore biosynthesis of the GPI anchor (Watanabe *et al.*, 1996). As shown in Figure 6A, incubation of the radiolabeled donor of GlcNAc, UDP-[6-³H]GlcNAc, with lysates of JY5 cells transfected with GST-tagged PIG-A resulted in synthesis

of GlcNAc-PI and its subsequent deacetylation to glucosaminyl phosphatidylinositol (GlcN-PI) (lane 1). Digitonin extracts of the same lysates supported synthesis of GlcNAc-PI but not its conversion to GlcN-PI (lane 2). We collected the protein complexes from the digitonin extract with glutathione beads, and incubated the complexes with UDP-[6-³H]GlcNAc. The complexes synthesized GlcNAc-PI (lane 3), presumably using endogenous PI associated with them. To characterize the *in vitro* product as GlcNAc-PI, we tested its sensitivity to PI-specific phospholipase C (PI-PLC) and to GlcNAc-PI deacetylase. Standard GlcNAc-PI and GlcN-PI prepared with cell lysates were sensitive to PI-PLC (lanes 4 and 5). The product generated by the protein complexes was also sensitive to PI-PLC (lanes 6 and 7). The same product was incubated with wild-type or deacetylase mutant CHO cell lysates to assess its sensitivity to GlcNAc-PI deacetylase (lanes 8 and 10). Incubation with wild-type cell lysates resulted in the generation of GlcN-PI (lane 8), which was sensitive to PI-PLC (lane 9). In contrast, incubation with the deacetylase mutant cell lysates did not generate GlcN-PI (lane 10). Therefore, the *in vitro* product had the predicted sensitivities to PI-PLC and GlcNAc-PI deacetylase, confirming its identity as GlcNAc-PI.

To confirm further that four proteins constitute the GlcNAc transferase complex, we transfected GST-tagged PIG-A, PIG-H, PIG-C, hGPI1 or msALDH individually into JY25 cells and collected GST-tagged proteins from digitonin extracts of the cells. Complexes collected with glutathione beads from cells transfected with GST-tagged PIG-A, PIG-H, PIG-C or hGPI1 showed GlcNAc transferase activity *in vitro* (Figure 6B, lanes 1–4), whereas those from cells transfected with GST-tagged msALDH showed only trace activity (lane 5). These results confirmed that GPI-GnT is a complex consisting of at least four proteins, namely PIG-A, PIG-H, PIG-C and hGPI1.

We finally asked whether the first and second enzymes are independent of one another and tested whether mutants deficient in components of the GlcNAc-PI synthetic complex are also defective in GlcNAc-PI deacetylation. Incubation of lysates of wild-type and of class A, C and H mutant cells with [³H]GlcNAc-PI revealed that the GlcNAc-PI synthesis mutants all had deacetylase activities comparable with that of wild-type cells (data not shown). The PIG-A, PIG-H and PIG-C proteins are therefore not necessary for GlcNAc-PI deacetylase activity, and the two enzymes are separable. Consistent with this, the yeast *gpi1*, *gpi2* and *gpi3* mutants all have GlcNAc-PI deacetylase activity (Leidich *et al.*, 1994, 1995).

Effects of various phospholipids and ceramide on GPI-GlcNAc transferase activity

We added various phospholipids and ceramide to the reaction mixture at a concentration of 100 μ M to test their effects on the production of GlcNAc-PI. The amount of [³H]GlcNAc-PI synthesized was quantitated and expressed as a percentage of the amount synthesized with endogenous PI. As shown in Figure 7, addition of bovine PI enhanced synthesis of GlcNAc-PI ~5-fold, whereas additions of phosphatidylethanolamine (PE), ceramide (Cer) or phosphatidic acid (PA) had no effect (88, 85 and 111% of control, respectively). Phosphatidylcholine (PC), phos-

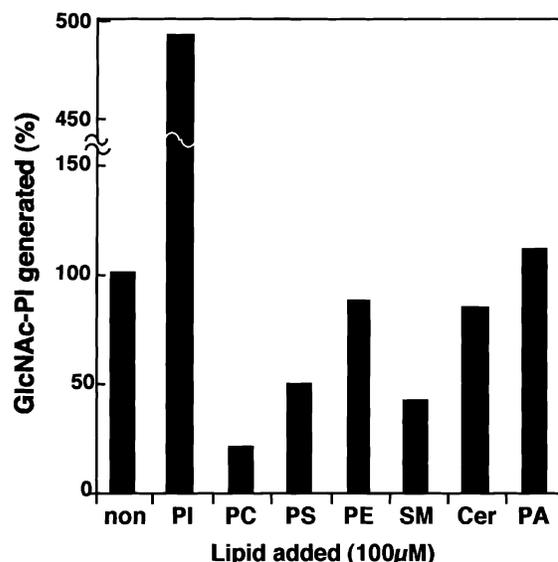


Fig. 7. Influence of phospholipids and ceramide on GPI-GnT activity. The enzyme reactions were carried out at 37°C for 60 min using glutathione beads bearing enzyme complexes derived from 2×10^7 cells in the absence or presence of 100 μ M of exogenous phospholipid or ceramide. The amount of GlcNAc-PI synthesized without exogenous lipid was taken as 100%.

phatidylserine (PS) and sphingomyelin (SM), however, were inhibitory (22, 50 and 42% of control, respectively).

The substrate specificity of GPI-GnT

We next examined the PI selectivity of the GPI-GnT enzyme complex. As shown in Figure 8, GPI-GnT used bovine PI more efficiently than it used soybean PI. In this experiment, addition of 100 μ M bovine PI enhanced synthesis of GlcNAc-PI 8-fold, whereas addition of 100 μ M soybean PI only doubled the amount of GlcNAc-PI formed. Addition of 1 μ M bovine PI had the same effect as addition of 100 μ M soybean PI.

We next examined whether the enzyme complex also catalyzed GlcNAc transfer to the lyso form of bovine PI. As shown in Figure 9, standard lyso PI and GlcNAc-lyso PI moved more slowly than PI and GlcNAc-PI in the solvent system used (lanes 1–4) (Masterson *et al.*, 1990). GlcNAc-lyso PI was synthesized upon addition of bovine lyso PI and reached a maximum at 10 μ M (lanes 5–7), indicating that bovine lyso PI acted as a substrate although its efficiency was much lower than that of bovine PI. Synthesis of GlcNAc-PI from endogenous PI was inhibited with increasing lyso PI concentration (lanes 5–7), probably due to the competition between PI and lyso PI. At higher lyso PI concentrations, the synthesis of GlcNAc-lyso PI was also abolished (lanes 8 and 9). Lyso PC also inhibited the GlcNAc transferase activity to a similar extent (data not shown). One explanation for this is that high concentrations of lyso phospholipids may affect the activity of the enzyme complex.

We also examined the effects of polar analogs of PI on the GlcNAc transferase activity. Inositol, glycerophosphate, inositol monophosphate and glycerophosphoinositol at 1 mM had little inhibitory effect on GlcNAc-PI synthesis (data not shown), suggesting that the fatty acyl chains of PI are more important than the polar head group for recognition of the lipid by the enzyme.

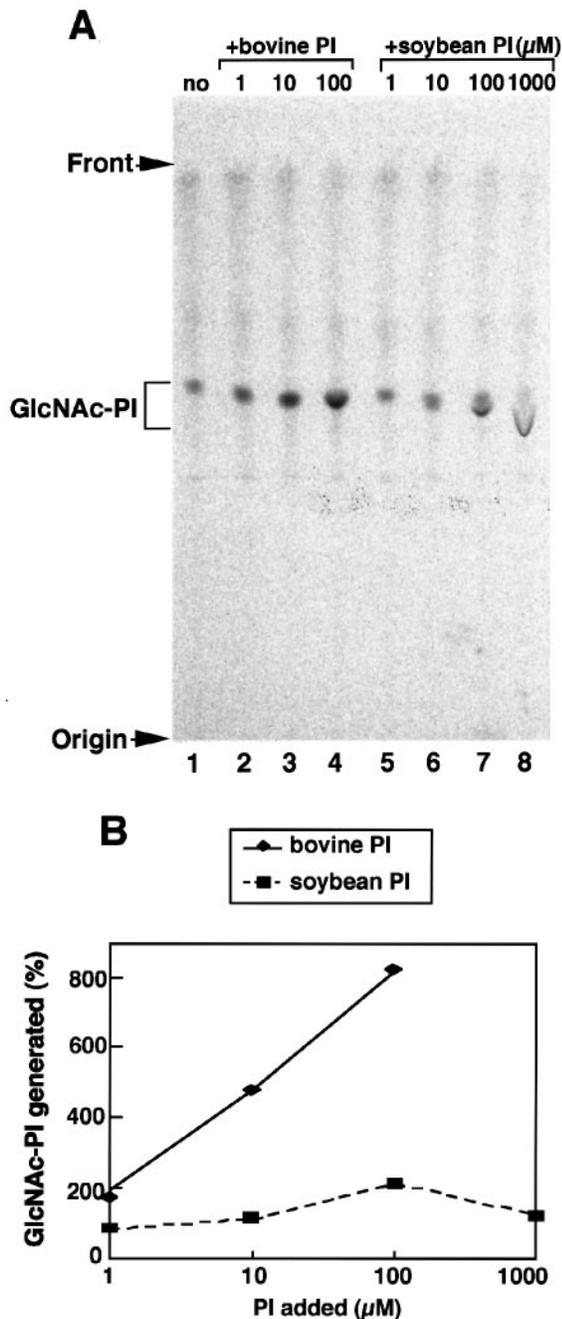


Fig. 8. GPI-GnT uses bovine PI more efficiently than soybean PI. (A) The enzyme reactions were carried out using glutathione beads bearing enzyme complexes derived from 10^7 cells in the absence (lane 1) or presence of bovine or soybean PI at 1 μM (lanes 2 or 5), 10 μM (lanes 3 or 6), 100 μM (lanes 4 or 7) and 1000 μM (lane 8). Radiolabeled glycolipids were analyzed by thin-layer chromatography. The migration of GlcNAc-PI was retarded at 1000 μM soybean PI (lane 8) due to a large amount of PI migrating slightly ahead of GlcNAc-PI. (B) The amounts of radioactivity in the spots of GlcNAc-PI were quantitated and expressed as a percentage of the amount synthesized without addition of exogenous PI. Solid and dotted lines are for bovine and soybean PI, respectively.

Discussion

The major finding of this study is that the first step in the biosynthesis of GPI anchors is mediated by a complex of four proteins. We cloned a cDNA for a human homolog of yeast Gpi1p and found that the hGPI1 protein forms a

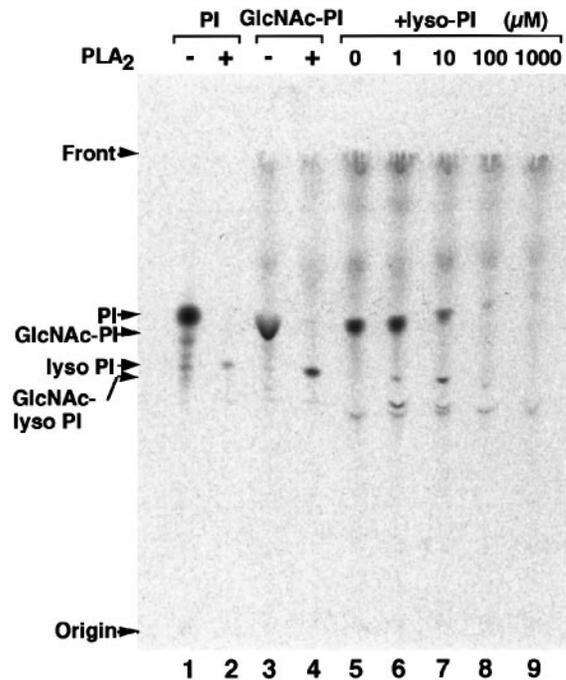


Fig. 9. Lyso PI is an inefficient substrate for GPI-GnT. Lyso PI from bovine liver was added at 1–1000 μM to the enzyme complex derived from 4×10^7 cells (lanes 6–9). Inositol-labeled PI or GlcNAc-PI synthesized *in vitro* were treated with PLA₂ to generate lyso PI and GlcNAc-lyso PI (lanes 2 and 4) or buffer only (lanes 1 and 3). The identities of the glycolipid spots are indicated on the left.

GPI-GnT complex with three other proteins (PIG-A, PIG-H and PIG-C) in the ER. In both yeast and mammalian systems, three genes are known to be involved in the first step, whereas their mutual correspondence and functions were not entirely clear. Our present study demonstrated that the three yeast genes involved in the first step, *GPI1*, *GPI2* and *GPI3*, have their mammalian counterparts in *hGPI1*, *PIG-C* and *PIG-A*, respectively. There is no obvious homolog of the fourth mammalian gene, *PIG-H*, in the *S.cerevisiae* genome, and it is not yet known whether the function of PIG-H is substituted for by another yeast protein, or whether only three proteins are sufficient for GlcNAc-PI synthesis. The present study also demonstrated that four mammalian gene products are directly or closely involved in transfer of GlcNAc, or in the regulation of the process in the ER, eliminating the possibility that some of these proteins might regulate the expression of the others.

PIG-A, *PIG-H* and *PIG-C* are required for the first step in GPI anchor biosynthesis because mutations in each of these genes lead to a complete defect in that reaction. Whether *hGPI1* is also required is as yet unclear because no *hGPI1*-deficient mutant cell line exists. The temperature-sensitive *S.cerevisiae gpi1* mutant is blocked in GPI anchoring at non-permissive temperature, and membranes from *gpi1* cells grown at permissive temperature lack *in vitro* GlcNAc-PI synthetic activity (Leidich *et al.*, 1994). Disruption of *GPI1*, however, yields viable haploid cells that are temperature-sensitive for growth and defective in GlcNAc-PI synthesis *in vitro*, but which incorporate [³H]inositol into proteins at only 40% of the wild-type levels (Leidich and Orlean, 1996). This indicates that Gpi1p is not essential for transfer of GlcNAc to PI *in vivo*.

Indeed, in *S.cerevisiae*, the function of Gpi1p *in vivo* can be bypassed, albeit weakly, by overexpression of *GPI2*, which leads to partial suppression of the temperature sensitivity of both the *gpi1* and *gpi1::URA3* null mutants (Leidich *et al.*, 1995). A functional relationship between Gpi1p and Gpi2p is also suggested by the fact that the combination of the *gpi1* and *gpi2* mutations is lethal. Taken together, the present results showing that hGPI associates with PIG-C as well as with PIG-A and PIG-H, and the genetic interactions between the *S.cerevisiae* *GPI1* and *GPI2* genes, are consistent with the notion that the role of Gpi1p may be to stabilize the GlcNAc-PI synthetic complex, rather than to participate in catalysis of GlcNAc transfer. The importance of Gpi1p's role may vary among different organisms. Thus, the protein is required for growth of *S.cerevisiae* at elevated temperatures, but disruption of the *Schizosaccharomyces pombe* *gpi1*⁺ gene yielded haploids that were not viable at 25 and 37°C (Colussi and Orlean, 1997).

It is intriguing but not clear why the first step is mediated by a complex of four proteins. PIG-A may contain the catalytic site for GlcNAc transfer because it has homology with the bacterial GlcNAc transferase (Kawagoe *et al.*, 1994; Vossen *et al.*, 1995). Consistent with the proposed cytoplasmic orientation of GlcNAc-PI in the ER membrane (Vidugiriene and Menon, 1993), PIG-A has a single transmembrane domain near its carboxy-terminus, and its large amino-terminal portion, which shows GlcNAc transferase homology, faces the cytoplasm, while its small carboxy-terminal portion is lumenally oriented. PIG-H does not have a hydrophobic segment that can act as a transmembrane domain, but seems to be a peripheral membrane protein facing the cytoplasm (Watanabe *et al.*, 1996). We cannot predict the function of PIG-H because it shows no homology to proteins of known function. The hydrophathy profile of PIG-C/Gpi2p indicates that the molecule is very hydrophobic and has six or seven transmembrane domains (Leidich *et al.*, 1995; Inoue *et al.*, 1996). The hydrophobicity of PIG-C/Gpi2p may reflect a role for the protein in recognition of the fatty acyl chains of PI, given that GPI-GnT showed selective usage of certain types of PI as a substrate. The unusually complex organization of GPI-GnT raises the possibility that these proteins may have regulatory functions in addition to GlcNAc transferase activity.

We tested whether the first and second step enzymes are coupled because GlcNAc-PI is deacetylated rapidly in trypanosomes (Doering *et al.*, 1989), mammalian cells (Hirose *et al.*, 1991) and yeast (Costello and Orlean, 1992). We recently cloned *PIG-L*, which is involved in this second step, and showed that PIG-L is an ER membrane protein with a large domain oriented towards the cytoplasm. Although deacetylase activity has not been demonstrated directly, PIG-L may be deacetylase itself, or closely involved in deacetylation, because its overexpression enhances deacetylase activity, and because the PIG-L protein has the same membrane orientation as GlcNAc-PI and GlcN-PI (Nakamura *et al.*, 1997). The precipitated GlcNAc-PI synthetic complex mediated the first reaction *in vitro*, but not the second. Consistent with this, PIG-L was not found in the complex. Furthermore, we showed that PIG-A, PIG-H and PIG-C are not necessary for deacetylase activity. Therefore, it seems that the first and

second step enzymes are independent of one another. This does not exclude the possibility that the deacetylase forms a loose complex with GPI-GnT in the ER membrane; however, association between two enzymes, if it occurs at all, must be weaker than the interactions between the four proteins involved in the first step.

The isolated GPI-GnT used bovine PI ~100-fold more efficiently than soybean PI. The difference between bovine and soybean PI is in their fatty acyl chains; bovine PI contains mainly stearic and arachidonic acid while soybean PI contains mainly linoleic and palmitic acid. There is a report that soybean PI enhanced synthesis of GlcNAc-PI and GlcN-PI only slightly less efficiently than bovine PI when cell lysate was used as a source of enzymes (Stevens and Raetz, 1991). In that study, the first and second reactions were not differentiated. Although the precise reason for the difference is not clear, it may be that the presence of a larger amount of endogenous PI associated with the membrane-bound enzymes than with the detergent-solubilized enzymes lowered the degree of stimulation obtained with exogenous PI. In *Trypanosoma brucei*, 20% of PI does not contain stearate, and this fraction is not used for GPI biosynthesis (Doering *et al.*, 1994). From the present study, as well as from previous work, it is clear that GPI-GnT enzymes of trypanosomes and mammals both recognize the fatty acyl chains of PI. Fatty acids are also important in deacetylation, as shown in studies using partially purified *T.brucei* GlcNAc-PI deacetylase (Milne *et al.*, 1994). The facts that the polar analogs of PI such as glycerophosphoinositol did not inhibit GlcNAc-PI synthesis significantly, and that bovine lyso PI was a very inefficient substrate *in vitro*, suggest that the fatty acid in the sn-2 position is critical for an efficient reaction. A further possible reason for the utilization of selected fatty acyl chains is that this may be a prerequisite for the subsequent remodeling reactions that are known to occur in trypanosomes (Masterson *et al.*, 1990), in yeast (Conzelmann *et al.*, 1992) and in mammalian cells (Singh *et al.*, 1994).

We further investigated the influence of other phospholipids on GPI-GnT activity to make sure that the enhancement of GlcNAc-PI synthesis by PI is due to its specific utilization as a substrate, rather than to stabilization of the enzyme by certain phospholipids, as has been found with some membrane-bound enzymes (Jensen and Schutzbach, 1985). The former was confirmed because PI was the only lipid that enhanced the reaction. Unexpectedly, 100 µM PC inhibited GlcNAc-PI synthesis by nearly 80%. This is in contrast to a report that synthesis of GlcNAc-PI and GlcN-PI in cell lysates was not affected by addition of exogenous PC at a concentration similar to that used here (Stevens and Raetz, 1991). These results may reflect different *in vitro* environments of the enzymes. The isolated detergent-solubilized enzyme may allow access of PC to a substrate-binding site, resulting in competition with endogenous PI, whereas the enzyme in the integrated membrane may not.

Our study has demonstrated that active GPI-GnT can be isolated from mammalian cells. It should be possible to isolate protozoan and yeast GPI-GnTs in a similar way. The isolated enzyme complexes will allow us to identify subunits that bind UDP-GlcNAc and PI, and to test inhibitory compounds.

Materials and methods

Cloning of hGPII

We searched an expressed sequence tag (EST) database (Boguski *et al.*, 1993) for a human homolog of *S.cerevisiae* GPII. Three 5' sequences (R24980, T97832, F07500) were identified that encoded peptides showing high homology to amino acids 340–430 of Gpi1p. To identify longer clones, we took the corresponding 3' sequence (R45240) of R24980, used it to perform a blastn search in an EST database and found six 3' sequences (R44534, T97729, Z40563, T97512, R44536, R41932). Clone 33898 containing R44534 had the longest insert of 2483 bp, and its 5' sequence (R24831) encoded a peptide with homology to amino acids 200–260 of Gpi1p. We purchased a plasmid of clone 33898 from the I.M.A.G.E. Consortium through Research Genetics, Inc. (Huntsville, AL). To obtain the sequence of the 5' end of hGPII, we amplified it from a pCEV4-based cDNA library prepared from KT3 cells (a gift from Dr N.Itoh) (Itoh *et al.*, 1991) by PCR. For this, we synthesized two 5' primers corresponding to the pCEV4 vector, SR1 (5'-GC-TTTGCTGACCCCTGCTTGC) and SR2 (5'-CCTCAGTGGATGT-TGCCTTAC), and two hGPII-specific 3' primers, R1 (5'-GGAACAG-ACGCCACAGTGAGGAC) and R2 (5'-GTAGGCAGGAGGAAGA-GCAGGAT), designed from the sequence of clone 33898. The first 20 cycles with primers SR1 and R2 consisted of 30 s of denaturation at 95°C, 30 s of annealing at 65°C and 2 min of extension at 72°C. The second 20 cycles, performed under the same conditions, used 2 µl of the product of the first amplification, and the SR2 and R1 primers. The product was subcloned and sequenced. Based on its sequence, we synthesized two new specific primers, 5' F1 (5'-CGTGTGCGTCT-CGGCGGACAG) and 5' R1 (5'-CAGGGTCTCCGCCTCCGTGTA), which lie within the newly amplified region, to screen pools of the same cDNA library by PCR. Positive fractions of the cDNA library were screened further by colony hybridization and positive clones were isolated.

Expression vectors containing tagged PIG-A, PIG-H, PIG-C and hGPII

To express GST- or FLAG-tagged-PIG-A, PIG-H and PIG-C in JY5 or JY25 cells, we used pMEEB-GST-PIG-A, pMEEB-FLAG-PIG-A, pMEEB-GST-PIG-H, pMEEB-FLAG-PIG-H (Watanabe *et al.*, 1996) and pMEEB-GST-PIG-C (Inoue *et al.*, 1996). pMEEB is an expression vector that uses the SR α promoter and bears the Epstein–Barr virus oriP and a hygromycin resistance gene. To construct an expression vector for FLAG-PIG-C, we replaced PIG-A cDNA in pMEEB-FLAG-PIG-A with PIG-C cDNA by digestion with *Sall* and *Xba*I and ligation of the *Sall*–*Xba*I fragment of pMEEB-GST-PIG-C. All these plasmids express proteins tagged at the amino-terminus. To add GST to the amino-terminus of hGPII, we amplified an hGPII fragment lacking an initiation codon but bearing a *Sall* site at the 5' end using 5' primer (5'-CGTGCAGTGTCTCAAGGCCCTTCTCCC) and 3' primer R2. This fragment was ligated to the rest of hGPII at the *Stu*I site to prepare full-length hGPII bearing a *Sall* site at the 5' end (termed *Sall* hGPII). We then digested pMEEB-GST-PIG-A with *Sall* and *Xba*I to remove PIG-A and replaced it with a *Sall*–*Xba*I fragment of *Sall* hGPII. To tag the carboxy-terminus of hGPII with GST, we amplified an hGPII fragment lacking a termination codon but bearing an *Mlu*I site at the 3' end using 5' primer 5'-GGGAGAAGCCGCATCGGGCATCT and 3' primer 5'-CACGCGTGTCTGCTTGTCCCTCTCT. The fragment was ligated to the rest of the 5' fragment of hGPII at the *Ehe*I site to prepare full-length hGPII bearing an *Mlu*I site at the 3' end (termed hGPII *Mlu*I). We then digested pMEEB-PIG-A-GST with *Xho*I and *Mlu*I to remove PIG-A, and replaced it with a *Xho*I–*Mlu*I fragment of hGPII *Mlu*I.

Transfection

Human B lymphoblastoid JY25 cells were transfected with GST-tagged hGPII DNA (15 µg) by electroporation at 250 V and 960 µF, and selected in hygromycin B (200 µg/ml)-containing medium for 1 week. Such cells were then transfected again with various combinations of FLAG-tagged-PIG-A, PIG-H and PIG-C DNAs (15 µg each) by electroporation. As a control, DNA encoding FLAG-tagged msALDH was co-transfected. In other experiments, JY25 cells were first transfected with DNA encoding FLAG-tagged PIG-C, PIG-L or msALDH, selected with hygromycin B and then transfected with combinations of two, three or four of GST-tagged-PIG-A, PIG-H, PIG-C and hGPII cDNAs.

Analysis of the protein complexes

Transfected cells (3.5×10^8) were suspended in water containing 0.1 mM TLCK and 1 µg/ml leupeptin, and homogenized in a tight pestle Dounce

homogenizer. Membranes collected by centrifugation at 100 000 g for 1 h were solubilized in 1% (w/v) digitonin in 50 mM HEPES-NaOH (pH 7.4) containing 25 mM KCl, 5 mM MgCl₂, 0.1 mM TLCK and 1 µg/ml leupeptin at 4°C for 1 h. After centrifugation at 100 000 g for 30 min to remove insoluble material, FLAG-tagged proteins were collected from the supernatant with anti-FLAG M2 affinity gel (Eastman Kodak, Rochester, NY). The proteins isolated by their immunoaffinity and the digitonin-insoluble material solubilized in SDS-PAGE sample buffer were separated by SDS-PAGE, transferred to PVDF membrane, and submitted to Western blot analysis using biotinylated anti-FLAG monoclonal antibody M2 plus horseradish peroxidase (HRP)–streptavidin conjugates (Amersham), or with anti-GST antibody (Pharmacia Biotech) plus HRP-conjugated anti-goat IgG antibody (Organon Teknika, N.V.), and visualized by chemiluminescence (Renaissance, DuPont). The digitonin-insoluble material contained only trace amounts of epitope-tagged protein, indicating that solubilization of transfected proteins with 1% (w/v) digitonin was nearly complete.

Analysis of GPI-GnT activity in vitro

JY cells expressing GST-tagged proteins were cultured for 3 h in the presence of 5 µg of tunicamycin/ml (WAKO, Osaka, Japan) to inhibit N-glycan synthesis. The cells were lysed hypotonically as described (Hirose *et al.*, 1991), the cell lysates (10⁷ cell equivalents) were solubilized with 1% (w/v) digitonin in buffer [50 mM HEPES-NaOH (pH 7.4), 25 mM KCl, 5 mM MgCl₂, 0.1 mM TLCK, 1 µg/ml leupeptin, 5 mM MnCl₂, 1 mM ATP, 0.5 mM dithiothreitol (DTT) and 0.2 µg/ml tunicamycin], and insoluble materials were removed by centrifugation. Protein complexes bearing GST tags were collected by adsorption to glutathione–Sepharose 4B (Pharmacia). The glutathione beads bearing protein complexes were washed three times and suspended in the above buffer. Cell lysates, their digitonin extracts and glutathione beads bearing the protein complexes were incubated with 0.2 µM UDP-[6-³H]GlcNAc (14 µCi/ml, American Radiolabeled Chemicals, MO) for 60 min at 37°C in buffer [50 mM HEPES-NaOH (pH 7.4), 25 mM KCl, 5 mM MgCl₂, 0.1 mM TLCK, 1 µg/ml leupeptin, 5 mM MnCl₂, 1 mM ATP, 0.5 mM DTT and 0.2 µg/ml tunicamycin]. The reaction was terminated by addition of chloroform/methanol (1:1 v/v). Insoluble material was removed by centrifugation, and the solution was evaporated with a vacuum centrifuge. The dried materials were extracted with *n*-butanol, or by using the method of Bligh and Dyer (1959). Extracted glycolipids were separated by thin-layer chromatography on Kieselgel 60 (Merk, Germany) with a solvent system of chloroform/methanol/1 M NH₄OH (10:10:3 by vol.) and radiolabeled lipids were detected by image analysis (Inoue *et al.*, 1996). To quantitate the amounts of GlcNAc-PI generated, the intensities of the spots were measured by Fuji Image Analyzer BAS 1500 (Fuji Film Co., Tokyo) after 1–3 days exposure, and expressed as percentages of the amount synthesized in the control reaction as indicated in the respective figure legends. Bovine liver and soybean PI, fresh egg yolk PC, bovine brain PS, egg yolk PE, bovine brain SM and Cer and synthetic PA (distearoyl) were purchased from Sigma, dissolved at 50 mM in the above buffer containing 1% (w/v) digitonin and added to the enzyme assay at 1–1000 µM. The lyso PI from bovine liver was purchased from Avanti Polar Lipids, Inc. The radiolabeled PI (myo-inositol-2-³H) was purchased from American Radiolabeled Chemicals.

Enzymatic treatments of lipids

Treatment of samples with *Bacillus thuringiensis* PI-PLC (Funakoshi, Tokyo, Japan) was done as previously described (Hirose *et al.*, 1991). To assess sensitivity to GlcNAc-PI deacetylase, samples suspended in 10 µl of 99.5% ethanol were incubated with lysates (10⁷ cell equivalents) of wild-type and deacetylase mutant CHO cells for 1 h at 37°C (Nakamura *et al.*, 1997). Standard lyso PI and GlcNAc-lyso PI were prepared by incubating PI and GlcNAc-PI overnight at 37°C with 2500 U/ml bee venom phospholipase A₂ (Sigma) in 100 mM Tris–HCl, pH 7.4, containing 1 mM CaCl₂ and 0.05% NP-40 (Heise *et al.*, 1996). After treatments, glycolipids were extracted and analyzed by thin-layer chromatography.

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References

- Bessler, M., Hillmen, P., Longo, L., Luzzatto, L. and Mason, P.J. (1994) Genomic organization of the X-linked gene (PIG-A) that is mutated in paroxysmal nocturnal haemoglobinuria and of a related autosomal pseudogene mapped to 12q21. *Hum. Mol. Genet.*, **3**, 751–757.
- Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem.*, **37**, 911–917.
- Boguski, M.S., Lowe, T.M. and Tolstoshev, C.M. (1993) dbEST—database for 'expressed sequence tags'. *Nature Genet.*, **4**, 332–333.
- Colussi, P.A. and Orlean, P. (1997) The essential *Schizosaccharomyces pombe* *gpi1⁺* gene complements a bakers' yeast GPI anchoring mutant and is required for efficient cell separation. *Yeast*, **13**, 139–150.
- Conzelmann, A., Fankhauser, C. and Desponds, C. (1990) Myoinositol gets incorporated into numerous membrane glycoproteins of *Saccharomyces cerevisiae*; incorporation is dependent on phosphomannomutase (SEC53). *EMBO J.*, **9**, 653–661.
- Conzelmann, A., Puoti, A., Lester, R.L. and Desponds, C. (1992) Two different types of lipid moieties are present in glycosylphosphatidylinositol-anchored membrane proteins of *Saccharomyces cerevisiae*. *EMBO J.*, **11**, 457–466.
- Costello, L.C. and Orlean, P. (1992) Inositol acylation of a potential glycosyl phosphoinositol anchor precursor from yeast requires acyl coenzyme A. *J. Biol. Chem.*, **267**, 8599–8603.
- Doering, T.L., Masterson, W.J., Englund, P.T. and Hart, G.W. (1989) Biosynthesis of the glycosyl phosphatidylinositol membrane anchor of the trypanosome variant surface glycoprotein. Origin of the non-acetylated glucosamine. *J. Biol. Chem.*, **264**, 11168–11173.
- Doering, T.L., Pessin, M.S., Hart, G.W., Raben, D.M. and Englund, P.T. (1994) The fatty acids in unremodelled trypanosome glycosylphosphatidylinositols. *Biochem. J.*, **299**, 741–746.
- Englund, P.T. (1993) The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. *Annu. Rev. Biochem.*, **62**, 121–138.
- Ferguson, M.A.J. (1994) What can GPI do for you? *Parasitol. Today*, **10**, 48–52.
- Ferguson, M.A.J. and Williams, A.F. (1988) Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. *Annu. Rev. Biochem.*, **57**, 285–320.
- Heise, N., Raper, J., Buxbaum, L.U., Peranovich, T.M. and de Almeida, M.L. (1996) Identification of complete precursors for the glycosylphosphatidylinositol protein anchors of *Trypanosoma cruzi*. *J. Biol. Chem.*, **271**, 16877–16887.
- Hirose, S., Ravi, L., Hazra, S.V. and Medof, M.E. (1991) Assembly and deacetylation of *N*-acetylglucosaminyl-plasmanylinositol in normal and affected paroxysmal nocturnal hemoglobinuria cells. *Proc. Natl Acad. Sci. USA*, **88**, 3762–3766.
- Inoue, N., Watanabe, R., Takeda, J. and Kinoshita, T. (1996) PIG-C, one of the three human genes involved in the first step of glycosylphosphatidylinositol biosynthesis is a homologue of *Saccharomyces cerevisiae* GPI2. *Biochem. Biophys. Res. Commun.*, **226**, 193–199.
- Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y. and Nagata, S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*, **66**, 233–243.
- Jensen, J.W. and Schutzbach, J.S. (1985) Activation of dolichyl-phosphomannose synthase by phospholipids. *Eur. J. Biochem.*, **153**, 41–48.
- Kamitani, T., Chang, H.M., Rollins, C., Waneck, G.L. and Yeh, E.T.H. (1993) Correction of the class H defect in glycosylphosphatidylinositol anchor biosynthesis in Ltk⁺ cells by a human cDNA clone. *J. Biol. Chem.*, **268**, 20733–20736.
- Kawagoe, K., Takeda, J., Endo, Y. and Kinoshita, T. (1994) Molecular cloning of murine PIG-a, a gene for GPI-anchor biosynthesis, and demonstration of interspecies conservation of its structure, function and gene locus. *Genomics*, **23**, 566–574.
- Kawagoe, K., Kitamura, D., Okabe, M., Taniuchi, I., Ikawa, M., Watanabe, T., Kinoshita, T. and Takeda, J. (1996) GPI-anchor deficient mice: implications for clonal dominance of mutant cells in paroxysmal nocturnal hemoglobinuria. *Blood*, **87**, 3600–3606.
- Kinoshita, T., Inoue, N. and Takeda, J. (1995) Defective glycosyl phosphatidylinositol anchor synthesis and paroxysmal nocturnal hemoglobinuria. *Adv. Immunol.*, **60**, 57–103.
- Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.*, **157**, 105–132.
- Leidich, S.D. and Orlean, P. (1996) Gpi1, a *Saccharomyces cerevisiae* protein that participates in the first step in GPI anchor biosynthesis. *J. Biol. Chem.*, **271**, 27829–27837.
- Leidich, S.D., Drapp, D.A. and Orlean, P. (1994) A conditionally lethal yeast mutant blocked at the first step in glycosyl phosphatidylinositol anchor synthesis. *J. Biol. Chem.*, **269**, 10193–10196.
- Leidich, S.D., Kostova, Z., Latek, R.R., Costello, L.C., Drapp, D.A., Gray, W., Fassler, J.S. and Orlean, P. (1995) Temperature-sensitive yeast GPI anchoring mutants *gpi2* and *gpi3* are defective in the synthesis of *N*-acetylglucosaminyl phosphatidylinositol: cloning of the GPI2 gene. *J. Biol. Chem.*, **270**, 13029–13035.
- Lu, C.F., Montijn, R.C., Brown, J.L., Klis, F., Kurjan, J., Bussey, H. and Lipke, P.N. (1995) Glycosyl phosphatidylinositol-dependent cross-linking of alpha-agglutinin and beta-1,6-glucan in the *Saccharomyces cerevisiae* cell wall. *J. Cell Biol.*, **128**, 333–340.
- Masterson, W.J., Doering, T.L., Hart, G.W. and Englund, P.T. (1989) A novel pathway for glycan assembly: biosynthesis of the glycosylphosphatidylinositol anchor of the trypanosome variant surface glycoprotein. *Cell*, **56**, 793–800.
- Masterson, W.J., Raper, J., Doering, T.L., Hart, G.W. and Englund, P.T. (1990) Fatty acid remodeling: a novel reaction sequence in the biosynthesis of trypanosome glycosyl phosphatidylinositol membrane anchors. *Cell*, **62**, 73–80.
- McConville, M.J. and Ferguson, M.A.J. (1993) The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.*, **294**, 305–324.
- Milne, K.G., Field, R.A., Masterson, W.J., Cottaz, S., Brimacombe, J.S. and Ferguson, M.A.J. (1994) Partial purification and characterization of the *N*-acetylglucosaminyl-phosphatidylinositol de-*N*-acetylase of trypanosomes. *J. Biol. Chem.*, **269**, 16403–16408.
- Miyata, T., Takeda, J., Iida, Y., Yamada, N., Inoue, N., Takahashi, M., Maeda, K., Kitani, T. and Kinoshita, T. (1993) Cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. *Science*, **259**, 1318–1320.
- Nakamura, N., Inoue, N., Watanabe, R., Takahashi, M., Takeda, J., Stevens, V.L. and Kinoshita, T. (1997) Expression cloning of PIG-L, a candidate *N*-acetylglucosaminyl-phosphatidylinositol deacetylase. *J. Biol. Chem.*, **272**, 15834–15840.
- Schönbacher, M., Horvath, A., Fassler, J. and Riezman, H. (1995) The yeast *spt14* gene is homologous to the human PIG-A gene and is required for GPI anchor synthesis. *EMBO J.*, **14**, 1637–1645.
- Singh, N., Zoeller, R.A., Tykocinski, M.L., Lazarow, P.B. and Tartakoff, A.M. (1994) Addition of lipid substituents of mammalian protein glycosylphosphoinositol anchors. *Mol. Cell. Biol.*, **14**, 21–31.
- Stevens, V.L. and Raetz, C.R. (1991) Defective glycosyl phosphatidylinositol biosynthesis in extracts of three Thy-1 negative lymphoma cell mutants. *J. Biol. Chem.*, **266**, 10039–10042.
- Sugiyama, E., DeGasperi, R., Urakaze, M., Chang, H.M., Thomas, L.J., Hyman, R., Warren, C.D. and Yeh, E.T.H. (1991) Identification of defects in glycosylphosphatidylinositol anchor biosynthesis in the Thy-1 expression mutants. *J. Biol. Chem.*, **266**, 12119–12122.
- Takeda, J., Miyata, T., Kawagoe, K., Iida, Y., Endo, Y., Fujita, T., Takahashi, M., Kitani, T. and Kinoshita, T. (1993) Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell*, **73**, 703–711.
- Thomas, J.R., Dwek, R.A. and Rademacher, T.W. (1990) Structure, biosynthesis, and function of glycosylphosphatidylinositols. *Biochemistry*, **29**, 5413–5422.
- Vidugiriene, J. and Menon, A.K. (1993) Early lipid intermediates in glycosyl-phosphatidylinositol anchor assembly are synthesized in the ER and located in the cytoplasmic leaflet of the ER membrane bilayer. *J. Cell Biol.*, **121**, 987–996.
- Vossen, J.H., Ram, A.F. and Klis, F.M. (1995) Identification of SPT14/CWH6 as the yeast homologue of hPIG-A, a gene involved in the biosynthesis of GPI anchors. *Biochim. Biophys. Acta*, **1243**, 549–551.
- Watanabe, R., Kinoshita, T., Masaki, R., Yamamoto, A., Takeda, J. and Inoue, N. (1996) PIG-A and PIG-H, which participate in glycosylphosphatidylinositol-anchor biosynthesis, form a protein complex in the endoplasmic reticulum. *J. Biol. Chem.*, **271**, 26868–26875.

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