GPI1 Stabilizes an Enzyme Essential in the First Step of Glycosylphosphatidylinositol Biosynthesis*

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Attachment of glycosylphosphatidylinositol (GPI) is essential for the surface expression of many proteins. Biosynthesis of glycosylphosphatidylinositol is initiated by the transfer of N-acetylgalcosamine from UDP-N-acetylgalcosamine to phosphatidylinositol. In mammalian cells, this reaction is mediated by a complex of PIG-A, PIG-H, PIG-C, and GPI1. This complexity may be relevant for regulation and for usage of a particular phosphatidylinositol. However, the functions of the respective components have been unclear. Here we cloned the mouse GPI1 gene and disrupted it in F9 embryonal carcinoma cells. Disruption of the GPI1 gene caused a severe but not complete defect in the generation of glycosylphosphatidylinositol-anchored proteins, indicating some residual biosynthetic activity. A complex of PIG-A, PIG-H, and PIG-C decreased to a nearly undetectable level, whereas a complex of PIG-A and PIG-H was easily detected. A lack of GPI1 also caused partial decreases of PIG-C and PIG-H. Therefore, GPI1 stabilizes the enzyme by tying up PIG-C with a complex of PIG-A and PIG-H.

Many eukaryotic cell surface proteins are post-translationally anchored to the membrane by carboxyl-terminal linkage to glycosylphosphatidylinositol (GPI)1 in the endoplasmic reticulum (1, 2). The basic backbone structure of GPI that is conserved in all GPI-anchored proteins found in protozoa, yeast, slime mold, fish, and mammals is formulated as EtN-P-6-Man, EtN-P, Man, and GlcN, respectively) (3). GPI anchor synthesis is essential for mouse embryogenesis (4, 5, 15–17). In affected hematopoietic cells and glucosamine (GlcNAc), respectively) (3). GPI anchor synthesis is essential for mouse embryogenesis (4, 5, 15–17). In affected hematopoietic cells and glucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI) to yield GlcNAc-PI (1, 11). Genes involved in this step have been cloned by means of complementation of mutants derived from mammalian cells and yeast Saccharomyces cerevisiae: PIG-A, PIG-H, and PIG-C from mammalian cells (12–14), and GPI1, GPI2 and GPI3/SPT14/CWH6 from yeast (5, 15–17). PIG-A and PIG-C are homologues of GPI1 and GPI2, respectively, whereas PIG-H and GPI1 are not similar to each other (12, 16). A mammalian GPI1 homologue has also been cloned, based on the sequence homology (18). A PIG-H homologue is not found in the S. cerevisiae genome. It was shown that PIG-A, PIG-H, PI, and GPI1 proteins form a complex in the endoplasmic reticulum that has UDP-GlcNAc:PI GlcNAc transferase (GPI-GnT) activity in vitro (18). Because PIG-A has homology to a bacterial GlcNAc transferase and other glycosyltransferases, this protein is thought to be a catalytic subunit (17, 19, 20). However, functions of three other proteins cannot be predicted from their sequences. Class A, C, and H mutant cells, corresponding to PIG-A, PIG-H, and PIG-C mutations, do not express GPI-anchored proteins on the cell surface, and their membranes do not have GPI-GnT activity, indicating that these three proteins are essential for the enzyme (21, 22). Similarly, S. cerevisiae gpi1 and gpi3 mutants are defective in GPI anchoring, indicating essential roles of GPI1 and GPI3 in GPI synthesis (5). In contrast, S. cerevisiae gpi1 is not essential for growth, and the gpi1-disrupted mutant showed temperature-sensitive growth. Moreover, the gpi1-disrupted cells incorporated a significant amount of inositol to proteins and generated mature Gas1p, a major GPI-anchored protein. Therefore, GPI1 may have a regulatory function rather than a direct role in the enzymatic function (15).

As described above, human GPI1 (hGPI1) is contained in the GPI-GnT complex (18). It has also been shown that hGPI1 physically associates with three other subunits, suggesting a role for hGPI1 in complex formation (18). More recently, it was reported that human and mouse GPI1 cDNAs complemented S. cerevisiae gpi1 and Schizosaccharomyces pombe gpi1 mutants (23), indicating that mammalian GPI1 is functionally homologous to yeast Gpllp (24). To clarify the function of GPI1 in GPI-GnT, in the present study, we generated GPI1 knockout mouse cells and found that GPI1 is necessary for stable formation of the GPI-GnT complex and stable expression of the PIG-C and PIG-H proteins.

EXPERIMENTAL PROCEDURES

Cells and Culture—Mouse embryonal carcinoma F9 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum on 0.1% gelatin-coated dishes. Mouse GPI-deficient mutant lymphoma T1M1 (Thy-1−) and S49 (Thy-1−) cells were gifts from Dr. R. Hyman (Salk Institute, San Diego, CA) and were cultured and transfected as described previously (25).

The synthesis of GPI is initiated by the transfer of N-acetylgalcosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI) to yield GlcNAc-PI (1, 11). Genes involved in this step have been cloned by means of complementation of mutants derived from mammalian cells and yeast Saccharomyces cerevisiae: PIG-A, PIG-H, and PIG-C from mammalian cells (12–14), and GPI1, GPI2 and GPI3/SPT14/CWH6 from yeast (5, 15–17). PIG-A and PIG-C are homologues of GPI1 and GPI2, respectively, whereas PIG-H and GPI1 are not similar to each other (12, 16). A mammalian GPI1 homologue has also been cloned, based on the sequence homology (18). A PIG-H homologue is not found in the S. cerevisiae genome. It was shown that PIG-A, PIG-H, PI, and GPI1 proteins form a complex in the endoplasmic reticulum that has UDP-GlcNAc:PI GlcNAc transferase (GPI-GnT) activity in vitro (18). Because PIG-A has homology to a bacterial GlcNAc transferase and other glycosyltransferases, this protein is thought to be a catalytic subunit (17, 19, 20). However, functions of three other proteins cannot be predicted from their sequences. Class A, C, and H mutant cells, corresponding to PIG-A, PIG-H, and PIG-C mutations, do not express GPI-anchored proteins on the cell surface, and their membranes do not have GPI-GnT activity, indicating that these three proteins are essential for the enzyme (21, 22). Similarly, S. cerevisiae gpi1 and gpi3 mutants are defective in GPI anchoring, indicating essential roles of GPI1 and GPI3 in GPI synthesis (5). In contrast, S. cerevisiae gpi1 is not essential for growth, and the gpi1-disrupted mutant showed temperature-sensitive growth. Moreover, the gpi1-disrupted cells incorporated a significant amount of inositol to proteins and generated mature Gas1p, a major GPI-anchored protein. Therefore, GPI1 may have a regulatory function rather than a direct role in the enzymatic function (15).

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Plasmids—To express mouse GPI, its cDNA was cloned to a pMEEB expression vector. To express GST-tagged PIG-A, GST-tagged PIG-H, and FLAG-tagged PIG-C at the same time, each fragment was cloned between the PGK promoter and the PGK poly(A) signal in pBS-PGKtk (7). Three constructs were tandemly blunted-ligated in pBS-PGKtk and introduced into DH5α by electroporation. This plasmid resisted G418. The surface expression of GPI-anchored proteins on human PIG-A-deficient JY5 cells, Pig-c-deficient TIM1 cells (Thy-1−c), and Pig-h-deficient S49 cells (Thy-1−h). To express FLAG-tagged gPPII, it was cloned between the PGK promoter and the PGK poly(A) signal in pBS-PGKtk and then ligated with pBS-PGKneo to generate pBS-FLAG-gPPII/neo. All of the other constructs used in this study were described previously (25).

Preparation of cDNA and Genomic Clones of mGPII—We designed degenerate primers on the basis of hGPII and yeast Gpi1p amino acid sequences to clone mGPII cDNA (15, 18). Two primers, forward primer HF1 (5′-GGTATTTGAGTCCAG-3′) and reverse primer HR1 (5′-GGAAGGTAAGGAGNARNGTNCDDAT-3′) (D, G, A, and T), and template DNA derived from a mouse testis cDNA library were used for 35 cycles of amplification reaction under the conditions of 94 °C (1 min), 55 °C (30 s), and 68 °C (1.5 min) for denaturation, annealing, and extension, respectively. A sequence of the PCR product was confirmed to be homologous to hGPII. To obtain the 5′ and 3′ regions, we screened 96 subpools of a cDNA library, each of which contained 2 × 106 independent clones containing the same forward primer and the reverse primer, showing a positive PCR band. Using DNAs of these pools, forward vector primer, and HR1 primer in PCR, fragments containing the 5′ region were cloned from the subpools. The longest clone was named pHG5c7. Using HF1 primer and reverse vector primer, fragments containing the 3′ region were cloned. All subpools showed the same fragments. The longest 5′ and 3′ fragments were ligated in the EcoR1 site to generate full-length cDNA. An analysis by 5′ rapid amplification of cDNA ends with the Marathon Race kit (Clontech) using RNA from F9 cells confirmed that this cDNA contained the longest 5′ sequence.

To obtain genomic clones of mGPII, we screened 1 × 106 plaque-forming units of lambda FIXII from a mouse 129/SvJ liver genomic library (Stratagene) using a 1.4-kb pGH5c7 fragment, which contained the 5′ region of mGPII cDNA, as a probe. We obtained eight positive clones.

Disruption of the mGPII Gene in F9 Embryonal Carcinoma Cells—A 9.4-kb HindIII genomic fragment of mGPII was blunted and transferred to the SmaI site of pBS, the EcoR1-XhoI fragment was then transferred to pPNT (26), and the plasmid was named pPNT-18HdelF. A 2.1-kb XhoI-EcoRI fragment of mGPII containing exon 6 of mGPII was blunted and transferred to pBS. Its XhoI-NotI fragment and a 1.8-kb XhoI-XhoI fragment derived from PGKpuro were ligated in tandem in the XhoI-NotI site in pPNT-18HdelF. This plasmid was then used as the first targeting vector. A 3.0-kb EcoRV fragment containing a region extending from the middle of exon 6 to exon 10 was transferred to the SmaI site of pBS. Its XhoI-XhoI fragment and a 1.9-kb XhoI-XhoI fragment derived from PKne were ligated in the XhoI-NotI site of pPNT-18HdelF. The generated plasmid was used as the second targeting vector.

F9 cells (2 × 105) were electroporated with 50 µg of NotI-cut targeting vector at 230 V and 500 microfarads using a GenePulser (Bio-Rad) and seeded on plates coated with 0.1% gelatin. Positive selection was started the next day with 2 mg/ml puromycin or 380 µg/ml G418. After 2 days, negative selection with 2 µg gancyclovir was performed. About 8–11 days after transfection, we screened colonies for recombinants, using PCR primers PGK-PA2970 (5′-CCTGAGAAGACAGAT-CAGCAGCTCTG-3′) and MGPI-KO154 (5′-TGGTTTATACACATCTTGACATAGT-3′) for the first targeting and PGK3254 (5′-CCTGAGCAGGAAGCAAGGAGAACAGC-3′) and MGPI-KO154 for the second targeting. PCSs were started at 93 °C (1 min) followed by annealing at 62 °C (1 min) and extension at 68 °C (3 min) for 35 cycles using Long Template PCR enzyme and buffers (Roche Molecular Biochemicals). Positive colonies were confirmed by Southern blot analysis.

Northern Blot Analysis—For Northern blot analysis of mouse Pig-c, Pig-a, Pig-H, and Pig-C mRNA, 2 µg of poly(A)+ RNA was loaded on each lane. After running, blots were hybridized with a digoxigenin-labeled probe, washed, and chemiluminescent detection was performed. The band intensities were quantified using a Fuji Image analyzer BAS1500 (Fuji Film Co., Tokyo Japan). The intensity of the hybridization bands was determined by measuring chemiluminescence with a Fuji Image analyzer (Fuji Photo Film Co., Tokyo, Japan). The band intensities in Western blotting were quantified using a Fuji Image analyzer BAS1500 (Fuji Film Co., Tokyo Japan). Treatments of lipids with Pinto thianthinis PS-phospholipase C (PLC) (Funakoshi, Japan) and acetylation of lipids were done as described previously (28). Moderate alkaline hydrolysis was done as described previously (21).

Immunoprecipitation and Western Blotting—Transfected cells (2 × 106) were hypotonically lysed in water containing 0.1 mM TLM and 1 mM leupeptin, mixed with an equal volume of a lysate buffer (100 mM HEPES/NaOH (pH 7.4), 50 mM KCl, 10 mM MgCl2, 20% glycerol, 0.1 mM TLM, and 1 µg/ml leupeptin) and stored at −80 °C until used. After thawing on ice, the cell lysates were further treated using a tight pestle Dounce homogenizer. Membranes and cytosol fractions were divided by centrifugation at 100,000 × g for 1 h, and supernatants were used for immunoprecipitation. For the cytosol fractions, Nonidet P-40 was added at 1% and used for immunoprecipitation. Anti-FLAG M2 affinity gel beads (Sigma) and glutathione–Sepharose beads (Amer sham Pharmacia Biotech) were used to collect FLAG- and GST-tagged proteins, respectively. Proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting. Western blotting was carried out with biotinylated anti-FLAG monoclonal antibody M2 plus horseradish peroxidase-conjugated streptavidin (Amersham Pharmacia Biotech) or with anti-GST antibody (Amersham Pharmacia Biotech) plus horseradish peroxidase-conjugated anti-gont IgG antibody (Organon Teknika) and visualized with chemiluminescence (Renaissance; DuPont). The band intensities in Western blotting were quantified using chemiluminescence with a Fuji Image analyzer BAS1500 (Fuji Photo Film Co., Tokyo Japan).

Fluorescence in situ Hybridization—Fluorescence in situ hybridization was carried out with a biotinylated genomic DNA probe for mGPII and a biotinylated cDNA probe for hGPII prepared as described previously (10).

RESULTS

Cloning of mGPII—The full-length mGPII cDNA contained 3062 nucleotides except a poly(A) tract, encoding a 581-amino acid protein that had an 89% amino acid identity to hGPII (GenBank accession number for mGPII cDNA is AB008895). Using a mGPII cDNA probe, we obtained eight genomic clones of mGPII, mapped them, and sequenced around the exons (GenBank accession numbers for the genomic sequences are AB008915 to AB008921). Exon sequences were exactly the same as that of the cDNA. The mGPII gene had 11 exons, starting a codon in exon 2 and a stop codon in exon 11 (Fig. 1A). This structure was similar to a partial structure of hGPII determined by analyzing a sequence obtained from GenBank™ (Fig. 1B). Their corresponding exons had the same lengths.

The amino acid sequence of mGPII was very different from that published recently as mouse GPII (24) (GenBank accession number AF030178), having only a 93% amino acid identity. We think that the published sequence is not that of mouse.
Role of GPI1 in GPI Synthesis

FIG. 1. Genomic structures of mGPI1 and hGPI1. The genomic structure of mGPI1 (A) was determined from overlapped restriction maps of genomic lambda clones and sequences of regions containing exons. The genomic structure of hGPI1 (B) was determined by analyzing nucleotide sequences from the GenBank accession numbers of genomic sequences of hGPI1 are Z98881 and Z98883. ■ and □, noncoding and coding exon regions, respectively. Exon numbers are indicated. Restriction enzyme sites are as follows: Bsn, BamHI; E, EcoRI; H, HindIII; RV, EcoRV; X, XhoI; and Sp, SpeI.

FIG. 2. Disruption of mGPI1 in F9 cells. A, targeting vectors for homologous recombination. The first and second targeting vectors were designed to disrupt exon 2 to exon 8 and exon 2 to the middle of exon 6, respectively, and to contain the puromycin (puro) and neomycin (neo) resistance genes, respectively. The HSV thymidine kinase gene (tk) was included at the 5′ end to select against random integration. All selection marker genes were driven by the PGK promoter and had a PGK poly(A) signal. Restriction enzyme site: Xh, XhoI. B, structures of disrupted mGPI1 after homologous recombination. To screen homologous recombinants, PCR primers were used as indicated (arrows). Probes A and B, which were used for Southern blotting, are in bold. C, Southern blotting of targeted mutants. Samples of genomic DNA (5 μg) were cut with XbaI (left panel) and SpeI (right panel) and probed with probes A and B (see B), respectively. Lanes 1, F9 (wild type); lanes 2, single knockout mutant; lanes 3, double knockout mutant. D, Northern blotting of mGPI1 knockout mutant. Samples of total RNA (30 μg) were separated in a 1% formaldehyde-agarose gel, transferred to a nylon membrane, and probed with radiolabeled mGPI1 cDNA. The membrane was reprobed with EF-1α cDNA as a control. Lane 1, F9; lane 2, mGPI1 knockout mutant.

GPI1 because: 1) our cDNA sequence and genomic exon sequence were exactly the same, 2) many mouse expressed sequence tag (EST) nucleotide sequences in the current databases matched exactly with our sequence, 3) the mGPI1 gene was successfully disrupted using our mGPI1 DNA fragments, and 4) their sequence was simply that of an EST clone and was not confirmed in mouse cDNA or DNA.

Disruption of mGPI1 in the F9 Embryonal Carcinoma Cell Line—We used the F9 cell line to generate mGPI1-disrupted cells because it has a relatively high frequency of homologous recombination and is easier to culture than embryonic stem cells. We designed two targeting vectors: the vector for the first targeting had the puromycin resistance gene in place of exon 2 and the vector for the second targeting had the neomycin resistance gene in place of exon 2 to a part of exon 6 (Fig. 2A). Colonies grown after antibiotic selection were screened with PCR (Fig. 2B) and confirmed with genomic Southern blotting (Fig. 2C). Bands corresponding to the wild type allele decreased after the first targeting (lanes 2) and disappeared after the second targeting with generation of the predicted targeted bands (lanes 3). After the second targeting, no 3.1-kb RNA band of mGPI1 was seen in Northern blotting (Fig. 2D), indicating that both alleles were disrupted.

Characterization of mGPI1 Knockout Mutants—We first assessed the surface expression of Thy-1, a GPI-anchored protein on mGPI1-knockout F9 cells, and found that the expression decreased greatly but remained at a low level (Fig. 3A). The surface Thy-1 expression was restored upon transfection of mGPI1 cDNA but not upon transfection of an empty vector (Fig. 3B). To confirm that mGPI1 knockout cells have some ability to generate GPI-anchored proteins, we transfected a cDNA of CD59. CD59 was more efficiently expressed on the cell surface, whereas the expression of endogenous Thy-1 stayed very low (Fig. 3C). These results showed that mGPI1 knockout cells still have some GPI-GnT activity.

We next measured GPI-GnT activity in vitro using cell lysates and microsomes of mGPI1 knockout cells (Fig. 4). In contrast to the significant surface expression of GPI-anchored proteins on mGPI1 knockout cells, their lysates and microsomal membranes did not generate GlcNAC-PI upon incubation with UDP-GlcNAc (lanes 2 in Fig. 4A and B), whereas they had comparable activities of dolichol phosphate glucose (Dol-P-Glc) synthase. This defect was restored by transfection of mGPI1 cDNA (lanes 3) but not by transfection of an empty vector (lanes 4). The lysates of mGPI1 knockout cells gave several radioactive spots, one of which had the same mobility as GlcN-PI (lane 2), the second intermediate derived from GlcNAC-PI by deacetylation. To determine whether it is GlcN-PI or a non-relevant material, we first tested for the presence of PI by treatment with PI-PLC (Fig. 5A). The spot was resistant to PI-PLC (lanes 3 and 4), whereas the GlcN-PI generated by the wild type cells was sensitive, as shown by a great decrease of the intensity (lanes 1 and 2), indicating that the spot did not contain PI. We next tested acetylation that would convert GlcN-PI to GlcNAC-PI (Fig. 5B). GlcN-PI generated by the wild type cells was acetylated to GlcNAC-PI, resulting in a decrease of GlcNAC-PI and a slight increase of GlcNAC-PI (lanes 1 and 2); however, the spot generated by mGPI1 knockout cells did not shift to the GlcNAC-PI position (lanes 3 and 4). As a final test, we treated the spot with mild alkali (Fig. 5C). If it contained diacylglycerol, then the acyl chains would be removed, causing a mobility shift or disappearance from the lipid fraction. The spot was resistant (lanes 3 and 4), whereas GlcN-PI and GlcNAC-PI were sensitive (lanes 1 and 2). All of these results indicated that the spot was not GlcN-PI. Other spots seen above and below the area corresponding to GlcNAC-PI and GlcN-PI should also be non-relevant. They are not related to N-glycans because a 10-fold higher concentration of tunicamycin did not affect them (data not shown). Therefore, the membranes of mGPI1 knockout cells did not have a detectable level of GPI-GnT activity in vitro.

We thought that the weak expression of Thy-1 on mGPI1 knockout cells (Fig. 3A) might be due to inefficient recognition of GPI attachment signals of Thy-1 by GPI transamidase in the presence of a limited amount of GPI. To test this, we replaced the GPI attachment signal sequence of CD59 with that of Thy-1...
and compared it with wild-type CD59. As shown in Fig. 6, CD59 bearing the GPI attachment signal of Thy-1 was processed much less efficiently than CD59 bearing its own signal. GPI1 Is Required for the Stable Formation of GPI-GnT Complexes and for the Full Expressions of PIG-C and PIG-H but not PIG-A—The above results demonstrated that a very low level of GPI-GnT activity was expressed in the absence of mGPI1. A possible explanation for this is that the GPI-GnT complex cannot be formed efficiently in the absence of mGPI1. Another possible explanation is that a protein complex of PIG-A, PIG-C, and PIG-H is formed even in the absence of mGPI1 but has a very weak activity. To test the former possibility, we stably transfected mGPI1 knockout cells with a vector bearing cDNAs for FLAG-tagged PIG-C and GST-tagged PIG-A and PIG-H. A vector for free GST was also cotransfected to the same cells. A clonal cell population was isolated by limiting dilution and further transfected with either mGPI1 cDNA or an empty vector. Therefore, two cells would potentially express tagged PIG-C, PIG-A, and PIG-H and free GST at the same levels, with the sole difference being the presence or absence of mGPI1. From digitonin extracts of these cells, we precipitated FLAG-tagged PIG-C with anti-FLAG beads, and coprecipitations of GST-tagged PIG-A and PIG-H were assessed by Western blotting with anti-GST (Fig. 7A). In the presence of mGPI1, GST-tagged PIG-A and PIG-H were clearly coprecipitated (lane 1), indicating the formation of complexes of PIG-A, PIG-H, PIG-C, and mGPI1. In contrast, almost no bands of GST-tagged PIG-H and PIG-A were seen in the absence of mGPI1 (lane 2). These cells expressed comparable levels of free GST in the

![Fig. 3. Flow cytometric analysis of knockout mutants of F9 cells.](image)

A. decrease in surface Thy-1 expression after the disruption of mGPI1. Cells were stained with biotinylated anti-Thy1 antibody (thick lines) or control antibody (thin lines). Left panel, F9; middle panel, single knockout mutant; right panel, mGPI1 knockout mutant. B. restoration of the surface Thy-1 expression with mGPI1 cDNA. Left panel, mock transfectants of mGPI1 knockout cells; right panel, mGPI1 stable transfectant of mGPI1 knockout cells. C, transfection of CD59 cDNA to mGPI1 knockout mutant. Endogenous Thy-1 (left panel) and human CD59 (right panel) were stained after stable transfection of CD59 cDNA into mGPI1 knockout cells. Thick lines, anti-Thy1 (left panel) and anti-CD59 antibodies (right panel); thin lines, control antibodies.

![Fig. 4. In vitro GPI-GnT assay with cell lysates and microsomes.](image)

Cell lysates (A) were prepared from 1 × 10^7 cells and labeled with UDP-[6-3H]GlcNAc for 1 h. In B, microsomes (100 μg) were used under the same reaction conditions. The same samples were also incubated with UDP-[6-3H]glucose to measure Dol-P-Glc synthase to assess the amount of membranes (lower panel). The radiolabeled lipids were extracted and resolved in TLC. Lanes 1, F9; lanes 2, mGPI1 knockout mutant; lanes 3, mGPI1-transfected knockout cells; lanes 4, mock-transfected knockout cells.

![Fig. 5. Characterization of GlcNAc-labeled products.](image)

Lysates (from 2 × 10^7 cells) of wild type (lanes 1 and 2 in all panels) and mGPI1 knockout (lanes 3 and 4 in all panels) F9 cells were labeled with UDP-[6-3H]GlcNAc for 1 h. A, extracted radiolabeled lipids were treated with PI-PLC (lanes 2 and 4) or a reaction buffer (lanes 1 and 3), and reextracted lipids were analyzed by TLC. B, the same samples as in A were treated with acetic anhydride (lanes 2 and 4) or a buffer (lanes 1 and 3). C, the same samples as in A and B were treated with mild alkali (lanes 2 and 4) or a neutral buffer (lanes 1 and 3).
cytoplasm (bottom panel). Therefore, only a trace amount, if any, of the protein complex was formed in the absence of mGPI1.

To evaluate the amounts of expressed FLAG-tagged PIG-C, we analyzed the immunoprecipitates with anti-FLAG beads by Western blotting against anti-FLAG antibody (Fig. 7A, lanes 3 and 4). The amount of FLAG-PIG-C was significantly lower in the absence (lane 4) than in the presence (lane 3) of mGPI1, whereas a nonspecific band (asterisk) and a light chain of the antibody (L) showed comparable intensities. We also evaluated the amounts of expressed GST-tagged PIG-A and PIG-H by collecting them from Nonidet P-40 extracts with glutathione-Sepharose beads followed by Western blotting against anti-GST antibody (lanes 5 and 6). The amount of GST-tagged PIG-H was significantly lower in the absence (lane 6) than in the presence (lane 5) of mGPI1, whereas the amounts of GST-tagged PIG-A were comparable. The intensities of these bands and bands of free GST collected from the cytoplasm were quantitated by an image analyzer. Expression levels of PIG-C and PIG-H decreased to one-third of the wild type levels in the absence of mGPI1, whereas PIG-A and a control free GST were expressed at similar levels.

These results were from cloned cells. To eliminate the chance that an unusual event might have occurred due to the use of cloned cells, we used cells without limiting dilution. For this we transiently cotransfected mGPI1 knockout cells with a vector bearing cDNAs for FLAG-tagged PIG-C and GST-tagged PIG-A and PIG-H in combination with mGPI1 cDNA or an empty vector and assessed the complex formation and expressions of three tagged proteins. We obtained results very similar to those with the cloned cells (data not shown).

To test the association between PIG-A and PIG-H in the absence of GPI1, we cotransfected GST-tagged PIG-H and FLAG-tagged PIG-A into mGPI1 knockout cells (Fig. 7B, lane 1). As a positive control, GST-tagged GPI1 and FLAG-tagged PIG-A were cotransfected (lane 2). As negative controls, GST-tagged ALDH (aldehyde dehydrogenase, a control endoplasmic reticulum membrane protein (29)) and FLAG-tagged PIG-A (lane 3) and GST-tagged PIG-H and FLAG-tagged ALDH (lane 4) were cotransfected, respectively. FLAG-tagged PIG-A was efficiently coprecipitated with GST-tagged PIG-H (lane 1), but not with GST-tagged ALDH (lane 3). FLAG-tagged ALDH was not coprecipitated with GST-tagged PIG-H (lane 4). Therefore, PIG-A and PIG-H associated specifically and efficiently in the absence of GPI1. This indicated that the efficient association of PIG-C with a complex of PIG-A and PIG-H is dependent upon GPI1.

Because GPI1 is an endoplasmic reticulum membrane protein (18), it is unlikely that the GPI1 protein affects transcriptions of the PIG-C and PIG-H genes. In fact, the expression levels of endogenous mouse PIG-c mRNA assessed by Northern blotting did not change between wild type and mGPI1 knockout cells (data not shown). It is likely that GPI1 affects the stability of the PIG-C and PIG-H proteins. However, decreased levels of PIG-C and PIG-H were still 30% of the wild type levels, which does not fully explain a nearly complete lack of complexes of PIG-A, PIG-C, and PIG-H. Therefore, GPI1 must function in the maintenance of the GPI-GnT complex itself.

To see whether the expression of GPI1 is conversely influenced by PIG-C and PIG-H, we cotransfected FLAG-tagged

FIG. 6. GPI attachment signals influenced the surface expressions of GPI-anchored proteins. MGP11 knockout F9 cells were transiently transfected with cDNAs of CD59 (A) and CD59 bearing the GPI attachment signal from Thy-1 (B). The surface CD59 expressions were determined 2 days after transfection. Thin lines, anti-CD59 antibodies; thick lines, isotype-matched control antibodies.

FIG. 7. The effects of mGPI1 disruption on GPI-GnT complexes. A, mGPI1 knockout F9 cells were stably transfected with FLAG-tagged PIG-C, GST-tagged PIG-A, GST-tagged PIG-H, and free GST simultaneously. The transfected cell was further transfected with mGPI1 cDNA (lanes 1, 3, and 5) or an empty vector (lanes 2, 4, and 6). Cytosol and membrane fractions from lysed cells (2 × 10^6) were separated. Proteins were immunoprecipitated from the solubilized membranes with the indicated beads. The immunoprecipitates were analyzed by Western blotting with anti-FLAG or anti-GST antibodies. Lanes 1 and 2, Western analysis with anti-GST of anti-FLAG immunoprecipitates; lanes 3 and 4, Western analysis of expression levels of FLAG-tagged PIG-C; lanes 5 and 6, Western analysis of expression levels of GST-tagged PIG-A and GST-tagged PIG-H. Size markers (in kDa) are shown on the right. B, association of PIG-A and PIG-H in the absence of GPI1. MGP11 knockout mutants were transfected with GST-tagged PIG-H and FLAG-tagged PIG-A (lane 1), GST-tagged GPI1 and FLAG-tagged PIG-A (lane 2), GST-tagged ALDH and FLAG-tagged PIG-A (lane 3), and GST-tagged PIG-H and FLAG-tagged ALDH (lane 4). GST-tagged proteins were collected from digitonin-solubilized membrane fractions with glutathione-Sepharose beads (top and middle panels) and Western blotted with anti-GST antibodies (top panel) or anti-FLAG antibodies (middle panel). After precipitation with glutathione-Sepharose beads, FLAG-tagged proteins in the supernatants were immunoprecipitated with anti-FLAG beads and Western blotted with anti-FLAG antibodies (bottom panel).
Role of GPI1 in GPI Synthesis

hGPI1 and free GST, as a control, into Pig-c-deficient TIM1 cells (Thy-1 c) and Pig-h-deficient S49 cells (Thy-1 h), obtained the cloned cells, and transfected them with PIG-C and PIG-H cDNA, respectively, or with an empty vector. An analysis by fluorescence-activated cell sorting confirmed that PIG-C-transfected TIM1 (Thy-1 c) cells and PIG-H-transfected S49 cells (Thy-1 h) but not vector transfectedants restored the surface expression of Thy-1 proteins (data not shown). However, the amounts of expressed FLAG-tagged GPI1 did not change significantly in the presence (Fig. 8A, lane 1) and absence (lane 2) of PIG-C or in the presence (Fig. 8B, lane 1) and absence (lane 2) of PIG-H. Therefore, it seems that GPI1 influences the stability of PIG-C and PIG-H, but not PIG-A, but that the stability of GPI1 is not influenced by PIG-C and PIG-H.

Chromosomal Localization of mGPI1 and hGPI1—We investigated the chromosomal localization of mGPI1 and hGPI1 using fluorescence in situ hybridization. With a 15.2-kb genomic fragment from a lambda clone as a probe, we localized mGPI1 to mouse chromosome 17B (Fig. 9A). Using a 2.9-kb hGPI1 cDNA probe, we localized hGPI1 to human chromosome 16p13.3 (Fig. 9B). These mouse and human chromosome regions are syntenic (30). The autosomal location of mammalian GPI1 is consistent with the notion that only PIG-A is X-linked among all GPI-anchor synthesis genes (1).

In this report, we present direct evidence that GPI1 is necessary for the stable formation of GPI-GnT. We disrupted mouse GPI1 in F9 embryonal carcinoma cells and found that the generation of GPI-anchored proteins was severely affected in the absence of GPI1. This defect was accounted for by a decrease of complexes of PIG-C with PIG-A and PIG-H to a nearly undetectable level, whereas an association between PIG-A and PIG-H was still seen. In the absence of GPI1, the levels of PIG-C and PIG-H were only one-third of the wild-type levels, indicating that GPI1 is needed to maintain normal levels of these proteins. The level of PIG-A was not affected by the absence of GPI1. These partial losses of PIG-C and PIG-H alone cannot fully account for the nearly complete lack of the complex of these components. Therefore, it is indicated that GPI1 is required for the efficient association of PIG-C with a complex of PIG-A and PIG-H. This is consistent with our previous report that GPI1 directly associates with each of the other three proteins (18).

Among the four proteins participating in GPI-GnT, PIG-A, PIG-C, and PIG-H are essential for activity because their mutant cells are completely defective in GPI-GnT (21, 22), whereas GPI1 is not essential because GPI1 knockout cells still had some ability to generate GPI-anchored proteins. This phenotype of mGPI1 knockout F9 cells is similar to that of gpi1-disrupted S. cerevisiae that was lethal at a higher temperature but was viable and incorporated some inositol to proteins at 25 °C (15). Expression of mammalian GPI1 in gpi1-disrupted S. cerevisiae restored growth at a higher temperature and synthesis of GlcNAc-PI in vitro (24), indicating that mammalian and yeast GPI1 proteins have conserved sites for association with other components.

In the absence of GPI1, proteins that are normally GPI-anchored would compete for a limited amount of GPI Proteins bearing different carbohydrate-terminal GPI attachment signals may have different affinities for GPI transamidase that recognizes the GPI attachment signal and replaces it with GPI (2). The mGPI1 knockout F9 cells expressed a very low level of Thy-1 on the surface but expressed CD59 quite efficiently.

**Fig. 8.** Lack of PIG-C or PIG-H had no effect on GPI1. A, TIM1 (Thy-1 c) mutants were transfected with FLAG-hGPI1 and GST simultaneously, cloned with limiting dilution, and then retransfected with PIG-C cDNA (lane 1) or an empty vector (lane 2). Membrane fractions were immunoprecipitated with anti-FLAG beads and blotted with anti-FLAG antibodies (top panel). GST proteins were detected in the cytosol fractions by Western blotting (bottom panel). B, S49 (Thy-1 h) mutants were transfected with FLAG-hGPI1 and GST simultaneously, cloned with limiting dilution, and then retransfected with PIG-H cDNA (lane 1) or an empty vector (lane 2). Membrane fractions were immunoprecipitated with anti-FLAG beads and blotted with anti-FLAG antibodies (top panel). GST proteins were detected in the cytosol fractions by Western blotting (bottom panel).

**Fig. 9.** Chromosomal localization of mGPI1 and hGPI1. Fluorescence in situ hybridization fluorescent spots were detected in mouse chromosome 17B (A) and in human chromosome 16p13.3 (B). The arrows and bars indicate positive signals of probes.
indicating that the GPI attachment signal of CD59 functioned more efficiently than that of Thy-1. A possible reason for this is that the signal sequence of CD59 contains asparagine at the ω site (31) to which GPI is linked, whereas the signal of Thy-1 has cysteine at the ω site (32). There are reports that the asparagine ω site functions more efficiently than the cysteine ω site in the GPI attachment reaction (33–35).

We localized the mouse GPI1 gene to chromosome 17B and localized human GPI1 to chromosome 16p13.3. This autosomal location is consistent with the fact that two homologous recombinations were required to eliminate mGPI1. This is also consistent with the observation that most, if not all, patients with paroxysmal nocturnal hemoglobinuria lost the ability for biosynthesis of GlcNAc-PI in their affected blood cells due to somatic mutation of the PIG-A gene in the hematopoietic stem cell (36). Because PIG-A is X-linked, a single somatic mutation should cause the GPI-GnT deficiency, whereas two somatic mutations of GPI1 should occur in the same cell to cause the mutant phenotype. The latter probability would be extremely low. It is difficult to predict the outcome of a hereditary lack of GPI1. Although a complete lack of GPI synthesis causes embryonic lethality (7, 8), no information is available about the effects of a partial GPI deficiency.

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