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The Initial Enzyme for Glycosylphosphatidylinositol Biosynthesis Requires PIG-Y, a Seventh Component

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Biosynthesis of glycosylphosphatidylinositol (GPI) is initiated by an unusually complex GPI-*N*-acetylglucosaminyltransferase (GPI-GnT) consisting of at least six proteins. Here, we report that human GPI-GnT requires another component, termed PIG-Y, a 71 amino acid protein with two transmembrane domains. The Burkitt lymphoma cell line Daudi, severely defective in the surface expression of GPI-anchored proteins, was a null mutant of PIG-Y. A complex of six components was formed without PIG-Y. PIG-Y appeared to be directly associated with PIG-A, implying that PIG-Y is the key molecule that regulates GPI-GnT activity by binding directly to the catalytic subunit PIG-A. PIG-Y is probably homologous to yeast Eri1p, a component of GPI-GnT. We did not obtain evidence for a functional linkage between GPI-GnT and ras GTPases in mammalian cells as has been reported for yeast cells. A single transcript encoded PIG-Y and, to its 5′ side, another protein PreY that has homologues in a wide range of organisms and is characterized by a conserved domain termed DUF343. These two proteins are translated from one mRNA by leaky scanning of the PreY initiation site.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) acts as a membrane anchor for many cell surface proteins (Tiede *et al.*, 1999; McConville and Menon, 2000; Ikezawa, 2002). The basic backbone structure of GPI is conserved in all GPI-anchored proteins found in protozoa, yeast, plants, and mammals. In mammalian cells, over 150 different proteins are GPI-anchored, including cell surface enzymes, receptors, and adhesion molecules (Kinoshita *et al.*, 1995). GPI-anchored proteins are enriched in "lipid rafts," allowing their functional linkages to signal transduction systems (Simons and Toomre, 2000). GPI anchor synthesis is essential for the growth of yeast (Leidich *et al.*, 1994), but not for that of mammalian cells. It is, however, essential for mouse embryogenesis (Nozaki *et al.*, 1999) and the development of skin (Tarutani *et al.*, 1997) as shown by studies with gene knockout mice, indicating that GPI-anchored proteins can have a critical role in cell-to-cell interactions. In humans, somatic mutation of *PIG-A*, an X-linked gene involved in GPI biosynthesis, in hematopoietic stem cells causes a clonal hematopoietic disease known as paroxysmal nocturnal hemoglobinuria (PNH; Takeda *et al.*, 1993).

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Abbreviations used: GPI, glycosylphosphatidylinositol; GPI-GnT, GPI-*N*-acetylglucosaminyltransferase; GlcNAc, *N*-acetylglucosamine; GlcN, glucosamine; PI, phosphatidylinositol; IRES, internal ribosome entry site; DAF, decay accelerating factor.

GPI is synthesized in the endoplasmic reticulum (ER) from phosphatidylinositol (PI) through multiple steps and becomes attached to proteins bearing the C-terminal GPI attachment signal sequence (Kinoshita and Inoue, 2000). Biosynthesis of GPI is initiated by the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to generate *N*-acetylglucosaminyl-PI (GlcNAc-PI). This reaction is mediated by GPI-*N*-acetylglucosaminyltransferase (GPI-GnT). In mammalian cells, this enzyme consists of at least six proteins, PIG-A, PIG-H, PIG-C, GPI1, PIG-P, and DPM2 (Watanabe *et al.*, 2000). In *Saccharomyces cerevisiae*, GPI-GnT has been reported to have five components, Gpi1p, Gpi2p, Gpi3p, Gpi15p, and Eri1p. Such a complex structure is unusual for glycosyltransferases and suggests regulation of the biosynthetic pathway at this step. It has been reported that GPI-GnT is associated with, and negatively regulated by, Ras2p in *S. cerevisiae* (Sobering *et al.*, 2004). Whether an association between GPI-GnT and small GTPases such as Ras2p exists in other organisms is yet to be determined.

PIG-A is homologous to a bacterial GnT involved in lipopolysaccharide biosynthesis and to many other glycosyltransferases and is most likely to be a catalytic component (Kinoshita *et al.*, 1997; Kostova *et al.*, 2000). Cells with mutations in PIG-A, PIG-H, PIG-C, or PIG-P are completely deficient in the surface expression of GPI-anchored proteins, indicating that these proteins are essential for GPI-GnT activity. The GPI1-knockout cell expresses a small amount of GPI-anchored proteins but its GPI-GnT activity is below the detectable level. GPI1 is important for the stable formation of a complex of PIG-A, PIG-H, and PIG-C (Hong *et al.*, 1999). DPM2 is one of the components of Dol-P-Man synthase, generating mannosyl donor for GPI (Maeda *et al.*, 1998) and is also a component of GPI-GnT. DPM2 is not essential for GnT but its presence enhances GnT activity (Watanabe *et al.*, 2000).

It has been reported that the Burkitt lymphoma-derived Daudi cell line is defective in the surface expression of the cellular prion protein because of a lack of GPI anchor formation (Morelon *et al.*, 2001). Here we report cloning of *PIG-Y*, the seventh component of GPI-GnT, and that Daudi cells are defective in GPI-GnT because of a defect in PIG-Y.

MATERIALS AND METHODS

Cells, Culture Conditions, and Transfection

Daudi cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium supplemented with 10% FCS. Human B-lymphoblastoid JY25 and JY5 cells (Hollander *et al.*, 1988), GPI1-knockout F9 cells, and HeLa cells were cultured in DMEM supplemented with 10% FCS. Daudi cells were transfected by electroporation at 350 V and 250 μF in a Gene Pulser (Bio-Rad, Hercules, CA) using 15–20 μg plasmid DNA. Transfectants were selected and maintained in the culture medium supplemented with 0.5 μg/ml puromycin and 600 μg/ml hygromycin B. JY5 and JY25 cells were electroporated at 250 V and 960 μF and then selected and maintained with 0.3 μg/ml puromycin and 400 μg/ml hygromycin B. F9 cells were electroporated at 270 V and 500 μF and then selected and maintained with 4 μg/ml blasticidin S and 500 μg/ml hygromycin B. HeLa cells were transiently transfected with Lipofectamin 2000 (Invitrogen, Carlsbad, CA).

Plasmids

Expression plasmids pMEEB-GST-PIG-A, pMEEB-GST-FLAG-PIG-A, pMEEB-GST-PIG-H, pMEEB-GST-PIG-C, pMEEB-GPI1-GST, pMEEB-GST-ALDH, pME-pyori-FLAG-PIG-P, pME-puro18sf+, pME-Br18sf+ (blasticidin resistance gene), and pMEEB-FLAG-GPI1 were described previously (Watanabe *et al.*, 2000). pMEEB-GST-PIG-P was generated from pMEEB-GST-PIG-A by replacing its *Sal*I-*Xba*I fragment bearing PIG-A with a *Sal*I-*Xba*I fragment bearing PIG-P. pCXN2-Flag-H-Ras-V12, -H-Ras-N17, and -R-Ras-V38-IE, and pCAP-Flag-K-Ras-V12, pCAP-FLAG-K-Ras-V12, and pIRM21-Flag-Rap1-V12 were gifts from Dr. M. Matsuda, Osaka University (Ohba *et al.*, 2000). PME-puro-oriP-Flag-H-Ras-V12, -H-Ras-N17, -R-Ras-V38-IE, -K-Ras-V12, and -Rap1-V12 were generated by inserting an *Eco*RI-*Not*I fragment bearing FLAG-Ras from each Ras plasmid to the *Eco*RI-*Not*I site of pME-puro and by replacing the *Bgl*II-*Sfi*I fragment of pME-puro with a *Bam*HI-*Sfi*I fragment bearing oriP from pMEEB-GST-PIG-A.

Purification of GPI-GnT Complex

GPI-GnT complex was affinity-purified from 3×10^9 cells of JY5 transfected with pMEEB-GST-FLAG-PIG-A as described previously (Watanabe *et al.*, 2000). Ninety percent of the purified sample was subjected to SDS-PAGE (16.5% Tris-tricin gel, Bio-Rad) and transferred to PVDF membrane. The N-terminal sequences of proteins revealed by staining were determined with a 494 sequencer (Applied Biosystems, Foster City, CA). Ten percent of the sample was subjected to SDS-PAGE/silver staining. To analyze formation of a complex containing PIG-A and other components in the absence of PIG-Y, Daudi cells transfected with pMEEB-GST-FLAG-PIG-A (10^7 cells) together with a vector bearing PIG-Y or a control protein, aldehyde dehydrogenase (ALDH) were used.

Assay for GPI-GnT

When we used cell lysate as an enzyme source, we preincubated cells (10^7) for 2 h with 5 μg/ml tunicamycin and prepared the lysate by hypotonic lysis and destruction with a Teflon homogenizer. When using purified GPI-GnT complex, we prepared glutathione-beads bearing protein complexes as described above. The cell lysate and the protein complex were incubated in 100 μl of a reaction mixture consisting of 2 μCi of UDP-6 [^{3}H]GlcNAc (American Radiolabeled Chemicals, St. Louis, MO), Bovine PI (100 μM; Sigma; only for protein complexes), 50 mM HEPES-NaOH (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothreitol, 0.2 μg/ml tunicamycin, and protease inhibitor cocktail, Complete EDTA free (Roche, Indianapolis, IN) for 2 h at 37°C. The reactions were terminated by adding 1 ml of chloroform:methanol (1:1) and the solutions were then dried. The lipids extracted by 1-butanol partitioning were separated by TLC and analyzed by an Image Analyzer BAS 1500 (Fuji Film, Tokyo, Japan).

Cloning Human PIG-Y cDNA, Genomic Sequences, and S. cerevisiae Eri1 cDNA

We searched the Expressed Sequence Tag (EST) database (National Center for Biotechnology Information, Bethesda, MD) using a tBLASTn program for sequences corresponding to the amino acid sequence of PIG-Y and found several ESTs. On the basis of their sequences, we synthesized the primers hPIGY-Uxho (5′-CACCTCGAGAGAATGTTTCTGTCTCTTCCTACGTT) and hPIGY-Lnot (5′-TAAAGCGGCCGCTCAATTATGCCTGAAGAGTTTAAT) and amplified the coding region by PCR from the human T lymphoma KT-3 cDNA library (a gift from Dr. S. Nagata, Osaka University; Itoh *et al.*, 1991). We subcloned the PCR products into the *Xho*I-*Not*I site of pME-puro to make pME-puro-PIG-Y. To make pME-puro-HA-PIG-Y and pME-puro-GST-PIG-Y, we replaced DPM2 sequence in pME-puro-HA-DPM2 and pME-puro-GST-DPM2 with PIG-Y sequence. To make pMEEB-HA-PIG-Y and pMEEB-FLAG-GST-PIG-Y, we replaced the *Sal*I-*Not*I fragment bearing PIG-H or GPI8 in pMEEB-HA-PIG-H or pMEEB-FLAG-GST-GPI8 with a *Sal*I-*Not*I fragment bearing PIG-Y from pME-puro-HA-PIG-Y. For cotransfection studies, pME-oriP-HA-PIG-Y was generated by replacing the *Bgl*II-*Sfi*I fragment of pME-puro-HA-PIG-Y with a *Bam*HI-*Sfi*I fragment bearing oriP from pMEEB-GST-PIG-A. We also inserted a *Xho*I-*Not*I fragment bearing HA-PIG-Y into pME-Br 18sf+ to make pME-Br-HA-PIG-Y. On the basis of the sequences found in the database, we synthesized primers Hind-PRL-PreYU (5′-CCCAAGCTTTATTGCGGGACGAACGGGAAGCCGGGAGCTCGGC) and hYgenom-XbaIL (5′-GCTCTAGAATAGGAAAGTGTCAACATGTTTATTGCT) and amplified the full-length PreY-PIG-Y cDNA (1316 base pairs) by PCR from the KT-3 cell cDNA library. The PCR fragment was cloned into *Hin*dIII-*Xba*I site of pRL-CMV (Promega, Madison, WI) to generate pRL-CMV-KT3. We used primers Hind-PRL-PreYU and PreYL (5′-CTAGCGCTGCTCCACTTCTTCTTGCTT) to amplify the genomic sequence spanning PreY and PIG-Y from Jurkat cells. The PCR fragment digested with *Hin*dIII-*Bam*HI was triple ligated with *Bam*HI-*Xba*I fragment from pRL-CMV-KT3 and *Hin*dIII-*Xba*I digested pRL-CMV to generate pRL-CMV-gPIG-Y. This construct was used for RT-PCR analysis (see below). To generate pRL-CMV-PreYF-PIG-YF, we did sequential PCR using the KT3 cells cDNA library as a template as follows. With the primers Hind-PRL-PreYU and FLAG-PreYL, we obtained fragment 1; with the primers FLAG-PreYU (5′-GACTACAAGGACGACGATGACAAATAGTTCATAATTTAAAAAAATTAA) and FLAG-YL (5′-TCATTTGTCATCGTCGTCCTTGTAGTCATTATGCCTGAAGAGTTTAATA), we obtained fragment 2; and with the primers FLAG-YU (5′-GACTACAAGGACGACGATGACAAATGATGCAACTAGAGTCAATATGCT) and hYgenom-XbaIL, we obtained fragment 3. We combined these three fragments by PCR one by one, and inserted the digested PCR fragment into the *Hin*dIII-*Xba*I site of pRL-CMV. To make variants a and b of the PreY start site, we amplified sequences containing modified sites using primers Hind-PRL-PreYU and PreY-MM-PstL (5′-GCCTGCAGCGTGCTCCACTCATCATGGTGGCAGCCGGAGACCAGGCCTC) for variant a, and primers Hind-PRL-PreYU and PreY-X-PstL (5′-AGCCTGCAGCGTGCTCCACTCAGCAAGGTCTGGCAGCCGGAGACCAGGC) for variant b, and replaced the *Hin*dIII-*Pst*I fragment of pRL-CMV-PreYF-PIG-YF with the digested PCR fragments.

On the basis of the published Eri1 sequence, we synthesized primers yPIGY-Usal (5′-CACGTCGACAGACCACGTGACCAAGGCTTTTTGGTT) and yPIGY-Lnot (5′-TAAAGCGGCCGCTTATTCATCTTTACTACTTGCGAA) and amplified the coding region by PCR from *S. cerevisiae* genomic DNA. The digested PCR fragment was inserted in place of the *Sal*I-*Not*I fragment bearing PIG-Y into pMEEB-HA-PIG-Y.

FACS Analysis

Cells were stained for CD59 (biotinylated 5H8), CD55 (biotinylated IA10), and CD48 (phycoerythrin [PE]-conjugated; PharMingen, San Diego, CA). Isotype control and secondary reagents used were biotinylated IgG1 and PE-streptavidin (Biomeda, Foster City, CA). Some cells were stained after PI-PLC (Molecular Probes, Eugene, OR) treatment for 1 h (1U/ml) to remove GPI-anchored proteins. Stained cells were analyzed in a FACS caliber (Becton Dickinson, Mountain View, CA).

Membrane Topology of PIG-Y Protein

CHO cells were transiently transfected with pME-pyori-GST-PIG-Y and cultured on glass coverslips for 2 d. Fixation, membrane permeabilization, and staining were done as previously described (Maeda *et al.*, 2001). Slides were mounted in Moviol and studied under a confocal microscope (Carl Zeiss, Thornwood, NY).

RT-PCR Analysis

For RT-PCR of PIG-Y in Daudi cells, we used primers hPIG-Y-Uxho and hPIG-Y-Lnot. For analysis of Daudi cells transfected with genomic PIG-Y sequence, we isolated polyA RNA after DNase treatment of total RNA and used primers PRL-U (5′-CAGATCACTAGAAGCTTTATTGCGG) corresponding to the vector's CMV promoter and hYgenom-XbaIL corresponding to the 3′ end of the PIG-Y-coding exon.

Analysis of Protein Interactions

To analyze the interaction of PIG-Y with other components of GPI-GnT, we cotransfected 15 μg of pME oriP-HA-PIG-Y together with 15 μg of pMEEB-GST-PIG-A, -GPI1, -PIG-H, -PIG-C, -PIG-P, or -ALDH into JY5 and JY25 cells. Cells (10^7) were solubilized in lysis buffer (1% digitonin, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and protease inhibitor cocktail-Complete EDTA free; Roche). After removal of insoluble materials by centrifugation at 10,000 $\times$ *g* for 5 min and at 100,000 $\times$ *g* for 1 h, the soluble fraction was mixed with glutathione beads and agitated for 1 h. The beads were collected and super-

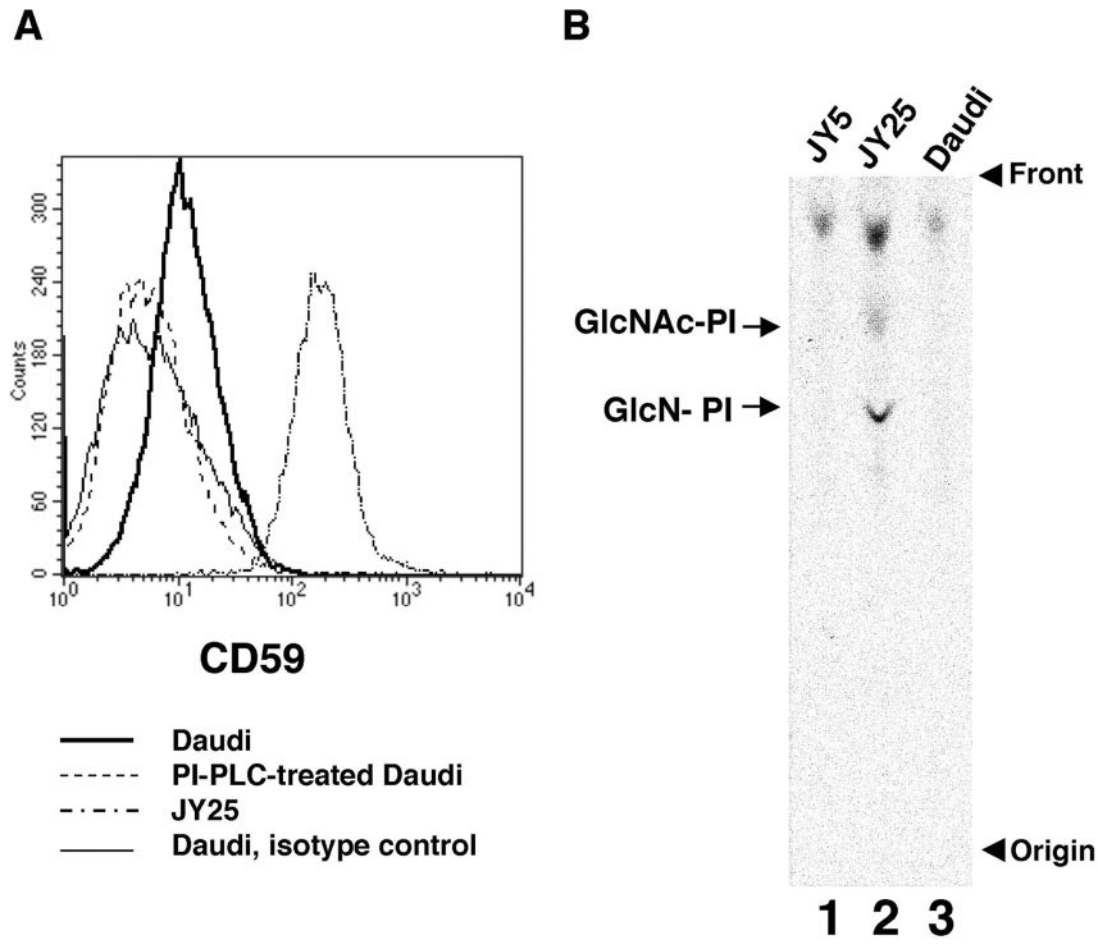

Figure 1. (A) FACS analysis of CD59. Thick line, Daudi cells; broken line, PI-PLC treated Daudi Cells; dotted line, JY25 cells as a wild-type control; thin line, isotype-matched negative control staining of Daudi cells. (B) In vitro assay for the early steps in GPI biosynthesis. Cell lysates were incubated with radiolabeled UDP-GlcNAc. The lipids were extracted and analyzed by TLC. Identities of spots are indicated on the left. Lane 1, mutant JY5 cells; lane 2, wild-type JY25 cells; lane 3, Daudi cells.

natants mixed with anti-HA beads (Roche) and agitated for 1 h. Both bead samples were washed with lysis buffer, eluted with 2× sample buffer, and analyzed by SDS-PAGE and Western blotting, using goat anti-GST antibody (Pharmacia, Piscataway, NJ) plus horseradish peroxidase (HRP)-conjugated proteinG (Bio-Rad) or mouse anti-HA (Sigma) plus sheep anti-mouse-HRP (Amersham, Indianapolis, IN). To analyze the interaction of PIG-Y with PIG-A in GPI1 knockout F9 cells, we permanently cotransfected pME-Br-HA-PIG-Y and pMEEB-GST-PIG-A together with or without pMEEB-FLAG-GPI1. Samples of 2×10^7 cells were analyzed as described above. To analyze GPI-GnT complex within Daudi cells, we cotransfected 15 μg each of pMEEB-GST-FLAG-PIG-A, pMEEB-GST-PIG-H, -GPI1, -PIG-C, and -PIG-P together with or without pME-oriP-HA-PIGY. Samples of 10^7 cells were analyzed as described above. We used M2 anti-FLAG-beads (Eastman Kodak, Rochester, NY) to capture FLAG-tagged proteins and for detection, we used anti-FLAG antibody M2 (Sigma) plus sheep anti-mouse-HRP (Amersham).

Rabbit Polyclonal Antibody against Human PreY

We immunized rabbits with affinity-purified, amino-terminally His-tagged human PreY. To purify the antibody from the antisera with an antigen column, we expressed human PreY as a maltose-binding protein fusion protein and coupled it to HiTrap *N*-hydroxysuccinimide-activated HP columns (Amersham).

RESULTS

Analysis of GPI Anchor Biosynthesis in Daudi Cells

Daudi cells are severely defective in the surface expression of GPI anchored proteins, such as CD59 (Figure 1A). PI-PLC removed the partially expressed proteins, indicating that they were GPI-anchored. To determine the defective biosynthetic step in Daudi cells, we measured the activities of the early GPI-anchor biosynthetic enzymes (Figure 1B). Lysates of wild-type B-lymphoblastoid cells, JY25, generated the first and second intermediates, GlcNAc-PI and GlcN-PI (lane 2). In contrast, Daudi cell lysates were defective in the synthesis of GlcNAc-PI (lane 3), like the lysates of PIG-A-defective JY5 cells (lane 1). Transfection with each of the six known components of GPI-GnT did not normalize Daudi cells, indicating that this mutant represents a new gene involved in GPI-GnT activity.

Cloning PIG-Y, a New Component of GPI-GnT

Expression cloning using Daudi mutant cells was tried several times without success, most probably owing to low transfection efficiency. To identify the new component of GPI-GnT, we isolated the GPI-GnT complex and searched for a new protein. We transfected JY5 cells with cDNA encoding PIG-A double-tagged with FLAG and glutathione *S*-transferase (GST), and isolated GPI-GnT complex by two-step affinity purification. Analysis by SDS-PAGE and silver staining of the complex demonstrated seven specific bands that included the tagged PIG-A. Among the smaller sized

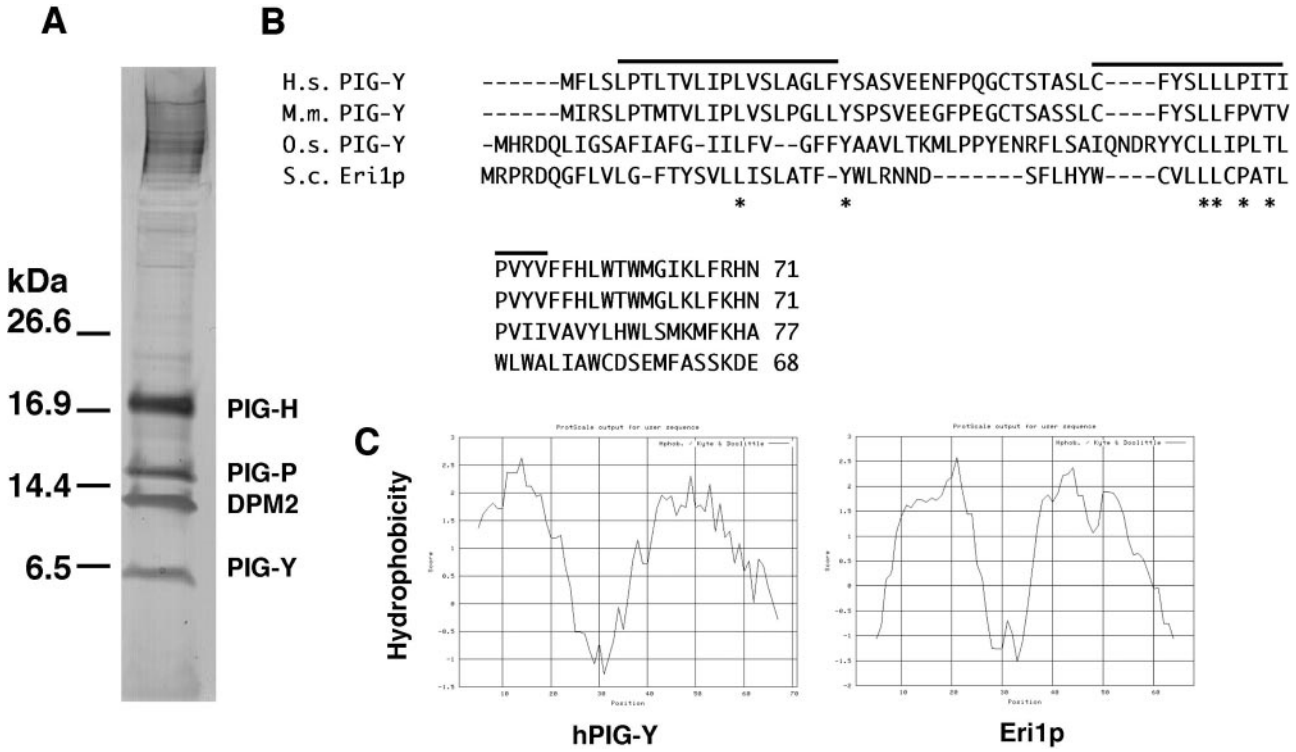

Figure 2. (A) Purification of GPI-GnT complexes. GST-FLAG-PIG-A was isolated by two-step affinity purification from the digitonin lysates of 5×10^7 cells of JY5 transfectant. Samples of the purified proteins were analyzed by SDS-PAGE (16.5% gel) and silver staining. (B) Alignment of amino acid sequences of human (H. s.) PIG-Y and its homologues of mouse (M. s.), rice (O. s.), and yeast (S. c.). Two putative transmembrane regions in human PIG-Y are overlined. *Amino acid conserved in all four sequences. (C) Hydropathy profiles of human PIG-Y and *S. cerevisiae* Eri1p (Kyte and Doolittle, 1982).

proteins in particular, the new band was clearly seen when the samples were developed in 16.5% gel (Figure 2A). We determined the N-terminal sequence using a sample derived from 10^9 cells and obtained the sequence MFLSLPTLTV. We searched databases with this sequence, found a full sequence and cloned a cDNA that encoded a 71-amino acid protein, which we termed *PIG-Y* (for phosphatidylinositol-glycan-class Y; Figure 2B; the DDBJ/EMBL/GenBank accession number of human *PIG-Y* cDNA is AB206972). PIG-Y was a hydrophobic protein consisting of two putative transmembrane domains predicted by the TMpred program (Hofmann and Stoffel, 1993). In databases, mouse and rice homologues of PIG-Y were identified, which exhibited 79 and 25% amino acid identity with human PIG-Y, respectively.

Recently, it was reported that *S. cerevisiae*'s gene termed *ERI1* (Sobering *et al.*, 2003) is involved in the first step in GPI biosynthesis (Sobering *et al.*, 2004). The Eri1p sequence was not hit with an intensive homology search, such as PSI-BLAST, with human PIG-Y sequence. However, the hydropathy profile of Eri1p was quite similar to that of PIG-Y and the amino acid identity was 22% (Figure 2, B and C). Therefore, Eri1p can probably be regarded as the yeast homolog of human PIG-Y.

To determine the membrane orientation of the N-terminus of PIG-Y, we prepared PIG-Y tagged with GST at the N-terminus, confirmed its functional activity by complementation of Daudi cells (see next section for complementation of Daudi cells with PIG-Y) and then transiently expressed it in CHO cells. After the selective permeabilization of the plasma membrane alone by 0.0005% digitonin (Figure 3, a–c) or permeabilization of both the plasma membrane and the ER membrane by 0.1% Triton X-100 (Figure 3, d–f), we stained the transfected CHO cells for a GST tag, BiP an authentic ER luminal protein and Hsc70 a cytosolic protein. The GST at the N-terminus showed staining after permeabilization of the plasma membrane alone, indicating that the N-terminus was cytoplasmically oriented (Figure 3b). Considering this together with the hydrophobicity profile (Figure 2C), it appeared that the C-terminus also faced the cytoplasmic side.

PIG-Y Restored GPI-GnT Activity in Daudi Cells

To determine whether Daudi cells are defective in PIG-Y, we transfected *PIG-Y* cDNA and analyzed the surface expression of GPI anchored proteins and GPI-GnT activity in vitro. *PIG-Y* cDNA restored the surface expression of CD59, DAF, and CD48 (Figure 4A), but an empty vector did not. GPI-GnT activity was also restored (Figure 4B, lane 2). It was also found that Daudi cells had no PIG-Y transcript, as assessed by RT-PCR (Figure 4C, lane 1). From these results we concluded that *PIG-Y* is the gene responsible for defective GPI expression in Daudi cells. Yeast Eri1p was not functional in Daudi cells (Figure 4A).

A Single Transcript Encodes Two Proteins: PreY and PIG-Y

According to the database, *PIG-Y* mRNA consists of 1316 bases, excluding polyA, and *PIG-Y* translation starts at the 517th base. In the region 5′ to *PIG-Y*, there was another coding region (hypothetical protein MGC14156, GenBank), which we termed *PreY* (Figure 5A). Analysis of the genomic sequence demonstrated that a 1316-base mRNA was derived

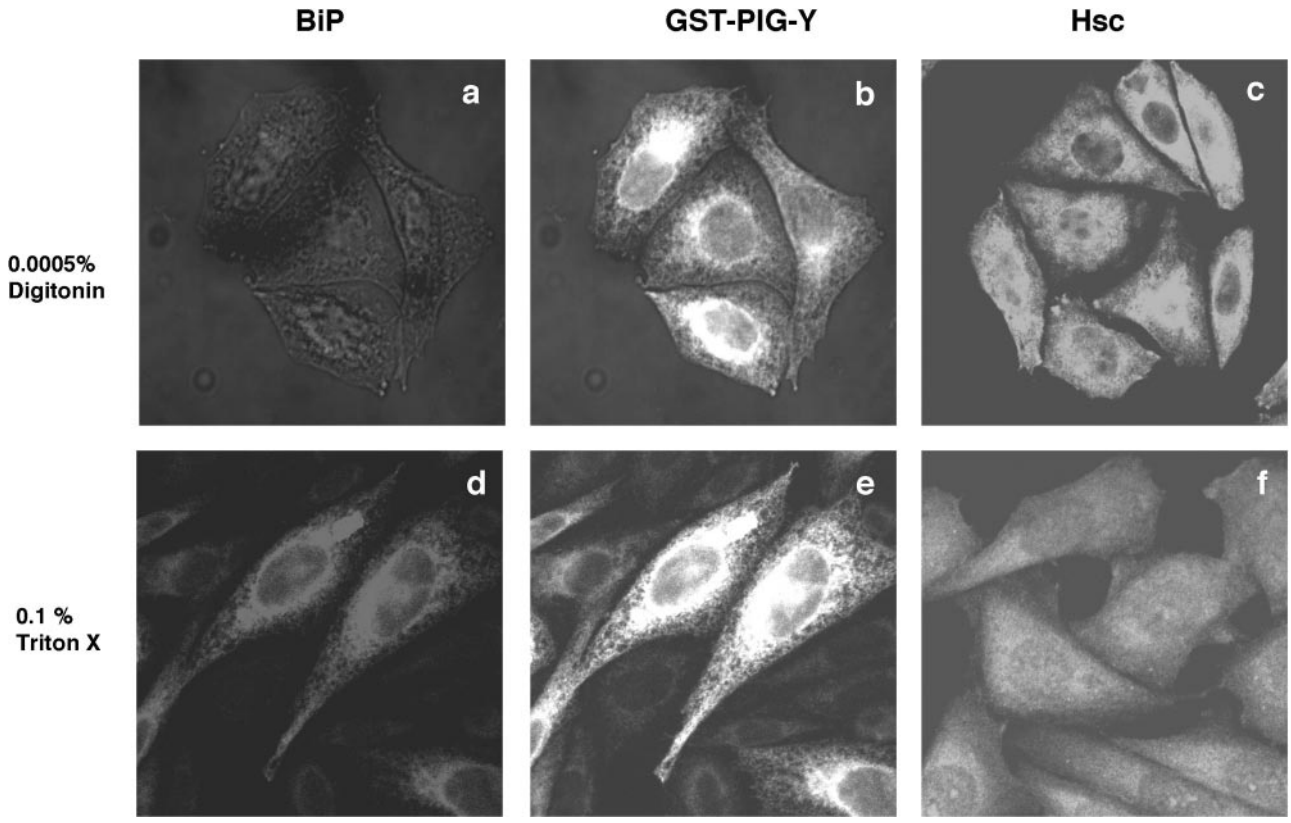

Figure 3. Membrane orientation of the N-terminus of PIG-Y. Human PIG-Y tagged at N-terminus (GST-PIG-Y) was expressed in CHO cells. After selective permeabilization of the plasma membrane (a–c) or permeabilization of both the plasma membrane and the ER membrane (d–f), cells were stained for tagged PIG-Y by anti-GST antibody (b and e), an endogenous ER luminal protein BiP (a and d), and a cytosolic protein Hsc70 (c and f).

from two exons separated by a 1468-base intron. PreY was encoded in the 3′ part of exon 1 and the 5′ part of exon 2 (Figure 5A). PreY and PIG-Y were separated by the intervening sequence of 101 bases.

Using a database search and both Northern blotting (Figure 5B) and RT-PCR (Figure 5C) analyses, we failed to find a shorter message containing the PIG-Y sequence. This indicated that PIG-Y is translated from the 1316-base mRNA. To examine how these two proteins are translated from one message, we considered two hypotheses. One is that there could be an internal ribosome entry site (IRES) between two open reading frames; the other is that PIG-Y could be separately initiated by leaky scanning of PreY initiation codon by ribosome (Kozak, 2002). To test the former, we made reporter constructs, in which two types of luciferase sequences were tandemly linked with intervening sequences of various lengths derived from 5′UTR of *PIG-Y*. As a positive control, we used IRES from Encephalomyocarditis virus (Jang *et al.*, 1988). The sequence between PreY and PIG-Y had no IRES activity (unpublished data). To test the latter hypothesis, we modified the 1316-base pair cDNA in three ways: 1) only the FLAG sequences were introduced at the C-termini of PreY and PIG-Y so that expression levels could be assessed by Western blotting (normal construct); 2) a few bases around the initiation codon of PreY were changed so that the initiation codon strictly matched Kozak's rule (Kozak, 1987; variant a); and 3) the initiation codon of PreY was destroyed by one base change (variant b; Figure 6A). We transfected these constructs into HeLa cells and assessed the expression of FLAG-tagged PreY and PIG-Y by Western blotting (Figure 6B). The normal construct produced both PreY and PIG-Y (lane 1). Variant a produced only PreY (lane 2), whereas variant b produced only PIG-Y (lane 3). It appeared, therefore, that the scanning ribosomal complex sometimes passed through the initiation codon of PreY and initiated translation at the downstream AUG start codon for PIG-Y in the normal construct. Optimization of the upstream PreY initiation codon resulted in the lack of PIG-Y synthesis. From these results, we concluded that the second of our hypotheses was true.

The function of PreY is not known. PreY, predicted to be a cytoplasmic soluble protein, had homologues in a wide range of species, and was characterized by a domain named DUF343 of unknown function (Bateman *et al.*, 2004; Figure 6A). It is clear that PreY is not required for the biosynthesis of GPI, because transfection of PIG-Y sequence alone restored the surface expression of GPI-anchored proteins on Daudi cells (Figure 4A), which are defective in both PreY and PIG-Y at the mRNA level (unpublished data). We prepared rabbit polyclonal antibodies against human PreY and tested expression of PreY in HeLa and Daudi cells. We detected PreY in HeLa but not Daudi cells (unpublished data).

Six Other Components of GPI-GnT Can Make a Complex without PIG-Y

To examine the function of PIG-Y within the GPI-GnT complex, we analyzed the formation of the complex without PIG-Y. GST-FLAG-PIG-A and four other FLAG-tagged com-

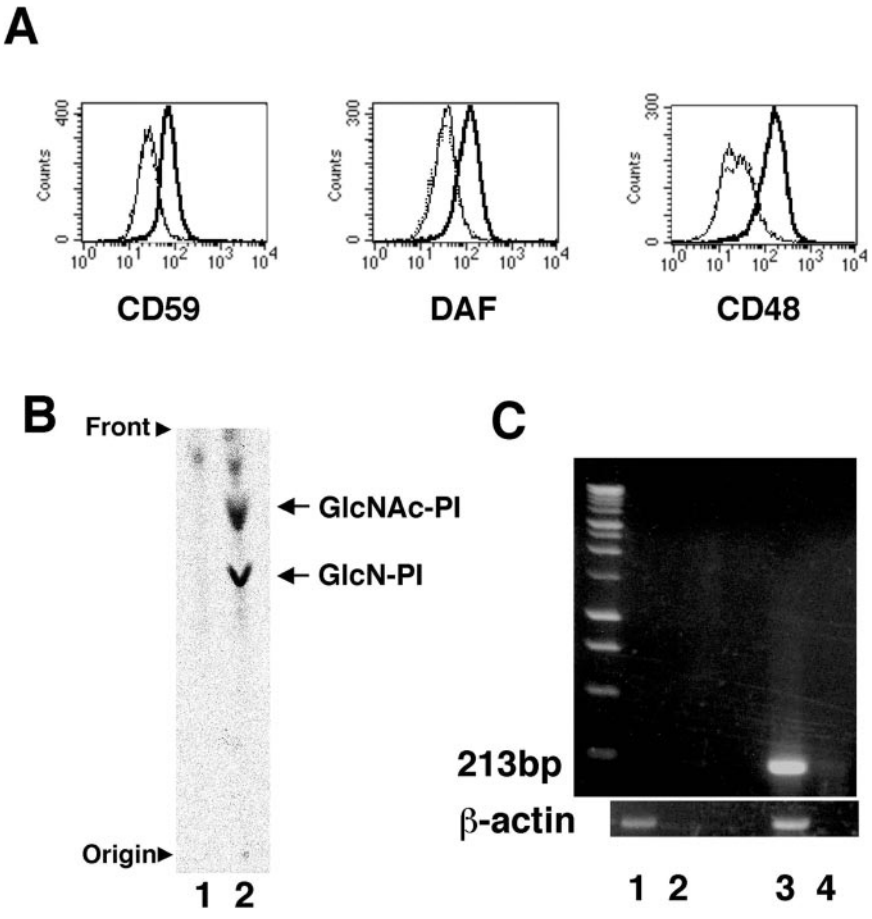

Figure 4. (A) FACS analysis of Daudi cells transfected with PIG-Y cDNA (thick lines), a control vector (thin lines), or ERI1 cDNA (dotted lines). Cells were stained by anti-CD59, anti-DAF, or anti-CD48 antibodies. (B) In vitro assay for the early steps in GPI biosynthesis. Lane 1, Daudi cells transfected with a control vector; lane 2, Daudi cells transfected with PIG-Y cDNA. (C) RT-PCR analysis of PIG-Y in Daudi cells and JY25 cells. Samples of RNA from Daudi (lanes 1 and 2) and JY25 (lanes 3 and 4) cells were incubated in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of reverse transcriptase. PCRs were done for the amplification of the coding region of human PIG-Y (top panel) and β-actin (bottom panel).

ponents were coexpressed with or without HA-PIG-Y in Daudi cells. Associations between PIG-A and the four other proteins in the presence or absence of PIG-Y were assessed by coprecipitation (Figure 7A). When PIG-A was precipitated with glutathione beads, GPI1, PIG-C, PIG-H, and PIG-P were coprecipitated similarly, whether with (lane 1) or without (lane 2) PIG-Y, indicating that the complex of five proteins was formed normally without PIG-Y. This was further confirmed when we analyzed PIG-A-containing complexes isolated by two-step affinity purification from Daudi cells transfected with GST-FLAG-PIG-A (Figure 7B). The silver-stained bands of the five components were equally visible with (lane 1) or without (lane 2) PIG-Y. We then analyzed the GPI-GnT activity of the purified complexes (Figure 7C). The complex without PIG-Y did not have detectable GPI-GnT activity (lane 2), whereas the one with PIG-Y generated GlcNAc-PI from UDP-GlcNAc and PI (lane 1), indicating that PIG-Y is critically important for GPI-GnT activity.

PIG-Y Associates Directly with PIG-A

To determine the component with which PIG-Y directly associates, we pairwisely cotransfected *HA-PIG-Y* with each of the GST-tagged other components into JY5 and JY25 cells and analyzed coprecipitation of PIG-Y with the cotransfected partner component. In PIG-A-deficient JY5 cells, PIG-Y was not coprecipitated with any other components (Figure 8A, lanes 2–6). When PIG-A was transfected, PIG-Y was coprecipitated with PIG-A (lane 1). In wild-type JY25 cells, PIG-Y was coprecipitated with each of all other components (lanes 8–11), but the amount of coprecipitated PIG-Y was much higher when PIG-A was overexpressed (lane 7). These results suggested that PIG-Y associated directly with PIG-A and indirectly with other components through the GnT complex. To investigate the direct association of PIG-Y and PIG-A further, we cotransfected HA-PIG-Y and GST-PIG-A into GPI1-knockout F9 cells, in which the association of PIG-A with PIG-C and PIG-H is inefficient because of a lack of stabilizer component GPI1 (Hong *et al.*, 1999). Under these conditions, HA-PIG-Y was efficiently coprecipitated with GST-PIG-A (Figure 8B, lane 1). Taken together, the above results indicate that PIG-Y directly associates with PIG-A.

PIG-Y Had No Association with Ras Family Members

There is a report that Eri1p, the PIG-Y homolog of *S. cerevisiae*, was associated with the active form of Ras2p in a cAMP-dependent manner and inhibited downstream signaling. It was also reported that a yeast mutant defective in Eri1p exhibited a hyper Ras phenotype, which was suppressed by Ras2 deletion. In addition, Ras2 overexpression significantly inhibited GPI-GnT activity in yeast, suggesting that Ras2p negatively regulates GPI biosynthesis (Sobering *et al.*, 2003, 2004). To examine whether this is also true in mammalian cells, we stably cotransfected HA-tagged PIG-Y and FLAG-tagged active forms of human Ras family proteins (H-ras, K-ras, R-ras, and Rap1) into Daudi cells and tested whether PIG-Y is coprecipitated with Ras proteins. We also analyzed in vitro the GPI-GnT activity of the transfectants. There was no detectable association between PIG-Y

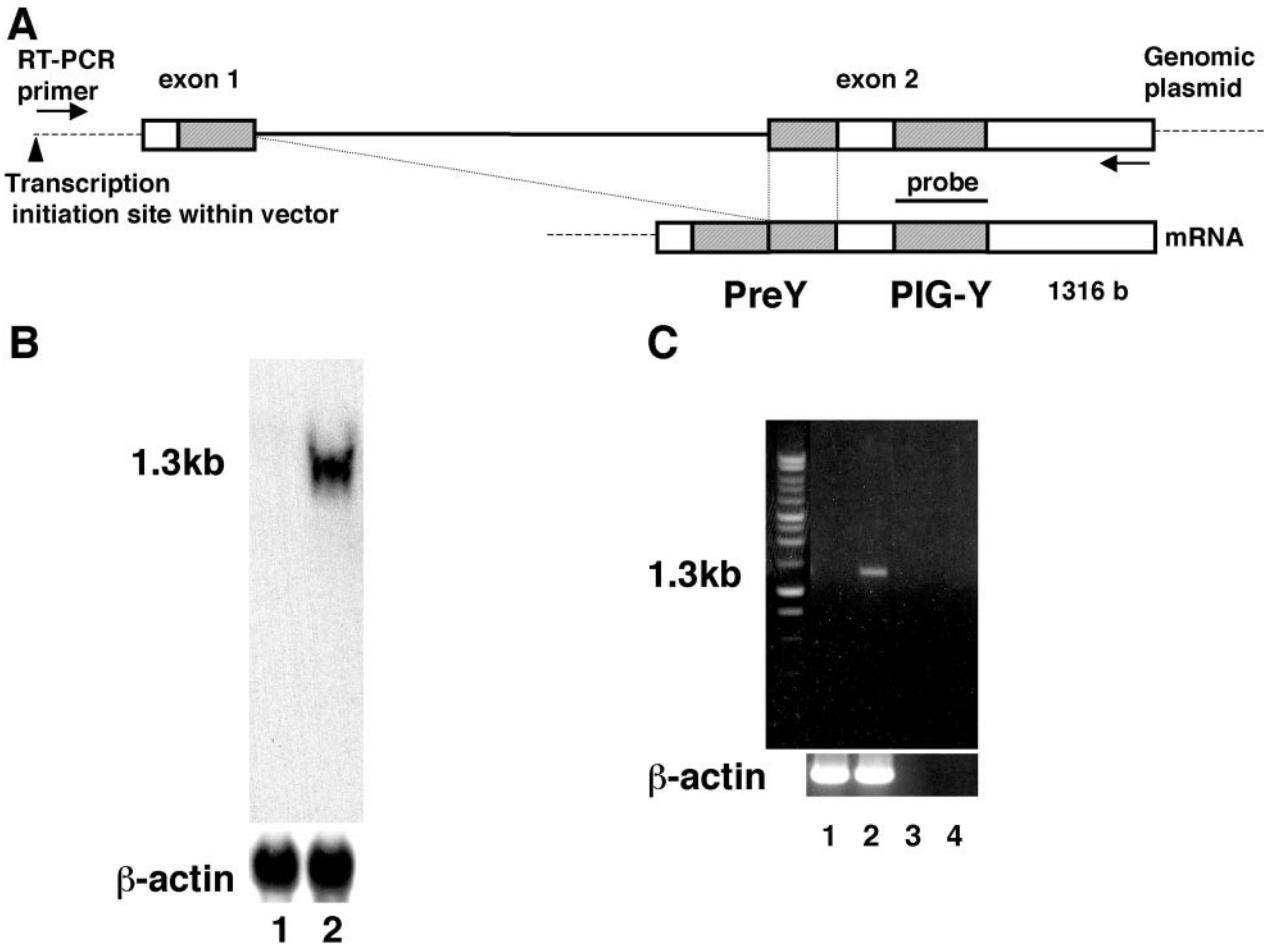

Figure 5. One gene encodes PreY and PIG-Y. (A) Plasmid bearing genomic PreY-PIG-Y sequence (top) and PreY-PIG-Y mRNA (bottom). The probe for northern blotting and primers for RT-PCR are shown. Boxes, exons; shaded areas, coding regions; open areas, noncoding regions; bar, intron; broken bars, plasmid regions. (B) Northern blot analysis of PIG-Y. Total RNAs from Daudi (lane 1) and HeLa (lane 2) cells were blotted against human PIG-Y cDNA probe (top panel). The same membrane was rehybridized with β-actin cDNA (bottom panel). (C) RT-PCR analysis of PIG-Y in Daudi cells restored with PIG-Y genomic sequence shown in A (lanes 2 and 4) or nontransfectant (lanes 1 and 3). Samples of Poly(A) RNA were incubated in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of reverse transcriptase. PCRs were done for the amplification of human full PIG-Y transcript (top panel) and β-actin (bottom panel).

and any of Ras proteins (unpublished data). Overexpression of active forms of the Ras family of proteins did not affect GPI-GnT activity (Figure 9). We also tested whether PIG-Y deficient Daudi cells exhibited the hyper Ras phenotype. Based on ERK phosphorylation and Raf-GST pulldown assays, there was no significant difference in Ras signaling between PIG-Y-deficient and -rescued cells (unpublished data). These results suggested that in mammalian cells, the GPI-GnT complex had no physical or functional association with the Ras signaling pathways.

DISCUSSION

PIG-Y, the Seventh Component of GPI-GnT Complex

In the present study, we found that human GPI-GnT contains a seventh component, termed PIG-Y, and that the severely decreased surface expression of GPI-anchored proteins on Burkitt lymphoma Daudi cells is due to a lack of PIG-Y. PIG-Y homologues are present in other mammalian species and plants, and Eri1p, a component of *S. cerevisiae* GPI-GnT, is very probably the functional homolog of PIG-Y. Therefore, PIG-Y/Eri1p can be regarded as a general component of GPI-GnT.

We demonstrated that PIG-Y is directly associated with PIG-A, the catalytic component of GPI-GnT. PIG-Y is a hydrophobic, 71-amino acid protein that has two transmembrane domains, with its N- and C-termini localized in the cytoplasm. The second transmembrane domain and the C-terminal cytoplasmic region are relatively well conserved among species and possibly form a region involved in the association with PIG-A.

A complex of the other enzyme components can be formed normally without PIG-Y but it lacks GPI-GnT activity in vitro, indicating that the association of PIG-Y with PIG-A in the complex is critical for the enzyme to become active. One possible mechanism is that the association with PIG-Y may cause a conformational change of the complex into its active form. The other possibility is that PIG-Y is involved in the recruitment of one of the two substrates. We assumed that if the latter possibility was true, a high concentration of PI or UDP-GlcNAc might overcome the lack of PIG-Y and lead to detectable enzyme activity of PIG-Y-less

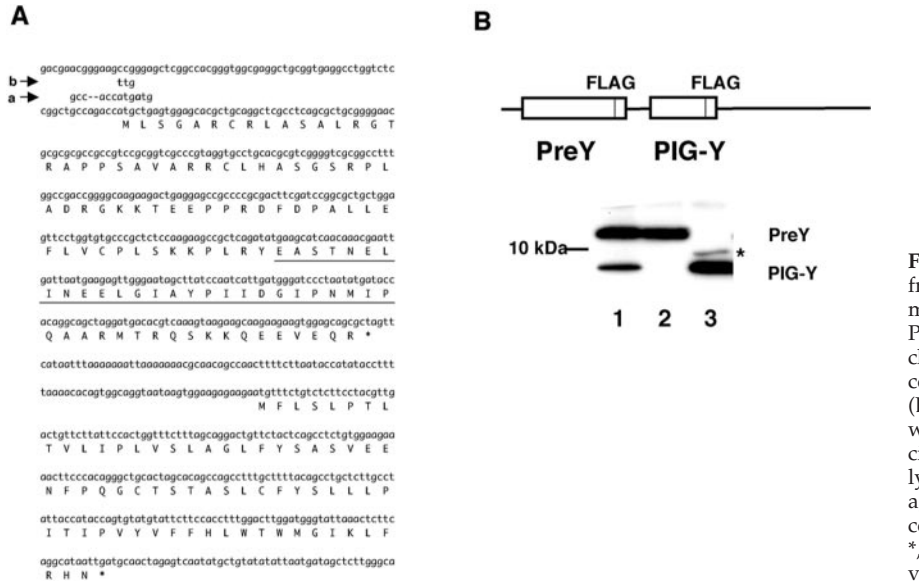

Figure 6. PreY and PIG-Y are translated from one long mRNA. (A) Sequences of human cDNA of PreY-PIG-Y, and PreY and PIG-Y proteins. Mutations in two variants to change stringency of the initiation codon according to Kozak's rule are shown (a and b). (B) The PreY-PIG-Y expression vector shown was transfected into HeLa cells. Immunoprecipitates with anti-FLAG beads were analyzed by SDS-PAGE/Western blotting against anti-FLAG antibodies. Lane 1, normal construct; lane 2, variant a; lane 3, variant b. *, a band of unknown origin seen only with variant b.

complex. The addition of 100 mM PI, known to enhance GPI-GnT eightfold (Watanabe *et al.*, 1998), to the PIG-Y-less complex did not lead to detectable enzyme activity, and culturing Daudi cells in high glucosamine medium, known to increase intracellular UDP-GlcNAc (Marshall *et al.*, 2004), did not lead to the expression of GPI-GnT activity (our unpublished results), indicating that the latter possibility seems less likely.

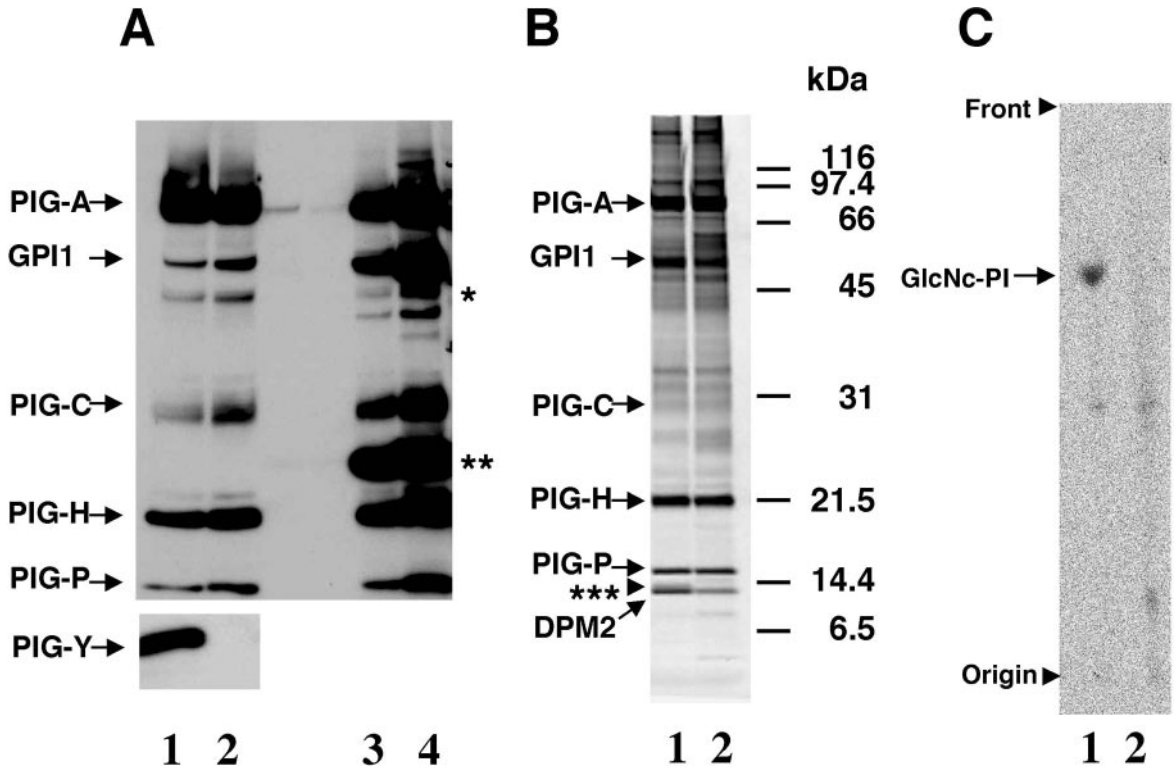

Figure 7. GPI-GnT complex is formed without PIG-Y. (A) GST-FLAG-PIG-A and the five FLAG-tagged GPI-GnT components were cotransfected with (lanes 1 and 3) or without HA-PIGY (lanes 2 and 4) into Daudi cells. Precipitates pull downed with glutathione beads (lanes 1 and 2) and second precipitates by anti-FLAG beads (lanes 3 and 4) were analyzed by SDS-PAGE/Western blotting against anti-FLAG (top panel) and anti-HA (bottom panel) antibodies. *Nonspecific band; **immunoglobulin light chain. (B and C) Protein complexes containing GST-FLAG-PIG-A were isolated by two-step affinity purification from digitonin lysates of 5×10^7 cells of Daudi cells transfected with GST-FLAG-PIG-A, and HA-PIG-Y (lane 1) or the empty vector (lane 2). Samples of purified proteins were used for SDS-PAGE and silver staining (B) or in vitro GPI-GnT assay (C). ***The bands of HA PIG-Y and DPM2 were overlapped each other (B, lane 1).

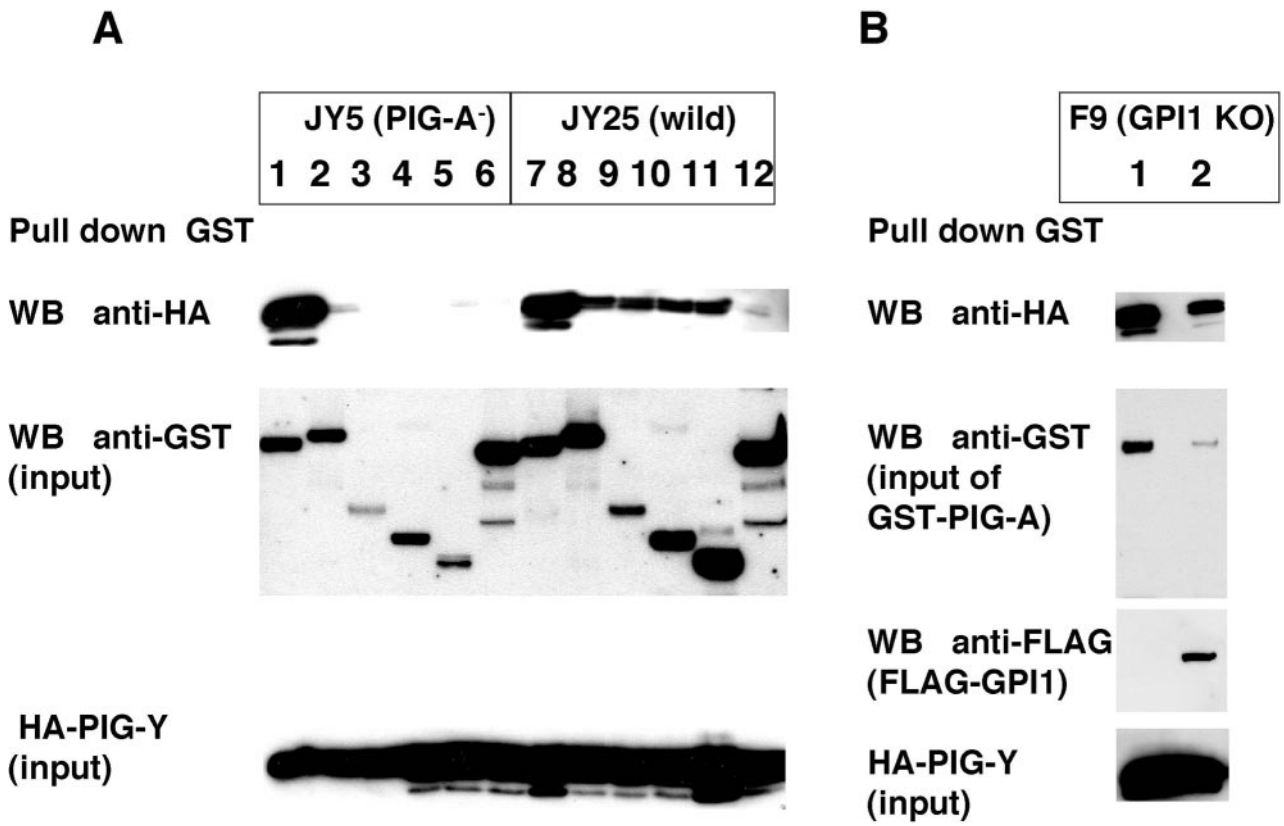

Figure 8. PIG-Y directly binds to PIG-A. (A) HA-PIG-Y was cotransfected with GST-PIG-A (lanes 1 and 7), GST-GPI1 (lanes 2 and 8), GST-PIG-C (lanes 3 and 9), GST-PIG-H (lanes 4 and 10), GST-PIG-P (lanes 5 and 11), or GST-ALDH, as a negative control (lanes 6 and 12) into JY5 (lanes 1–6) and JY25 (lanes 7–12) cells. Precipitates with glutathione beads (top and middle panels) and supernatant further precipitated with anti-HA beads (bottom panel) were analyzed by SDS-PAGE/Western blotting against anti-HA (top and bottom panels) and anti-GST (middle panel) antibodies. (B) HA-PIG-Y was cotransfected with GST-PIG-A into GPI1 knockout F9 cells not restored (lane 1) or restored with FLAG-GPI1 (lane 2). Precipitates with glutathione beads (top two panels) and supernatant further precipitated with anti-HA beads (bottom panel) were analyzed by SDS-PAGE/Western blotting against anti-HA (top and bottom panels), anti-GST (upper middle panel), and anti-FLAG (lower middle panel) antibodies.

It is reasonable that GPI biosynthesis is regulated at the initial reaction step. The unusually complex nature of GPI-GnT as a glycosyltransferase is very likely to be relevant to regulation. These results suggest that GPI-GnT activity might increase with an increase of PIG-Y expression and hence, GPI-GnT activity might be regulated by PIG-Y.

A Single Transcript Encodes Two Proteins: PreY and PIG-Y

We found that PIG-Y is translated from a 1316-base mRNA, which also contains an upstream PreY coding sequence. PreY has homologues in a wide range of species and also has a domain termed DUF343, with an unknown function but widely present in a range of organisms from bacteria to mammals. We made a rabbit polyclonal antibody against human PreY and detected endogenously expressed PreY in HeLa cells. Therefore, two proteins must be translated from one mRNA. Translations of several mammalian proteins involved in gene expressions or stress response, like apoptosis (Vagner *et al.*, 2001; Stoneley and Willis, 2004), are known to be regulated by IRES activity. However, no IRES activity was found in the sequence between PreY and PIG-Y. According to Kozak's rule, −3 adenine in the initiation codon of *PreY* matched the rule, whereas +4 cytosine did not, indicating that the AUG initiation codon for PreY is not optimal. Optimization of the initiation codon for PreY resulted in a loss of PIG-Y synthesis (Figure 6), consistent with a leaky scanning mechanism (Kozak, 2002). The initiation site of PIG-Y resides more than 400 nt downstream and there are two in-frame optimal AUG codons and one out-frame optimal AUG codon within the *PreY* coding sequence. To our knowledge, such a long distance scanning has not been reported for cellular mRNAs (Kozak, 2002).

GPI-anchor biosynthesis is essential for embryogenesis as shown in Piga knockout mice (Nozaki *et al.*, 1999). PIG-Y would be essential for embryogenesis because a lack of PIG-Y severely affects GPI-anchor biosynthesis. Translation of such an important gene must be assured and in this regard it seems unusual that it is dependent on the leaky scanning mechanism. We believe that there must be some regulation of their translation. Production of two proteins via leaky scanning would appear to be difficult to regulate, but there are reports that it might be possible for dual initiation via leaky scanning to be subject to regulation (Spotts *et al.*, 1997).

No Functional Association of GPI-GnT with Ras Family Members in Human System, a Major Difference from the S. cerevisiae System

Eri1p has been reported to be a component of the GPI-GnT complex in *S. cerevisiae* and deletion of *ERI1* caused the

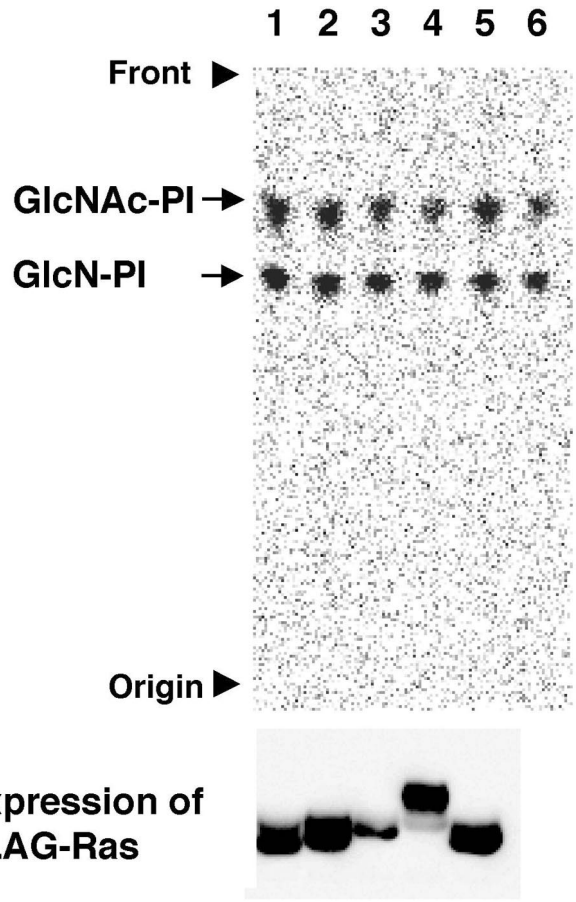

Figure 9. Overexpression of active forms of Ras-family proteins did not affect GPI-GnT activity. HA-PIG-Y was permanently cotransfected with FLAG-H-RasV (lane 1), FLAG-H-RasN (lane 2), FLAG-K-RasV (lane 3), FLAG-R-RasV (lane 4), FLAG-Rap1V (lane 5), or only HA-PIG-Y was transfected (lane 6) into Daudi cells. Top, GPI-GnT activity in cell lysate from the transfectants; bottom, precipitates with anti-FLAG beads analyzed by SDS-PAGE/Western blotting against anti-FLAG antibodies, showing expression level of Ras family proteins.

hyper Ras phenotype (Sobering *et al.*, 2003, 2004). It was shown that Ras2p bound to the GPI-GnT complex in a GTP and effector loop-dependent manner and, through this interaction, these enzymes inhibited each other's function. Indeed, Ras2p overexpression significantly inhibited GPI-GnT activity in yeast (Sobering *et al.*, 2004). As Eri1p is probably a human PIG-Y homolog, we examined whether there is physical and/or functional association between PIG-Y/GPI-GnT and Ras family members in mammalian cells. We stably cotransfected HA-tagged PIG-Y and FLAG-tagged active or dominant negative forms of human Ras proteins (H-Ras, K-Ras, R-Ras, and Rap1) into Daudi cells and tested whether PIG-Y was coprecipitated with Ras proteins and also analyzed in vitro GPI-GnT activity of the transfectants. There was no detectable association between PIG-Y and any of the Ras proteins (unpublished data), and overexpression of either form of Ras family proteins did not affect GPI-GnT activity (Figure 9). In addition, PIG-Y deficient Daudi cells failed to display the hyper Ras phenotype. These results indicated that in mammalian cells, the GPI-GnT complex had no physiological association with Ras signaling pathways.

In yeast, other GPI biosynthesis mutants, such as Gaa1, also showed hyper Ras phenotype in the presence of complete GPI-GnT complex (Sobering *et al.*, 2004). In contrast to the situation in mammalian cells where GPI biosynthesis is not essential at a cell level, in yeast GPI biosynthesis is essential for survival, and thus only the mutant with a leaky phenotype can be analyzed (Leidich *et al.*, 1994). Proteins derived from GPI-anchored proteins are major yeast cell wall components, so when GPI biosynthesis is affected, the cell wall becomes fragile, inducing signals to plasma membrane localizing molecules to activate downstream molecules (Toh-e and Oguchi, 2001). It seems that the hyper Ras phenotype is not the result of failure to bind GPI-GnT complex but the result of a cell wall defect caused by GPI-anchored protein deficiency, the mechanism of which may be quite complex (Heinisch *et al.*, 1999). In yeast, Ras2p is activated by nutritional starvation, which causes morphological changes of the yeast into a filamentous form (Gancedo, 2001). It has been proposed that Ras2p regulates changes in cell wall architecture through inhibition of the GPI-GnT, which would have the dual effect of decreasing GPI-anchor proteins at the cell surface and increasing chitin by diverting the pool of UDP-GlcNAc from GPI-anchor production to chitin biosynthesis (Sobering *et al.*, 2004). On this point, together with the fact that Ras/cAMP signaling is specific to the yeast system (Toda *et al.*, 1985), the mechanisms in yeast and humans are very different.

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REFERENCES

Bateman, A. *et al.* (2004). The Pfam protein families database. Nucleic Acids Res. *32*, D138–D141.

Gancedo, J. M. (2001). Control of pseudohyphae formation in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. *25*, 107–123.

Heinisch, J. J., Lorberg, A., Schmitz, H. P., and Jacoby, J. J. (1999). The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae*. Mol. Microbiol. *32*, 671–680.

Hofmann, K., and Stoffel, W. (1993). TMbase—a database of membrane spanning proteins segments. Biol. Chem. Hoppe-Seyler *347*, 166.

Hollander, N., Selvaraj, P., and Springer, T. A. (1988). Biosynthesis and function of LFA-3 in human mutant cells deficient in phosphatidylinositol-anchored proteins. J. Immunol. *141*, 4283–4290.

Hong, Y., Ohishi, K., Watanabe, R., Endo, Y., Maeda, Y., and Kinoshita, T. (1999). GPI1 stabilizes an enzyme essential in the first step of glycosylphosphatidylinositol biosynthesis. J. Biol. Chem. *274*, 18582–18588.

Ikezawa, H. (2002). Glycosylphosphatidylinositol (GPI)-anchored proteins. Biol. Pharm. Bull. *25*, 409–417.

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.

Jang, S. K., Krausslich, H. G., Nicklin, M. J., Duke, G. M., Palmenberg, A. C., and Wimmer, E. (1988). A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. *62*, 2636–2643.

Kinoshita, T., and Inoue, N. (2000). Dissecting and manipulating the pathway for glycosylphosphatidylinositol-anchor biosynthesis. Curr. Opin. Chem. Biol. *4*, 632–638.

Kinoshita,T.,Inoue,N.,andTakeda,J.(1995).Defectiveglycosylphosphatidylinositol anchor synthesis and paroxysmal nocturnal hemoglobinuria. Adv. Immunol. *60*, 57–103.

Kinoshita, T., Ohishi, K., and Takeda, J. (1997). GPI-anchor synthesis in mammalian cells: genes, their products, and a deficiency. J. Biochem. *122*, 251–257.

Kostova, Z., Rancour, D. M., Menon, A. K., and Orlean, P. (2000). Photoaffinity labelling with P^3-(4-azidoanilido)uridine 5′-triphosphate identifies Gpi3p as the UDP-GlcNAc-binding subunit of the enzyme that catalyses formation of GlcNAc-phosphatidylinositol, the first glycolipid intermediate in glycosylphosphatidylinositol synthesis. Biochem. J. *350*, 815–822.

Kozak, M. (1987). An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. *15*, 8125–8148.

Kozak, M. (2002). Pushing the limits of the scanning mechanism for initiation of translation. Gene *299*, 1–34.

Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. *157*, 105–132.

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.

Maeda, Y., Tomita, S., Watanabe, R., Ohishi, K., and Kinoshita, T. (1998). DPM2 regulates biosynthesis of dolichol phosphate-mannose in mammalian cells: correct subcellular localization and stabilization of DPM1, and binding of dolichol phosphate. EMBO J. *17*, 4920–4929.

Maeda, Y., Watanabe, R., Harris, C. L., Hong, Y., Ohishi, K., Kinoshita, K., and Kinoshita, T. (2001). PIG-M transfers the first mannose to glycosylphosphatidylinositol on the lumenal side of the ER. EMBO J. *20*, 250–261.

Marshall, S., Nadeau, O., and Yamasaki, K. (2004). Dynamic actions of glucose and glucosamine on hexosamine biosynthesis in isolated adipocytes: differential effects on glucosamine 6-phosphate, UDP-*N*-acetylglucosamine, and ATP levels. J. Biol. Chem. *279*, 35313–35319.

McConville, M. J., and Menon, A. K. (2000). Recent developments in the cell biology and biochemistry of glycosylphosphatidylinositol lipids. Mol. Membr. Biol. *17*, 1–16.

Morelon, E., Dodelet, V., Lavery, P., Cashman, N. R., and Loertscher, R. (2001). The failure of Daudi cells to express the cellular prion protein is caused by a lack of glycosyl-phosphatidylinositol anchor formation. Immunology *102*, 242–247.

Nozaki, M., Ohishi, K., Yamada, N., Kinoshita, T., Nagy, A., and Takeda, J. (1999). Developmental abnormalities of glycosylphosphatidylinositol-anchor-deficient embryos revealed by Cre/loxP system. Lab. Invest. *79*, 293–299.

Ohba, Y., Mochizuki, N., Matsuo, K., Yamashita, S., Nakaya, M., Hashimoto, Y., Hamaguchi, M., Kurata, T., Nagashima, K., and Matsuda, M. (2000). Rap2 as a slowly responding molecular switch in the Rap1 signaling cascade. Mol. Cell. Biol. *20*, 6074–6083.

Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. Nat. Rev. Mol. Cell. Biol. *1*, 31–39.

Sobering, A. K., Romeo, M. J., Vay, H. A., and Levin, D. E. (2003). A novel Ras inhibitor, Eri1, engages yeast Ras at the endoplasmic reticulum. Mol. Cell. Biol. *23*, 4983–4990.

Sobering, A. K., Watanabe, R., Romeo, M. J., Yan, B. C., Specht, C. A., Orlean, P., Riezman, H., and Levin, D. E. (2004). Yeast Ras regulates the complex that catalyzes the first step in GPI-anchor biosynthesis at the ER. Cell *117*, 637–648.

Spotts, G. D., Patel, S. V., Xiao, Q., and Hann, S. R. (1997). Identification of downstream-initiated c-Myc proteins which are dominant-negative inhibitors of transactivation by full-length c-Myc proteins. Mol. Cell. Biol. *17*, 1459–1468.

Stoneley, M., and Willis, A. E. (2004). Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. Oncogene *23*, 3200–3207.

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.

Tarutani, M., Itami, S., Okabe, M., Ikawa, M., Tezuka, T., Yoshikawa, K., Kinoshita, T., and Takeda, J. (1997). Tissue specific knock-out of the mouse Pig-a gene reveals important roles for GPI-anchored proteins in skin development. Proc. Natl. Acad. Sci. USA *94*, 7400–7405.

Tiede, A., Bastisch, I., Schubert, J., Orlean, P., and Schmidt, R. E. (1999). Biosynthesis of glycosylphosphatidylinositols in mammalian and unicellular microbes. Biol. Chem. *380*, 503–523.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985). In yeast, RAS proteins are controlling elements of adenylate cyclase. Cell *40*, 27–36.

Toh-e, A., and Oguchi, T. (2001). Defects in glycosylphosphatidylinositol (GPI) anchor synthesis activate Hog1 kinase and confer copper-resistance in *Saccharomyces cerevisiae*. Genes Genet. Syst. *76*, 393–410.

Vagner, S., Galy, B., and Pyronnet, S. (2001). Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. EMBO Rep. *2*, 893–898.

Watanabe, R., Inoue, N., Westfall, B., Taron, C. H., Orlean, P., Takeda, J., and Kinoshita, T. (1998). The first step of glycosylphosphatidylinositol biosynthesis is mediated by a complex of PIG-A, PIG-H, PIG-C and GPI1. EMBO J. *17*, 877–885.

Watanabe, R., Murakami, Y., Marmor, M. D., Inoue, N., Maeda, Y., Hino, J., Kangawa, K., Julius, M., and Kinoshita, T. (2000). Initial enzyme for glycosylphosphatidylinositol biosynthesis requires PIG-P and is regulated by DPM2. EMBO J. *19*, 4402–4411.