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-acetylg glucosaminyltransferase (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 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 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-acetylg glucosaminyltransferase (GPI-GnT). In mammalian cells, this enzyme consists of at least six proteins, PIG-A, PIG-H, PIG-C, PIG-P, 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 enzyme 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. PIG1 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 Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Plasmids

Expression plasmids pMEEB-GST-PIG-A, pMEEB-GST-FLAG-PIG-A, pMEEB-GST-PIG-H, pMEEB-GST-PIG-P, pMEEB-GST-PIG-C, pMEEB-GST-PIG-ALDH, pME-pyori-FLAG-PIG-P, pME-puro18sf, pME-Br18sf (baculovirus 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 SalI-XbaI fragment bearing PIG-A with a SalI-XbaI fragment bearing PIG-P. pCXN2-Flag-H-Ras-V12, -H-Ras-N17, and -R-Ras-V12, and -P-CAP-Flag-K-Ras-V12, pCAP-Flag-K-Ras-V12, and p1MR2-Flag-Rap1-V12 were gifts from Dr. M. Matsuda, Osaka University (Obiha et al., 2000). PMU-puro-ori-Flag-H-Ras-V12, -H-Ras-N17, -R-Ras-V12, -K-Ras-V12, and -Kap-Ras-V12 were generated by inserting an EcoRI-NorI fragment bearing Flag-RAS from each Ras plasmid to the EcoRI-NorI site of pME-puro by replacing the Bgl II/SfiI fragment of pME-puro with a BamHI/SfiI fragment bearing oriP from pME-GST-P-PIG-A.

Purification of GPI-GnT Complex

GPI-GnT complex was affinity-purified from 3 x 10^11 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-tricine 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 GPI-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-[3H]GlcNAc (American Radio-labeled 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 MgCl2, 1 mM ATP, 15 μM dibucaine, 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 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 hPIG-Y-Uss (5'-CACCTCGAGGAGAAGTTTCTGCCTCCTCTCTAAT-3') and hPIG-Y-Lnot (5'-AAACCCGGGGCTGCATTTCTGCAGAAGTTTAAT-3') 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 Xhol-Not site of pME-puro to make pME-puro-PIG-Y. To make a PIG-Y-His-PG-LcRNA-FLAG-P1 and pME-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 Sall-NorI fragment bearing PIG-H or GFP11 in pME-puro-HA-PG or pMEEB-FLAG-GST-PIG1 with a Sall-NorI fragment bearing PIG-Y. For preparation of PIG-Y from pME-oriP-PIG-Y, we first cotransfection studied pME-oriP-HA-PIG-Y was generated by replacing the Bgl II/SfiI fragment of pME-puro-HA-PIG-Y with a BamHI/SfiI fragment bearing oriP from pME-GST-P-PIG-A. We also inserted a XhoI-NorI fragment bearing HA-PIG-Y into pME-pbr B18. After electroporation the pME-pbr-HA-PIG-Y. On the basis of the sequence, we synthesized primers Hind-PRL-PreYU (5'-CAGTACAAAGGCGCAAGTACAATAATGTTCAATCATGCTCGTCTCTTCAGTTTGCTCGGCGGAGACCAGGCCG) and hYgenom-ExbII (5'-GCTCCTAGATAGGAAATGCTGACAATAGTTGAATGGTCTGC) and amplified the full-length PreY-PIG-Y cDNA (1516 base pairs) by PCR from the KT-3 cDNA library. The PCR fragment was cloned into HindIII-Xhol site of pRL-CMV (Promega, Madison, WI) to generate pRL-CMV-KT3. We used primers Hind-PRL-PreYU and PreY-ExbII (5'-CTAGCCGCTGCTACCACTTCTCTCTCTCTCTCTC) to amplify the genomic sequence spanning PreY-PIG-Y from Jurkat cells. The PCR fragment digested with HindIII-BamHI was triple ligated with BamHI-Xhol fragment from pRL-CMV-KT3 and HindIII-Xhol digested pRL-CMV to generate pRL-CMV-gpIG-Y. This construct was used for RT-PCR analysis (see below). To generate pRL-CMV-PreY-PIG-Y, we did sequential PCR using the KT-3 cells cDNA library as a template with as follows. With the primers Hind-PRL-PreYU and FLAG-PreY1, we obtained fragment 1; with the primers FLAG-PreYU (5'-GACTACAAAGGCGCAAGTACAATAATGTTCAATCATGCTCGTCTCTTCAGTTTGCTCGGCGGAGACCAGGCCG) and hYgenom-ExbII, we obtained fragment 2; and with the primers FLAG-PreYU (5'-GACTACAAAGGCGCAAGTACAATAATGTTCAATCATGCTCGTCTCTTCAGTTTGCTCGGCGGAGACCAGGCCG) for variant a, and primers Hind-PRL-PreYU and PreY-ExbII (5'-AGCCTGACGGCTGCACCAAGAATGTTCAATCATGCTCGTCTCTTCAGTTTGCTCGGCGGAGACCAGGCCG) for variant b, and replaced the HindIII-PsrI fragment of pRL-CMV-PreY-PIG-Y with the digested PCR fragment. On the basis of the published GPI-GnT-Q1 sequence (Nagata et al., 2000), we synthesized primers gPIG-Y-Uss (5'-CACCTCGACCAACGCACTGCACAGAACAGGGTTTGGTT-3') and gPIG-Y-Lnot (5'-AAACCCGGGGCTGCATTTCTGCAGAAGTTTAAT-3') and amplified the coding region by PCR from S. cerevisiae genomic DNA. The digested PCR fragment was inserted in place of the SalI-NorI fragment bearing PIG-Y into pME-pHA-PIG-Y.

FACS Analysis

Cells were stained for CD59 (biotinylated SH8), CD55 (biotinylated IA10), and CD4 (phycoerythrin (PE)-conjugated; PharMingen, San Diego, CA). Isotype control secondary reagents were used for background subtraction. 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-Uss 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'-CAGATCAGAAGGCACTGTTCAATCATGCTCGTCTCTTCAGTTTGCTCGGCGGAGACCAGGCCG) and amplified the coding region by PCR from S. cerevisiae genomic DNA. The digested PCR fragment was inserted in place of the SalI-NorI fragment bearing PIG-Y into pME-pHA-PIG-Y.
natants mixed with anti-HA beads (Roche) and agitated for 1 h. Both bead samples were washed with lysis buffer, eluted with 2 x 10^6 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 x 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, -PIG-H, -PIG-C, and -PIG-P together with or without pMEEB-FLAG+PIG-Y. 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...
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 hydrophyt 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.

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.
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-
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 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).
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 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).
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 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.

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 × 10⁷ 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).
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 adenosine 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
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 Er11p is probably a human PI(4,5)P2-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 transfecants. 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 data 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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