PIG-A and PIG-H, Which Participate in Glycosylphosphatidylinositol Anchor Biosynthesis, Form a Protein Complex in the Endoplasmic Reticulum

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Reika Watanabe†, Taroh Kinoshiba‡, Ryuichi Masaki§, Akitsugu Yamamoto§, Junji Takeda∥, and Norimitsu Inoue¶*

From the †Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565 and the ‡Department of Physiology, and Cell Biology Division of Liver Research Center, Kansai Medical University, Moriguchi, Osaka 570, Japan

Many eukaryotic cell surface proteins are bound to the membrane via a glycosylphosphatidylinositol (GPI) anchor. Assembly of the GPI anchor precursor is a sequential addition of components to phosphatidylinositol (PI) in the endoplasmic reticulum (ER). The first step is the transfer of N-acetylglucosamine (GlcNAc) to PI from UDP-GlcNAc to generate GlcNAc-PI. This simple process, however, is regulated by at least three genes because in both mammals and yeasts, there are three mutants of different complementation classes. To clarify this complexity, we analyzed the products of two cloned human genes, PIG-A and PIG-H. Here we demonstrate 1) that PIG-A is an ER transmembrane protein with a large cytoplasmic domain that has homology to a bacterial GlcNAc transferase and a small lumenal domain; 2) that PIG-H is a cytoplasmically oriented, ER-associated protein; and 3) that they form a protein complex. We also show that part of the small lumenal domain of PIG-A plays an essential functional role in targeting itself to the rough ER. Taken together with the cytoplasmic orientation of GlcNAc-PI, these results indicate that PIG-A and PIG-H are subunits of the GPI GlcNAc transferase that transfers GlcNAc to PI on the cytoplasmic side of the ER.

Many eukaryotic proteins are bound to the cell surface membrane by a glycosylphosphatidylinositol (GPI) anchor. Its common core structure consists of ethanolamine phosphate, three mannos, glucosamine, and inositol phospholipid (3, 4). The anchor is linked to the carboxyl terminus via an amide bond with ethanolamine. This linkage is formed by the transfer of a synthesized GPI anchor precursor to protein in the endoplasmic reticulum (ER) following the cleavage of a glycosylphosphatidylinositol (GPI) anchor. Many mutant mammalian cell lines (5, 6) and yeasts (7–9) that are deficient in biosynthesis or attachment of the GPI anchor have been established. They have been characterized biochemically and grouped into complementation classes.

The first reaction step is the transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to PI to form the first intermediate GlcNAc-PI. GlcNAc-PI is thought to face the cytoplasm because those associated with intact microsomes prepared from murine thymoma cells and trypanosomes are sensitive to PI-specific phospholipase C acting from outside (10, 11). Mammalian cells belonging to three complementation classes (A, C, and H) cannot synthesize the first intermediate, indicating that at least three genes are involved in this step (5). Human cDNAs that restore synthesis of the GPI anchor in class A (12) and H (13) mutants have been cloned. The genes were termed PIG-A and PIG-H (or PIG-F) for phosphatidylinositol glycan class A and class H, respectively. A gene for class C, PIG-C, has not been cloned. The PIG-A cDNA consists of 4568 base pairs and encodes a predicted protein of 484 amino acids (12). There is a hydrophobic segment near the carboxyl terminus that may be a transmembrane domain. The carboxyl-terminal portion of about 40 residues is hydrophilic. There is no amino-terminal hydrophobic signal sequence. A region spanning amino acids 304–395 has homology to the bacterial GlcNAc transferase, RfAK, which is involved in the synthesis of lipopolysaccharides, suggesting that PIG-A protein has a catalytic site for the transfer of GlcNAc (14). The PIG-H cDNA contains 1394 base pairs and encodes a predicted protein of 188 amino acids (13). There is no amino-terminal hydrophobic signal sequence. There is a hydrophobic region in the middle of the molecule, but whether or not it is a transmembrane domain remains unclear. No homology has been found to known DNA and protein sequences in the data bases.

There are also three complementation groups of yeast mutants in the first step: gpi1, gpi2, and gpi3 (7), and their genes have been cloned (15). GPI3 is identical to independently characterized SPT14 (15) and CWH6 (16) and is homologous to mammalian PIG-A (17). Neither GPI1 nor GPI2 is homologous to PIG-H (15), and there is no structural homologue of PIG-H in the entire genome of Saccharomyces cerevisiae. If GPI1 or GPI2 is homologous to PIG-C, then either four genes, GPI1, GPI2, GPI3, and PIG-H functional homologue in yeast, as well as PIG-A, PIG-H, PIG-C, and the GPI1 or GPI2 homologue in mammals, are involved in this step or different combinations of three genes are involved in yeast and mammals.

The nucleotide sequences of PIG-H, GPI1, and GPI2 have not
provided clues as to their functions, nevertheless, their gene products may form a protein complex with the presumably catalytic PIG-A/PIG3 gene product. In this study we demonstrated that PIG-A and PIG-H form a complex with a cytoplasmic orientation in the ER. This orientation is in agreement with the notion that the first step of GPI anchor synthesis occurs on the cytoplasmic side of the ER membrane.

MATERIALS AND METHODS

Construction and Expression of Tagged PIG-A and PIG-H—We used the vector pME18Sf (a gift from Dr. K. Maruyama) that contains SRα promoter to express tagged PIG-A and PIG-H in human and murine cell lines. To modify the vector for expression in human B-lymphoblastoid cell lines containing the human restriction enzymes HindIII and XmaI, the replication origin of Epstein-Barr virus, was obtained from pEB (12) and inserted into pME18Sf to generate pMEEB. For expression in murine thymoma cells, an Sfi1 fragment containing neo- mycin resistance gene derived from pcD2 was inserted into pME18Sf to generate pMNEe.

To fuse glutathione-S-transferase (GST) as a tag for the amino terminus, we fused PIG-H, and microsomal aldehyde dehydrogenase (masALDH), we amplified the GST gene from pGEX-4T2 (Pharmacia Biotech Inc.) by PCR using a 5′ primer (5′-CCCTCGAGGACCATGTC-CCCTTACTAGTTGA) containing Kozak’s consensus sequence (18) on the 5′ side of the start codon and a 3′ primer (5′-CCCTAGTCGACGATGACACCCGAACACGAT) containing a SalI site on the 3′ side of GST. The PCR products of 0.8 ml were digested with HindIII and SalI, and ligated with a SalI fragment of GST (Biomeda) and a 5′ part of PIG-H, or orvP, respectively, in 0.8 ml of Hepes-KOH at pH 7.6 containing 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 μg/ml aprotinin) using a tight pestle Dounce homogenizer and fractionated by differential centrifugation into cytoplasm, nuclei, and mitochondria plus membranes (29).

PIG-A and PI-GH mutants were disrupted in 2 ml of buffer C (10 mM HEPES-KOH at pH 7.6 containing 1 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 μg/ml aprotinin) using a tight pestle Dounce homogenizer and fractionated by differential centrifugation into cytoplasm, nuclei, and mitochondria plus membranes (29).

Immunofluorescence and Immunoelectron Microscopic Analyses of PIG-A and PIG-H—Immunofluorescence and immunoelectron microscopic analyses of PIG-A and PIG-H—Immunofluorescence and immunoelectron microscopic analyses of PIG-A and PIG-H (Biomeda) and studied under a fluorescence microscope (BX50-FLA; Olympus Corp.). They were mounted on glass slides with Gel/Mount (Biomedical) and studied under a fluorescence microscope (BX50-FLA; Olympus Corp.).

Three million COS-7 cells were transfected with GST-tagged PIG-A and PIG-H mutant by electroporation at 960 μF and 250 V. Two days later, transfectants were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Ultrathin cryosections were stained with goat anti-GST antibody and gold-conjugated anti-IgG successively, then studied under an electron microscope (21).

Localization of PIG-A and PIG-H—JY5 cells (1 × 10⁶) expressing GST-tagged PIG-A were disrupted in 2 ml of buffer C (10 mM HEPES-KOH at pH 7.6 containing 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 μg/ml aprotinin) using a tight pestle Dounce homogenizer and fractionated by differential centrifugation into cytoplasm, nuclei, and mitochondria plus membranes (29).

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The fractions were incubated with 1% Nonidet P-40 to solubilize membranes followed by centrifugation to eliminate insoluble materials. GST-tagged proteins were collected from the supernatants by adsorption to glutathione-Sepharose 4B (Pharmacia Biotech) at room temperature for 30 min or 4°C overnight, eluted from the beads with reducing SDS-PAGE sample buffer, and Western-blotted. The bands of GST-tagged proteins recognized by anti-GST antibodies were visualized by incubation with horseradish peroxidase-conjugated anti-IgG antibody (Organon Teknika N.V.) and detected by chlormium reactions (Renaissance, DuPont).

Determination of Membrane Orientation of PIG-A and PIG-H by Proteinase K Protection Assay—Microsomes were prepared from cells expressing PIG-A or PIG-H tagged by GST at either the amino or the carboxyl terminus. The ER-enriched microsomes prepared by means of sucrose density gradient centrifugation as described above were incubated with 200 μg/ml proteinase K at 4°C for 30 min. After inactivating the proteinase K by PMSF (4 mM), the microsomes were solubilized with 1% Nonidet P-40. The membranes were solubilized with Nonidet P-40 and incubated with proteinase K in parallel, to confirm the susceptibility of the substrate proteins to the enzyme. GST-tagged proteins were collected with glutathione-Sepharose and Western-blotted with anti-GST antibodies. PDI remaining unbound to glutathione-Sepharose was also Western-blotted with anti-PDI antibodies.

Affinity Precipitation of PIG-A and PIG-H Complex—GST-tagged PIG-A, PIG-H, or ALDH was transfected into JY5 cells. The transfected cells were selected in hygromycin B. They were then transfected with FLAG-tagged-PIG-A, FLAG-tagged-ALDH and PIG-H, or FLAG-tagged-PIG-A and -PIG-H, respectively. The efficiency of second transfections to the cells expressing PIG-H or ALDH were estimated to be nearly 90%. This was done by assessment of the surface expression of CD25, because transfaction of PIG-A should restore it on JY5 cells (12). The membranes were prepared from 3.5 × 10⁶ cells by a tight pestle. The cytoplasmic orientation of simian virus 40 tag and antigen (20) by electroperoration at 960 μF and 250 V. They were cultured on 14-mm diameter glass coverslips in 24-well plates for 2 days, washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 1 h. Thereafter, they were incubated with 5% bovine serum albumin in PBS for 1 h, then stained with goat anti-GST antibody (Pharmacia Biotech), fluorescein isothiocyanate-conjugated donkey anti-goat IgG antibody (Chemicon International, Inc.), rabbit anti-protein-disulfide isomerase (PDI) antibody (21), and rhodamine-conjugated donkey anti-rabbit IgG (Chemicon International, Inc.). They were mounted on glass slides with Gel/Mount (Biomedical) and studied under a fluorescent microscope (BX50-FLA; Olympus Corp.).

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we confirmed that GST-tagged proteins rather than free proteins liberated from them (28 kDa) were not detected (Fig. 2). So proteins of the predicted molecular mass, but free GST proteins (Fig. 1, a). The four GST-tagged proteins were detected as fusion proteins tagged at the amino and the carboxyl termini were termed GST-PIG-A and PIG-A-GST, respectively, and those of PIG-H fusion proteins were functional, because the deficient surface expression of GPI-anchored proteins of class A and class H mutant cells was fully complemented by transfected with PIG-A and PIG-H coding sequence. VD and TR are dipeptides connecting GST to the amino and carboxyl termini, respectively. Panel b, restoration of the surface expression of CD59 on class A mutant JY5 cells by transfection with GST-tagged PIG-A cDNA. Line 1, JY5 cells; line 2, GST-PIG-A-transfected JY5; line 3, PIG-A-GST-transfected JY5; line 4, wild-type cell line JY25. Panel c, restoration of the surface expression of Thy-1 on class H mutant S49 Thy-1^-h cells by transfection with GST-tagged PIG-H cDNA. Line 1, S49 Thy-1^-h cells; line 2, GST-PIG-H-transfected S49 Thy-1^-h cells; line 3, PIG-H-GST-transfected S49 Thy-1^-h cells; line 4, parental cell line S49.

Expression of PIG-A and PIG-H Tagged by GST at Either the Amino or the Carboxyl Terminus—To analyze the intracellular localization of PIG-A and PIG-H, we prepared expression plasmids of GST-tagged PIG-A and PIG-H and transfected them into cells. GST was fused to either the amino or the carboxyl terminus of PIG-A and of PIG-H. Fusion proteins of PIG-A tagged at the amino and the carboxyl termini were termed GST-PIG-A and PIG-A-GST, respectively, and those of PIG-H were similarly termed GST-PIG-H and PIG-H-GST, respectively (Fig. 1a).

All four fusion proteins were functional, because the deficient surface expression of GPI-anchored proteins of class A and class H mutant cells was fully complemented by transfection with PIG-A and PIG-H fusion plasmids, respectively (Fig. 1, b and c).

To examine whether the GST-tagged proteins rather than free proteins liberated from them are functional, we analyzed the intactness of GST-tagged proteins by Western blotting (Fig. 2). The four GST-tagged cell proteins were detected as fusion proteins of the predicted molecular mass, but free GST proteins liberated from them (28 kDa) were not detected (Fig. 2). So we confirmed that GST-tagged proteins rather than free proteins liberated from them were responsible for complementing activities.

**ER Localization of PIG-A and PIG-H**—As all intact PIG-A and PIG-H fusion proteins were functional, we examined their subcellular localization by indirect immunofluorescence staining using anti-GST antibodies. To facilitate determination of localization, we used adherent cells as recipients. HeLa cells transfected with the fusion constructs were double-stained with anti-GST and anti-PDI antibodies 2 days later. Both GST-PIG-A and PIG-A-GST were stained in a reticular manner in regions surrounding the nuclei, and they were colocalized with endogenous PDI, an ER marker (Fig. 3, panels b and c). Both GST-PIG-H and PIG-H-GST also showed similar staining profiles (Fig. 3, panels d and e). In contrast, GST itself did not show reticular staining, but it was widely distributed in the cytosol and the nuclei (Fig. 3, panel a). These results suggested that PIG-A and PIG-H are localized in the ER.

To confirm the ER localization of PIG-A and PIG-H, we fractionated cells expressing GST-tagged PIG-A and PIG-H and assessed them by Western blotting with anti-GST antibody. GST-PIG-A was expressed in human class A mutant B-lymphoblastoid JY5 cells, and GST-PIG-H was expressed in murine class H mutant thymoma S49 Thy-1^-h cells. Both GST-PIG-A (Fig. 4) and GST-PIG-H (data not shown) were found in the membrane fraction (lane 3) and detergent-soluble nuclei (lane 2) but not in the cytosol (lane 4). When the postnuclear supernatants were further fractionated by sucrose density gradient centrifugation, both fusion proteins were found with the ER enzyme markers, NADPH-cytochrome c reductase or Dol-P-Man synthase, whereas they were separated from the plasma membrane marker, alkaline phosphodiesterase I, and the Golgi marker, α-mannosidase II (Fig. 5). Therefore, we confirmed the ER localization of PIG-A and PIG-H.

Immunoelectron microscopy further supported the ER localization of PIG-A. To enable detection of PIG-A after fixation, we expressed GST-PIG-A in COS-7 cells at a very high level, then detected it with anti-GST antibody and gold-conjugated anti-IgG. Gold particles were distributed mostly on the cytoplasmic side of the rough ER and the outer membrane of nuclei (Fig. 6).

**Orientation of PIG-A and PIG-H in the ER Membrane**—The first intermediate of the GPI anchor biosynthesis, GlcNAc-P1, is oriented to the cytoplasm on the ER (10, 11). To examine whether or not the orientation of PIG-A and PIG-H on the ER are compatible with this, microsomes obtained from cells transfected with fusion constructs were assayed by means of proteinase K protection. Endogenous PDI, a luminal protein, was used as a marker of the intactness of the vesicles. After protease K digestion of the microsomes, the GST fusion proteins released by proteolysis and those protected and then detergent-
solubilized were collected by glutathione-Sepharose and Western-blotted against anti-GST antibody.

When microsomes bearing GST-PIG-A (Fig. 7a, lanes 1) were processed as described above, the band of GST-PIG-A (80 kDa, lane 1) disappeared and a dense band appeared at 27 kDa (lanes 2), whereas most PDI was protected, indicating intact vesicles (lane 2, lower panel). The 27-kDa peptide that was not degraded in the presence of Nonidet P-40 (lane 3) should be GST itself, because it was the same size as GST and because recombinant GST was resistant to proteinase K. The presence of some GST in the absence of proteinase K (lane 1) should be due to degradation of GST-PIG-A during sample preparation. Thus, the amino-terminal portion of PIG-A was accessed by the enzyme acting from the cytoplasmic side. This is in agreement with the results of the immunoelectron microscopy shown in Fig. 6, demonstrating that the GST portion of GST-PIG-A expressed in COS-7 cells is on the cytoplasmic side of the ER. In contrast, when microsomes bearing PIG-A-GST (Fig. 7a, lanes 4–6) were digested, a 35-kDa peptide was protected (lane 5). Since this peptide was degraded to 27-kDa GST in the presence of detergent (lane 6), it should have contained an 8-kDa peptide derived from the carboxyl terminus of PIG-A. Judging from this size, the hydrophobic segment and the carboxyl-terminal hydrophilic portion should have been protected. Therefore, PIG-A is a transmembrane protein with its large amino-terminal portion oriented to the cytoplasm and its small carboxyl-terminal portion oriented to the ER lumen.

Digestion of microsomal vesicles with proteinase K completely degraded both GST-PIG-H and PIG-H-GST (Fig. 7b, lanes 2 and 5), whereas major fraction of PDI was protected, indicating intact vesicles (Fig. 7b, lower panel). Detection of intact GST-PIG-H in this experiment was inefficient (lane 1), presumably due to inefficient capture by glutathione-Sepharose or inefficient binding of anti-GST antibody or both. Thus, both the amino and carboxyl termini of PIG-H were oriented to the cytoplasmic side.

Roles of the Short Carboxyl-terminal, Lumenal Domain of PIG-A—The cytoplasmic orientations of GlcNAc-PI, PIG-H,
and the large amino-terminal portion of PIG-A that contains a region homologous to a bacterial GlcNAc transferase are all strong evidence for the notion that the first step of GPI anchor biosynthesis occurs on the cytoplasmic side. To determine the functional roles of the carboxyl-terminal, luminal domain of PIG-A, we established four mutants (M1–M4) in which various lengths of the carboxyl-terminal portion were deleted (Fig. 8a). M1, which contained 415 amino-terminal residues, lacked the transmembrane and the entire luminal domain. M2, which contained 442 residues, bore the transmembrane domain but lacked the entire luminal domain. M3 consisted of 450 residues, having 23 lumenal residues next to the transmembrane domain. M4 consisted of 465 residues, having 8 lumenal residues immediately carboxyl-terminal to the transmembrane domain. Wetransfected them into class A mutant JY5 cells and determined the orientation mutants of PIG-A to determine whether or not the loss of functions was due to mislocation of the proteins. The fully active mutant M4 was absolutely membrane-bound (Fig. 9a) and distributed in ER-enriched fractions 3, 4, and 5 (Fig. 9b), coexisting with Dol-P-Man synthase (data not shown). The inactive mutant M2, which lacked the entire luminal domain, although it was mostly membrane-bound (Fig. 9a), was distributed in membrane fractions lighter than M4 (Fig. 9b). The mutant M1, which lacked the transmembrane and the luminal domains, was found in both the cytoplasm and the membrane (Fig. 9c). The partially active M3 was widely distributed in membrane fractions (Fig. 9b). Immunoelectron microscopic analysis of COS-7 cells expressing M2 demonstrated that it was not present in the Golgi apparatus but that it was associated with a membrane structure that may be the smooth ER (Fig. 10). Only a small amount of gold particles were bound to the rough ER and the outer membrane of the nucleus. These results suggested that the 23 luminal residues are necessary for targeting PIG-A to the rough ER, a membrane compartment

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Protein Complexes Containing PIG-A and PIG-H—The membrane orientation of PIG-A and PIG-H are in line with the notion that they are subunits of a protein complex that mediates the first reaction step. To test the possibility of complex, we coexpressed differentially tagged PIG-A and PIG-H and solubilized them with 1% digitonin. They were affinity-precipitated using glutathione-Sepharose, then Western-blotted against anti-FLAG antibodies and anti-GST antibodies. As shown in Fig. 11a, glutathione-Sepharose precipitated FLAG-tagged PIG-A together with GST-PIG-H from the digitonin extract of cells cotransfected with FLAG-PIG-A and GST-PIG-H (lane 1). Complexes were disrupted when the cells were solubilized with Nonidet P-40 (lane 4).

To confirm the specificity of the association of PIG-A with PIG-H, we analyzed whether msALDH, a nonrelevant ER membrane protein, associates with PIG-A or PIG-H. msALDH has molecular size similar to that of PIG-A and, like PIG-A, has a large amino-terminal portion in the cytoplasm and a transmembrane domain near its carboxyl terminus, hence the same topology as PIG-A (21). GST-tagged msALDH (GST-ALDH) was expressed in the ER of JY5 (data not shown). JY5 cells expressing GST-ALDH, FLAG-PIG-A, and FLAG-PIG-H were solubilized with digitonin and affinity-precipitated using glutathione-Sepharose, then Western-blotted against biotinylated anti-FLAG antibodies and anti-GST antibodies (Fig. 11b, lanes 3 and 4). A lack of coprecipitation of ALDH with PIG-A and PIG-H, lanes 1 and 3, glutathione-Sepharose precipitate (lane 1) and its supernatant (lane 3) from digitonin lysate of JY5 cells expressing GST-ALDH, FLAG-PIG-A, and FLAG-PIG-H (lane 1). Complexes were disrupted when the cells were solubilized with Nonidet P-40 (lane 4).
Glutathione-Sepharose precipitated neither FLAG-PIG-A nor FLAG-PIG-H together with GST-ALDH (lane 1), all the FLAG-PIG-A and FLAG-PIG-H remaining in the supernatants (lane 2). When JY5 cells expressing FLAG-ALDH, GST-PIG-A, and FLAG-PIG-H were similarly treated, glutathione-Sepharose precipitated FLAG-PIG-H but not FLAG-ALDH together with GST-PIG-A as shown in lanes 2 (precipitate) and 4 (supernatant). Thus, msALDH did not coprecipitate with PIG-A or PIG-H.

We finally tested whether the ER localization of PIG-A or PIG-H depends on the expression of the other. GST-PIG-H was normally localized in the ER when expressed in class A mutant JY5 cells (data not shown), which do not express PIG-A mRNA (12). GST-PIG-A was normally localized in the ER when expressed in class H mutant S49 Thy-1 h cells (data not shown). Therefore, PIG-A and PIG-H are expressed in the ER independently of each other.

**DISCUSSION**

In this study we showed that the products of two genes, PIG-A and PIG-H, which participate in the first step of GPI anchor biosynthesis, are ER membrane proteins that together form a protein complex. This suggests that these proteins are involved in an enzyme complex that mediates the transfer of GlcNAc to PI.

We determined the subcellular localization and orientation of PIG-A and PIG-H using GST as a tag and anti-GST antibody as a probe based on the observation that both PIG-A and PIG-H tagged by GST at either termini were functional (Fig. 1). PIG-A was an ER transmembrane protein consisting of a large amino-terminal domain of about 400 residues that resides on the cytoplasmic side of the ER, a transmembrane domain near the carboxyl terminus, and a small carboxyl-terminal domain within the ER lumen (Figs. 3–7). A hydrophathy profile of PIG-A indicated that there is no hydrophobic amino-terminal signal sequence for translocation through the ER membrane, that there is a hydrophobic region of 27 residues near the carboxyl terminus, and that the 42 carboxyl-terminal residues are hydrophilic. These characteristics are consistent with the orientation determined by the protease K protection assay. The overall domain organization of PIG-A is shared by many transmembrane proteins that have large cytoplasmic domains, such as cytochrome _b_ and msALDH (21, 25).

PIG-H was also localized to the ER (Figs. 3 and 5). Both its amino and carboxyl termini were on the cytoplasmic side of the ER (Fig. 7). Consistent with this, there is no hydrophobic amino-terminal signal sequence, nor is there a typical hydrophobic segment for a transmembrane domain in the PIG-H sequence. Therefore, most of PIG-H would be on the cytoplasmic side of the ER. Data base search did not reveal any significant homology between PIG-H and known sequences (13), so we have no information regarding the function of PIG-H.

The large amino-terminal domain of PIG-A is, as a whole, homologous to RfaK, a bacterial GlcNAc transferase of 381 amino acids (16.9% amino acid identity) (16). In particular, the region spanning amino acids 304–395 of PIG-A has a higher similarity to the stretch between amino acids 280 and 372 of RfaK (27% amino acid identity), which characterizes a family of glycosyltransferases that includes a number of bacterial, yeast, and plant proteins, such as RfaG, RfaB, KdtA, sucrose synthases, and glycogen synthases (14). Therefore, it is likely that the large cytoplasmic domain of PIG-A is a catalytic domain of a GlcNAc transferase that mediates the first reaction.

The small luminal domain of PIG-A was also important for function. The most carboxyl-terminal 19 residues were not essential, whereas the proximal 23 residues that are conserved in mouse and human PIG-A (14) were (Fig. 8). The non-functional mutant M2 that lacked the entire luminal domain was found in membrane fractions at a lower density than those in wild-type PIG-A (Fig. 9). It was located in the smooth ER by electron microscopy (Fig. 10). Therefore, these 23 luminal residues play a role in targeting and/or the retention of PIG-A to the proper membrane compartment, the rough ER.

PIG-A appeared to form a protein complex with PIG-H (Fig. 11). In preliminary studies, the GST-PIG-A-containing protein complex that was partially purified with glutathione-Sepharose showed the activity to transfer GlcNAc from UDP-GlcNAc to PI. This suggests that the first reaction step is mediated by an enzyme complex. The complex was dependent on weak interactions because it was detected in the digitonin extract but not in the Nonidet P-40 extract (Fig. 11). It is not clear whether PIG-H is associated directly with PIG-A or whether they are the only components of the complex, because at least one more gene, _PIG-C_, is involved in the same step. _PIG-C_ must be cloned to clarify these points. Since genetic data suggest that products of the yeast _GPI1_ and _GPI2_ genes interact (15) and hence a protein complex also participates in yeasts, identification and characterization of mammalian homologues of _GPI1_ and _GPI2_ would also be important.

The results with the carboxyl-terminal deletion mutants were consistent with data obtained from analyzing of somatic mutations of PIG-A in patients with paroxysmal nocturnal hemoglobinuria (26, 27). Paroxysmal nocturnal hemoglobinuria is an acquired genetic disorder of hematopoietic stem cells characterized by complement-mediated hemolysis (2). Mono- or oligoclonal mutant cells bearing somatic mutation in _PIG-A_ appear in multiple hematopoietic, but not in non-hematopoietic lineages (2, 28). Many GPI-anchored proteins are not expressed on the surface of those mutant cells. In particular, deficient expression of GPI-anchored complement regulatory proteins on erythrocytes renders them very sensitive to complement, leading to intravascular hemolysis. About 80 somatic mutations in _PIG-A_ determined to date are widely distributed in the coding regions and splice sites (2, 29). Whereas most coding region mutations reside within the cytoplasmic and transmembrane domains, two are within the luminal domain. One is substitution of the AT dinucleotide with C in codon 450, causing a frameshift and a premature stop codon at position 455 (26). The other is a deletion of G in codon 459 that changes the latter sequence due to a frameshift (27). These mutations would result in mutant proteins of PIG-A resembling M3 mutant protein and hence would cause mislocation of the PIG-A proteins, resulting in a GPI anchor deficiency.

The orientation of PIG-A indicates that the transmembrane and luminal domains of PIG-A must translocate across the ER membrane. Whether it occurs posttranslationally or cotranslationally is not known. However, it seems that the transmembrane domain acts as an efficient signal for membrane translocation because GST, which is a 27-kDa cytoplasmic protein by itself, was translocated into the ER lumen when it was connected to the carboxyl terminus of PIG-A (Fig. 7).

This study provided evidence that the first step of GPI anchor biosynthesis is mediated by a protein complex containing PIG-A and PIG-H that is localized in the ER. At present it is not clear why the transfer of GlcNAc to PI is mediated by a protein complex. The cytoplasmic orientation of PIG-H and of a large putative catalytic domain of PIG-A is consistent with a report that GlcNAc-PI faces the cytoplasm (10); together, they indicate that the first reaction occurs on the cytoplasmic side of the ER.

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Note Added in Proof—We recently demonstrated that human PIG-C is homologous to yeast GPI2.

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PIG-A and PIG-H, Which Participate in Glycosylphosphatidylinositol Anchor Biosynthesis, Form a Protein Complex in the Endoplasmic Reticulum
Reika Watanabe, Taroh Kinoshita, Ryuichi Masaki, Akitsugu Yamamoto, Junji Takeda and Norimitsu Inoue

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