Requirement of PIG-F and PIG-O for Transferring Phosphoethanolamine to the Third Mannose in Glycosylphosphatidylinositol*

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Many eukaryotic proteins are anchored by glycosylphosphatidylinositol (GPI) to the cell surface membrane. The GPI anchor is linked to proteins by an amide bond formed between the carboxyl terminus and phosphoethanolamine attached to the third mannose. Here, we report the roles of two mammalian genes involved in transfer of phosphoethanolamine to the third mannose in GPI. We cloned a mouse gene termed Pig-o that encodes a 1101-amino acid GPI-O protein bearing regions conserved in various phosphodiesterases. Pig-o knock-out F9 embryonal carcinoma cells expressed very little GPI-anchored proteins and accumulated the same major GPI intermediate as the mouse class F mutant cell, which is defective in transferring phosphoethanolamine to the third mannose due to mutant Pig-f gene. PIG-O and PIG-F proteins associate with each other, and the stability of PIG-O was dependent upon PIG-F. However, the class F cell is completely deficient in the surface expression of GPI-anchored proteins. A minor GPI intermediate seen in Pig-f knockout but not class F cells had more than three mannoses with phosphoethanolamines on the first and third mannoses, suggesting that this GPI may account for the low expression of GPI-anchored proteins. Therefore, mammalian cells have redundant activities in transferring phosphoethanolamine to the third mannose, both of which require PIG-F.

Many proteins on the eukaryotic cell surface are anchored by glycosylphosphatidylinositol (GPI) (1–5). The common backbone, EtNP-6Man, EtNP-1,6Man, GlcN, and P are phosphoethanolamine, mannose, glucosamine, and phosphate, respectively, is assembled in the endoplasmic reticulum (ER) by the sequential additions of sugar and EtNP components to phosphatidylinositol (GPI) to the cell surface membrane. The GPI anchor is linked to proteins by an amide bond formed between the carboxyl terminus and phosphoethanolamine attached to the third mannose. Therefore, mammalian cells have redundant activities in transferring phosphoethanolamine to the third mannose, both of which require PIG-F.

Saccharomyces cerevisiae (8, 9) and in mammalian cells (10), the first mannose (Man1) is modified by EtNP at position 2. The second mannose (Man2) of mammalian and yeast GPIs can also be modified by EtNP at position 6 (11, 12). EtNP on the third mannose (Man3) is transferred from phosphatidylethanolamine (13, 14), whereas donors for EtNP on Man1 and Man2 have not been clarified.

In yeast, two homologous gene products involved in side chain modification of mannose residues were characterized. Gpi7p is involved in the addition of a side chain, probably EtNP, to Man2, which is not essential for growth (12). Mcd4p, which is essential for growth, is probably involved in EtNP transfer to Man1 (15, 16). Mouse F9 cells defective in Pig-n, a mouse homologue of MCD4, accumulate GPI precursors without EtNP on Man1 (16). YW3548 (17) inhibits the addition of EtNP to Man1 in both yeast and mammalian cells by inhibiting Mcd4p and Pig-n (8, 16), indicating that yeast Mcd4p is homologous to MCD4 and that PIG-O and PIG-F could be involved in transferring EtNP to Man1. Mcd4p and Gpi7p are large proteins of about 120 kDa having an amino-terminal lumenal domain and multiple transmembrane domains in the carboxyl-terminal portion. They have regions conserved in phosphodiesterases and nucleotide pyrophosphatases within the amino-terminal lumenal domain (12, 15). It is likely that they are involved in transfer of EtNP to mannosyl, although their enzyme activities remain to be demonstrated.

In the yeast genome, there is a third gene, YLL031c, homologous to MCD4 and GPI7 (12, 15). Although YLL031c has not been shown to be involved in GPI biosynthesis, its disruption in yeast caused a phenotype similar to those of other GPI anchoring mutants; namely, germinated spores had lethal growth defect after one to two generations with heterogeneous bud sizes (18). Therefore, it is likely that YLL031c is involved in EtNP transfer to Man3. Man1 is modified at position 2, whereas Man2 and Man3 are modified at position 6. Consistent with these different positions of modification, Gpi7p and YLL031c share an amino acid identity (38%) that is higher than that of Gpi7p and Mcd4p (24%) and that of YLL031c and Mcd4p (21%) (12, 16).

Mammalian GPI biosynthesis mutant cells of complementation class F are defective in EtNP transfer to Man3 (10, 19, 20), for which mutation in Pig-f is responsible (21, 22). However, PIG-F protein does not have structural similarity to YLL031c, and yeast has a Pig-F homologue, YDR302w. Here, we report molecular cloning of a mouse YLL031c homologue termed Pig-o (phosphatidylglycan, class O) and show that PIG-O and PIG-F are involved in the addition of EtNP to Man3. We also show that mammalian cells have another mechanism of adding EtNP to Man3 that requires PIG-F but not PIG-O.
EXPERIMENTAL PROCEDURES

Cells and Reagents—Mouse embryonal carcinoma F9 cells were obtained from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum on 0.1% gelatin-coated dishes. Gaa1 knockout F9 cells were cultured in the same medium (23). Mouse GPI-deficient class B and F mutant lymphoma cells S1A (Thy-1 2 b) and EL4 (Thy-1 2 f) (24) were gifts from Dr. R. Hyman (Salk Institute, San Diego, CA). Chinese hamster ovary (CHO) cells were cultured in F-12 medium supplemented with 10% fetal calf serum. CHO and EL4 (Thy-1 2 f) cells (10^7 in 1xHeBS) (25) were transfected by electroporation at 260 V and 960 μF and at 250 V and 960 μF, respectively. BE49385A, the same compound as YW3548 (17), was provided by Banyu Pharmaceutical Co. (Tokyo, Japan) (16).

Preparation of cDNA and Genomic Clones of Pig-0—We searched the GenBank database using the BLAST software (26) for sequences homologous to yeast YLL031c and found a mouse genomic sequence. We designed two primers, 5′-ATAGACGCTCTGCGGTTTGACTTTGCC (forward) and 5′-CATTATCTTCCACTATAGCATGGCTGG (reverse), based on the genomic sequence and amplified its cDNA from a mouse testis cDNA library (a gift from Dr. H. Nojima, Osaka University). The sequence of the PCR product coincided with the corresponding genomic sequences. Using a forward primer designed in a vector and the reverse primer above, fragments containing the 5′ region were amplified and cloned from the cDNA library. The longest clone was 1.2 kb long. Using the forward primer above and a reverse vector primer, a 3.3-kb fragment containing the 3′ region was cloned. The longest 5′ and the 3′ fragments were ligated to generate a full-length cDNA. Exon positions in the genomic sequence were determined.

Disruption of Pig-0 Gene in F9 Cells—A 2.0-kb NotI-XbaI genomic fragment containing the 5′ region flanking exon 1 was amplified by PCR with forward primer 5′-TGACAGCGCGCCGCCCTCAGTGGCTGAG-
Fig. 2. Disruption of Pig-o. A, targeting vectors for homologous recombinations. The first and second targeting vectors were designed to disrupt exons 1–3 and to contain neomycin (neo) and puromycin (puro) resistance genes, respectively. Herpes simplex virus thymidine kinase gene (tk) was included at the 3' end to select against random integration. All marker genes were driven by PGK promoter and had PGK poly(A) signals. Exons are numbered. B, the structures of intact (middle) and disrupted (top and bottom) Pig-o genes. To screen homologous recombinations, PCR primers were used as indicated (arrows). The forward primer was outside the targeting vectors. The genomic structure of Pig-o was obtained from mouse genomic sequence in the GenBank™ data base. The start codon is located in exon 1. Filled boxes, noncoding exons; open boxes, coding exons. The expected XhoI fragments detected by Southern blotting are indicated by thick bars with lengths (kb). RI, EcoRI; RV, EcoRV; K, KpnI; Not, NotI; Sal, SalI; Xb, XbaI; Xh, XhoI. C, Southern blotting of targeted mutants. Samples of 5 μg of genomic DNA were cut with XhoI and probed with Pig-o cDNA bearing exons 1–6. Lane 1, F9 (wild-type); lane 2, single knockout mutant; lane 3, double knockout mutant; D, PCR of genomic DNAs. DNAs (350 ng) of wild-type F9 (lanes 1 and 5), single knockout cells (lanes 2 and 6), and double knockout cells (lanes 3 and 7) were used in PCRs with exon 1 (lanes 1–3) and exon 6 (lanes 5–7) primer sets. Lane 4, size markers.

CAGTAATGCAGCG and reverse primer 5'-TCCCAATTTGACAGATTGTTGCATTAGCATTCCGC and cloned into pPNT (27). (The resulting plasmid was named pPNT-B.) A 6.9-kb SalI-EcoRI genomic fragment containing exons 4–10 was amplified by PCR with forward primer 5'-GTGCTCTTACTGAAGATTCATCTCTCCGGC. This fragment and a 1.9-kb XbaI-SalI fragment of PGKpuro were ligated together in the XbaI-EcoRI site in pPNT-B. The resulting plasmids were used as the first and second targeting vectors to delete exons 1–3. The gene targeting strategy was followed as described (28). Briefly, F9 cells (2 × 10⁷) were electroporated with 50 μg of NotI-cut targeting vector at 230 V and 500 μF using a GenePulser (Bio-Rad) and seeded on plates coated with 0.1% agarose. Cells were collected after 2 days, transfected with 100,000 × g for 2 h and dissolved in 1% Nonidet P-40–150 mM NaCl. GST-tagged Pig-o and FLAG-tagged Pig-F were affinity-precipitated with glutathione beads (Amersham Pharmacia Biotech) and anti-FLAG antibody M2 beads (Sigma), respectively, and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Western blotting was carried out with biotinylated anti-FLAG monoclonal antibody M2 plus horseradish peroxidase-conjugated streptavidin (Amersham Pharmacia Biotech) or with anti-GST antibody (Amersham Pharmacia Biotech) plus HRP-conjugated anti-GST antibody (Organon Teknika, N.V.) and visualized with chemiluminescence (Renaissance, DuPont) (31). The band intensities were quantitated by measuring chemiluminescence with a Fuji Image analyzer LAS1000 (Fuji Film Co., Tokyo, Japan) (28). Fractions were also characterized by assaying membrane marker enzymes, alkaline phosphodiesterase I for the plasma membrane, α-mannosidase II for the Golgi, and dolichol phosphate mannose synthase for the ER, as described (32).

Analysis of Protein Complexes—CHO cells (2 × 10⁷) were cotransfected with 10 μg of pMEori-FLAG-Pig-F and 40 μg of pMEEB-GST-Pig-o, pMEEB-GST-aldolase dehydrogenase (ALDH), or pMEEB-GST-Pig-n (16). After culture for 2 days, transfected cells were hypotonically lysed...
Fig. 3. Surface expression of GPI-anchored proteins on Pig-o knockout F9 cells. Cells were stained with biotinylated anti-Thy-1 antibody (thick lines) or control antibody (thin lines). A, the expression of GPI-anchored proteins on Pig-o knockout cells decreased but remained (middle panel). Left panel, F9 (wild-type); right panel, Gaa1 knockout F9 cell. B, restoration of the surface expression of Thy-1 on Pig-o knockout cells by Pig-o cDNA (left panel) but not Pig-n (second panel from left), PIG-F (second panel from right), or ALDH (right panel) cDNAs.

in 50 mM HEPES/NaOH (pH 7.4) containing 0.1 mM TLCK and 1 μg/ml leupeptin for 20 min on ice and further disrupted by sonication. Membranes were obtained by centrifugation at 100,000 × g for 1 h. The collected membranes were solubilized in 1% digitonin in 50 mM HEPES/NaOH (pH 7.4), 25 mM KCl, 5 mM MgCl2, 0.1 mM TLCK, and 1 μg/ml leupeptin. Insoluble materials were removed by centrifugation at 100,000 × g for 1 h, and supernatants were used for affinity precipitation. Precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting as described above.

Stabilities of Pig-O in Class F Cells and of Pig-F in Pig-o Knockout F9 Cells—ELS (Thy-1-f) cells were transfected with 15 μg of PMEEB-GST-Pig-o, 8 μg of PME-FLAG-Pig-o (a vector for GST-tagged dolichol phosphate mannosyltransferase) (30, 33)), and 2 μg of PGKpu and selected for 2 weeks. Stable transfected cells (1 × 107) were incubated with 20 μg of pMEnor-Pig-F or an empty vector and cultured for 2 days. Pig-o knockout cells were transfected with 8 μg of pMEnor-FLAG-Pig-F, 15 μg of PMEEB-FLAG-ALDH and 2 μg of PGKpu and selected for 2 weeks. Stable transfected cells (1 × 107) were incubated with 20 μg of pMEEB-Pig-o or an empty vector and cultured for 2 days. Cells were treated in 1% Nonidet P-40 solution and assessed for GST-tagged proteins.

In Vivo Mannose Labeling and Characterization of Mannolipids—Cells (2 × 107) were briefly washed twice with glucose-free medium and incubated for 1 h in 1 ml of a medium containing 20 mM HEPES/NaOH (pH 7.4), 100 μg/ml glucose, 10% dialyzed fetal calf serum, and 10 μg/ml tunicamycin. Then, 40 μCi/ml [3H]mannose (Amersham Pharmaceuticals Biotech) was added, and the cells were further incubated in the same medium for 45 min. After five washes with phosphate-buffered saline, lipids were extracted from pelleted cells with chloroform/methanol/water (10:3:1) and partitioned into thin lines control antibody (thick lines) or control antibody (thin lines). A, the expression of GPI-anchored proteins on Pig-o knockout cells decreased but remained (middle panel). Left panel, F9 (wild-type); right panel, Gaa1 knockout F9 cell. B, restoration of the surface expression of Thy-1 on Pig-o knockout cells by Pig-o cDNA (left panel) but not Pig-n (second panel from left), PIG-F (second panel from right), or ALDH (right panel) cDNAs.

RESULTS

Cloning of Pig-o—We found in the GenBank™ data base human and mouse DNA sequences (accession numbers AC004472 and AC005259, respectively) that have homology to yeast YLL031c. We then amplified and cloned a corresponding mouse cDNA from a testis library. We obtained a 4.2-kb cDNA that encodes 1101 amino acids (Fig. 1A) and named the gene Pig-o (for phosphatidylinositol glycan-class O) (DDBJ/GenBank™/EMBL accession number AB038560). Both Pig-o and its human orthologue PIG-O consisted of 10 exons, the latter being localized on chromosome 9p13 (35). Pig-O had 27% amino acid identity to YLL031c (Fig. 1A) and several regions highly conserved in two other homologues, Gpi7p and Mcd4p (12, 15). Pig-o had two potential N-linked glycosylation sites (Asn-45 and Asn-276) and regions conserved in various phosphodiesterases and nucleotide pyrophosphatases (Fig. 1A) (12, 15). There was one predicted transmembrane domain near the amino terminus (amino acids 12–34) that was followed by a hydrophilic portion of about 400 amino acids (Fig. 1B). Within the carboxyl-terminal portion, there were 15 transmembrane domains, as predicted by analyses with the SOSUI (36) and TMHMM (37) programs. Therefore, it is predicted that Pig-O, like Mcd4p and Gpi7p, is a membrane protein with a large hydrophilic domain near the amino terminus and multiple transmembrane domains in the carboxyl-terminal portion.

Disruption of Pig-o in F9 Cells—To see whether Pig-o is involved in GPI anchor biosynthesis, we disrupted Pig-o with homologous recombination in F9 embryonal carcinoma cells. We used targeting vectors in which about a 1.3-kb 5′ flanking region and exons 1–3 were replaced by neomycin or puromycin resistance gene (Fig. 2A). A region encoded by exons 1–3 that corresponds to the amino-terminal 25% of the protein contains regions conserved in YLL031c and various phosphodiesterases and nucleotide pyrophosphatases (Fig. 1A). After the first and second homologous recombinations, genomic DNAs were Southern blotted against a CDNA probe containing exons 1–6 (Fig. 2C). The probe should recognize a 9.3-kb fragment before

Peptated against the remaining pellets once more. A total of 800 μl of solution was dried up in vacuum and used as purified lipid.

20914

PIG-F and PIG-O in Biosynthesis of GPI

... a solvent consisting of isopropanol/hexane/water (50: 25: 20). Tubes were sealed and sonicated in an ultrasonic bath for 10 s and centrifuged at 12,000 × g for 10 min. Supernatants were decanted to new tubes. Extraction was re...
PIG-F and PIG-O in Biosynthesis of GPI

Fig. 4. A, ER localization of PIG-O and PIG-F. CHO cells transfected with GST-tagged PIG-o and FLAG-tagged PIG-F were disrupted, and supernatants were obtained after centrifugation at 10,000 × g. They were further separated by sucrose density gradient centrifugation. Fractions were characterized by assaying total protein and membrane marker enzymes (top panel). GST-tagged PIG-O and FLAG-tagged PIG-F were affinity-precipitated and detected by Western blotting. B, association of PIG-O and PIG-F. CHO cells cotransfected with FLAG-tagged PIG-F and GST-tagged PIG-O (lane 1), ALDH (lane 2), and Pig-n (lane 3) were disrupted and dissolved in 1% digitonin. From the digitonin extracts, GST-tagged proteins (top and middle panels) and FLAG-tagged PIG-F (bottom panel) were affinity-precipitated. Precipitates were analyzed by Western blotting with anti-GST (top panel) or anti-FLAG (middle and bottom panels) antibodies.

Fig. 5. A lack of PIG-F affects the stable expression of PIG-O. A, class F mutant cells were stably cotransfected with GST-tagged PIG-o and dolichol phosphate mannose1 and then retransfected with PIG-F cDNA (lane 1) or a mock vector (lane 2). Two days after transfection, cells were dissolved in 1% Nonidet P-40, and amounts of GST-tagged proteins were assessed by affinity precipitation with glutathione beads and Western blotted with anti-GST antibodies. B, PIG-o knockout mutants were stably cotransfected with FLAG-tagged PIG-F and ALDH and then retransfected with Pig-o cDNA (lane 1) or a mock vector (lane 2). FLAG-tagged proteins were immunoprecipitated with anti-FLAG beads and Western blotted with anti-FLAG antibodies.

disruption and 7.4- and 7.3-kb fragments after the first and second disruptions, respectively (Fig. 2B). As expected, the 9.3-kb fragment disappeared after the second disruption with the appearance of 7.4/7.3-kb fragments, indicating that PIG-o was completely knocked out (Fig. 2C). To confirm that there was no contamination by nontargeted cells in double disruptant cells, we amplified exon 1 and exon 6 by PCR from genomic DNA (Fig. 2D). Exon 1 was amplified from DNA of wild-type and single disruptant cells (Fig. 2D, lanes 1 and 2) but not double disruptant cells (lane 3), whereas exon 6 was amplified from all of these cells (lanes 5–7). Taken together, the results show that both alleles of PIG-o were successfully disrupted.

Fig. 6. Impaired GPI biosynthesis in Pig-o knockout F9 cells. Cells were radiolabeled with [3H]mannose, and extracted lipids were analyzed by TLC. Lane 1, F9 wild-type cell; lane 2, Gaa1 knockout F9; lane 3, class B mutant; lane 4, class F mutant; lane 5, Pig-o knockout F9; lane 6, Pig-o knockout F9 transfected with Pig-o cDNA; lane 7, Pig-o knockout F9 transfected with GST-tagged Pig-o cDNA; lane 8, Pig-o knockout F9 transfected with PIG-F cDNA; lane 9, Pig-o knockout F9 transfected with FLAG-tagged PIG-F cDNA; lane 10, Pig-o knockout F9 transfected with a mock vector. Positions of various lipids, origin, and front are indicated. DPM, dolichol phosphate mannose. See Fig. 7 for structures of GPI intermediates.

Transfection of Pig-o cDNA into Pig-o knockout cells normalized the surface Thy-1 expression as expected (Fig. 3B, left panel). Transfections of cDNA of Pig-o (16), a homologous gene involved in transfer of EtNP to Man1, and Pig-f cDNA (22), as well as a control ALDH cDNA, did not restore the surface Thy-1 expression (Fig. 3B, right three panels).

Pig-o Is an ER Membrane Protein and Forms a Complex with PIG-F—To begin to clarify the functional relationships between PIG-O and PIG-F, we determined the subcellular localization of these two proteins. We cotransfected cDNAs of GST-tagged PIG-O and FLAG-tagged PIG-F into CHO cells and separated the postnuclear membranes with sucrose density gradient centrifugation. Most of the PIG-O and PIG-F was found in fraction 5, which contained the ER membranes, but not in the fractions containing the plasma membranes and the Golgi membranes (Fig. 4A).
We next tested whether the two proteins associate with each other. FLAG-tagged PIG-F was cotransfected with GST-tagged Pig-o, ALDH, and Pig-n separately into CHO cells. ALDH (38) and Pig-n (16) are ER membrane proteins. After the membranes had been dissolved with 1% digitonin, the GST-tagged proteins were precipitated with glutathione beads, and coprecipitation of FLAG-tagged proteins was analyzed by Western blotting (Fig. 4B). FLAG-tagged PIG-F was coprecipitated (Fig. 4B, middle panel) with GST-tagged PIG-O (Fig. 4B, lane 1) but not with GST-tagged ALDH (lane 2) or GST-tagged Pig-n (lane 3). Therefore, PIG-O specifically associated with PIG-F. It was noted that much of PIG-F existed without association with PIG-O (Fig. 4B, lane 1, middle versus bottom panel).

PIG-F Stabilizes PIG-O—Because PIG-O and PIG-F were bound with each other, one might affect the expression of the other. To investigate this, we made a stable transfectant of class F cells (19, 20) in which GST-tagged PIG-O and dolichol phosphate mannose 1, an ER membrane protein (33), were expressed. Then the transfectant cells were transiently transfected with PIG-F cDNA or an empty vector and expression of GST-tagged proteins was assessed (Fig. 5A). Expression of GST-tagged PIG-O was much higher in the presence (Fig. 5A, lane 1) than in the absence (lane 2) of PIG-F. The difference was three to five times in repeated experiments, indicating that PIG-F stabilizes PIG-O. To test whether PIG-O stabilizes PIG-F, we transfected FLAG-tagged PIG-F together with FLAG-tagged ALDH into Pig-o knockout cells and assessed the expression of FLAG-tagged PIG-F in the presence and absence of Pig-o cDNA (Fig. 5B). The expression of PIG-F was not affected by PIG-O because a ratio of PIG-F to ALDH did not change with (Fig. 5B, lane 1) or without (lane 2) PIG-O. These results showed that PIG-O was associated with PIG-F in the ER, that stable expression of PIG-O was dependent upon PIG-F and that PIG-F was stable in the absence of PIG-O.

Transfection of GST-tagged PIG-O without PIG-F cDNA caused a weak but significant expression of GST-tagged PIG-O in class F cells (Fig. 5A, lane 2). However, the surface expression of Thy-1 was not restored at all under these conditions (data not shown). Therefore, PIG-O requires the presence of PIG-F to restore GPI anchor biosynthesis, suggesting a functional role of PIG-F in addition to its ability to stabilize PIG-O.

The Same Major GPI Intermediate Accumulates in Pig-o Knockout Cells and Class F Mutant Cells, but Minor GPls Are Different—To determine the step in GPI biosynthesis at which
**Pig-o** is involved, we metabolically labeled Pig-o knockout cells and other cells with \(^{3}H\)mannose (Fig. 6). In wild-type F9 cells, H8, a mature GPI anchor competent for transfer to proteins, was seen (Fig. 6, lane 1). Gaa1 knockout cells (23), in which a gene for GPI transamidase component is disrupted, accumulated various GPI intermediates (lane 2). Class B (lane 3) and F (lane 4) cells accumulated major spots, B and H6, respectively, which were previously characterized as GPsIs containing two and three mannosides with EtNP on Man1, respectively (19, 20, 39). See Fig. 7 for a schematic representation of the structures of various GPI intermediates and mature GPsIs. Pig-o knockout cells accumulated H6 as a major spot (Fig. 6, lane 5), similarly to class F cells (lane 4).

Pig-o knockout cells accumulated two minor spots, KO-1 and KO-2 (Fig. 6, lane 5) whereas class F cells accumulated one, F-1 (lane 4). KO-1 and F-1 had similar mobilities. GPI biosynthesis in Pig-o knockout cells was normalized by transfection of cDNAs of Pig-O and GST-tagged Pig-O (Fig. 6, lanes 6 and 7), indicating that the GST-tagged Pig-O used in the experiments shown in Figs. 4 and 5 was functional. Transfection of Pig-o cDNA (Fig. 6, lane 8), PIG-F cDNA (lane 9), and an empty vector (lane 10) had no effect. These results suggest that Pig-o knockout and class F cells accumulate the same major GPI but different minor GPsIs.

To clarify this difference, we characterized KO-1, KO-2, and F-1 as well as H6. KO-1 and KO-2 were completely cleaved and H6 was partially cleaved by GPI-phospholipase D, showing that they are GPsIs (Fig. 8A). We purified H6, KO-1, KO-2, and F-1 from TLC plates. Purified H6 from Pig-o knockout and class F cells (Fig. 8B, lanes 3 and 5) as well as unpurified H6 from Gaa1 and Pig-o knockout cells (lanes 1 and 6), had the same mobility. After digestion with jack bean \(\alpha\)-mannosidase, they migrated to the same position that aligned with H5 (Fig. 8B, lanes 2 and 4), consistent with their identity as H6. PIG-O, therefore, like PIG-F, is involved in transfer of EtNP to Man3.

Purified KO-1 and F-1 showed similar migration (Fig. 8C, lanes 3 and 5). After digestion with \(\alpha\)-mannosidase, their migration shifted similarly (Fig. 8C, lanes 2 and 4), suggesting that KO-1 and F-1 are the same glycolipids. The number of mannosides removed by \(\alpha\)-mannosidase was not clear but may be two, as judged from the extent of mobility shift. Purified KO-2 (Fig. 8C, lane 7) migrated more slowly than a GPI termed H7, seen in Gaa1 knockout cells (lane 8). KO-2 was sensitive to \(\alpha\)-mannosidase and migrated to a position similar to that of H7 (Fig. 8C, lane 6), indicating that KO-2 had one or more unmodified mannosides at its terminus. H7 has three mannosides, with EtNPs on Man1 and Man3, and is thought to be competent for transfer to proteins. It is suggested, therefore, that KO-2 would have at least one more mannose than H7 and be able to anchor proteins. This idea that KO-2 may account for the residual surface expression of GPI-anchored proteins on Pig-o knockout cells is consistent with the fact that class F cells, which are completely deficient in the expression of GPI-anchored proteins, have no KO-2.

To further confirm that KO-1 and KO-2 have EtNP on Man1, we treated wild-type and Pig-o knockout cells with 10 \(\mu\)mol YW3548/BE49385A, a drug that inhibits addition of EtNP to Man1 (16, 17). In the presence of the drug, wild-type F9 cells accumulated H7 and H7 as reported previously (Fig. 9, lanes 3 and 4) (16), indicating that EtNP addition to Man1 was inhibited under these conditions. The drug inhibited generation of KO-1 and KO-2 (Fig. 9, lanes 1 and 2), indicating that they had EtNP on Man1.

**DISCUSSION**

In the present study, we cloned and characterized Pig-o and analyzed the roles of Pig-O and PIG-F in GPI biosynthesis. The first conclusion of this study is that PIG-O and PIG-F act
together in transferring EtNP to Man3. This is based on two results: 1) Pig-o knockout cells and class F cells defective in PIG-F accumulated the same major GPI intermediate, H6, which bears three mannoses lacking EtNP on Man3; and 2) PIG-O and PIG-F formed a protein complex in the ER, and the stability of the former was dependent upon the latter. The second conclusion is that although this mechanism of EtNP transfer to Man3 is predominant, there must be a second mechanism that also requires PIG-F but not PIG-O. This conclusion is supported by two observations: 1) Pig-o knockout cells express a low level of GPI-anchored proteins, whereas class F cells are completely deficient in the surface expression of GPI-anchored protein; and 2) Pig-o knockout but not class F cells generated a minor GPI with more than three mannoses that has EtNPs on Man1 and Man3.

Association of PIG-O and PIG-F—Both PIG-O and PIG-F were found exclusively in the ER, where the GPI anchor is synthesized, and they specifically associated with each other. PIG-F, which is a very hydrophobic protein, may associate with the carboxyl-terminal hydrophobic region of PIG-O.

The expression of PIG-O was dependent upon PIG-F, i.e. the level of PIG-O expression was at least three times higher in the presence than in the absence of PIG-F. However, the expression of PIG-F was not affected by a lack of PIG-O. Presumably, most PIG-O was associated with PIG-F, whereas some PIG-F may exist free from PIG-O. These results suggest that PIG-O acts together with PIG-F in the ER and that PIG-F may have at least one more partner that is involved in the second mechanism of transferring EtNP to Man3 (see below for further discussion).

Common and Different Phenotypes of Pig-o Knockout and Class F Mutant Cells—Pig-o knockout and class F cells share common defective phenotypes but they have some differences too. Both cells are deficient in the surface expression of GPI-anchored proteins. However, low levels of Thy-1 and Sca1 remained on the Pig-o knockout F9 cells. In contrast, class F cells are completely deficient in the surface Thy-1 expression. Therefore, PIG-O is involved in but not essential for GPI-anchoring of proteins, whereas PIG-F is essential for it. Both Pig-o knockout and class F cells did not generate a mature GPI, H8, but accumulated a GPI intermediate, H6. H6 has three mannoses and EtNP modification on Man1 but lacks EtNP on Man3 (10). Therefore, both cells are defective in the transfer of EtNP to Man3 (see Fig. 7 for the pathway). Class F cells generated one minor GPI, termed F-1, whereas Pig-o knockout cells generated two minor GPs, termed KO-1 and KO-2. We concluded that F-1 and KO-1 are the same GPI (see below for discussion), but KO-2 was seen only in Pig-o knockout cells.

Possible Structures of GPs, KO-1/F-1 and KO-2—KO-1 and F-1 had similar mobilities on TLC, slightly slower than that of H7. H7 bears three mannoses with EtNPs on Man1 and Man3. After the treatment with α-mannosidase, KO-1 and F-1 behaved similarly and became a GPI migrating slightly more slowly than H6. H6 bears three mannoses with EtNP on Man1. KO-1 must have EtNP on Man1 because its generation was inhibited by YW3548/BE49385A. Although we did not analyze the number of mannose residues in KO-1 and F-1, we speculate based on these results that they have three or four mannoses with EtNP on Man1 and Man2 (Fig. 7).

KO-2 migrated more slowly than H7, and after α-mannosidase treatment, its product behaved similarly to H7 on TLC. KO-2 has EtNP on Man1 because its generation was inhibited by YW3548/BE49385A. KO-2, therefore, has more than three, most likely four, mannoses with EtNP on Man1 and Man3.

Two Mechanisms of Transfer of EtNP to Man3 in Mammalian Cells—The complex of PIG-O and PIG-F should be responsible for the addition of EtNP to Man3 in H6 to generate H7 and subsequently H8. It is very likely that PIG-O bears a catalytic site because three family members, Mcd4p/Pig-n, Gpi7p, and PIG-O, correspond to EtNP additions to Man1, Man2, and Man3, respectively, and because they have regions with homology to various phosphodiesterases and nucleotide pyrophosphatases. Consistent with the idea that these regions are involved in the catalytic site, the temperature-sensitive mutant allele of MCD4 had a mutation within one of these regions (15). These regions are within the luminal domains, suggesting that transfer of EtNP to Man3 occurs on the luminal side of the ER.

PIG-F is required for the stable expression of PIG-O. It is unlikely that this is the only role of PIG-F because PIG-O expressed in the absence of PIG-F did not cause the surface Thy-1 expression in class F cells. PIG-F may play some role in the addition of EtNP to Man3.

In the absence of PIG-O, GPI-anchored proteins were expressed at low levels. Because KO-2 may have Man3 with EtNP on it, it seems possible that KO-2 accounts for the residual GPI-anchoring in Pig-o knockout cells. Generation of KO-2 should require PIG-F because it was not seen in class F cells. Consistent with the notion that KO-2 is competent for attachment to proteins, class F cells are completely deficient in the surface expression of GPI-anchored proteins. Because PIG-F may not be a catalytic component, it would act together with a catalytic component other than PIG-O to generate KO-2. Because Gpi7p is responsible for addition of EtNP to Man2 at position 6 in yeast and EtNP on Man3 is also at position 6, a mammalian homologue of Gpi7p seems to be a candidate of the second partner for PIG-F. Human and mouse sequences homologous to Gpi7p are found in the GenBank™ data base. They
should be cloned and characterized to determine whether they represent a functional homologue of Gpi7p and act with PIG-F in transferring EtnP to Man3.

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Note Added in Proof—Two papers describing functions of yeast orthologues of PIG-F and PIG-O were recently published (Taron, C. H., Wiedman, J. M., Grimme, S. J., and Orlean, P. (2000) Mol. Biol. Cell 11, 1611–1630; Flury, I., Benachour, A., and Conzelmann, A. (May 22, 2000) J. Biol. Chem. 10.1074/jbc.M003844200).

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