A Conserved Proline in the Last Transmembrane Segment of Gaa1 Is Required for Glycosylphosphatidylinositol (GPI) Recognition by GPI Transamidase

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Glycosylphosphatidylinositol (GPI)-anchored proteins are synthesized as precursor proteins that are processed in the endoplasmic reticulum by GPI transamidase (GPIT). Human GPIT is a multisubunit membrane-bound protein complex consisting of Gaa1, Gpi8, phosphatidylinositol glycan (PIG)-S, PIG-T, and PIG-U. The enzyme recognizes a C-terminal signal sequence in the proprotein and replaces it with a preformed GPI lipid. The nature of the functional interaction of the GPIT subunits with each other and with the proprotein and GPI substrates is largely unknown. We recently analyzed the GPIT subunit Gaa1, a polytopic protein with seven transmembrane (TM) spans, to identify sequence determinants in the protein that are required for its interaction with other subunits and for function (Vainauskas, S., Maeda, Y., Kurniawan, H., Kinoshita, T., and Menon, A. K. (2002) J. Biol. Chem. 277, 30535–30542). We showed that elimination of the C-terminal TM segment of Gaa1 allows the protein to interact with Gpi8, PIG-S, and PIG-T but renders the resulting GPIT complex non-functional. We now show that GPIT complexes containing C-terminally truncated Gaa1 possess a full complement of subunits and are able to interact with a proprotein substrate but cannot co-immunoprecipitate GPI. We go on to show that mutation of a conserved proline residue centrally located within the C-terminal TM span of Gaa1 is sufficient to abrogate the ability of the resulting GPIT complex to co-immunoprecipitate GPI. We suggest that the putative dynamic hinge created by the proline residue provides a structural basis for the interaction of GPI with GPIT.

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Glycosylphosphatidylinositol (GPI) anchors are attached to ER-translocated proproteins bearing a C-terminal signal sequence for GPI attachment. The GPI signal sequence consists of a tripeptide of small amino acids (termed ω, ω + 1, and ω + 2), a hydrophilic spacer sequence of 7–10 amino acids, and a C-terminal hydrophobic stretch (1). The ω amino acid is the GPI attachment site. GPI anchor attachment is catalyzed by GPI transamidase (GPIT), a multisubunit, ER membrane-bound protein complex (2–11). A sulfhydryl group in GPIT activates the carbonyl function of the ω amino acid, resulting in the formation of an enzyme-substrate complex and concomitant cleavage of the amide bond between the ω and ω + 1 residues. Nucleophilic attack by GPI on the activated carbonyl regenerates GPIT and yields a GPI-anchored protein (1, 12–14).

Mammalian GPIT consists of at least five components: Gaa1, Gpi8, PIG-S, PIG-T, and PIG-U (4–7, 9). All five subunits are required for function. Gpi8 is the likely enzymatic component of the GPIT complex, since it shares sequence homology with a family of plant vacuolar endopeptidases and possesses a critical sulfhydryl residue that is probably involved in catalysis (3, 6, 15). Furthermore, chemical and site-specific photocross-linking studies show that Gpi8 can be cross-linked to proproteins, indicating that it is in physical proximity to at least one of the substrates of GPIT (16, 17). PIG-U contains a sequence motif found in yeast and mammalian fatty acid elongases. This motif is important for PIG-U function, and it has been suggested to play a role in recognizing long chain fatty acids in GPI (9). Unlike Gpi8 and PIG-U, the other subunits (Gaa1, PIG-S, and PIG-T) share essentially no homology with any proteins of known function, and their functional role in GPIT action is unclear. As part of our overall objective to determine the physical and functional architecture of GPIT and to understand the role of the various subunits in GPIT action, we recently identified sequence determinants in Gaa1 that are required for its interaction with other GPIT subunits and for function (18). Gaa1 is a polytopic membrane protein with seven transmembrane (TM) spans and a large, N-glycosylated luminal loop between the first two TM spans (Fig. 1). The luminal loop is required for Gaa1 to interact with Gpi8, PIG-S, and PIG-T. However, the entire protein appears to be required for function, since removal of the last TM segment yields GPIT complexes that are nonfunctional (18).

In this study, we analyzed why deletion of the last TM segment of Gaa1 renders the protein nonfunctional. We considered three possibilities: (i) Gaa1 is involved in binding PIG-U (this was not tested in our previous work, since PIG-U was discovered only subsequently); (ii) Gaa1 participates in recognition of the proprotein; and (iii) Gaa1 is needed for recognition/presentation of GPI. We speculated that removal of the last TM segment of Gaa1 could conceivably affect any or all of these possible interactions with other subunits or GPIT substrates. To investigate these possibilities, we prepared different epitope-tagged variants of Gaa1 (Fig. 1) and tested the ability of these constructs to interact with PIG-U, proprotein,
and GPI substrates. We used transient transfection to introduce the proteins into HeLa cells and immunoprecipitation to probe interactions with other components. Our results show that deletion of the last TM segment of Gaa1 does not affect recruitment of PIG-U or proprotein to the GPI complex but abrogates GPI binding. Further analyses indicate that a putative hinge created by a proline residue centrally located within the TM region may provide structural basis for the interaction of GPI with GPI. These results provide the first experimental data on how GPIs are associated with the GPI signal sequence of decay-accelerating factor protein (a gift from Vicky Stevens, Emory University) for 30 min at 37 °C, and the resulting supernatant was ultra-

GPI temperature gradients and GPI substrates. We used transient transfection with different FLAG-Gaa1 constructs or FLAG-PIG-A. Two days later, the transfected HeLa cells (1–2 × 10^7 cells) were scraped off the culture dish 48 h post-transfection, washed once with PBS, resuspended in 1 ml of MSB buffer (20 mm Hepes-KOH, pH 7.6, 200 mM NaCl, 1% (w/v) digitonin or Nonidet P-40, and 1× protease inhibitor mixture (Calbiochem)) and incubated on ice for 60 min. The cell lysate was clarified by medium speed centrifugation at 10,000 × g for 10 min, and the resulting supernatant was ultra-

Endoglycosidase H (Endo H) and Peptide-N-Glycosidase F (PNGase F) Treatment—Endo H and PNGase F (New England Biolabs) were used for digestion of protein N-linked glycans. Protein samples were denatured by adding 0.1 volume of 10× denaturant buffer (5% SDS, 10% β-mercaptoethanol), followed by incubation for 5 min at 100 °C. Then, 0.1 volume of 10× Endo H reaction buffer (0.5 mM sodium citrate, pH 5.5, at 25 °C) or 10× PNGase F reaction buffer (0.5 mM sodium phosphate, pH 7.5, at 25 °C, 10% Nonidet P-40) was added to the denatured sample, followed by incubation with 0.5 μl of 500 units/μl Endo H or PNGase F for 1 h at 37 °C. Glycosidase-treated samples were analyzed by SDS-PAGE and immunoblotting.

Flow Cytometry—Mouse GAA1-knockout F9 cells were a generous gift from Dr. Tomohiko Kusunoki (Osaka University). The cells (1 × 10^7) were suspended in 400 μl of culture medium (high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum) and electroporated with 25 μg each of plasmids at 500 microfarads and 250 V using Gene Pulser Xcell (Bio-Rad). Two days after electroporation, an aliquot of the sample was taken for immunoblotting analysis of the expressed proteins as described under “Immunoprecipitation and Immunoblots,” whereas the remaining cells were stained with anti-Thy-1 antibody followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG and analyzed by FACS Calibur (Becton Dickinson).

Phosphatidylinositol-specific Phospholipase C (PI-PLC) Treatment—HeLa cells were transiently transfected with HA-GPI cDNA. Two days post-transfection, cells (~1 × 10^7) were washed twice with PBS and incubated in 1 ml of PBS with Staphylococcus aureus PI-PLC (a gift from Vicky Stevens, Emory University) for 30 min at 37 °C. After treatment, the incubation buffer was collected, and the cells were washed and centrifuged at 10,000 × g for 10 min, and the resulting supernatant was ultra-

RESULTS AND DISCUSSION

The constructs used in this paper are illustrated schematically in Fig. 1. PIG-U Is Present in GPI Complexes Containing C-terminally Truncated Gaa1—Previous analyses of a series of C-terminal truncation variants of Gaa1 led us to the conclusion that the large luminal loop between the first and second TM spans of Gaa1 is responsible for bringing Gaa1 into a complex with Gpi8, PIG-S, and PIG-T, whereas the last TM segment is necessary for generating a functional GPI complex (18). We specifically showed that the D2 construct that lacks the last TM segment of Gaa1 formed a nonfunctional, ER-localized GPI complex with Gpi8, PIG-S, and PIG-T. We speculated that the last TM segment of Gaa1 is required for substrate binding or possibly for mediating the interaction of Gaa1 with yet other, hitherto unidentified, subunits of the GPI complex.

Kinosita and colleagues (9) recently identified PIG-U, a highly hydrophobic, polytopic membrane protein, as a fifth subunit of GPI. We considered the possibility that the last TM segment of Gaa1 is involved in recruiting PIG-U to GPI complex and that D2-containing GPI complexes are nonfunctional because they lack PIG-U. To test this idea, we co-transfected HeLa cells with cDNAs corresponding to PIG-U and either D1, D2, or FLAG-PIG-A (an irrelevant FLAG-tagged
membrane protein). Two days after transfection, the cells were lysed and treated with immobilized anti-V5 antibodies to immunoprecipitate PIG-U-V5 and any associated proteins. The immunoprecipitated (IP) samples and post-IP supernatants (material not bound to the anti-V5 beads) were analyzed by immunoblotting with anti-V5 antibodies (to reveal PIG-U-V5) and anti-FLAG antibodies (to reveal D1, D2, or PIG-A). Both D1 and D2 were co-immunoprecipitated with PIG-U (Fig. 2, lower panel, lanes 1 and 3), whereas FLAG-PIG-A was not (Fig. 2, lower panel, lane 5). The significant amount of D1 and D2 remaining in the post-IP supernatant (Fig. 2, lower panel, lanes 2 and 4), despite quantitative immunoprecipitation of PIG-U-V5 (Fig. 2, upper panel), is a result of protein overexpression and a presumed shortage of the endogenously expressed Gpi8, PIG-S, and PIG-T subunits. These results, together with our previous data, indicate that D2-containing GPIT complexes possess a full complement of known transamidase subunits and that the nonfunctionality of these complexes is probably due to defects in substrate recognition or catalysis.

The ability of the full-length and truncated Gaa1 variants to co-precipitate PIG-U was significantly reduced when lysates were prepared in the presence of 1% Nonidet P-40, compared with 1% digitonin (data not shown). Since Nonidet P-40 had no effect on the co-precipitation of the other GPIT components with Gaa1, it is possible that Gaa1, Gpi8, PIG-S, and PIG-T form a tightly associated core with which PIG-U is only weakly associated.

**FIG. 1. Schematic of transamidase subunit constructs and the free GPI H8.** Shown are the various constructs used in this paper. Epitope-tagged Gaa1 variant D1; the C-terminal truncation mutants D2, D4, and D5 (the truncation site is indicated by an open star); and point mutants of D1 (P609L and W611L) are shown in the top schematic. The middle schematic shows PIG-U-V5, whereas the bottom schematic illustrates FLAG-Gpi8, HA-GPI proprotein (with a C-terminal GPI attachment signal sequence), and FLAG-PIG-A, a component of the multisubunit GlcNAc-PI synthase enzyme used as a control in our studies. The free GPI molecule, H8, is also shown in the bottom schematic (filled triangle, phosphoethanolamine; open circle, mannose; shaded square, glucosamine; open square, inositol; the inositol residue is acylated and shown linked to phosphatidic acid). Protein N-glycosylation is indicated by a Y appended to the relevant structures (Gaa1 variants and pro-HA-GPI). All structures are shown in the context of the ER membrane bilayer. The membrane topology for all constructs except PIG-U-V5 is based on experimental data; the ER lumen is at the top of each membrane, and the cytoplasm is at the bottom. The structures are not drawn to scale.

GPIT Complexes Containing C-terminally Truncated Gaa1 Are Able to Interact with a Proprotein Substrate of the Enzyme—We next set out to test whether D2-containing GPIT complexes are able to interact with proproteins destined for GPI anchor attachment. Instead of relying on endogenously expressed proprotein precursors of GPI-anchored proteins, we used HA-GPI for our experiments. HA-GPI is a modified form of influenza hemagglutinin that contains the GPI attachment signal sequence, with a C-terminal His tag that enables co-precipitation with anti-V5 antibodies. Immunoprecipitates of D1, D2, and D4 contain both GPI-HA and PIG-U-V5, whereas the control FLAG-PIG-A immunoprecipitate lacks PIG-U-V5. The anti-V5 blots shown in Fig. 1 illustrate the ability of D1, D2, or D4 to co-precipitate PIG-U-V5 and any associated proteins. The anti-V5 antibodies (for PIG-U-V5) were used to detect PIG-U-V5, whereas the anti-FLAG antibodies (to reveal D1, D2, or PIG-A) were used as a control in our studies. The free GPI molecule, H8, is also shown in the bottom schematic (filled triangle, phosphoethanolamine; open circle, mannose; shaded square, glucosamine; open square, inositol; the inositol residue is acylated and shown linked to phosphatidic acid). Protein N-glycosylation is indicated by a Y appended to the relevant structures (Gaa1 variants and pro-HA-GPI). All structures are shown in the context of the ER membrane bilayer. The membrane topology for all constructs except PIG-U-V5 is based on experimental data; the ER lumen is at the top of each membrane, and the cytoplasm is at the bottom. The structures are not drawn to scale.

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intermediate in the transamidase reaction. The HA-GPI molecules that co-precipitate with GPIT in our analyses could be either proproteins with an intact GPI signal sequence or GPI-anchored proteins that have yet to exit the ER (see below). Although this is a point that remains to be explored in future work, it does not affect the conclusions presented here. We henceforth refer to GPIT-associated HA-GPI as a proprotein.

The association of substrate protein with GPIT has been reported previously (10, 16, 17, 21) in studies utilizing an in vitro translation system and model proproteins with altered C-terminal GPI attachment signal sequences, or utilizing inactive GPIT complexes for co-precipitation of proproteins with altered proproteins. Our results indicate that the interaction between GPIT and the proprotein is experimentally detectable even without recourse to mutant GPIT complexes or modified proproteins.

Fig. 3A shows that HA-GPI recovered in the post-IP supernatant migrates more slowly than HA-GPI that is immunoprecipitated with D1, D2, or D4. This suggests that HA-GPI in the IP fraction has N-glycans characteristic of the ER, whereas HA-GPI in the post-IP supernatants possesses higher molecular mass, Golgi-modified N-glycans. To verify this, we prepared cell lysates and anti-FLAG immunoprecipitates from HeLa cells co-transfected with cDNAs corresponding to D1 and HA-GPI and analyzed the samples by glycosidase treatment, SDS-

Fig. 4. The free GPI H8 is co-precipitated with intact Gaa1 but not with a C-terminally truncated Gaa1 variant. HeLa cells were transfected with cDNAs corresponding to D1, D2, or FLAG-PIG-A. Two days post-transfection, the cells were metabolically radiolabeled with [2-3H]mannose for 2 h. Cell lysates were prepared and subjected to immunoprecipitation with anti-FLAG antibodies. Approximately 5% of the immunoprecipitate was taken for SDS-PAGE-immunoblotting with anti-FLAG antibodies (bottom panel), whereas the rest was subjected to lipid extraction. Extracts corresponding to ~95% of the immunoprecipitated material and ~1.25% of the post-IP supernatant were analyzed by TLC. The chromatograms were visualized with a radioactivity scanner. The migration position of the fully characterized GPI moiety H8 is indicated. The cluster of radiolabeled peaks near the top of the chromatogram does not correspond to GPI structures. These peaks are routinely seen in metabolic labeling experiments and remain uncharacterized.

Fig. 5. H8 is co-precipitated with FLAG-Gpi8-containing GPIT complexes. HeLa cells were transfected with cDNA corresponding to FLAG-Gpi8. Two days post-transfection, the cells were metabolically radiolabeled with [2-3H]mannose for 2 h. Cell lysates were prepared and subjected to immunoprecipitation with anti-FLAG antibodies. Approximately 5% of the immunoprecipitate was taken for SDS-PAGE-immunoblotting with anti-FLAG antibodies. Approximately 5% of the immunoprecipitated material and ~1.25% of the post-IP supernatant were analyzed by TLC. The chromatograms were visualized with a radioactivity scanner (the y axis represents arbitrary units; the migration position of H8 is indicated).
PAGE, and immunoblotting. When the crude lysate was treated with Endo H or PNGase F, both Endo H-sensitive and -resistant forms of HA-GPI were observed (Fig. 3C). However, only the Endo H-sensitive form of HA-GPI was found co-immunoprecipitated with D1, indicating a specific interaction of the ER-localized HA-GPI protein precursor with GPIT.

**D2-containing GPIT Complexes Do Not Interact with GPI**—Since D2-containing GPIT complexes comprise all five known transamidase subunits and retain the ability to interact with the proprotein substrate of the enzyme, we considered the possibility that the nonfunctionality of these complexes is due to their inability to interact with GPI. To test this, we transfected HeLa cells with D1, D2, or FLAG-PIG-A (control) and metabolically labeled the cells with [2-3H]mannose to generate radiolabeled GPI lipids in situ. Digitonin extracts of the cells were incubated with anti-FLAG beads, and the IP material and post-IP supernatants were extracted with chloroform/methanol/water (10:10:3 by volume). The organic extracts were dried, partitioned between n-butyl alcohol and water, and the n-butyl alcohol phases were analyzed by TLC (Fig. 4). The TLC profile of radiolabeled lipids (visualized using a radioactivity scanner) in the post-IP supernatants from all transfectants displayed strong peaks corresponding to the phosphethanolamine-containing GPI H8 (Fig. 1) and its lysoderivative as well as a collection of fast migrating peaks that have been previously noted and remain to be characterized. The extent of radioactivity recovered in the lipid extract of all three post-IP supernatants was comparable. However, only the D1 IP sample contained H8, whereas IP material from D2 and FLAG-PIG-A did not contain detectable amounts of the lipid (Fig. 4). All three constructs were similarly expressed and recovered in the IP (Fig. 4, immunoblot), indicating that the variation in GPI recovery was not due to expression level. H8 was not recovered to the same extent as H8, suggestive of a weaker binding interaction.

We confirmed that the interaction we observed was between H8- and D1-containing GPIT complexes rather than between H8- and “free” overexpressed D1 by demonstrating that H8 could be co-immunoprecipitated with FLAG-Gpi8 (Fig. 5), PIG-T-V5, and PIG-U-V5 (data for the latter constructs are not shown). The cumulative data show that H8 can be recovered in association with functional GPIT complexes in digitonin extracts of cells but not with D2-containing GPIT complexes under the same conditions. These results offer an explanation for the nonfunctionality of D2 and point to a role for the last TM segment of Gaa1 in GPI recognition by GPIT.

**A Proline Residue in the Center of the Last TM Segment of Gaa1 Is Required for GPI Recognition by GPIT**—The nonfunctional D2 mutant lacks the last 56 amino acids of Gaa1 that form part of the connecting loop between last two TM domains, the last TM segment itself, and a short luminally exposed C terminus (Fig. 1). ClustalW multiple sequence alignment (22) of the Gaa1 orthologs revealed no significant homology in the loop region and limited, although important, homology in the last TM segment. As seen in Fig. 6, two highly conserved residues (proline and tryptophan) are evident in the more limited alignment shown on the right.

**Fig. 6. Sequence comparisons of the last TM segment of Gaa1 proteins.** The alignment on the left includes *Schizosaccharomyces pombe*, *Leishmania major*, and *Trypanosoma brucei* Gaa1; these are omitted from the alignment shown on the right. Both alignments indicate a conserved proline residue located roughly in the middle of the TM domain and frequently part of a GXXP or GXP motif; an additional conserved residue, tryptophan, is evident in the more limited alignment shown on the right.
the last TM segment. The conserved proline residue is of particular interest. Although proline occurs frequently at either end of TM α-helices, there is a significant occurrence of this “helix-breaking” residue within the transbilayer region (23, 24). Prolines within a GXXP or GXp motif located in the middle of a TM α-helix have been shown, via structural analyses and molecular dynamics simulations, to behave as a molecular hinge capable of dynamically kinking and swiveling the helix (25, 26). Fig. 6 shows that the last TM span of mammalian (mouse, rat, and human), Saccharomyces cerevisiae, Candida albicans, and Caenorhabditis elegans Gaα1 contains a GXXP motif.

To investigate whether the conserved proline and tryptophan residues (Pro609 and Trp611 in human Gaα1) are important for recognition of GPI, we replaced them individually with leucine. We confirmed that both the P609L and the W611L mutants of D1 were incorporated into GPIT complexes by demonstrating that they could be co-precipitated with the other GPIT subunits (not shown). To determine whether these mutations affected the ability of GPIT complexes to bind H8, HeLa cells were separately transfected with cDNAs corresponding to P609L and W611L, metabolically labeled with [2-3H]mannose on day 2 post-transfection, lysed in digitonin, and treated with anti-FLAG beads to immunoprecipitate GPIT complexes containing the mutant proteins. The IP material and post-IP supernatants were subjected to lipid extraction followed by TLC analysis. Fig. 7A shows that despite similar levels of protein expression and GPI radiolabeling, P609L-containing GPIT complexes did not bind H8 under our IP conditions, whereas W611L-containing complexes did. However, P609L-containing GPIT complexes could co-immunoprecipitate HA-GPIT, indicating that the defect specifically concerned recognition of the GPI substrate (Fig. 7B).

To assay the functional capability of the mutants, we separately transfected mouse Gaα1 knockout F9 cells with cDNAs corresponding to D1, P609L, and W611L. F9 cells are unable to express cell surface GPI-anchored Thy-1, and we hypothesized that expression of D1 or W611L in the cells would rescue cell surface Thy-1 expression, whereas expression of P609L would not. Transfection of F9 cells with D1 or W611L cDNA rescued Thy-1 expression as seen by fluorescence-activated cell sorting, whereas transfection with P609L cDNA or an empty vector control did not (data not shown). However, immunoblotting of the same samples showed that whereas D1 and W611L were expressed well, expression of P609L was very poor (data not shown). For reasons that are unclear, the expression level of P609L in F9 cells in several independent attempts was more than 10-fold lower than that of either D1 or W611L, despite all three proteins being comparably expressed in HeLa cells. Thus, whereas we can conclude that both D1 and W611L are functional, the experiments with P609L in F9 cells were uninformative.

Conclusion—The results described in this paper point to a critical role for the Gaα1 subunit in GPI recognition by GPIT. Elimination of the last TM segment of Gaα1 or mutation of a centrally located, conserved proline residue within the last TM segment of Gaα1 abrogates the ability of GPIT to co-immunoprecipitate GPI without affecting the ability of the enzyme to bind its other substrate, the proprotein. The functional importance of the proline residue may be due to its ability to kink and swivel the predicted α-helical structure of the last TM span, forming a dynamic hinge. Proline-induced hinges have been postulated to play a role in the insertion of antimicrobial peptides into membranes and in the gating of ion channels (24–26). In the case of GPIT, we suggest that a proline hinge in Gaα1 could provide a flexible structural accommodation for the GPI moiety, possibly in concert with PIG-U, a protein that has been proposed to be involved in recognizing long fatty acyl chains of GPIs by virtue of its functionally critical fatty acid elongase sequence motif.

The precise role of Gaα1 and/or PIG-U in GPI binding remains to be determined. These subunits may function to present GPI to Gπp, influence GPI binding to Gπp8, or contribute a GPI-binding activator site that communicates allosterically with another GPI binding site in Gπp8. Udenfriend and colleagues (27) have shown that GPI is an obligatory co-substrate for GPIT even when another nucleophile is present to attack the activated carbonyl intermediate formed in the first step of the transamidase reaction. These results support the possibility that Gaα1 and/or PIG-U may represent a regulatory binding site for GPI within GPIT. Evidence of two lipid binding sites has been recently presented for oligosaccharyltransferase, a multisubunit, membrane-bound ER enzyme that, like GPIT, handles both protein and lipid substrates (28). More work needs to be done to investigate these possibilities.

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