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Structural Requirements for the Recruitment of Gaa1 into a Functional Glycosylphosphatidylinositol Transamidase Complex*

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Glycosylphosphatidylinositol (GPI)-anchored proteins are synthesized on membrane-bound ribosomes, translocated across the endoplasmic reticulum membrane, and GPI-anchored by GPI transamidase (GPIT). GPIT is a minimally heterotetrameric membrane protein complex composed of Gaa1, Gpi8, PIG-S and PIG-T. We describe structure-function analyses of Gaa1, the most hydrophobic of the GPIT subunits, with the aim of assigning a functional role to the different sequence domains of the protein. We generated epitope-tagged Gaa1 mutants and analyzed their membrane topology, subcellular distribution, complex-forming capability, and ability to restore GPIT activity in Gaa1-deficient cells. We show that (i) detergent-extracted, Gaa1-containing GPIT complexes sediment unexpectedly rapidly at ~17 S, (ii) Gaa1 is an endoplasmic reticulum-localized membrane glycoprotein with a cytoplasmically oriented N terminus and a lumenally oriented C terminus, (iii) elimination of C-terminal transmembrane segments allows Gaa1 to interact with other GPIT subunits but renders the resulting GPIT complex nonfunctional, (iv) interaction between Gaa1 and other GPIT subunits occurs via the large lumenal domain of Gaa1 located between the first and second transmembrane segments, and (v) the cytoplasmic N terminus of Gaa1 is not required for formation of a functional GPIT complex but may act as a membrane-sorting determinant directing Gaa1 and associated GPIT subunits to an endoplasmic reticulum membrane domain.

Glycosylphosphatidylinositol (GPI)[1]-anchored proteins are synthesized as prepro-proteins with an endoplasmic reticulum (ER)-targeting N-terminal signal sequence and a C-terminal signal sequence that directs the attachment of the GPI anchor (1–5). Upon translocation of the prepro-protein across the ER membrane, the N-terminal signal sequence is removed by signal peptidase resulting in a pro-protein. The pro-protein is then processed by GPI transamidase (GPIT), a novel multisubunit enzyme that removes the C-terminal signal sequence and attaches a GPI molecule to the newly exposed C-terminal amino acid (6–8). The linkage between GPI and protein is an amide bond between the capping ethanolamine residue in the GPI structure and the C-terminal α-carboxylic acid of the protein (9). The GPIT-catalyzed reaction represents the final step in the assembly of a GPI-anchored protein and provides the critical post-translational modification of this class of proteins that allows them to gain entry into ER-derived transport vesicles for delivery to the cell surface (10, 11).

GPIT is a minimally heterotetrameric, membrane-bound protein complex containing the subunits Gpi8 (~45 kDa), Gaa1 (~67 kDa), PIG-S (~62 kDa), and PIG-T (~65 kDa). Gaa1 and Gpi8 were identified through genetic studies in yeast (12, 13), whereas PIG-S and PIG-T were identified more recently through co-immunoprecipitation experiments using epitope-tagged Gpi8 (14, 15). Photocross-linking experiments indicate that the complex may contain at least one other protein with a molecular mass of ~120 kDa that appears not to associate with the other components under immunoprecipitation conditions (16). Gpi8, Gaa1, PIG-S, and PIG-T are all required for transamidase function (12–15, 17, 18). Gpi8 appears to be the likely enzymatic component of the GPIT protein complex because it shares sequence homology with a family of plant vacuolar endopeptidases, one of which catalyzes the transamidation step in the maturation of concanavalin A (13, 19). Gaa1, PIG-S, and PIG-T share no homology with any proteins of known function, and their functional role in GPIT action is unclear.

There is no "soluble" assay for GPIT. Most measures of GPIT activity described thus far require the ER protein translocation apparatus and an intact ER membrane to generate an appropriate pro-protein substrate (2, 20–22). Isolated Gpi8 shows no activity against a variety of protein and peptide substrates (13). Nevertheless, photocross-linking experiments show that Gpi8, Gaa1, a ~120-kDa protein, and possibly PIG-S are in close physical proximity to the pro-protein substrates of GPIT as they undergo processing (16, 23). Other analyses using a mammalian cell-free translation-translocation system indicate that pro-proteins bind Gaa1 in the absence of Gpi8 but that binding of pro-proteins to Gpi8 does not occur in the absence of Gaa1.[2] Although these studies are suggestive, it is difficult to take them further to analyze the physical and functional architecture of GPIT and determine the role of its various constituent subunits.

In an attempt to dissect the GPIT complex from a structure-function perspective, we opted to analyze human Gaa1. Gaa1 is the most conspicuously hydrophobic of the known subunits of GPIT, and the results described above suggest that it may play a key role in substrate recognition. It is predicted to be a multispanning membrane protein of ~67 kDa with seven transmembrane (TM) domains, a large lumenal domain be-

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; GPIT, GPI transamidase; ER, endoplasmic reticulum; PIG, phosphatidylinositol glycan; ST, α2,6-sialyltransferase; TM, transmembrane; PBS, phosphate-buffered saline; Endo H, endoglycosidase H; CHO, Chinese hamster ovary.

[2] R. Chen and M. E. Medof, submitted for publication.

tween the first two TM segments, and a cytoplasmically oriented N terminus bearing a potential ER retrieval signal in the form of a di-arginine motif (24). The TM domains may play a role in recognizing the hydrophobic component of the GPI signal sequence in pro-proteins, whereas the lumenal domain may mediate the association of Gaa1 with the other GPIT subunits and possibly also GPI. Furthermore, the potential ER retrieval signal in Gaa1 may serve to localize the entire human GPIT complex to the ER because no known ER retention/retrieval motifs are evident in any of the other subunits of mammalian GPIT. To explore these possibilities, we created a series of epitope tagged C-terminal deletion variants of human Gaa1, expressed them in mammalian cells, and used them to analyze the role of Gaa1 in GPIT. Our results provide a clear functional delineation of the different sequence domains of Gaa1.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, fetal bovine serum, and penicillin-streptomycin were purchased from Invitrogen (San Diego, CA). Goat serum was purchased from Sigma. Restriction enzymes, DNA modifying enzymes, and DNA polymerase were purchased from MBI Fermentas and New England Biolabs. All of the other chemicals were of reagent grade and were used without further purification.

Antibodies—Anti-Gpi8 and anti-Gaa1 rabbit polyclonal antibodies were generated against *Escherichia coli*-expressed polypeptides corresponding to residues 31–322 of human Gpi8 and residues 60–205 of human Gaa1, respectively. Mouse monoclonal antibodies against the FLAG epitope (an 8-amino acid sequence consisting of DYKDDDDK), the V5 epitope (a 14-amino acid sequence consisting of GKPIPNPLLGLDST), and human calnexin were purchased from Sigma, Invitrogen, and Transduction Laboratories (Lexington, KY), respectively. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse and anti-rabbit IgGs conjugated with Alexa Fluor 568 or Alexa Fluor 488 were from Molecular Probes (Eugene, OR).

Construction of Mammalian Expression Vectors—N-terminally FLAG-tagged human Gaa1 cDNA subcloned into the pME18Sf vector (17) was used as a template to generate Gaa1 truncation mutants by PCR (see Figs. 3*A* and 8*A* for schematic figures of the mutants). For the production of constructs D1–D7, primers were designed so that the sense strand primer TR9 included an *Eco*RI site at the 5′ end for subcloning, and the antisense primers D1–D7 included nontemplated sequences encoding the His_6 epitope tag, a stop codon, and an *Xba*I restriction site at the 3′ end. After *Eco*RI and *Xba*I digestion, the PCR products were ligated to *Eco*RI/*Xba*I-digested pME18Sf vector. The resulting plasmids were named pME/D1–7. The Gaa1 open reading frame encompasses codons 1–621 (D1), 564 (D2), 524 (D3), 483 (D4), 446 (D5), 386 (D6), and 367 (D7). To generate Gaa1 constructs bearing a V5 epitope tag C-terminal to the His_6 sequence, sense GN5 and antisense GAV5 primers with *Bam*HI and *Xba*I sites, respectively, were used for PCR amplification of Gaa1 cDNA from pME18Sf/Gaa1. The PCR product was digested with *Bam*HI/*Xba*I and ligated into a *Bam*HI/*Xba*I pEF6/V5 His vector (Invitrogen). The resulting plasmid was named pEF/D1V5. The D10, D11, and D12 constructs were made by PCR using the sense strand primer D10 containing a *Sal*I site and antisense primers D6, D7, and D1, respectively. PCR-amplified products were digested with *Sal*I/*Xba*I and ligated to *Sal*I/*Xba*I-digested pME18Sf/Gaa1 vector. In the resulting constructs 18 residues at the N terminus of Gaa1 were replaced with Met-FLAG tag epitope, followed by a 3-amino acid (Val-Asp-Arg) linker to the first TM segment.

All substitutions of amino acid codons to Ala within the Gaa1 coding region in constructs pME/D24 (amino acids in positions 241–244 substituted with Ala residues) and pME/D35 (positions 354–358 substituted with Ala residues) were introduced by the primer-mediated mutagenesis method (25). In both cases, pME/D1 was used as the amplification template. PCR products with substitution mutations were digested by *Eco*RI/*Xba*I and subcloned into *Eco*RI/*Xba*I-digested pME18Sf vector.

To replace the N-terminal portion of α2,6-sialyltransferase (ST) with the N-terminal cytoplasmic portion of Gaa1, a PCR fragment was generated from pME18Sf/Gaa1 vector using primers GN5 and GN3. The PCR product thus obtained was digested with *Bam*HI/*Eco*RI and ligated into *Bam*HI/*Eco*RI Iip33-ST-V5-pcDNA 3.1 (a gift from Dr. Karen Colley, University of Illinois). The resulting N19-ST construct includes 19 N-terminal residues of human Gaa1, followed by Glu and Phe, and then the ST transmembrane region, stem region, and catalytic domain, fused with V5 epitope and His_6 sequence.

Cell Culture and Transfection—HeLa cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. The cells were passaged every 3–4 days. Exponentially growing cells were harvested by trypsinization and washed once with cytomix buffer (26). The cells were subsequently resuspended at a density of 1×10^7 cells/ml in the same buffer, and 400 μl of suspension were transferred to a 0.4-cm electroporation cuvette (Invitrogen) on ice. 50 μg of plasmid DNA was added to the suspension in the cuvette and mixed well. The mixture was then exposed to a single electric pulse of 300 V with a capacitance of 1,000 microfarads using an Invitrogen gene pulser system. The cuvette was immediately placed on ice for 10 min, and the cells were then suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and plated onto 100-mm plates. The cells were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h prior to harvesting for biochemical analyses and/or indirect immunofluorescence microscopy.

Immunoprecipitations and Velocity Gradient Sedimentation—Transfected HeLa cells ($1–2 \times 10^7$ cells) were harvested by scraping 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% digitonin or 0.5% Nonidet P-40, and 1× protease inhibitor mixture (Calbiochem, San Diego, CA)), and solubilized on ice for 30 min. The cell lysates were clarified by centrifugation ($10,000 \times g$ for 20 min at 4 °C). S10 supernatants were centrifuged at $100,000 \times g$ for 45 min at 4 °C. To each supernatant fraction, 30 μl of anti-FLAG M2 agarose (Sigma) slurry was added, and the sample was incubated at 4 °C for 4 h with gentle agitation. The agarose beads were pelleted by 15 s of centrifugation at $10,000 \times g$. The samples were washed four times for 5 min each time in 1.5 ml of buffer MSB with 10 mM dithiothreitol. Bound antigen was released from the anti-FLAG M2 agarose beads by incubation with FLAG peptide (200 μg/ml) in MSB buffer. Immunoprecipitated fractions were subjected to centrifugation on a sucrose gradient or directly analyzed by SDS-PAGE, followed by immunoblotting using chemiluminescence reagents (Pierce).

Sucrose gradients (4 ml) were centrifuged at 4 °C for 18 h at $192,000 \times g$, using a Beckman SW50.1 rotor. Following centrifugation, the gradients were fractionated into 350-μl aliquots, and the proteins were detected by SDS-PAGE and immunoblotting. Gradient performance and resolution were evaluated by analyzing standard proteins: bovine serum albumin (65 kDa, 4.2 S), yeast alcohol dehydrogenase (150 kDa, 7.6 S), catalase (250 kDa, 11 S), and ferritin (460 kDa, 17.7 S). The standard proteins were detected by SDS-PAGE/Coomassie staining.

Fluorescence Microscopy—Transiently transfected HeLa cells were plated onto poly-D-lysine-coated glass coverslips and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. After 24 h the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After three more washes with PBS, the plasma membrane was selectively permeabilized with digitonin at 3 μg/ml in cytomix buffer with 0.3 M sucrose for 5 min on ice. Alternatively, plasma membranes as well as intracellular membranes were permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature. After permeabilization, the cells were washed three times with PBS and incubated with 10% goat serum albumin in PBS for 60 min at room temperature to block nonspecific binding. The cells were then incubated for 1 h at room temperature with anti-FLAG monoclonal antibody at 1 μg/ml, anti-V5 monoclonal antibody at 1:500 dilution, or anti-calnexin monoclonal antibody at 1:1000 dilution, after which they were washed three times with PBS. Alexa Fluor 568 or Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500 dilution) was then added, and the cells were incubated for 1 h at room temperature. After four washes with PBS at room temperature, the coverslips were mounted onto glass slides (a drop of Vectashield (Vector Laboratories, Burlingame, CA) was included during mounting of the coverslip to prevent rapid photobleaching of the fluorescent conjugates) and taken for confocal microscopy using a Bio-Rad confocal microscope (type MRC 1000) with optical section chosen at 0.2 μm/section.

In Vitro Translation—*In vitro* translation reactions were performed using TNT Quick Coupled Transcription System (Promega, Madison, WI) as described by the manufacturer.

N-Glycosidase Treatment—Endoglycosidase H (Endo H) from New England Biolabs was used for carbohydrate digestion. The protein samples were denatured by the addition of 0.1 volume of 10× Endo H

denaturation 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 M sodium citrate, pH 5.5, at 25 °C) was added to the denatured sample, followed by incubation with 1 μl of 4,000 units/μl Endo H for 1 h at 37 °C. The samples were analyzed by SDS-PAGE to monitor molecular mass reductions accompanying deglycosylation.

Flow Cytometric and Immunoblotting Analyses—Mouse GAA1 knockout F9 cells (1×10^7) (17) suspended in 400 μl of culture medium (high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum) were electroporated with 25 μg each of plasmids at 500 microfarads and 250 V using a Gene Pulser (Bio-Rad). Two days after electroporation, the cells were stained with biotinylated anti-Thy-1 G7 antibody followed by phycoerythrin-conjugated streptavidin (Biomeda, Foster City, CA) and analyzed by FACScan (Becton Dickinson, San Jose, CA).

GAA1-deficient CHO D9PA91 cells (1×10^7) (27) were electroporated with 25 μg each of plasmids at 960 microfarads and 280V. Two days after the transfection, the cells were harvested, one-thirtieth of the sample was used for flow cytometric analysis, and the rest of the sample was used for immunoblotting. For cytometric analysis the cells were stained with biotinylated anti-CD59 5H8 antibody followed by phycoerythrin-conjugated streptavidin (Biomeda) and analyzed using a FACScan (Becton Dickinson). For immunoblotting analysis the cells were solubilized in 1 ml of buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride for 30 min on ice. The lysates obtained by centrifugation of the cells at 18,000 × *g* for 20 min were incubated with 10 μl of M2 anti-FLAG beads overnight. The precipitates were washed five times with the same buffer without protease inhibitors, heated with sample buffer for SDS-PAGE, and analyzed by immunoblotting using biotinylated M2 anti-FLAG antibody and horseradish peroxidase-conjugated streptavidin (Amersham Biosciences).

SDS-PAGE Analysis and Protein Sequencing—The proteins were resolved by SDS-PAGE using polyacrylamide slab gels made according to the method of Laemmli (28). Protein sequence analysis was performed at the Harvard Microchemistry Facility (golgi.harvard.edu/microchem/) by microcapillary reverse-phase high pressure liquid chromatography nanoelectrospray tandem mass spectrometry on a Finnegan LCQ DECA quadrupole ion trap mass spectrometer.

RESULTS

Gaa1 Is a Component of a ~17 S Protein Complex—The predicted molecular mass of a stoichiometric complex of the four known components of GPIT is ~240 kDa. To assess the size of the native GPIT complex directly, a digitonin extract of HeLa cell microsomes was analyzed by velocity sedimentation on a 5–30% sucrose gradient. Immunoblotting of the gradient fractions with anti-Gpi8 and anti-Gaa1 antibodies (Fig. 1*A*) showed a fast-sedimenting pattern centered roughly around the ~17.7 S (~460 kDa) apo-ferritin standard. A similar analysis of a denaturing SDS extract showed Gpi8 sedimenting at ~4 S, consistent with its ~45-kDa monomeric size. These data indicate that GPIT sediments as a ~17 S complex, larger than expected for a globular protein complex of ~240 kDa. Our results are consistent with recent blue native gel electrophoresis analyses in which yeast Gpi8p was found within high molecular mass complexes in the 430–650-kDa range (15).

The large sedimentation coefficient of the digitonin-extracted GPIT complex may be attributed to a nonglobular shape, bound detergent, the presence of multiple copies of one or more of the known subunits, the presence of additional, hitherto unidentified subunits, and/or interaction with other cellular components (29). We investigated the latter possibilities by expressing FLAG-tagged human Gaa1 in HeLa cells and using anti-FLAG antibodies to immunoprecipitate FLAG-Gaa1 complexes from a digitonin or Nonidet P-40 extract of the cells. Cells transfected with an empty vector were used to assess the specificity of the immunoprecipitation. Fig. 1*B* shows that PIG-S, PIG-T, Gpi8, and α- and β-tubulin are all co-immunoprecipitated with FLAG-Gaa1. The presence of the tubulin isoforms as well as PIG-S and PIG-T was confirmed by nanoelectrospray tandem mass spectrometric analysis of tryptic

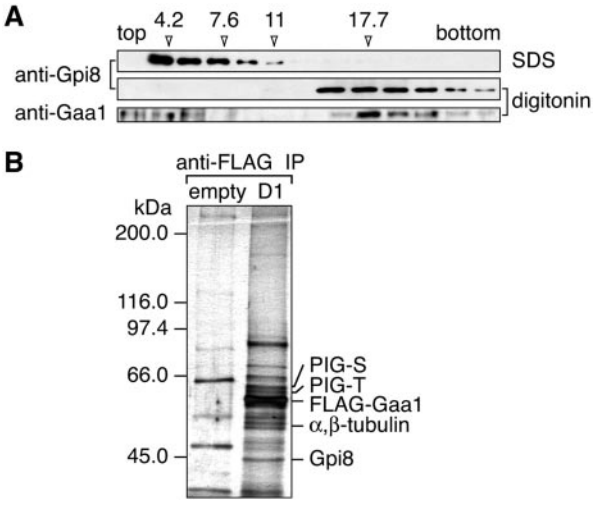

FIG. 1. **GPIT is a large complex sedimenting at ~17 S.** *A*, HeLa membranes were extracted using 0.5% SDS or 1.0% digitonin. The resulting precleared detergent extracts were analyzed by velocity sedimentation on 5–30% sucrose gradients. The fractions were collected, resolved by SDS-PAGE, and immunoblotted with anti-Gpi8 or anti-Gaa1 antibodies. The sedimentation coefficients of standard proteins are shown at the *top*. *B*, identification of proteins co-immunoprecipitated with FLAG-tagged Gaa1. HeLa cells were transiently transfected with an empty expression vector or a plasmid encoding FLAG-tagged Gaa1. Digitonin extracts of transfected cells were immunoprecipitated with anti-FLAG M2 agarose. After elution with FLAG peptide, the samples were resolved on a 7.5% SDS-polyacrylamide gel and visualized by silver stain.

peptides. The presence of tubulin in the immunoprecipitates is discussed below. Several other protein bands were also seen in the FLAG-Gaa1 immunoprecipitate, but these were deemed to be nonspecific because they were either found in control immunoprecipitates or seen to sediment separately from the immunoprecipitated GPIT complex on sucrose velocity gradients (see below). The lower intensity of bands corresponding to PIG-S, PIG-T, and Gpi8 compared with Gaa1 (Fig. 1*B*) is most likely due to the fact that FLAG-Gaa1 is overexpressed, and not all copies of the protein are integrated into GPIT complexes (see Fig. 4 and accompanying text). Other possible explanations are that the complex is not a stoichiometric combination of the four known subunits or that the subunits are not equally well stained with silver.

Membrane Topology of Gaa1—As a prelude to our mutagenesis study of Gaa1, we carried out experiments to verify the ER localization and membrane topology of the protein. Based upon predictive algorithms, Gaa1 is presumed to be an integral membrane protein with 7 TM domains and a large loop between the first and second TM segments. The N terminus of the protein is predicted to be oriented toward the cytoplasm, whereas the C terminus is expected to be oriented toward the ER lumen. Gaa1 constructs containing either an N-terminal FLAG epitope tag or a C-terminal V5 epitope tag were expressed in HeLa cells. Both expressed constructs displayed a reticular distribution pattern characteristic of the ER. The cells were fixed, treated with digitonin to permeabilize the plasma membrane or Triton X-100 to permeabilize all membranes, and labeled with antibodies to the FLAG or V5 epitope tags. As shown in Fig. 2, digitonin-permeabilized cells could be labeled with anti-FLAG antibodies, confirming the cytoplasmic orientation of the Gaa1 N terminus. In contrast, the V5 epitope tag could be labeled only after Triton X-100 permeabilization, implying that the Gaa1 C terminus is sequestered in the lumen of the ER. These data, together with evidence that an asparagine residue within a glycosylation sequon in the loop region of Gaa1 is *N*-glycosylated (data not shown, but see corresponding data

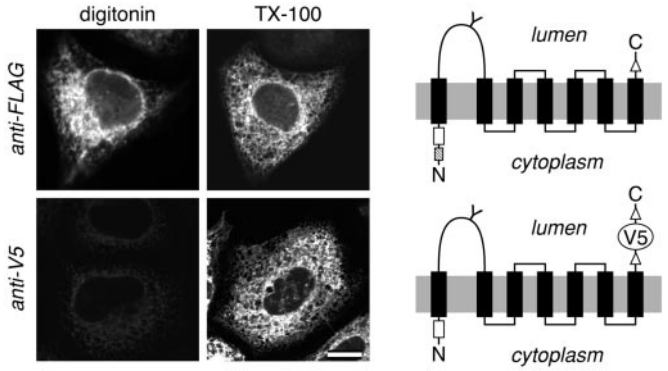

FIG. 2. **Gaa1 is an ER-localized membrane protein with a cytoplasmically oriented N terminus and a lumenally oriented C terminus.** Gaa1 constructs containing an N-terminal FLAG epitope tag or a C-terminal V5 epitope tag were expressed in HeLa cells. Both constructs contain C-terminal His_6 tags (indicated by the *open triangles*). The cells were fixed in 4% paraformaldehyde, selectively permeabilized with digitonin (3 μg/ml) or 0.3% Triton X-100, and stained with anti-FLAG or anti-V5 antibodies followed by Alexa Fluor 568 or Alexa Fluor 488-conjugated secondary antibodies. *Scale bar*, 10 μm.

for truncated Gaa1 variants below), indicate that the predicted topology of the protein is correct.

Gaa1 Variants Containing an Intact Lumenal Loop but Lacking All Except the First Two TM Domains Are Able to Form a Complex with Gpi8, PIG-S, and PIG-T—To identify structural features of Gaa1 required for its interaction with the other known components of GPIT, we created a series of N-terminally FLAG-tagged truncation mutants (Fig. 3*A*) in which we systematically removed six of the seven predicted membrane-spanning segments from the C terminus of Gaa1. These constructs (termed D2–D7), as well as similarly tagged full-length Gaa1 (termed D1), were transiently expressed in HeLa cells. The cells were extracted with Nonidet P-40, and the detergent extracts were incubated with anti-FLAG-agarose beads to immunoprecipitate the Gaa1 constructs and any associated proteins. Immunoblotting with anti-FLAG antibodies indicated that all constructs were well expressed, with the possible exception of D2 and D5, which were expressed at lower levels compared with D1 (Fig. 3*B*, *upper panel*). Immunoblotting with anti-Gpi8 antibodies indicated that Gpi8 was immunoprecipitated with all of the Gaa1 constructs except D7, a construct representing only the first N-terminal TM domain and lumenal loop (Fig. 3*B*, *lower panel*). Silver staining of SDS-PAGE-resolved immunoprecipitates showed that PIG-S and PIG-T were also co-immunoprecipitated with all the truncated constructs except D7 (data for D6 and D7 are shown in Fig. 3*C*). These results indicate that the five C-terminal TM domains of Gaa1 are not required for its interaction with the other GPIT subunits.

Sedimentation Behavior of Truncated Gaa1 Variants—We used velocity sedimentation analyses to investigate the possibility that truncated Gaa1 variants are incorporated into subcomplexes of GPIT corresponding to only two or three of the four known subunits. The constructs D1, D6, and D7 were transiently expressed in HeLa cells, immunoprecipitated from detergent extracts of the cells with anti-FLAG M2 agarose, and eluted from the resin with FLAG peptide. The eluates were loaded onto a sucrose gradient (5–30%) and centrifuged; gradient fractions were collected and analyzed by immunoblotting with anti-FLAG antibodies as well as with anti-Gpi8 (Fig. 4). The three constructs showed clearly distinct sedimentation behavior. The D7 distribution peaked toward the top of the gradient, consistent with the inability of D7 to interact with other GPIT subunits. Both D1 and D6 yielded a diffuse distribution spanning two-thirds of the sedimentation range covered by the gradient.

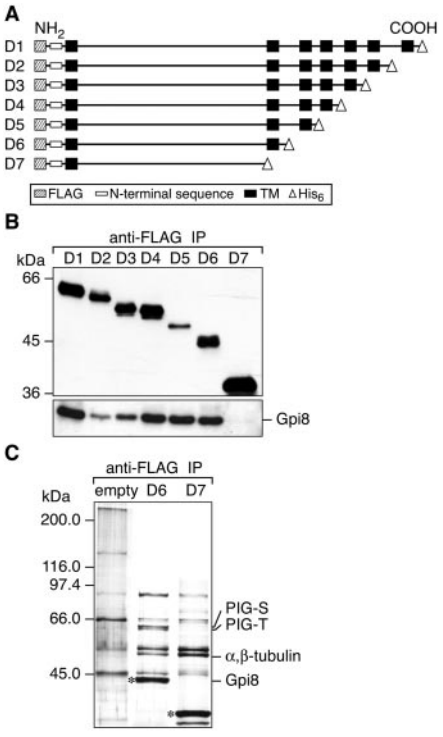

FIG. 3. **Truncated Gaa1 containing only one TM domain and lumenal loop binds Gpi8.** *A*, schematic of the epitope-tagged human Gaa1 truncation constructs used in this study. *Black boxes*, membrane-spanning segments; *hatched boxes*, FLAG tag; *open boxes*, cytoplasmically oriented N-terminal region of Gaa1; *open triangles*, His_6 tag. *B*, co-immunoprecipitation of Gpi8 with expressed Gaa1 constructs. HeLa cells were transiently transfected with expression vectors encoding the FLAG-tagged Gaa1 constructs D1-D7. Digitonin extracts of transfected cells were subjected to immunoprecipitation with anti-FLAG M2 affinity gel. After elution with FLAG peptide, the samples were resolved by SDS-PAGE and immunoblotted with anti-FLAG or anti-Gpi8 antibodies. *C*, analysis of proteins co-immunoprecipitated with D6 or D7. Digitonin extracts of transiently transfected cells were subjected to immunoprecipitation with anti-FLAG M2 affinity gel (cells transfected with an empty vector were similarly processed). The anti-FLAG-bound material was eluted with FLAG peptide, analyzed by SDS-PAGE on a 7.5% gel, and stained with silver.

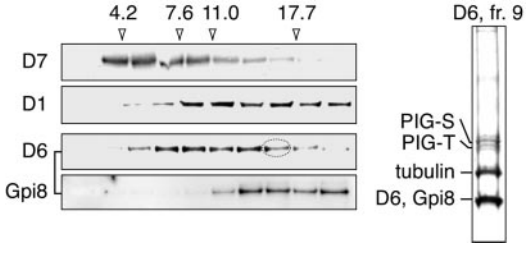

FIG. 4. **Sedimentation behavior of Gaa1 and truncated variants.** HeLa cells were transfected with expression vectors encoding the Gaa1 constructs D1, D6, and D7. Digitonin extracts of the cells were immunoprecipitated with anti-FLAG M2 affinity gel. The anti-FLAG-bound material was eluted with FLAG peptide and analyzed by sucrose gradient centrifugation. The fractions were analyzed by SDS-PAGE and immunoblotted with anti-FLAG and/or anti-Gpi8. The anti-Gpi8 blot shown in the *bottom panel* corresponds to the D6 gradient immediately above it. An SDS-PAGE/silver-stained profile of fraction 9 (indicated by a *dotted circle*) from the gradient analysis of D6 is shown in the *right panel*.

Immunoblotting revealed that only fractions corresponding to the more rapidly sedimenting material (≥11 S) in the D1 and D6 gradients were positive for Gpi8 (Fig. 4). Despite the overlapping but distinct sedimentation behavior of D1 and D6, the

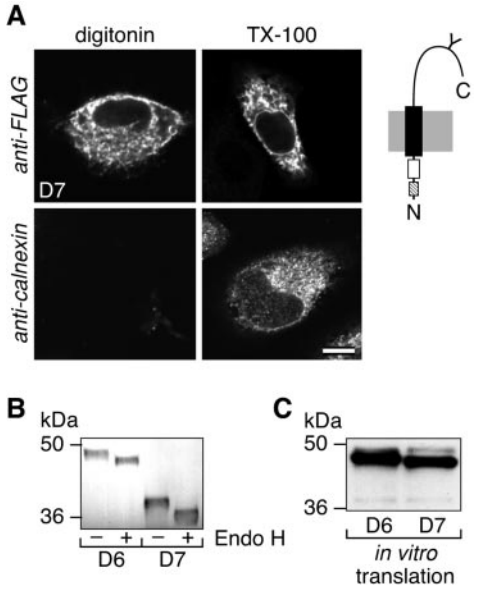

FIG. 5. **Subcellular localization and membrane topology of D7.** *A*, HeLa cells expressing D7 were fixed in 4% paraformaldehyde, selectively permeabilized with digitonin (3 μg/ml) or 0.3% Triton X-100, and stained with anti-FLAG or anti-calnexin antibodies followed by Alexa Fluor 568-conjugated secondary antibodies. *Scale bar*, 10 μm. *B*, endoglycosidase H treatment of *in vivo* expressed D6 and D7. *C*, *in vitro* translated D6 and D7. The proteins in *B* and *C* were visualized by SDS-PAGE and immunoblotting.

sedimentation profiles of co-immunoprecipitated Gpi8 were identical in the two samples (Fig. 4 and data not shown). The Gpi8-positive fractions were also positive for PIG-S, PIG-T, and tubulin as determined by SDS-PAGE and silver staining (Fig. 4). We could not identify fractions containing putative subcomplexes of Gaa1 with only one or two of the other GPIT subunits; the gradient fractions contained either D1 or D6 alone (<11 S) or D1 or D6 together with Gpi8, PIG-S, and PIG-T (>11 S). We also saw no evidence (via immunoblotting with anti-Gaa1) of endogenous Gaa1 participating in complexes containing epitope-tagged Gaa1 variants, suggesting that GPIT does not contain more than one copy of Gaa1/complex. The slightly slower sedimentation of the immunoprecipitated GPIT complexes (Gpi8/PIG-S/PIG-T-positive fractions) in comparison with that of the endogenous GPIT complex (Fig. 1) may be partly explained by the loss of a putative weakly interacting subunit during immunoprecipitation (see "Discussion"). The diffuse sedimentation profile of D1 and D6 is possibly a result of overexpression, resulting in the formation of small complexes between the overexpressed constructs and possibly ER chaperones (in the ~4–11 S range), as well as larger complexes involving GPIT components (>11 S; Fig. 4).

In Situ Proteolysis of the Lumenal Loop Accounts for the Inability of D7 to Interact with Other GPIT Subunits—The inability of D7 to interact with other GPIT subunits may be due to an absolute requirement for the second TM domain (present in the D6 construct, which interacts with the other GPIT subunits, but absent in D7), mislocalization of the protein, incorrect membrane topology, or differences in post-translational modification when compared with the other Gaa1 variants.

To evaluate these possibilities, D7 was transiently expressed in HeLa cells, and its subcellular distribution was inspected by indirect immunofluorescence microscopy. Fig. 5*A* shows that D7 displays an ER reticular staining pattern similar to that seen for full-length Gaa1 (Fig. 2). Because D7 does not interact with other GPIT subunits, this result indicates that it must independently contain the requisite targeting information for ER localization. The membrane topology of D7 was determined by assaying the orientation of the N-terminal FLAG tag with anti-FLAG antibody in permeable (Triton X-100-treated) and semipermeable (digitonin-treated) cells. Staining with antibodies against the ER lumen marker calnexin (30) served as a control for the integrity of ER membranes in digitonin-treated cells. The N-terminal FLAG epitope tag could be detected by antibody staining in both semi-permeabilized and permeabilized cells (Fig. 5*A*, *upper panels*), whereas calnexin could only be labeled in permeabilized cells (Fig. 5*A*, *lower panels*). These data indicate that D7 displays the same N-terminal membrane topology as full-length Gaa1. As an additional confirmation of membrane topology, we assayed the status of the single *N*-glycosylation site in the loop region of D6 and D7; endoglycosidase H treatment of D6 and D7 immunoprecipitated from HeLa cell extracts caused a shift to a lower molecular mass, indicating that both proteins were modified by high mannose *N*-glycans (Fig. 5*B*) and that the loop region containing the *N*-glycosylation sequon was oriented toward the ER lumen. Thus, the inability of D7 to interact with Gpi8, PIG-S, and PIG-T is not due to mislocalization or incorrect membrane topology but rather due to loss of a structural determinant(s) responsible for promoting interactions with the other GPIT subunits.

Analysis of the mobility of D6 *versus* D7 on SDS-PAGE indicated that although the two constructs differ by only 20 amino acids, D7 migrates substantially faster than D6, corresponding to a molecular mass difference of ~8 kDa. This observation raises the possibility that D7 is proteolytically processed in the cell in such a way that it is rendered unable to interact with other GPIT subunits. To test this, we translated both D6 and D7 *in vitro* and checked their mobility on SDS-PAGE. Fig. 5*C* shows that both the *in vitro* translated proteins run at ~43 kDa, with D7 migrating only slightly faster than D6 as expected for a 20-amino acid difference in sequence. Comparison of *in vitro* translated *versus* HeLa-expressed D7 indicates that a C-terminal segment is clipped from the lumenal loop of HeLa-expressed D7, leaving the *N*-glycosylation site intact. This proteolytic processing appears to be responsible for the inability of D7 to interact with other GPIT subunits.

The Lumenal Loop Mediates the Interaction of Gaa1 with Gpi8 and Other GPIT Subunits—The ability of severely truncated Gaa1 variants such as D6 to interact with other GPIT subunits suggests that the lumenal loop region between the first two TM domains may contain the relevant interaction domain. Loss of such a domain by proteolytic processing in the D7 construct prevents assembly of D7 into complexes containing the other subunits. Alignment of Gaa1 protein sequences from human, mouse, yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Leishmania major* revealed several short stretches of amino acids that are conserved in the lumenal loop of Gaa1 in all species. Two of the conserved stretches (amino acids 235–250 and 346–361 of human Gaa1) are located between the *N*-glycosylation site and second TM domain, *i.e.* the region that is proteolytically removed in D7. To investigate whether these regions are important for the interaction of Gaa1 with other GPIT components, we replaced the amino acids in these conserved sites with alanines. Gaa1 D24 and D35 constructs (amino acids substituted to Ala in positions 241–244 and 354–358, respectively) were transiently expressed in HeLa cells and immunoprecipitated with anti-FLAG M2-agarose as described above, followed by immunoblotting analysis using anti-FLAG and anti-Gpi8 antibodies. The D24 construct interacts with Gpi8, PIG-S, and PIG-T at a significantly reduced level compared with wild-type (Fig. 6 and data not shown), suggesting that the mutated site is structurally important but not crucial for the interaction of Gaa1 with other GPIT sub-

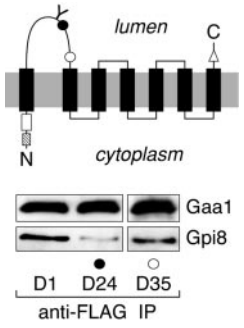

FIG. 6. **A conserved sequence in the Gaa1 lumenal loop influences the interaction of Gaa1 with Gpi8.** FLAG-tagged Gaa1 D24 and D35 constructs (amino acids in positions 241–244 and 354–358 of human Gaa1, respectively, substituted with alanines) were transiently expressed in HeLa cells and immunoprecipitated with anti-FLAG M2-agarose, followed by immunoblotting analysis using anti-FLAG and anti-Gpi8 antibodies.

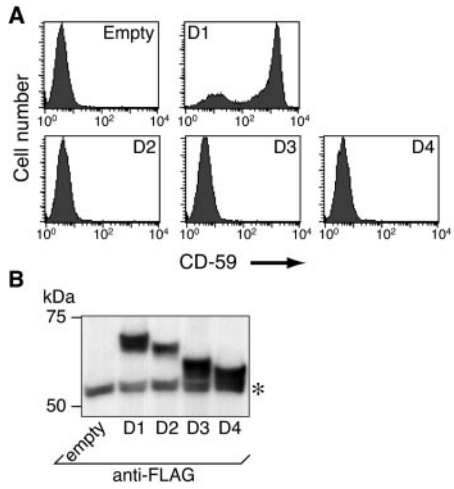

FIG. 7. **Elimination of only one C-terminal TM domain of Gaa1 renders the resulting GPIT complex nonfunctional.** *A*, CHO GAA1-deficient cells transfected with an empty vector or Gaa1 constructs D1, D2, D3, or D4 were analyzed 2 days after transfection for surface expression of GPI-anchored CD59 by flow cytometry. *B*, expression of Gaa1 constructs in the transfectants used in *A*. FLAG-tagged Gaa1 variants in detergent extracts of the transfectants were collected with anti-FLAG beads and analyzed by immunoblotting with anti-FLAG antibodies. The *band* marked with an *asterisk* is unrelated to the constructs and can also be seen in cells transfected with an empty vector.

units. Substitution of the five amino acids near the second TM domain D35 did not affect the co-immunoprecipitation of Gpi8, PIG-S, and PIG-T (Fig. 6 and data not shown).

GPIT Complexes Containing Truncated Gaa1 Variants Are Nonfunctional—The results presented above indicate that C-terminally truncated Gaa1 variants with intact lumenal domains are able to interact with Gpi8, PIG-S, and PIG-T to yield GPIT complexes similar in sedimentation behavior to endogenous GPIT. To assess whether the GPIT complexes containing truncated Gaa1 variants are functional, we expressed the Gaa1 variants D1–D7 in mouse F9 embryonal carcinoma cells in which chromosomal copies of the GAA1 gene had been disrupted by homologous recombination. The GAA1 knockout F9 cells were previously shown not to express cell surface GPI-anchored proteins, including Thy-1, except when transfected with Gaa1 cDNA (17). We used fluorescence-activated cell sorter analysis to test surface expression of Thy-1 in cells transfected with the various Gaa1 constructs, in conjunction with immunoblotting to assess the expression level of the individual constructs. With the exception of D2 and D3, all of the constructs were well expressed in the GAA1 knockout F9 cells, but only the D1 construct was able to restore surface expression of Thy-1 (data not shown). Because the expression of D3 was poor, and virtually no D2 was detected in GAA1 knockout F9 cells, we next tested whether these constructs were able to restore surface expression of GPI-anchored CD59 in GAA1-deficient CHO cells (27). Although immunoblotting analysis showed that D2 and D3 were well expressed (at levels comparable with that of D1) in the mutant CHO cells, they were unable to restore CD59 expression (Fig. 7). These data indicate (i) that epitope tagging does not affect the function of Gaa1 and (ii) that elimination of even the C-terminal TM domain of Gaa1 renders the resulting GPIT complex nonfunctional.

Deletion of the Cytoplasmically Oriented N-terminal Region of Gaa1 Blocks Proteolytic Processing of D7 and Eliminates Interaction of Gaa1 Variants with Tubulin—The observation that the severely truncated D7 construct is retained in the ER without integration into a GPIT complex suggests that it contains information for ER localization. The cytoplasmically oriented N terminus of Gaa1 (^{1}MGLLSDPVRRRALARLVLR) contains an arginine repeat motif (underlined) that may act as an ER retention/retrieval signal (24, 31, 32). This N-terminal region may also be responsible for the specific interaction and co-precipitation of α- and β-tubulin with Gaa1 constructs, including D7 (Figs. 1*B* and 3*C*). To assess the function of this sequence, we generated constructs D10, D11, and D12, which correspond to D6, D7, and D1, respectively, with the N-terminal sequence removed (Fig. 8*A*).

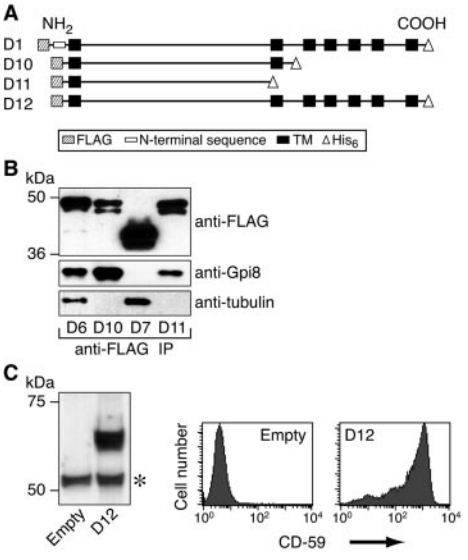

FIG. 8. **Analyses of Gaa1 variants lacking the cytoplasmically oriented N-terminal region.** *A*, schematic of the epitope-tagged, N-terminally deleted human Gaa1 truncation constructs used in this study. The full-length, nontruncated D1 construct is shown for comparison. *Black boxes*, membrane-spanning segments; *hatched boxes*, FLAG tag; *open boxes*, cytoplasmically oriented N-terminal region of Gaa1; *open triangles*, His_6 tag. *B*, SDS-PAGE and immunoblotting analysis of Gaa1 constructs expressed in HeLa cells. D6 and D7 (see Fig. 3*A*) have an intact N terminus; D10 and D11 are N-terminally deleted versions of D6 and D7. Immunoblots were carried out with anti-FLAG antibodies (*top panel*), anti-Gpi8 antibodies (*middle panel*), and anti-α-tubulin antibodies (*lower panel*). *C*, GAA1-deficient CHO cells transfected with an empty vector or the Gaa1 construct D12 were analyzed 2 days after transfection for surface expression of GPI-anchored CD59 by flow cytometry. Expression of D12 was monitored by immunoprecipitation and immunoblotting with anti-FLAG antibodies as in Fig. 7. The *band* marked with an *asterisk* is nonspecific.

Transient expression of D10 and D11 in HeLa cells followed by immunoprecipitation from detergent extracts of the cells showed that both constructs were able to interact with the other GPIT subunits but not with tubulin (Fig. 8*B*). Consistent

with this observation, D11 displayed only a minor sign of the proteolytic clipping seen in its parent construct D7, suggesting that removal of the N-terminal sequence may have prevented access of D11 to an ER membrane domain or other subcellular compartment containing the protease responsible for modifying D7. The data also confirm that proteolysis of D7 occurs in the cell rather than during sample work-up (in the latter case D11 would also have been expected to be proteolytically clipped).

Similar to results obtained with other C-terminally truncated Gaa1 constructs (Fig. 7), transfection of D10 and D11 into F9 Gaa1 knockout cells did not restore surface expression of GPI-anchored Thy-1, although both constructs were well expressed (data not shown). However, D12 was capable of restoring surface expression of CD59 in GAA1-deficient CHO cells at levels comparable with that seen with the full-length construct D1 (Fig. 8*C*). This suggests that elimination of the putative membrane-sorting/tubulin-binding motif at the N terminus of Gaa1 does not affect its ability to form a functional GPIT complex.

Removal of the N-terminal sequence in D7 to create the D11 construct largely abrogates but does not eliminate the lumenal proteolytic clipping event that causes D7 to lose its ability to interact with other GPIT components. Indeed, the low level of proteolysis seen with D11 is also seen with all Gaa1 constructs (including the full-length D1 construct), giving rise in each case to an N-terminal fragment of the same size (~37 kDa) as proteolyzed D7 (data not shown). These data suggest that Gaa1 constructs are processed via a single endoproteolytic cleavage event. The low yield of the fragment for all constructs, except D7, with an intact N terminus may be explained by suggesting that removal of the second TM domain makes the cleavage site in D7 more accessible to the protease. An alternative possibility is that Gaa1 constructs that are not included in GPIT complexes, *e.g.* D7 and the pool of each of the other constructs that sediments in Gpi8-negative fractions at <11 S (Fig. 4), are more likely to be targets for the protease. We think that this is unlikely because the yield of the proteolytic fragment is not proportional to the pool size of material excluded from GPIT complexes. Based on these results we speculate that the N-terminal sequence contains targeting information that causes Gaa1 to be transported to a subcellular compartment, likely a membrane domain within the ER, that contains the endoprotease responsible for clipping the lumenal loop.

Analysis of the Role of the Cytoplasmically Oriented N Terminus of Gaa1 as a Potential ER Sorting Signal—Indirect immunofluorescence microscopy showed that the subcellular distribution of D10 and D11 was dramatically different from that of the parent constructs D6 and D7 (Fig. 9, *upper panels*). The N-terminally deleted constructs appeared in a thick, perinuclear pattern suggestive of a collapse of the ER around the nucleus and away from the cell periphery. Identical results were obtained with D12 (data not shown). These data indicate that the N terminus of Gaa1 exerts a dramatic influence on the subcellular distribution of the protein and possibly also on the structure of the ER. The altered distribution and effect on cell morphology correlates strongly with the inability of these constructs to interact with tubulin. Further investigation of this unusual effect is underway.

We considered the possibility that the N terminus of Gaa1 acts as a sorting signal involved either in maintaining the ER localization of Gaa1 or in recruiting Gaa1 to an ER membrane domain. The latter possibility is suggested by the lack of proteolysis of D11 compared with D7. To test these ideas we generated N19-ST, a V5-epitope tagged variant of ST, a Golgi-localized type II membrane protein (33), modified with the 19-amino acid Gaa1 N terminus. Fig. 9 (*lower panels*) shows

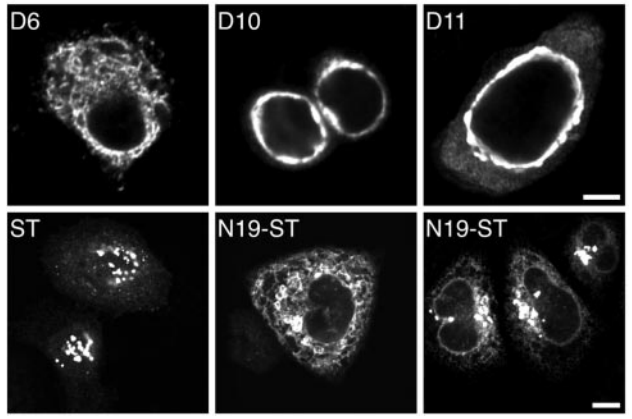

FIG. 9. **Analyses of the role of the N-terminal 18 amino acids of Gaa1 as a membrane sorting determinant.** *Upper panels*, localization of D6 (construct containing the cytoplasmically oriented N-terminal region of Gaa1), D10, and D11 (constructs lacking the N terminus of Gaa1). HeLa cells (24 h post-transfection) were fixed, permeabilized, and stained with anti-FLAG M2 and Alexa Fluor 568-conjugated anti-mouse Ig. *Scale bar*, 10 μm. *Lower panels*, non-Golgi distribution of a fusion protein consisting of ST fused with the cytoplasmic N terminus of Gaa1. HeLa cells transiently expressing either V5-tagged ST or the V5-tagged fusion protein N19-ST were fixed after 24 h of expression, permeabilized with 0.3% Triton X-100, incubated with anti-V5 primary antibody, and labeled with Alexa Fluor 488-conjugated anti-mouse Ig. *Scale bar*, 10 μm.

that ST is distributed as shown previously, in a compact collection of perinuclear fluorescence structures encompassing the Golgi apparatus. However, N19-ST displays a widely heterogeneous subcellular redistribution ranging from perinuclear Golgi staining to a distorted ER reticular distribution. The data clearly indicate that the Gaa1 N terminus has the ability to alter the compact Golgi localization of ST.

DISCUSSION

We describe structure-function analyses of Gaa1, the most hydrophobic of the GPIT subunits. Our approach involved analyzing the membrane topology, subcellular distribution, complex-forming ability, and functional capacity of various epitope-tagged human Gaa1 mutants to assign a role to the different sequence domains of the protein. Our main results are that (i) Gaa1 is a component of a rapidly sedimenting ~17 S protein complex that is larger than anticipated for a stoichiometric combination of the four known GPIT subunits, (ii) Gaa1 is a multispanning, ER-localized membrane glycoprotein with a cytoplasmically oriented N terminus and a lumenally oriented C terminus (implying an odd number of TM domains), (iii) truncation of C-terminal TM segments allows Gaa1 to interact with other GPIT subunits but renders the resulting GPIT complex nonfunctional, (iv) interaction between Gaa1 and other GPIT subunits occurs via the large lumenal domain of Gaa1 located between the first and second TM segments, and (v) the cytoplasmic N terminus of Gaa1 is not required for formation of a functional GPIT complex but may act as a membrane-sorting determinant directing Gaa1 and associated GPIT subunits to an ER membrane domain.

The precise function of Gaa1 in the GPIT complex is unknown. There may exist a functional role for the TM segments of Gaa1 in recognizing or stabilizing the hydrophobic segment of the GPI signal sequence in pro-proteins, possibly presenting the GPI signal sequence to Gpi8, and/or recognizing or positioning the GPI moiety. These ideas stem from the facts that (i) Gpi8 does not have to be directly membrane-anchored for function (17, 34, 35), (ii) introduction of charged residues in the hydrophobic region of the GPI attachment signal peptide dra-

matically reduces the efficiency of GPI anchoring, whereas substitution of any hydrophobic amino acid with another hydrophobic amino acid has no effect (36), and (iii) co-immunoprecipitation of pro-proteins with Gpi8 requires Gaa1.[2] These observations suggest that the hydrophobic transmembrane regions of GPIT components such as Gaa1 may recognize the GPI signal peptide. Functional analysis of GPIT complexes containing different Gaa1 truncated variants reveals that the deletion of even one C-terminal TM segment makes GPIT nonfunctional while allowing apparently normal complex formation. Poor homology of the C-terminal primary amino acid sequence between Gaa1 orthologues from different species and the similar organization of the membrane spanning segments in the C-terminal part of the proteins suggest that the structure and position of hydrophobic regions, rather than the specific sites between them, are important for recognition of the substrate(s).

How is the human GPIT complex retained in the ER? Yeast Gaa1p and Gpi16p (PIG-T) have dilysine motifs near their C terminus that likely serve as ER retrieval signals for the individual proteins as well as for the GPIT complex. No such motifs are present in any of the mammalian subunits, except for an arginine repeat motif in the cytoplasmically oriented N-terminal region of Gaa1. Deletion of the N-terminal region of Gaa1 dramatically altered the localization of the protein from a reticular network distribution to a collapsed distribution encircling the nucleus. Co-expressed epitope-tagged PIG-S and PIG-T were completely co-localized to the perinuclear structure with N-terminal Gaa1 deletants (data not shown), indicating that variations in Gaa1 localization influenced the subcellular distribution of other GPIT components and that Gaa1 may be responsible for GPIT retention in ER. Fusion of the Gaa1 N-terminal sequence with normally Golgi-localized α2,6-sialyltransferase shifted a significant fraction of the fusion protein from the Golgi to ER. The Gaa1 N-terminal sequence could not completely override the Golgi sorting signal present in ST, resulting in partial retention of the fusion protein in the Golgi. A similar shift in subcellular distribution was reported previously when the diarginine motif-containing cytoplasmic tail of the human invariant chain Iip33, a type II membrane protein, was fused to the N terminus of ST (33).

Our results suggest that the N terminus of Gaa1 may act not only as a simple ER sorting sequence but also as a subcompartmental targeting determinant within the ER. The proteolytic cleavage of D7, but not N-terminally truncated D11, suggests that cleavage of the expressed protein occurs within a specific subcompartment of the ER. Interestingly, a small amount of the same proteolytic product can be observed with all expressed Gaa1 variants; the function and mechanism of this cleavage is unknown. The identity of the ER subcompartment containing the endoprotease is a matter of speculation. It may be the mitochondria-associated ER membrane that was previously demonstrated to be enriched in GPI biosynthetic activities (37) and possibly to contain the protease responsible for cleaving the pro-form of the human serum protein haptoglobin (38).

Surprisingly, the overexpression of N-terminally truncated Gaa1 mutants induced considerable morphological changes in HeLa cells, whereas overexpression of Gaa1 variants with an intact N terminus did not cause any morphological alterations. Although more work needs to be done to understand this phenomenon at the mechanistic level, it is likely that it is related to the inability of the N-terminally truncated Gaa1 constructs to interact with tubulin and, by implication, with microtubules. The presence of tubulin in immunoprecipitates involving FLAG-tagged Gaa1 constructs is clearly due to a specific interaction requiring the presence of the cytoplasmically oriented N terminus of Gaa1. Similar results have been previously reported where overexpressed proteins with an arginine repeat motif-containing cytoplasmic N-terminal sequence have been shown to associate with microtubules and to display a microtubule cytoskeleton-like distribution (32, 39, 40). Further experiments will be necessary to define the nature and function of the interaction between tubulin and Gaa1, as well as to understand the basis for the morphological alterations caused upon removal of the Gaa1 N-terminal region.

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