Endoplasmic Reticulum Localization of Gaa1 and PIG-T, Subunits of the Glycosylphosphatidylinositol Transamidase Complex*

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After integration into the endoplasmic reticulum (ER) membrane, ER-resident membrane proteins must be segregated from proteins that are exported to post-ER compartments. Here we analyze how human Gaa1 and PIG-T, two of the five subunits of the ER-localized glycosylphosphatidylinositol transamidase complex, are retained in the ER. Neither protein contains a known ER localization signal. Gaa1 is a polytopic membrane glycoprotein with a cytoplasmic N terminus and a large luminal loop between its first two transmembrane spans; PIG-T is a type I membrane glycoprotein. To simplify our analyses, we studied Gaa1 and PIG-T constructs that could not interact with other subunits of the transamidase. We now show that Gaa1<sub>282</sub>, a truncated protein consisting of the first TM domain and luminal loop of Gaa1, is correctly oriented, N-glycosylated, and ER-localized. Removal of a potential ER localization signal in the form of a triple arginine cluster near the N terminus of Gaa1 or Gaa1<sub>282</sub> had no effect on ER localization. Fusion proteins consisting of different elements of Gaa1<sub>282</sub> appended to α<sub>2,6</sub>-sialyltransferase or transferrin receptor could exit the ER, indicating that Gaa1<sub>282</sub> and by implication Gaa1, does not contain any dominant ER-sorting determinants. The data suggest that Gaa1 is passively retained in the ER by a signalless mechanism. In contrast, similar analyses of PIG-T revealed that it is ER-localized because of information in its transmembrane span; fusion of the PIG-T transmembrane span to Tac antigen, a plasma membrane-localized protein, caused the fusion protein to remain in the ER. These data are discussed in the context of models that have been proposed to account for retention of ER membrane proteins.

The mechanism(s) by which ER<sup>1</sup>-resident proteins are sorted from those destined for post-ER compartments is a subject of ongoing debate (1–5). Possible scenarios to explain ER retention of proteins include trapping of ER residents within a matrix or oligomeric structure, signal-mediated retention, lack of an export signal, and signal-mediated retrieval from post-ER compartments. These scenarios must be viewed in the context of bulk anterograde membrane flow from the ER that has been hypothesized to nonselectively carry membrane and fluid content to post-ER compartments (6, 7). The two best characterized ER localization signals, the KDEL and dilysine motifs, are based on retrieval mechanisms that involve direct or indirect binding of the motif to the COPI coats of vesicles mediating retrograde transport from the Golgi to the ER. The KDEL motif is found on soluble proteins that reside within the ER lumen (8–11); the cytoplasmically exposed dilysine motif serves a similar function for ER-resident membrane proteins (12, 13). However, only a small fraction of any given type of molecule bearing a KDEL or dilysine motif appears to exit the ER and needs to be retrieved; the majority appear not to leave the ER at all. The question remains. How do ER resident proteins remain resident in the ER?

It is possible that many ER resident proteins are components of hetero- or homo-oligomeric complexes that in turn interact with other complexes to yield an aggregate that is too large to export efficiently. Consistent with this are observations of the slow lateral diffusion of oligosaccharyltransferase complexes that associate with the protein translocon (14). Alternatively, some ER residents, like ER chaperones, may be continuously engaged with protein traffic entering the ER such that their own exit is impeded. These interactions or the numerous quasi-interactions that are probably promoted by the high protein density in the ER may be sufficient to retain proteins if the rate of bulk flow of membrane and fluid departing the ER is low and if the proteins lack explicit export signals. We classify these possibilities under the heading of “signalless” retention mechanisms.

In contrast, it is possible to consider retention resulting from an explicit signal within the retained protein. For the particular case of ER membrane proteins, it is conceivable that sequence motifs in one or more of the topological domains of the protein (cytoplasmic, transmembrane (TM), or luminal) interact specifically with effectors to block exit of the protein from the ER. However, many ER membrane proteins appear to enjoy relatively unencumbered lateral diffusion in the membrane, suggesting that their presumed exclusion from ER-derived transport vesicles is not due to immobility in the membrane resulting from, for example, cytoskeletal association or large complex formation (15, 16). The few experimentally characterized ER retention motifs, such as cytoplasmic N-terminal and internal diarginine sequences, cytoplasmic C-terminal tyrosine-based sequences, or transmembrane domain sequences, are not universal and or share no sequence similarity between other proteins (1, 17–23). The mechanism by which these motifs promote retention is unknown.

Many ER-translocated proteins are covalently modified with a glycosylphosphatidylinositol (GPI) anchor (24). GPI anchor attachment is catalyzed by GPI transamidase (GPT), a membrane-bound protein complex composed of at least five sub-
units: Gpi8, Gaa1, PIG-S, PIG-T, and PIG-U (25–30). GPIT removes a C-terminal GPI-directing signal peptide from the translocated protein and attaches a preassembled GPI anchor via an amide linkage to the newly exposed C-terminal amino acid residue of the protein (31, 32). Gpi8 is presumed to form the catalytic center of GPIT (33, 34), but all subunits are essential for function. Recent data suggest that Gaa1 and possibly PIG-U may play a role in recruiting GPI to GPIT (28, 35), and it has been proposed that a β-propeller structure in the luminal domain of PIG-T gates access to the active site of Gpi8 (36). The role of PIG-S is unknown. Gpi8 and PIG-T are type I transmembrane proteins; the other three subunits have multiple transmembrane spans. Recent studies show that human Gaa1 has a cytoplasmically oriented N terminus and spans the ER membrane seven times (37); the membrane topology of PIG-S and PIG-U remains to be experimentally determined.

We are interested in understanding how the GPIT complex is assembled into a functional unit that is localized to the ER membrane. The individual subunits are presumably independently synthesized and integrated into the ER membrane and must remain in the ER long enough to encounter the other subunits in order to form the GPIT complex. The complex itself, after assembly, must remain in the ER in order to execute its catalytic function. Thus, individual subunits as well as the GPIT complex as a whole must be able to avoid entering the secretory pathway and exiting the ER.

To identify the mechanism(s) by which the subunits of GPIT are localized to the ER, we chose initially to analyze Gaa1 and PIG-T. We used fluorescence microscopy and glycosidase digestion to analyze the subcellular localization of epitope-tagged variants of these two proteins as well as that of fusion proteins prepared using elements of Gaa1 or PIG-T combined with variants of these two proteins as well as that of fusion proteins prepared using elements of Gaa1 or PIG-T combined with sequences from the Golgi protein. Thus, individual subunits as well as the GPIT complex as a whole must be able to avoid entering the secretory pathway and exiting the ER.

To replace the N-terminal domain of human transferrin receptor (TR) with the N-terminal cytoplasmic part of Gaa1, two PCR fragments were generated: (i) from pEF/DIV5 vector using T7 and GN3′ (5′-tgaggatactgacagtggctagg-3′) primers and (ii) from pSVT7/TR vector using primers TFR62 (5′-tgaggatactgaggaattgtcagtgg-3′) and TFR3. Amplified DNA products were digested with BamHI and EcoRI and ligated into BamHI/XbaI, respectively, and ligated into BamHI/XbaI pEF6/V5 His vector. The resulting Gaa119pTR3a-70 construct includes the N-terminal 19 residues of Gaa1, followed by Glu and His sequence. The same strategy was used to generate other chimeric proteins. The Gaa119pTR3a-70 construct contains N-terminal sequence of the Gaa1 (aa 1–55) fused to the ectodomain of TR (aa 81–760).

Human PIG-T cDNA was obtained from HeLa First Strand cDNA (Stratagene, La Jolla, CA) by PCR amplification with a sense primer 5′-tgaggatactgaggaattgtcagtgg-3′ and an antisense primer 5′-tgaggatactgaggaattgtcagtgg-3′. The PCR-amplified product was ligated into the mammalian expression vector pEF6/V5-His-TOPO (Invitrogen). The resulting pEF/PIT-V5 construct encodes PIG-T with His6 and His6 epitope tags. To generate constructs T545-V5 and T578-FLAG, the primers T545 (5′-tgaggatactgaggaattgtcagtgg-3′) and T578 (5′-tgaggatactgaggaattgtcagtgg-3′) were used, respectively. To generate Tac, T516–578 construct, the ectodomain of Tac was obtained from pCRF/Tac plasmid (38) using primers T7 and TACLUM3 (5′-tgaggatactgaggaattgtcagtgg-3′), and the C-terminal part of PIG-T was amplified with primers T514F (5′-tgaggatactgaggaattgtcagtgg-3′) and PIG-T5 (5′-tgaggatactgaggaattgtcagtgg-3′). The obtained PCR products were digested with BamHI/XbaI and ligated into BamHI/XbaI pEF6/V5 His vector.

**Cell Culture and Transfection—**HeLa cells were cultured at 37 °C in a humidified 5% CO2 atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. Cells were transfected with the construction of pCDNA3.1/STV5 His constructs by electroporation as described previously (37).

**Immunoprecipitations and Immunoblots—**Transfected HeLa cells (1–2 × 106) were harvested by scraping 48 h post-transfection, washed once with PBS, resuspended in 1 ml of MSB buffer (20 mm Hapes-KOH, pH 7.6, 200 mm NaCl, 1% digitonin or Nonidet P-40, and 1% protease inhibitor mixture (Calbiochem), and solubilized on ice for 30 min. The resulting cell lysate was centrifuged by centrifugation at 10,000 × g for 20 min at 4 °C. The 10,000 × g supernatant was incubated with 30 µl of anti-FLAG M2, or anti-V5 agarose (Sigma) slurry was added and incubated at 4 °C for 4 h with gentle agitation. The beads were pelleted by 15-s centrifugation at 10,000 × g. The samples were washed four times for 5 min each in 1.5 ml of buffer MSB. Bound antigen was released from the beads by incubation with FLAG or V5 peptide (200 µg/ml) in buffer MSB. Immunoprecipitated fractions were analyzed by SDS-PAGE, followed by immunoblotting using chemiluminescence reagents (Pierce).

**Pulse-Chase Labeling—**Transfected HeLa cells were analyzed 48 h post-transfection. The cells were pulse-labeled for 10 min with EXPRE’S® Protein Labeling Mix (1 ml/cell; PerkinElmer Life Sciences) and chased with a phenylalanine-free medium for 2–8 h at 37 °C. Cell lysates were prepared, and immunoprecipitations were carried out as described above. Immunoprecipitated fractions were resolved by SDS-PAGE, followed by electrophoresis, the gels were dried and radioimaged with luminescent imaging. The bands were quantitated using software (Amersham Biosciences).
ER Localization of GPI Transamidase Subunits

A Triple Arginine Motif in the Cytoplasmically Oriented N-terminal Sequence of Gaa1 Does Not Control ER Localization of the Protein—We previously suggested that the cytoplasmic N terminus of Gaa1 might function as an ER sorting determinant (37). Inspection of the primary sequence of human Gaa1 identified an internal triple arginine cluster (Arg⁹-Arg¹⁰-Arg¹¹) near the N terminus (Fig. 1A). This arginine cluster resembles the Arg-Xaa-Arg ER localization motifs recently identified in subunits of multimeric protein complexes displayed at the cell surface (18, 39, 40). Correct assembly of these protein complexes and their transport to the cell surface appears to be regulated through control of the trafficking of individual subunits, such that incompletely assembled complexes are retained in the ER. The secretory transport machinery is able to differentiate between unassembled units and assembled complexes because the Arg-Xaa-Arg motif in individual subunits can be masked after assembly of the protein into the complex or because the ability of the motif to signal ER retention/retrieval can be suppressed in some cases by phosphorylation of adjacent residues.

We hypothesized that the Arg⁹-Arg¹⁰-Arg¹¹ motif in Gaa1 acts as an ER localization signal. We tested this hypothesis by preparing a Gaa1 variant (denoted Gaa1(R⇄A)) in which the three arginines were replaced with alanine residues. HeLa cells were transfected with plasmids encoding wild type, V5-epitope-tagged Gaa1 (Fig. 1A), or similarly tagged Gaa1(R⇄A). The subcellular distribution of the transiently expressed proteins was determined by indirect immunofluorescence microscopy using anti-V5 antibodies. Fig. 1B shows that both wild type Gaa1 and Gaa1(R⇄A) display a reticular staining pattern consistent with ER localization. Gaa1 and Gaa1(R⇄A) were extracted from cells in detergent-containing buffer, treated with glycosidases, and analyzed by SDS-PAGE and immunoblotting. Fig. 1C shows that both proteins are modified with a single, endoglycosidase H-susceptible N-glycan, suggesting that the wild-type Gaa1 and Gaa1(R⇄A) are not exposed to Golgi N-glycan modification enzymes.

We considered the possibility that, without its arginine cluster, Gaa1(R⇄A) could potentially be exported from the ER but is unable to leave the compartment as a result of improper folding. Misfolding would mitigate the ability of the protein to pass ER quality control systems for secretion and potentially accelerate its rate of turnover. Thus, compared with wild-type Gaa1, Gaa1(R⇄A) may be turned over more rapidly by ER-associated degradation pathways. To test whether this was the case, we used [³⁵S]methionine/cysteine to pulse-label HeLa cells that were transiently expressing wild-type Gaa1 or Gaa1(R⇄A) and then chased the cells for various periods of time (up to 8 h), extracted proteins in detergent-containing buffer, and used immunoprecipitation, SDS-PAGE, and PhosphorImager analysis to determine the amount of radiolabeled Gaa1 or Gaa1(R⇄A) remaining in the cells. The SDS-PAGE/PhosphorImager data for one experiment are shown in Fig. 1D, whereas the quantitation of the rate of decay taken from three independent experiments is shown in Fig. 1E. The data show that Gaa1(R⇄A) turns over with a half-time of ~3.2 h, similar to the rate of turnover of wild-type Gaa1 (a single, monoeponential decay curve is plotted for both proteins in Fig. 1E).

This result suggests that it is unlikely that folding/degradation problems underlie the ER retention of Gaa1(R⇄A). Together with the immunofluorescence localization of Gaa1(R⇄A) to the ER and the Endo H-susceptibility of its N-glycan, these data suggest that the triple arginine motif (Arg⁹-Arg¹⁰-Arg¹¹) is not necessary for ER localization of Gaa1.

Gaa1null, a Construct Consisting of the N-terminal Cytoplasmic Tail, First TM Domain, and Part of the N-Glycosylated Luminal Loop of Gaa1, Is ER-localized—Co-immunoprecipitation experiments indicated that Gaa1(R⇄A), like Gaa1, interacts with Gpi8, PIG-T, and PIG-S (data not shown). It is therefore conceivable that Gaa1(R⇄A) is retained in the ER through its interactions with other subunits of the GPI transamidase and that these interactions render potential localization information in the triple arginine motif redundant. Alternatively, it is possible that full-length Gaa1 contains more than one ER localization signal, rendering individual signals redundant.

To clarify this issue, we investigated the subcellular local-

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**FIG. 1.** The cytoplasmic triple arginine motif is not required for ER localization of Gaa1. A, topology of the C-terminally V5 epitope-tagged human Gaa1 construct used in this study. N-Glycosylation of the protein at Asn⁶⁰³ is depicted. The V5 tag is a 14-amino acid sequence consisting of GKPPIPPLLLGDLST. B, localization of Gaa1 and Gaa1(R⇄A) (construct with Ala⁹-Ala¹⁰-Ala¹¹ instead of Arg⁹-Arg¹⁰-Arg¹¹). HeLa cells were fixed 24 h after transfection, permeabilized, and stained with mouse monoclonal anti-V5 antibodies and Alexa Fluor 568-conjugated anti-mouse Ig. Scale bar, 10 μm. C, Endo H and PNGase F treatment of in vivo expressed Gaa1 variants. The immunoblot was done with anti-V5 antibodies. D, pulse-chase labeling of Gaa1 and Gaa1(R⇄A) proteins that were transiently expressed in HeLa cells. Cells were labeled for 10 min with [³⁵S]methionine/cysteine and chased for up to 8 h. E, turnover of the radiolabeled Gaa1 and Gaa1(R⇄A). A single, monoeponential decay curve is plotted for both proteins from the results of three independent pulse-chase labeling experiments.
PIG-T and Gpi8 with Gaa1 and Gaa1282. HeLa cells were transiently transfected with expression vectors encoding Gaa1 and Gaa1282. Digitonin extracts of transfected cells were subjected to immunoprecipitation (IP) with anti-V5 affinity beads. 10% of the immunoprecipitated samples and 1.5% of the nonbound material (postimmunoprecipitation; Post-IP) were resolved by SDS-PAGE and immunoblotted with anti-V5, anti-PIG-T, or anti-Gpi8 antibodies. C, localization of Gaa1282 and Gaa119 (R→A). HeLa cells were fixed 24 h after transfection, permeabilized, and stained with mouse monoclonal anti-V5 antibodies and Alexa Fluor 568-conjugated anti-mouse Ig. Scale bar, 10 μm. D, Endo H treatment of in vivo expressed Gaa1282 and Gaa1282(R→A).

FIG. 2. Gaa1282 is correctly oriented, N-glycosylated, and ER-localized. A, schematic and membrane topology of the C-terminally V5 epitope-tagged human Gaa1282 construct. B, co-immunoprecipitation of PIG-T and Gpi8 with Gaa1 and Gaa1282. HeLa cells were transiently transfected with expression vectors encoding Gaa1 and Gaa1282. Digitonin extracts of transfected cells were subjected to immunoprecipitation (IP) with anti-V5 affinity beads. 10% of the immunoprecipitated samples and 1.5% of the nonbound material (postimmunoprecipitation; Post-IP) were resolved by SDS-PAGE and immunoblotted with anti-V5, anti-PIG-T, or anti-Gpi8 antibodies. C, localization of Gaa1282 and Gaa119 (R→A). HeLa cells were fixed 24 h after transfection, permeabilized, and stained with mouse monoclonal anti-V5 antibodies and Alexa Fluor 568-conjugated anti-mouse Ig. Scale bar, 10 μm. D, Endo H treatment of in vivo expressed Gaa1282 and Gaa1282(R→A).

A fusion construct, Gaa1-19-ST7-402, consisting of the first 19 amino acids of Gaa1 appended to ST7-402 (ST lacking the first 6 amino acids) (Fig. 3A), showed intense Golgi fluorescence and a background of reticular staining by indirect immunofluorescence microscopy when transiently expressed in HeLa cells (fluorescence microscopy data for this construct were described in a previous publication (37) but are reiterated here to provide an experimental control). Similar to the result obtained with ST, a fraction of the Gaa119-ST7-402 molecules displayed an Endo H-resistant glycan (Fig. 3C, lanes 4–6; the Endo H-resistant fraction is denoted by an asterisk in lane 5). A second fusion construct (Gaa155-ST30-402 (Fig. 3A)), consisting of the N-terminal sequence and first TM domain of Gaa1 (amino acids 1–55) fused to the ectodomain of ST (amino acids 30–402), behaved similarly to Gaa119-ST7-402. When transiently expressed in HeLa cells, the protein acquired an Endo H-resistant form (asterisk in Fig. 3C, lanes 4–6; the Endo H-resistant fraction is denoted by an asterisk in lane 5). Unlike ST (41), Gaa155-ST30-402 could not be detected in the cell surface by immunofluorescence microscopy; however, unlike ST (41), Gaa155-ST30-402 was not cleaved efficiently to release its ectodomain into the extracellular medium (data not shown). The export of a significant fraction of Gaa119-ST7-402 and Gaa155-ST30-402 to the Golgi indicates that the cytoplasmically disposed, 19-amino acid N-terminal sequence of Gaa1, alone or in concert with the first TM domain, does not contain a dominant ER localization signal.
Gaa119-ST7–402 contains a short remnant of the N-terminal cytoplasmic tail of ST that includes a cluster of lysine residues (Lys5-Lys6-Lys7) located proximal to the TM domain (Fig. 3A). It has recently been suggested that dibasic (R/K)K/R/K) motifs in the membrane-proximal region of the cytoplasmic tail of Golgi-resident glycosyltransferases act as ER exit signals by promoting interactions between the glycosyltransferase tail sequence and resident glycosyltransferases as ER exit signals by promoting interactions between the glycosyltransferase tail sequence and resident glycosyltransferases. However, the COPII vesicle coat (43). It is therefore possible that the export interactions between the glycosyltransferase tail sequence and resident glycosyltransferases act as ER exit signals by promoting the membrane-proximal region of the cytoplasmic tail of Golgi-resident glycosyltransferases as ER exit signals by promoting interactions between the glycosyltransferase tail sequence and resident glycosyltransferases. 

Gaa155-ST30–402, a fusion protein that is appended to the C terminus of Gaa1282. As with the other fusion proteins described above, a significant portion of Gaa1282-ST30–402 was detected in the Golgi and in an Endo H-resistant form (Fig. 3, B and C). These results clearly show that the N-terminal part of ST that includes a cluster of lysine residues (Lys5-Lys6-Lys7) located proximal to the TM domain (Fig. 3A). It has recently been suggested that dibasic (R/K)K/R/K) motifs in the membrane-proximal region of the cytoplasmic tail of Golgi-resident glycosyltransferases act as ER exit signals by promoting interactions between the glycosyltransferase tail sequence and resident glycosyltransferases as ER exit signals by promoting interactions between the glycosyltransferase tail sequence and resident glycosyltransferases.

To complete our analysis of the role of sequence elements of Gaa1282 that could play a role in ER-localization of the protein, we next prepared Gaa1282-ST30–402, a chimeric protein in which the luminal domain of ST (amino acids 30–402) was appended to the C terminus of Gaa1282. As with the other fusion proteins described above, a significant portion of Gaa1282-ST30–402 was detected in the Golgi and in an Endo H-resistant form (Fig. 3, B and C). These results clearly show that the N-terminal part of ST that includes a cluster of lysine residues (Lys5-Lys6-Lys7) located proximal to the TM domain (Fig. 3A). It has recently been suggested that dibasic (R/K)K/R/K) motifs in the membrane-proximal region of the cytoplasmic tail of Golgi-resident glycosyltransferases act as ER exit signals by promoting interactions between the glycosyltransferase tail sequence and resident glycosyltransferases as ER exit signals by promoting interactions between the glycosyltransferase tail sequence and resident glycosyltransferases.
arginine or lysine clusters that have been shown to act as ER localization signals for the membrane proteins (44), we hypothesized that the charged residues in the PIG-T cytoplasmic tail may be involved in its ER retention. To test this, we made a T545 construct (Fig. 5A, middle; compare with the image for intact, epitope-tagged PIG-T in the top panel) in which the C-terminal 33 amino acids were replaced with a V5 epitope tag. (We previously showed that the membrane-proximal cytoplasmic display of the V5 tag does not cause ER localization of proteins destined for post-ER compartments (38); see also below.) When transiently overexpressed in HeLa cells, the recombinant protein was found located only in the ER, suggesting that the C-terminal sequence is not required for ER localization (Fig. 5A, middle). However, like PIG-T, T545 interacts with other GPIT subunits as detected by co-immunoprecipitation of endogenous Gpi8 (Fig. 5B, lanes 1–4) and Gaa1 (data not shown); these interactions may be a contributing factor in its ER localization.

We prepared a second truncated construct, T514, consisting of only the luminal portion of PIG-T terminating in a C-terminal FLAG epitope tag. We tested whether this mutant was localized to the ER and able to bind other transamidase subunits. T514 did not co-precipitate Gpi8 (Fig. 5B, lanes 5 and 6) or Gaa1 (data not shown), suggesting that without its transmembrane domain PIG-T does not interact efficiently with other GPIT subunits. Although fluorescence microscopy showed an ER-Golgi staining pattern for T514 (Fig. 5A, bottom), the protein was secreted into the medium (Fig. 5C, lane 4) in the form of two higher molecular weight forms suggestive of N-glycan processing and, for the upper band, possibly O-glycan addition. Both secreted bands were Endo H-resistant but PNGase F-sensitive, consistent with this proposal. These results suggest that PIG-T without its membrane anchor and lacking the ability to complex with other GPIT subunits can be exported from the ER. We conclude that the TM domain of PIG-T may be required directly or indirectly (by facilitating interaction between PIG-T and other GPIT subunits) for localizing PIG-T in the ER.

To test whether the TM domain plays a direct role in ER retention of PIG-T, we prepared a chimeric construct, Tac−,T516–578 consisting of the 237-amino acid ectodomain of human interleukin-2 receptor (Tac) appended to the TM domain and C-terminal cytoplasmic tail of PIG-T (amino acids 516–578) (Fig. 6A). When HeLa cells expressing Tac−,T516–578 were assessed by indirect immunofluorescence microscopy, the fusion protein was found located in the ER (Fig. 6B, lower panels), whereas Tac was displayed, as expected, at the cell surface (Fig. 6B, top panels). Unlike Tac, Tac−,T516–578 appears not to acquire high molecular weight O-glycans (Fig. 6C, lanes 1 and 3), and its N-glycans are Endo H-sensitive (Fig. 6D). These data are consistent with the conclusion that Tac−,T516–578 is ER-localized. Co-precipitation experiments show that Gpi8 does not interact with Tac−,T516–578 (Fig. 6C, lanes 3 and 4). Immunoblotting of the Tac−,T516–578 IP fraction with anti-Gaa1 antibodies revealed that the chimeric protein does not interact with Gaa1 either (data not shown), indicating that ER retention of the fusion protein is unlikely to be due to its complexation with GPIT subunits. We conclude that the TM domain and cytoplasmic tail of PIG-T can confer ER localization to a type I membrane protein destined for the cell surface.

**DISCUSSION**

In this paper, we explore the mechanisms by which the human GPI transamidase subunits Gaa1 and PIG-T are retained in the ER. ER localization of these two membrane proteins as well as that of the three other known GPIT subunits (Gpi8, PIG-S, and PIG-U) must occur at two levels: the first involves ER retention of the individual proteins prior to assembly into the GPIT complex, and the second involves retention of the complex itself. Our studies focus on the first issue. We show that there is no primary sequence element in Gaa1 that can act as a dominant ER localization signal. In contrast, we show that the single TM domain in PIG-T is sufficient to ER-localize the ectodomain of Tac, a protein that is normally exported to the plasma membrane. Thus, PIG-T contains ER localization information, whereas Gaa1 does not.

In order to identify ER localization information within Gaa1.
and PIG-T, it was necessary to generate and study variants of these proteins that do not interact with other GPIT subunits. Thus, our information on Gaa1 relied on analyses of Gaa1 C-terminal constructs, an ER-localized truncated construct that displays the same membrane topology and N-glycosylation as Gaa1 but does not interact with other GPIT subunits. Similarly, our results concerning the ER retention of PIG-T rely on T514 and Tac C-terminal constructs, PIG-T-derived proteins that do not interact with other GPIT subunits. It is important to note that our conclusions concerning the absence (for Gaa1) or presence (for PIG-T) of ER localization signals rely ultimately on the ability to observe protein export from the ER. We view export of a fusion protein (e.g., Gaa155-ST30–402) or of a tagged, albeit truncated, construct (e.g., T514) as evidence that the protein is correctly folded and that the ER retention of simpler variants of these constructs (e.g., Gaa1292 or T545) is due to one of the retention mechanisms described in the Introduction rather than a result of the protein being targeted for degradation. In the case of the intact Gaa1 molecules described in Fig. 1, we explicitly assess and rule out the latter possibility by measuring protein turnover. The approaches discussed here contrast with those used to assess sorting signals for proteins located in post-ER compartments; in these situations, protein folding is less of an issue, since the protein reporters being tested have typically already negotiated ER quality control mechanisms en route to their final destination.

Our results contribute to the ongoing debate about the nature of signal-mediated protein transport in the secretory pathway. The controversy concerning whether protein transport in the secretory pathway occurs by a passive (“bulk flow”) mechanism, active (receptor-mediated) mechanism, or both raises similar questions about retention mechanisms of ER-resident proteins. The vectorial flow of proteins from the ER to the plasma membrane would suggest that active mechanisms are required to retain ER-resident proteins, whereas selective sorting of proteins for export would imply that the absence of positive ER export signals and an inefficient bulk flow pathway would be sufficient to retain ER residents (45).

Recent studies addressing the importance of sorting signals for COPII-dependent export of proteins from the ER provide support for the idea of passive ER retention of membrane proteins that lack functional ER exit signals. For example, the exit of wild-type cystic fibrosis transmembrane conductance regulator from the ER is blocked by mutation of a diacidic ER exit motif, which is required for protein binding to COPII vesicle coats (46). Similar results were obtained when a diacidic motif in Gap1p, the general amino acid permease, was mutated to other amino acids (45). Also, mutations of a novel export motif, FNX2LLX3L, in the C terminus of the human pituitary vasopressin V1b/V3 receptor abolished export of the protein (47). Thus, the lack of positive exit codes or retention signals appears to be one way to retain membrane proteins in the ER. Our data on the ER localization of Gaa1 are consistent with this idea.

The molecular basis for the TM-mediated ER retention of PIG-T is unclear. Like other ER-retained membrane proteins (15, 16), PIG-T diffuses freely in the plane of the ER membrane (green fluorescent protein-tagged PIG-T has a lateral diffusion coefficient of $D = 1.5 \times 10^{-9} \text{cm}^2\text{s}^{-1}$ and a mobile fraction of $\sim 77\%$), suggesting that it is not a stable component of a large matrix or supercomplex. The length and hydrophobicity of TM segments has been proposed to provide sorting information for some tail-anchored and integral membrane proteins (20, 22, 48–52), and it is conceivable that something similar holds true for PIG-T. Protein sequence alignment reveals that the N-terminal portion of the PIG-T TM domain (SFY544; conserved amino acids are shown in boldface type) is highly conserved within the family of PIG-T orthologs; however, since the sequence does not possess significant homology with other membrane proteins in the data bases, it is difficult to make generalizations. Further investigations and other approaches will be needed to determine the mechanism involved in the TM-mediated ER retention of PIG-T.

Acknowledgments—We thank Paul Gleeson, Karen Colley, and Thomas Waldmann for the transferrin receptor, sialyltransferase, and Tac constructs, respectively; Kazuhiro Ohishi and Taroh Kinoshita for the anti-PIG-T antibody; Ania Pottekat for contributing part of Fig. 6A; the Kilmle laboratory for use of their confocal microscope; Laura van der Ploeg for preparing the final figures; and Ania Pottekat and Christian Frank for comments on the manuscript. A. K. M. continues to acknowledge the stimulus provided by Alec Leamas (Assistant Librarian).

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