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Membrane Topogenesis of the Three Amino-Terminal Transmembrane Segments of Glucose-6-phosphatase on Endoplasmic Reticulum

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We investigated the membrane topogenesis of glucose-6-phosphatase (G6Pase), a multispanning membrane protein, on the endoplasmic reticulum. In COS-7 cells, the first transmembrane segment (TM1) with weak hydrophobicity is inserted into the membrane in the N-terminus-out/C-terminus-cytoplasm orientation. The following TM2 is inserted depending on TM3. TM3 has the same orientation as TM1. In contrast to data from living cells, the full-length molecule and N-terminal fusion constructs were not inserted into the membrane in a cell-free system. Addition of a signal recognition particle did not improve G6Pase insertion. When the 37-residue N-terminal segment was deleted, however, TM2 and TM3 were correctly inserted. We concluded that the three N-terminal TM segments are inserted into the membrane dependent on the two signal-anchor sequences of TM1 and TM3. TM1 is likely to be an unconventional signal sequence that barely functions *in vitro*. The 37-residue N-terminal segment inhibits the signal function of the following TM3 in cell-free systems.

Glucose-6-phosphatase (G6Pase) is a key enzyme of gluconeogenesis in the liver (1). Deficiency of the enzyme induces glycogen storage disease type 1a, whose clinical symptoms are severe hypoglycemia, hyperlipidemia, hepatomegaly, kidney enlargement, and growth retardation (2). The activity of G6Pase is used as an endoplasmic reticulum (ER) marker. The enzyme activity is sequestered in the ER lumen and the latency is released by treatment with detergent. Based on the amino acid sequence, it has been predicted to possess nine transmembrane (TM) segments. Epitope-tagging and protease treatment experiments have demonstrated that the N-terminus is in the luminal side and the C-terminus is in the cytosolic side of the ER membrane (3). Of the three potential glycosylation sites in human G6Pase, only Asn^{96} is glycosylated (4). Other membrane-bound phosphatases, human PAP type 2b (PAP2b) and rat Dri 42 protein, which have 94% identity with each other, have six membrane spanning segments (5–7).

G6Pase is a typical multispanning membrane protein and is thus expected to be integrated into the ER membrane similarly to other membrane proteins in the secretory pathway. Many membrane proteins are synthesized by ribosomes bound to the ER membrane. The ribosomes synthesizing the membrane proteins are targeted to the ER membrane via the signal recognition particle (SRP) mediated pathway, and nascent polypeptides are cotranslationally integrated into the membrane, mediated by so-called translocon (8). ER-targeting of the nascent polypeptide is determined by a signal sequence that has a hydrophobic character. Among them, signal peptide and type II signal-anchor sequences mediate translocation of the following portion to result in the N_{cyto}/C_{out} topology, whereas the type I signal-anchor sequence (SA-I sequence) mediates the translocation of the N-terminal portion and results in the N_{out}/C_{cyto} orientation (9). In multispanning membrane proteins, the TM segments are sequentially inserted into the translocon as the nascent polypeptide chain is emerging from the ribosome. Some hydrophobic segments with a strong topogenic character determine the particular orientation irrespective of the context (e.g., an internal SA-I sequence can confer the transmembrane disposition onto the preceding sequence, which cannot be inserted into membrane by itself) (10, 11). ER targeting of signal sequences is primarily determined by the hydrophobic region. The hydrophobic regions of signal-anchor sequences are

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composed of 17 to 27 residues and those of signal peptides are composed of 7 to 14 residues (12). The longer hydrophobic region and less positive charges in the N-terminal domain often cause N-terminal translocation and result in an SA-I sequence (9, 13).

In the process of investigation of the membrane topogenesis of G6Pase, we determined that in COS-7 cells, both TM1 and TM3 have the signal-anchor function and TM2 is inserted into the membrane depending on the following TM3. TM1, which is much less hydrophobic than the other known signal-anchor sequences, does not reveal the signal function in the cell-free system. Furthermore, the N-terminal sequence inhibits the signal-function of TM3. The difference in apparent topogenic functions in the two expression systems, *in vivo* and *in vitro*, suggests that there are unconventional mechanisms of membrane protein integration.

MATERIALS AND METHODS

Materials. Anti-human G6Pase antibodies were prepared by immunizing the 75-residue (1–75) N-terminal peptide expressed in *E. coli* into rabbit. Green fluorescent protein (GFP) antibodies were obtained either from Clontech or as described previously (14). The preparation of rough microsomal membranes (RM) (15), SRP (16), and rabbit reticulocyte lysate (17) was as previously described.

Construction of expression plasmids. cDNA coding for human G6Pase was amplified by polymerase chain reaction (PCR) using the following primers containing the *Hin*dIII and *Xba*I sites: TT-GAAGCTTCCACCATGGAGGAAG and GATCTAGACAACGACT-TCTTGTGCG (initiation codon is underlined).

The DNA fragment was subcloned between the *Hin*dIII and *Xba*I sites of pRc/CMV (Invitrogen) to obtain pG6Pase. The human PAP2b cDNA (6, 18) was subcloned similarly. Plasmid pRc/CMV-SytII was previously described (19).

For GFP-fusion constructs, the DNA fragments obtained by PCR amplification, which possess the restriction enzyme sites indicated in parentheses, were ligated with the appropriate plasmid. The DNA fragment (*Hin*dIII/*Xba*I) coding for Met^{1}-Gln^{55}, Met^{1}-His^{119}, and Met^{1}-Arg^{147} of G6Pase, and the GFP fragment (20) (*Xba*I/*Apa*I) were inserted into pRc/CMV (*Hin*dIII/*Apa*I). For the glycosylation site mutants, the constructs were made by the method of Kunkel (21). N13 and N72 constructs were made by point mutation of I13N and V74S, respectively. The N63 and N84 constructs were made by inserting the three codons encoding Asn-Thr-Ser after Leu^{62} and Arg^{83}, respectively. Mutations of N96D, N203D, and N276D were made to disrupt the potential glycosylation sites. The glycosylation sites included in each construct are indicated in the figures. For the signal peptide fusion, the signal peptide including the processing site of bovine prolactin (Met^{1}-Ser^{40}, *Nco*I, and *Eco*RI) and the fragment for G6Pase (*Eco*RI and *Xba*I) were ligated with the pCITE2b plasmid (11). For N-terminal deletions, DNA fragments encoding the sequences from Met^{5}, Met^{13}, Met^{17}, Met^{21}, Met^{29}, and Met^{37} to the termination codon (*Hin*dIII/*Xba*I) were amplified by PCR and subcloned into pRc/CMV (*Hin*dIII/*Xba*I) (20).

Transfection and immuno-fluorescence microscopy of COS-7 cells. COS-7 cells were cultured in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum at 37°C under an atmosphere of 10% CO_2 in air. Transfection was performed using FuGene 6 reagent (Roche Molecular Biochemicals) and cells were analyzed 24 h after transfection. Immunofluorescence microscopy was performed as described (20). Fluorescent images were observed with a confocal laser scanning microscope.

Cell fractionation and enzyme treatments. Cell fractionation of rat liver and cultured cells was performed as described previously (22). Liver from a rat that was fasted overnight was homogenized in 9 volumes (V/W) of H-buffer (0.25 M sucrose, 5 mM Hepes, pH 7.4) in a Potter homogenizer. Homogenate was centrifuged at 8000*g* for 6 min and the supernatant was centrifuged further at 105,000*g* for 30 min to separate the cytoplasmic supernatant and the microsomal precipitates. COS-7 cells, which were confluent in a 10-cm culture dish, were suspended in 500 μl of H-buffer and homogenized by passing 20-times through a 27-gauge needle. The homogenate was then fractionated as described above. Aliquots of the microsomal membranes or total cell lysate were treated with endoglycosidase H (EndoH, New England Biolabs) at 37°C for 60 min under denaturing conditions. Aliquots of microsomal membranes were treated with 400 μg/ml proteinase K (ProK, Merck) at 4°C for 40 min. The reaction was terminated with 10% trichloroacetic acid. For the alkali extraction, 100 μg of the membranes was suspended in 100 μl of 100 mM Na_2CO_3 (pH 11.0) and incubated on ice for 30 min, and then centrifuged at 100,000*g* for 5 min. Proteins in the supernatant were precipitated with trichloroacetic acid. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and subsequent immunoblotting using antibodies to the N-terminus of G6Pase, GFP, or the N-terminal domain of calnexin (SPA-865, SressGen Biotechnologies). The immuno-reacted bands were visualized with peroxidase conjugated second antibodies and ECL-reagent (Amersham/Pharmacia Biotech) and LAS 1000 plus (Fuji).

In vitro analysis. Plasmids that were linearized by either *Eco*RI for pRc/CMV vector or *Xho*I for pCITE2b vector were transcribed by T7-RNA polymerase. The mRNAs were translated in the reticulocyte lysate cell-free system as previously described (23). For EndoH treatment, aliquots (20 μl) of the reaction mixture were treated under denaturing conditions. For salt extraction, aliquots (20 μl) were adjusted to 50 μl with final concentrations of 500 mM NaCl, 5 mM $Mg(oAc)_2$, and 30 mM Hepes (pH 7.4) and then overlaid onto a 100-μl cushion of the same buffer containing 1.25 M sucrose. After centrifugation at 50000 rpm (RP100AT2 rotor, Hitachi Co.) for 5 min, 50 μl of supernatant and membrane precipitate was recovered. The proteins synthesized *in vitro* were analyzed by SDS–PAGE and subsequent image analysis (FLA-2000, Fuji). The protein bands were quantified using ImageGauge software (Ver.2.5.2, Fuji). For the SRP titration experiment, mRNA was translated in the wheat germ extract (S23 fraction) (24) in the presence or absence of RM. Where indicated, the purified SRP was included.

RESULTS AND DISCUSSION

When human G6Pase was expressed in COS-7 cells, two bands were observed by immunoblotting analysis (Fig. 1A, lane 1). Both of the bands were recovered in the microsomal membrane fraction (lane 3), but not in the cytoplasmic fraction. The G6Pase was still bound to the membrane after alkali extraction (lane 6). The larger polypeptide was shifted down by EndoH treatment (lane 4), indicating that it was glycosylated. The glycosylation ratio in the COS-7 cells decreased over time following the transfection (Fig. 1B). This is most likely due to overexpression in the cells. We used the cells 20 h after the transfection in the following experiments. At this time point, half of the expressed full-length G6Pase was glycosylated. In contrast, a single band was observed by the immunoblotting analysis of rat hepatocytes (Fig. 1C, lane 1). The band was recovered in the microsomal membrane fraction (lane 3) and

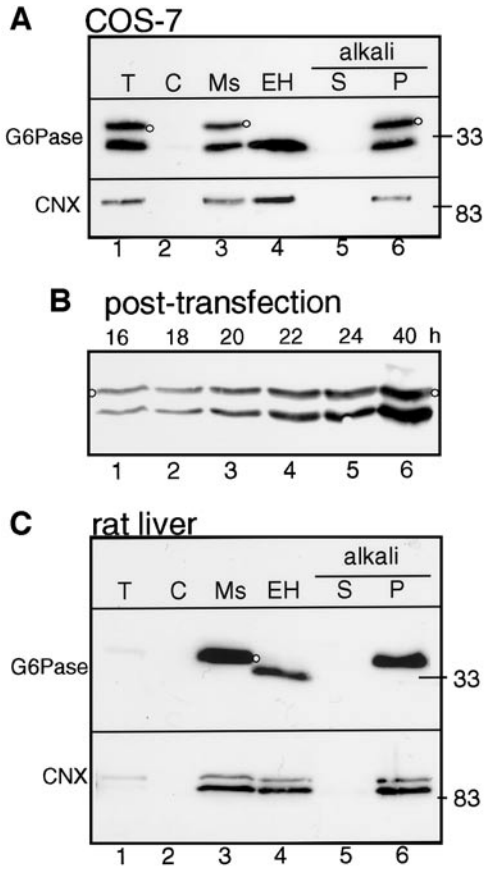

FIG. 1. Glycosylation status of G6Pase in cells. (A) Expression of human G6Pase in COS-7 cells. Total cell lysate (T), cytosol (C), and microsomal membranes (Ms) were obtained from COS-7 cells transfected with the expression construct. Aliquots of microsomes were treated with EndoH (EH). Other aliquots were extracted with alkali solution (0.1 mM Na_2CO_3) and separated into supernatant (S) and membrane pellet (P). Proteins were analyzed by SDS–PAGE and subsequent immunoblot analysis using antibodies to G6Pase and calnexin (CNX). The glycosylated form is indicated by an open circle. (B) The glycosylation status of G6Pase in COS-7 cells after transfection. The transfected cells were cultured for the indicated times (h). Twenty-five percent of the cells on the 3.5-cm dish were subjected to immunoblot analysis. (C) Glycosylation status of rat liver G6Pase. Total cell lysate (T), cytosol (C), and microsomal membrane (Ms) were analyzed as in A.

associated with the membrane in an alkaline-resistant manner (lane 6). The band shifted down following treatment with EndoH (lane 4), indicating that it was fully glycosylated. The fractionation behaviors in COS-7 cells and hepatocytes were same as calnexin, an integral membrane protein of ER (CNX lanes).

Although the human G6Pase has been predicted to possess nine TM segments, the two N-terminal segments are not very hydrophobic (Figs. 2A and 2B). In contrast, human PAP2b, another membrane-bound phosphatase, had substantially hydrophobic peaks in the N-terminal portion (not shown). To elucidate the topogenic functions of the N-terminal TM segments, we constructed GFP fusion proteins, which included one, two, or three N-terminal TM segments (Fig. 2C). The constructs contained potential glycosylation sites at the endogenous site (Asn^{96}) and/or a created site (Asn^{13}). The GFP fusion proteins were expressed in COS-7 cells and their glycosylation status was monitored by EndoH treatment. While wild-type fusion construct TM1-GFP was not glycosylated, the (N13)TM1-GFP construct, which possessed the glycosylation site in the N-terminal domain, was almost completely glycosylated (Fig. 2D, lane 2). Glycosylation was confirmed by EndoH treatment (lane 3). In TM1-2-GFP constructs, the N13-construct was efficiently glycosylated (lane 6), while the N96-construct was only weakly glycosylated (lane 4). The N13N96-construct of the TM1-2-GFP fusion was slightly di-glycosylated and mainly mono-glycosylated (lane 8). This is consistent with the efficient glycosylation of only N13, thus indicating that TM2 does not possess a start-transfer function. In TM1-3-GFP constructs, the N96-construct was efficiently glycosylated (lane 10) and the N13N96-construct was also efficiently di-glycosylated (lane 12). It is clear that TM2 was inserted into the membrane. The N-terminal domain was translocated by TM1, which is not a very hydrophobic segment (lanes 10 and 12). The correct insertion of TM2 required TM3 function (Fig. 2E).

To confirm the transmembrane topology of TM1 in Fig. 2E, we performed ProK treatment experiments (Fig. 3A). Following ProK treatment, almost all the membrane bound TM1-GFP constructs were degraded (lane 4). Upon alkaline extraction, 50% of the constructs were extracted into the supernatant, indicating that the association with the membrane was not as tight as that of the full-length G6Pase. This is probably due to the low hydrophobicity of TM1. The degradation profile of calnexin, an ER integral membrane protein, was also monitored as a control. It shifted down following treatment with ProK, while the N-terminal fragment located in the lumen was completely resistant to the protease treatment, indicating that the membrane was fully intact (CNX in lane 4). Therefore, the GFP domain of the construct was on the cytoplasmic side of the membrane. Immunofluorescent microscopy indicated that the (N13)TM1-GFP construct was co-localized with the reticular pattern of calnexin (Fig. 3B). These data demonstrate that TM1 is a signal-anchor sequence for ER targeting and integration in the N_{out}/C_{cyto} orientation (Fig. 2E). The other GFP fusion proteins that possess TM1-2 and TM1-3 remained in the GFP domain on the cytoplasmic side of the membrane, where they were accessible to the protease, as in the case of the TM1 construct (lanes 11 and 16).

We then examined the membrane insertion of G6Pase in a cell free system. The full-length G6Pase was barely glycosylated in the cell-free system (Fig. 4A, lane 2), while PAP2b (lane 7) and mouse synaptotagmin II (Syt II, lane 12) were efficiently glycosylated.

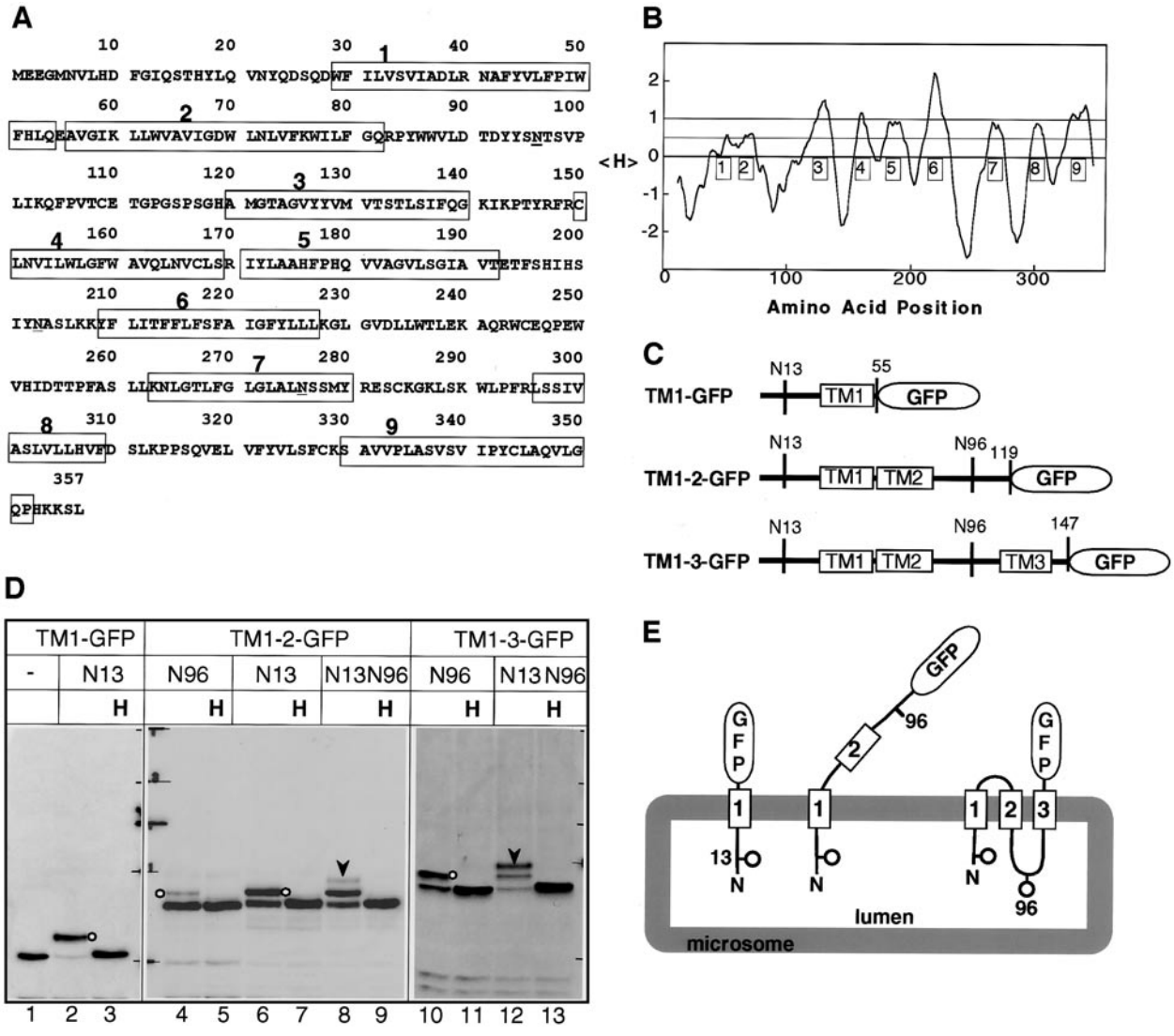

FIG. 2. Topogenic functions of N-terminal TM segments. (A) Amino acid sequence of human G6Pase. The hydrophobic segments are indicated by boxes. (B) Hydrophobicity plot of human G6Pase. The sequence was analyzed by TopPred II using the default parameters (28). The potential TM segments are indicated with numbers. (C) N-terminus-GFP fusion proteins used. The numbers at the left of the GFP indicate amino acid residues of G6Pase at fusion points. The positions of the endogenous glycosylation site of Asn^{96} (N96) and the created site of Asn^{13} (N13) are indicated. (D) Glycosylation status of the constructs in COS-7 cells. The total extract of COS-7 cells transfected with the indicated constructs was analyzed by immunoblotting. Aliquots were treated with EndoH (H lanes). Arrowhead and open circle indicate the diglycosylated and monoglycosylated forms, respectively. N13, N96, and N13N96 indicate the included glycosylation sites in the constructs. (E) Membrane topology of the constructs. TM1 is inserted into the membrane in an N_{out}/C_{cyto} topology. TM2 is not inserted into the membrane by itself, and is inserted only in the presence of TM3. Circles indicate the glycosylated sites in the lumen.

Furthermore, G6Pase was completely extracted into the supernatant, even with a high salt buffer (Fig. 4A, lane 4), whereas a substantial amount of PAP2b and Syt II were associated with the membrane (Fig. 3B, lanes 10 and 15). The N-terminal GFP fusion constructs were barely, if at all, glycosylated in the cell free system as in the case of full-length G6Pase (B). These results raised the unexpected conclusion that TM1 and TM3 cannot exert their topogenic function in the reticulocyte lysate cell free system. Three lots of the reticulocyte lysate preparations were examined with similar results. To examine the effect of the translation system and externally added SRP, G6Pase was synthesized in a wheat germ extract system supplemented with purified SRP (Fig. 4C). While PAP2b and Syt II were glycosylated depending on the dose of SRP, the G6Pase was barely glycosylated despite the addition of SRP (Fig. 4C).

To examine whether G6Pase can be inserted into RM when directed to the membrane by an authentic signal peptide, the signal peptide of bovine prolactin, which included the processing sequence, was fused to the

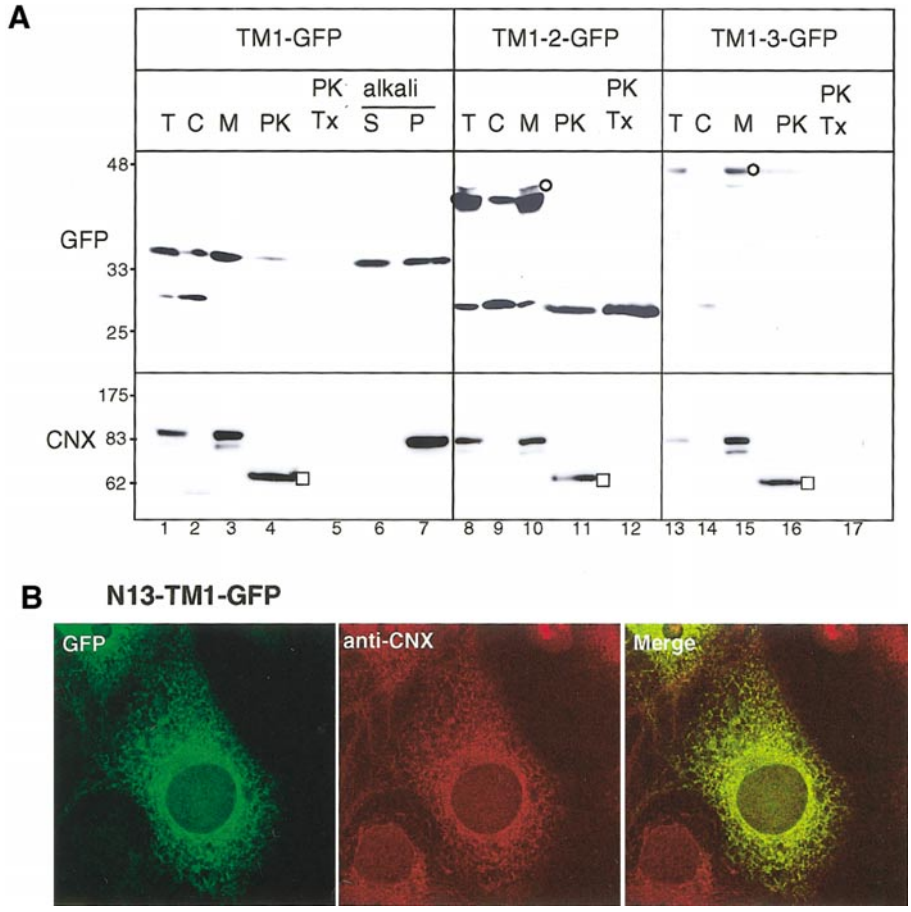

FIG. 3. ER targeting and topology of the N-terminal fusion proteins. (A) Protease sensitivity of the constructs on RM. Total cell extract (T), cytoplasmic supernatant (C), and microsome (M) were obtained and subjected to immunoblotting analysis using anti-GFP antibodies and anti-calnexin (CNX) antibodies. Aliquots were treated with proteinase K (PK) in the presence and absence of 1% Triton X-100 (Tx). The microsomal membranes were extracted under alkali conditions into supernatant (S) and pellet (P). Open circles indicate the glycosylated forms of GFP-fusion proteins. Squares indicate the N-terminal domain of calnexin protected by the membrane. (B) Fluorescent images of (N13)TM1-GFP protein expressed in COS-7 cells. GFP signal (green) and immunostained calnexin (red), and their merged images are indicated.

N-terminus of G6Pase (Fig. 4D). When synthesized in a reticulocyte lysate cell free system in the presence of RM, the fusion protein was processed and glycosylated (D, lane 2). Upon high salt extraction, the processed and glycosylated forms were present in the membrane precipitates (open and closed circles, respectively in lane 5), while the precursor form was present in the supernatant (lane 4). The mutants in which the other two potential glycosylation sites, but not N96, were disrupted produced the same pattern (lane 7). When the glycosylation site of N96 was disrupted, the mutant was no longer glycosylated (lane 10). Thus, G6Pase polypeptide can be inserted into RM and the authentic site (N96) was glycosylated when it was targeted to the translocon by the added N-terminal signal peptide. The two N-terminal TM segments are not very hydrophobic, whereas TM3 is more hydrophobic and actually insert the preceding TM2. Because TM3 seems to possess the necessary characteristics of a conventional signal-anchor sequence, it should function in the cell-free system. To examine the possibility that the N-terminal sequence influences the function of the following TM segment in the cell-free system, constructs were made in which the N-terminal sequences were deleted (Fig. 5A). When expressed in a reticulocyte lysate system, the △2-37 construct was as efficiently glycosylated as PAP2b. The other longer constructs, however, were not so efficiently glycosylated (A and B). There was also an inhibitory effect of the N-terminal sequence in the N-terminal fusion (TM1-3-GFP) constructs (compare lanes 8 and 10 in panel D). The (1–147)-GFP construct was barely glycosylated (lane 10), while the (38–147)-GFP construct was substantially glycosylated (lane 8). When the glycosylation sites of N96 shifted toward TM2 region of the (38–146)-GFP construct (Figs. 5C and 5D), those sites were not glycosylated, indicating that TM2 was inserted into the membrane despite the N-terminal deletion and had a

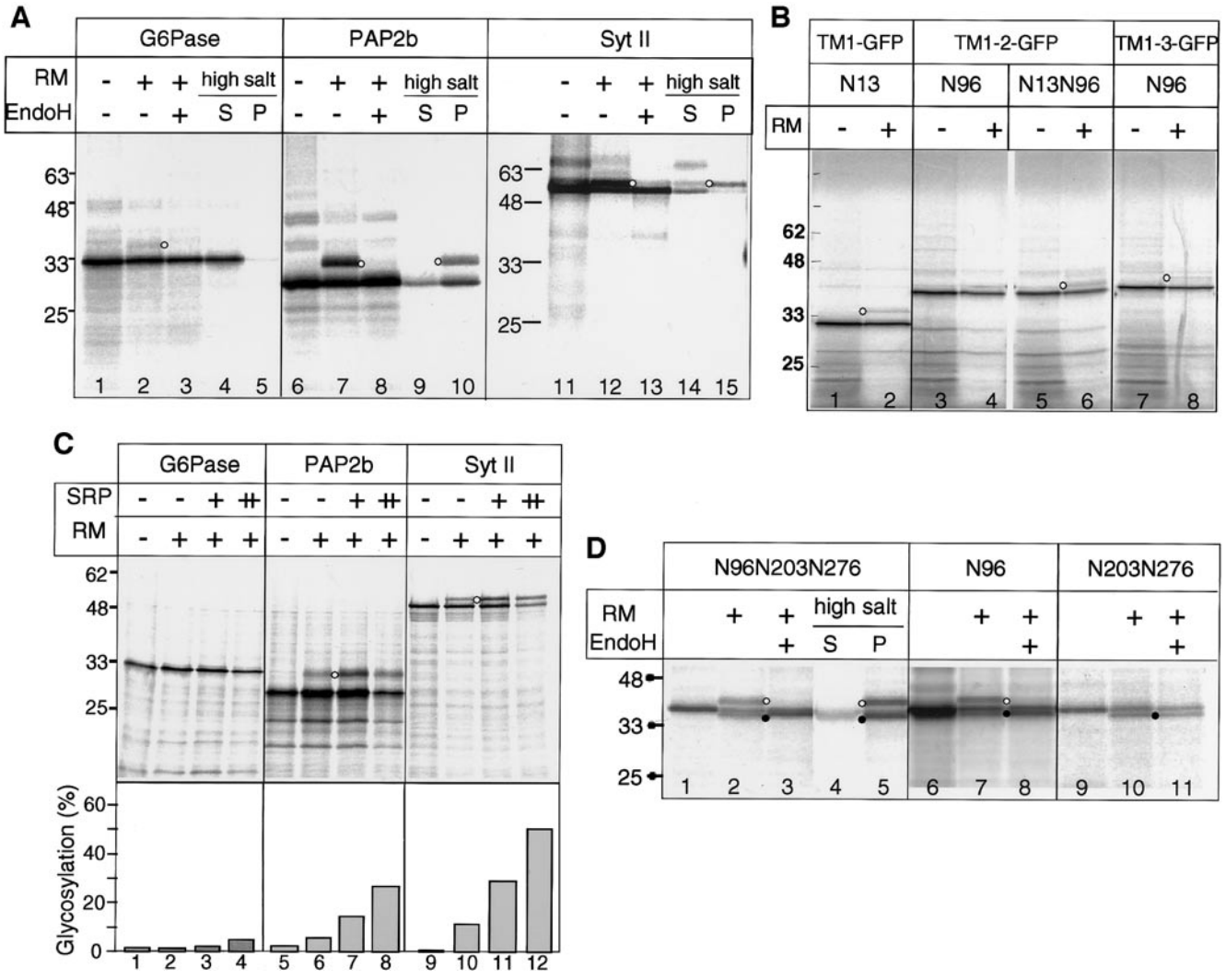

FIG. 4. GFP constructs were not integrated into RM in the cell free system. (A) Full length G6Pase was barely integrated in the cell-free system. G6Pase, PAP2b, and Syt II were expressed in the cell-free system in the absence (−) or presence (+) of RM. Aliquots were treated with EndoH. Other aliquots were extracted under high-salt conditions to separate the membrane precipitates (P) and supernatants (S). The proteins were analyzed by SDS–PAGE and subsequent image analysis. Open circles indicate the glycosylated forms. (B) The N-terminal fusion constructs used in Fig. 2 were expressed in the reticulocyte lysate cell-free system in the absence (−) or presence (+) of RM. Open circles indicate trace amounts of the glycosylated forms. (C) Effect of SRP on the integration. The three proteins in A were expressed in the wheat germ lysate system in the absence (−) or presence (+) of RM and the indicated amount of SRP (2 U, +; 4 U, ++). Glycosylated and nonglycosylated forms were quantified and the efficiency (%) was calculated. (D) Membrane insertion of G6Pase fused with the signal peptide. Signal peptide including the processing sequence of bovine preprolactin was fused to the N-terminus of G6Pase. The included potential glycosylation sites are indicated at the top of the panel. Aliquots were treated with EndoH. Other aliquots were extracted under high-salt conditions to separate membrane (P) and supernatants (S). Closed and open circles indicate the processed-unglycosylated and the processed-glycosylated forms, respectively.

transmembrane orientation (Fig. 5D). These data suggest that the 37-residue N-terminal sequence inhibits the signal function of TM3 in cell-free systems.

The data obtained above indicate that the N-terminal portion of G6Pase is integrated into the membrane by the action of two signal-anchor sequences of TM1 and TM2 with the N_{out}/C_{cyto} orientation. TM1, which possesses much lower hydrophobicity than conventional signal-anchor sequences (9, 13), can mediate the insertion of the N-terminus into the lumen. TM2 cannot mediate insertion of the following portion and is inserted into the membrane by TM3. This TM3-dependent insertion of TM2 is an example of a well-defined "forced transmembrane orientation" of a weakly hydrophobic segment by the following signal-anchor sequence (10). Although the mechanism to ensure the function of TM1 should be clarified, the present study clarifies the topogenic process of the three N-terminal segments of human G6Pase. The hydrophobic segment of TM1 is interrupted by two charged residues, Asp^{38}

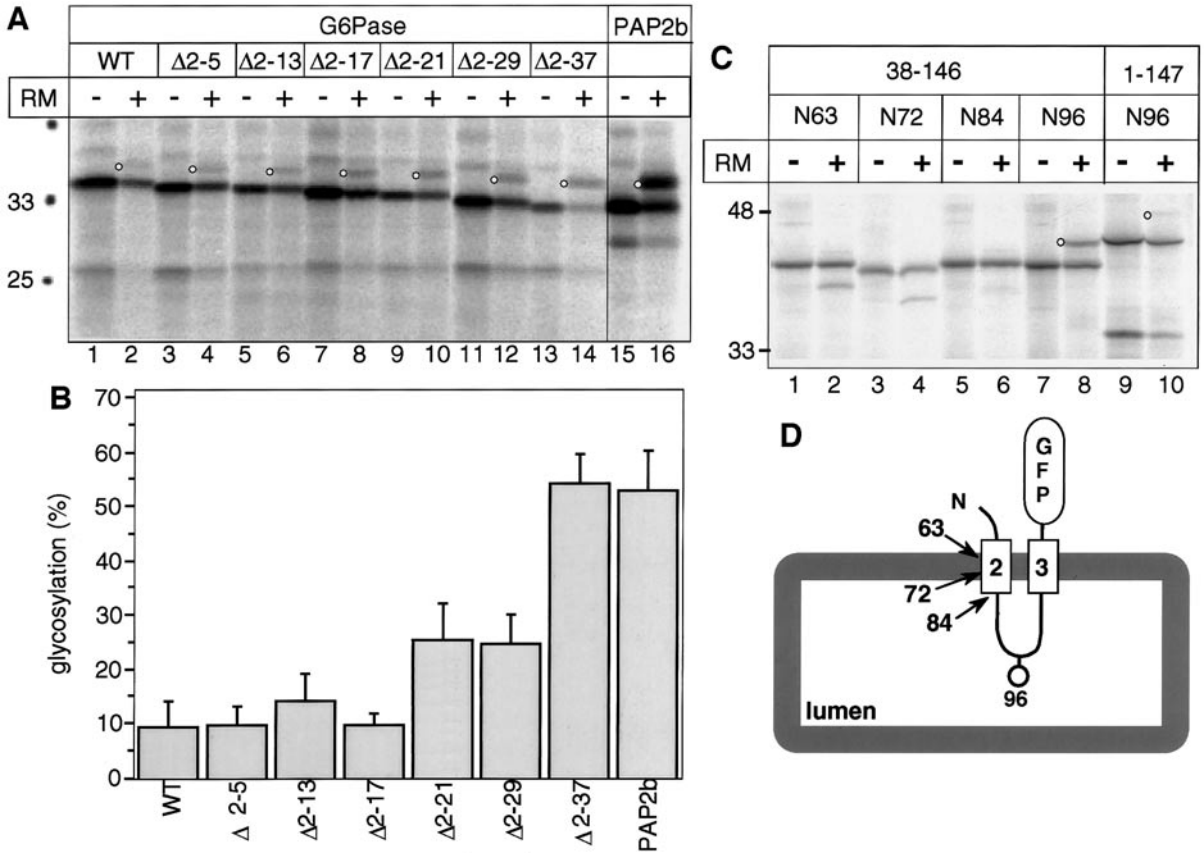

FIG. 5. Effect of N-terminal deletion on the integration efficiency. (A) N-terminal deleted mutants of G6Pase were expressed in the reticulocyte lysate system in the absence (−) or presence (+) of RM. PAP2b was expressed as a positive control. Open circles indicate the glycosylated forms. (B) Glycosylation efficiencies were determined. The experiments for each construct were carried out more than three times and the standard deviations are indicated by error bars. (C) TM1-3-GFP (1–147) and its N-terminal deleted mutant (38–146) were expressed in the reticulocyte lysate system in the absence (−) or presence (+) of RM. The glycosylation sites included in each construct are indicated (N63–N96). Open circles indicate the glycosylated forms. (D) Schematic presentation of membrane topology of the (38–146) construct. Only N96 was glycosylated in the lumen, and the others, N63–N84, were not, indicating that TM2 forms a transmembrane orientation.

and Arg[40]. Both segments on either side of these residues seem to be insufficient for the signal-anchor function. The two charges might compensate for each other by some unknown mechanisms to function as an SA-I sequence in the cell. The orientations of the three N-terminal TMs are consistent with previous reports: the tag sequence fused at the N-terminus is on the luminal side of the ER membrane (3) and the Asn[96] is the actual and only glycosylation site of human G6Pase (4).

The unexpected finding is that insertion of the full-length molecule was not reproduced in the cell-free system and that deletion of the 37-residue N-terminal segment, including a part of TM1, allowed the insertion of TM2 and TM3. We examined higher dosages of RM and several different preparations of RM and translation systems, but failed to improve the efficiency. In the cell-free system, the N-terminal sequence does not reveal a signal function, but further inhibits the function of the following signal sequences. The deletion of the 37-residue N-terminal segment enables the following signal sequence with sufficient hydrophobicity to target and mediate insertion of the following portion. In living cells, there is a mechanism that might overcome the inhibitory function of the N-terminal sequence as well as maintain the correct function of the signal-anchor function of TM1.

In a previous report, glycosylation of Asn[96] of human G6Pase was more than 50% in the cell-free system (4). We did not obtain such an efficient glycosylation *in vitro*. Thus far, we have used the cell-free translation-translocation system to assess the integration of various membrane proteins and the necessary activities of the preparations of RM have been established (25–27). It is possible that the difference is due to the following: (i) The authors of the previous report (4) used a C-terminal FLAG-tagged construct in the previous study. (ii) The transcription constructs differ from each other. The sequence of the non-coding region of mRNA might influence the membrane insertion process. (iii)

Some unknown factor(s) might be included in the preparations of RM and reticulocyte lysate used.

The heterologous cell-free system, using canine pancreas RM, has been thought to have the necessary functions, and has been able to reproduce various insertion modes of membrane proteins. Detailed studies of the insertion of G6Pase, which has been traditionally used as an ER marker enzyme, will provide new insights into the membrane topogenesis mechanism in the RM.

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