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Dissection of De Novo Membrane Insertion Activities of Internal Transmembrane Segments of ATP-Binding-Cassette Transporters: Toward Understanding Topological Rules for Membrane Assembly of Polytopic Membrane Proteins

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The membrane assembly of polytopic membrane proteins is a complicated process. Using Chinese hamster P-glycoprotein (Pgp) as a model protein, we investigated this process previously and found that Pgp expresses more than one topology. One of the variations occurs at the transmembrane (TM) domain including TM3 and TM4: TM4 inserts into membranes in an N_{in}-C_{out} rather than the predicted N_{out}-C_{in} orientation, and TM3 is in cytoplasm rather than the predicted N_{in}-C_{out} orientation in the membrane. It is possible that TM4 has a strong activity to initiate the N_{in}-C_{out} membrane insertion, leaving TM3 out of the membrane. Here, we tested this hypothesis by expressing TM3 and TM4 in isolated conditions. Our results show that TM3 of Pgp does not have de novo N_{in}-C_{out} membrane insertion activity whereas TM4 initiates the N_{in}-C_{out} membrane insertion regardless of the presence of TM3. In contrast, TM3 and TM4 of another polytopic membrane protein, cystic fibrosis transmembrane conductance regulator (CFTR), have a similar level of de novo N_{in}-C_{out} membrane insertion activity and TM4 of CFTR functions only as a stop-transfer sequence in the presence of TM3. Based on these findings, we propose that 1) the membrane insertion of TM3 and TM4 of Pgp does not follow the sequential model, which predicts that TM3 initiates N_{in}-C_{out} membrane insertion whereas TM4 stops the insertion event; and 2) "leaving one TM segment out of the membrane" may be an important folding mechanism for polytopic membrane proteins, and it is regulated by the N_{in}-C_{out} membrane insertion activities of the TM segments.

INTRODUCTION

ATP-binding cassette (ABC)[1] membrane transporters function as transport ATPases (for reviews see Higgins, 1992; Doige and Ames, 1993; Childs and Ling, 1994). Members of this family include mammalian P-glycoprotein (Pgp) and cystic fibrosis transmembrane conductance regulator (CFTR), yeast pheromone transporter (STE6), and bacterial hemolysin transporter (Hly B) (Higgins, 1992; Doige and Ames, 1993; Childs and Ling, 1994). They are polytopic membrane proteins with multiple putative transmembrane segments (Higgins, 1992).

The topological folding of Pgp, CFTR, and STE6 has been investigated. Topologies different from the hydropathy prediction were found with Pgp (Zhang and Ling, 1991; Zhang *et al.*, 1993; Skach *et al.*, 1993; Bibi and Béjà, 1994; Zhang, 1996) but not with CFTR (Chang *et al.*, 1994; Chen and Zhang, 1996) or STE6 (Geller *et al.*, 1996). One of the alterations found in Pgp

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[1] Abbreviations used: ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; MDR, multidrug resistance; PCR, polymerase chain reaction; Pgp, P-glycoprotein; RM, microsomal membranes; RRL, rabbit reticulocyte lysate; TM, transmembrane.

topology is located in the region including transmembrane (TM)3 and TM4 (Zhang *et al.*, 1993, see Figure 1A). In the alternative folding of Pgp (Figure 1A, model II), TM4 inserts into membranes in an N_{in}-C_{out} orientation opposite to the predicted N_{out}-C_{in} orientation whereas TM3 is in cytoplasm (Figure 1A, compare model I and II). On the other hand, TM3 and TM4 of CFTR insert in membranes in an orientation as expected (N_{in}-C_{out} for TM3 and N_{out}-C_{in} for TM4 as shown in model I of Figure 1A) (Chen and Zhang, 1996).

The biogenesis of TM segments in an integral membrane protein is a complicated process (for reviews see Walter and Johnson, 1994; Corsi and Schekman, 1996; Rapoport *et al.*, 1996; Schatz and Dobberstein, 1996; Johnson, 1997). A TM segment may function as an uncleavable signal-anchor sequence to generate a class II orientation (N_{in}-C_{out}) or as a stop-transfer sequence to generate a class III orientation (N_{out}-C_{in}) (von Heijne and Gavel, 1988). For polytopic membrane proteins, it has been proposed that the topology is generated by the sequential membrane insertion of signal-anchor and stop-transfer sequences (Blobel, 1980; Wessels and Spiess, 1988; Hartmann *et al.*, 1989; Lipp *et al.*, 1989; Skach and Lingappa, 1993). However, recent studies with Pgp suggested that the membrane-insertion process of each TM segment is more complicated than the prevailing sequential insertion model (Zhang and Ling, 1991; Skach *et al.*, 1993; Zhang *et al.*, 1993, 1995; Bibi and Béjà, 1994).

Recently, polytopic membrane proteins which were forced to adopt alternative topologies have been created both in bacteria (Gafvelin and von Heijne, 1994) and in mammalian cells (Gafvelin *et al.*, 1997). In the alternative topologies of these proteins, one or more TM segments were left out of the membrane to accommodate the proper membrane orientation of other TM segments as observed with the alternative topology of Pgp (see Figure 1A). Such a "leaving one out" strategy may be an important mechanism for polytopic membrane proteins to fold properly when topogenic signals from various TM segments contradict each other. That is, some TM segments will be forced to stay away from inserting into membranes in order for other TM segments to insert into membranes properly.

To understand how the alternative topology of Pgp is formed and how the "leaving one out" strategy is regulated, we further dissected the topogenesis mechanism of Pgp in this study. We investigated in detail the membrane insertion properties of isolated TM3 and TM4 of both CFTR and Pgp in microsomal membranes. Our results suggest that TM4 of Pgp has a strong de novo activity for the N_{in}-C_{out} membrane insertion even in the presence of TM3 whereas TM3 does not have de novo activity to initiate N_{in}-C_{out} insertion. TM3 and TM4 of CFTR, on the other hand, have a similar level of de novo N_{in}-C_{out} membrane insertion activity, and TM4 functions only as a stop-transfer sequence when following TM3. Thus, Pgp can form an alternative topology whereas CFTR cannot.

MATERIALS AND METHODS

Materials

pGEM-4z plasmid, SP6 and T7 RNA polymerase, RNase inhibitor (RNasin), ribonucleotides, RQ1 deoxyribonuclease, rabbit reticulocyte lysate (RRL), and dog pancreatic microsomal membranes (RM) were obtained from Promega (Madison, WI). [^{35}S]methionine and Amplify were purchased from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL), respectively. $m^7G(5')ppp(5')G$ cap analog was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Peptide N-glycosidase F (PNGase F) and restriction enzymes were obtained from Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), or Promega. pCR cloning vector was obtained from Invitrogen (San Diego, CA). All other chemicals were obtained from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Engineering Recombinant DNA Constructs

Polymerase chain reaction (PCR) was used to make Pgp-TM3R and Pgp-TM3,4R from pGPGP-N3 and pGPGP-N4 DNA templates (Zhang *et al.*, 1993) and CF-TM3R and CF-TM3,4R from CFTR-N3R and CFTR-N4R DNA templates (Chen and Zhang, 1996), respectively. The first PCR was primed with a common primer 5′-CACTTTTGCCAACCAG-3′ (B50) in the reporter domain and a second primer specific for each construct containing a Kozak translation initiation codon: 5′-GTGATGGAGTTTTTTCATGC-3′ for Pgp-TM3R and Pgp-TM3,4R, and 5′-GAGACCATGCAGATGAGAATAG-3′ for CF-TM3R and CF-TM3,4R. The PCR product encoding TM3 or TM3-TM4 was cloned and propagated using a pCR cloning vector (Invitrogen), released by *Eco*RI and *Bgl*II digestion, isolated by gel electrophoresis, and finally ligated into a vector containing a reporter cDNA linearized with *Eco*RI and *Bgl*II. The reporter used in this study is the ATP-binding domain of Pgp, the same as used in our previous studies (Zhang *et al.*, 1993; 1995; Chen and Zhang, 1996; and Zhang, 1996).

To engineer Pgp-TM4R and CF-TM4R, TM4 in Pgp-TM3,4R and CF-TM3,4R was deleted by a two-step PCR. In the first PCR, we used B50 primer (see above) and an opposite primer that contains a sequence fused between the sequences outside the N- and C-terminal end of TM3. These primers were: 5′-GCCTCGAGTTTTGTCACCAATTCC-3′ for Pgp-TM4R and 5′-GAGACCATGCAGATGAGAATAG-3′ for CF-TM4R, respectively. The first PCR product was then used as a primer together with SP6 primer to amplify the remaining sequence of Pgp-TM3,4R and CF-TM3,4R, respectively. The second PCR product encoding TM4 was cloned using the pCR cloning vector and ligated to a reporter as described above.

Pgp-TM3R(S), Pgp-TM4R(S), and CF-TM4R(S) were engineered using PCR as described above using Pgp-TM3R, Pgp-TM4R, and CF-TM4R as templates, respectively. The sense primers were SP6 primer for Pgp-TM3R(S), 5′-TCGTGATGACTCGAGGCTGG-3′ for Pgp-TM4R(S), and 5′-GAGACCATGGAGTTGTTACAG-3′ for CF-TM4R(S). The antisense primers were 5′-GTCTGGTTTGGTTAGCTTC-3′ for Pgp-TM3R(S), B50 primer (see above) for Pgp-TM4R(S), and 5′-CAAGATCTGAATCACAAGTCT-3′ for CF-TM4R(S). The PCR product was cloned, propagated, and ligated to a reporter as described above.

A two-step PCR was used to engineer CF1,2-P3,4R construct. In the first PCR, pGPGP-N4 (Zhang *et al.*, 1993) was used as a template to amplify the sequence encoding TM3 and TM4 of Pgp. The primers used were 5′-GTCAAGCCGTGTTCTAGATAAAGAGCTCAACA-3′ (the first 22 nucleotides are from CFTR and the last 10 nucleotides are from Pgp) and B50 primer (see above). The first PCR product was purified and used as a primer together with the SP6

primer to amplify the sequence encoding the N-terminal sequence including TM1 and TM2 of CFTR. The second PCR product was digested with *Pst*I and *Eco*RI, isolated, and then ligated into a vector containing the reporter-coding sequence and linearized with *Pst*I and *Eco*RI.

To engineer CF1,2-P3R fusion protein, a *Pst*I-*Xho*I fragment from CF1,2-P3,4R DNA was released, isolated, and ligated into pGEM-4z together with a *Xho*I–*Hin*dIII fragment from pGPGP-N3 DNA (Zhang *et al.*, 1993). All DNAs were sequenced to confirm the correct linkage and to eliminate any potential mutations due to cDNA manipulation and PCR.

In Vitro Transcription and Translation

About 6 μg of recombinant DNA linearized with *Hin*dIII was transcribed in the presence of 5 A_{260} U/ml cap analog $m^7G(5')ppp(5')G$ as described previously (Zhang and Ling, 1991). Removal of DNA templates with RQ1 deoxyribonuclease after transcription and purification of RNA transcripts was carried out according to Zhang and Ling (1991). Cell-free translations in RRL, proteolysis/membrane protection assay, limited endoglycosidase treatment, isolation of membrane fractions by centrifugation, as well as analysis using SDS-PAGE and fluorography, were performed as previously described (Zhang *et al.*, 1993).

RESULTS

De Novo N_{in}-C_{out} Insertion of Pgp TM4

We previously showed that in the folding that is different from prediction (Figure 1A, model II), TM3 of Pgp is in cytoplasm and TM4 is in the membrane in a N_{in}-C_{out} orientation (Zhang *et al.*, 1993). In this study, we tested whether the TM4 of Pgp has de novo signal sequence activity to initiate the N_{in}-C_{out} membrane insertion. A fusion construct encoding TM4 of Pgp followed by a glycosylation reporter was engineered (Figure 1B, Pgp-TM4R). As shown in Figure 2A, translation of Pgp-TM4R in the absence of microsomal membranes (RM) generated a 43-kDa protein (lane 1). In the presence of RM, an additional protein of 45-kDa was produced (Figure 2A, lane 2). Separation of soluble and membrane-associated proteins by centrifugation revealed that all of the 45-kDa proteins and about half of the 43-kDa protein were in the membrane pellet (Figure 2A, lanes 3 and 4). Endoglycosidase PNGase F treatment reduced the 45-kDa protein to 43 kDa (Figure 2A, lanes 5 and 6), suggesting that the 45-kDa protein is a glycosylated form of the 43-kDa protein. Thus, the C-terminal reporter of the 45-kDa protein is likely in the RM lumen (Figure 2C, N_{in}-C_{out} model).

To confirm lumenal location of the reporter peptide of the glycosylated Pgp-TM4R protein, we performed proteinase K digestion of the membrane fraction after translation. As shown in Figure 2B, a major peptide fragment of 39 kDa was protected from digestion (lane 1). However, digestion of membrane fractions permeabilized with Triton X-100 did not generate this peptide (Figure 2B, lane 2), suggesting that generation of the 39-kDa peptide fragment was due to the protection by the membranes. The proteinase K-resistant 39-kDa peptide was reduced to ~37 kDa by PNGase F (Figure 2B, lane 3), suggesting that it was glycosylated. Thus, the reporter of the glycosylated Pgp-TM4R protein is located in the RM lumen. Taken together, we conclude that the TM4 of Pgp has de novo activity to initiate N_{in}-C_{out} membrane insertion. This activity may be

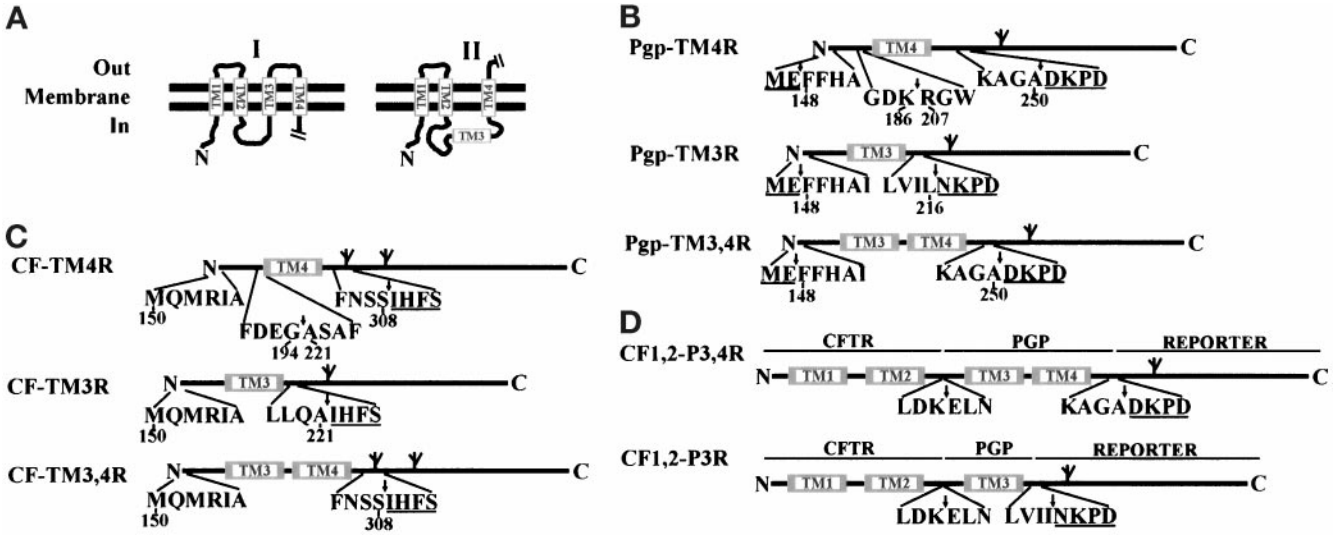

Figure 1. (A) Topological models of a partial sequence of Pgp. Model I is the predicted topology with all TM segments in the membrane. Model II is the alternative topology with TM3 in cytoplasm and TM4 in an N_{in}-C_{out} orientation. (B, C, and D) Diagram of constructs used to characterize TM3 and TM4 of Pgp (B and D) and CFTR (C). Each fusion site is indicated by an arrow. The potential glycosylation sites are shown as branched symbols. The underlined amino acids (in single-letter code) are residues from the reporter peptide. Note that the reporter peptide in CF-TM4R and CF-TM3,4R has two potential N-linked glycosylation sites (Chen and Zhang, 1996).

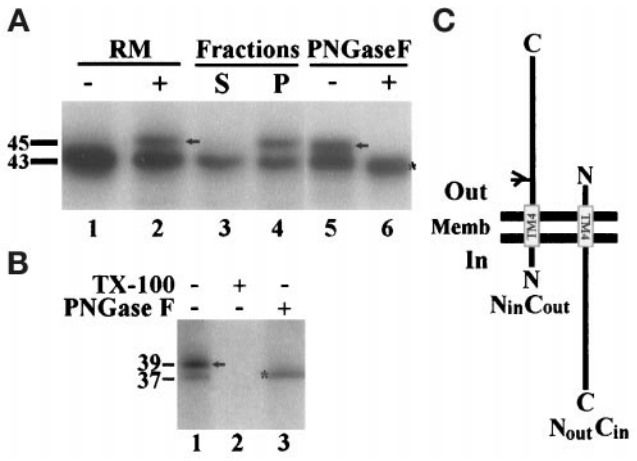

Figure 2. Translation and membrane orientation of Pgp-TM4R. (A) Translation of Pgp-TM4R. Pgp-TM4R RNA was translated in RRL in the absence (lane 1) or presence (lane 2) of RM. Lanes 3–4 and 5–6 represent proteins from separation of membrane (P) and soluble (S) fractions by centrifugation and endoglycosidase treatment, respectively. (B) Proteinase K digestion of membrane-associated Pgp-TM4R. Proteinase K digestion was performed in the absence (lane 1) or presence (lane 2) of Triton X-100 or further treated with endoglycosidase PNGase F (lane 3). The symbols (←) and (*) indicate glycosylated and deglycosylated proteins, respectively. (C) Orientations of Pgp-TM4R protein in the membrane. "In" represents the cytoplasmic side and "out" represents the lumenal side.

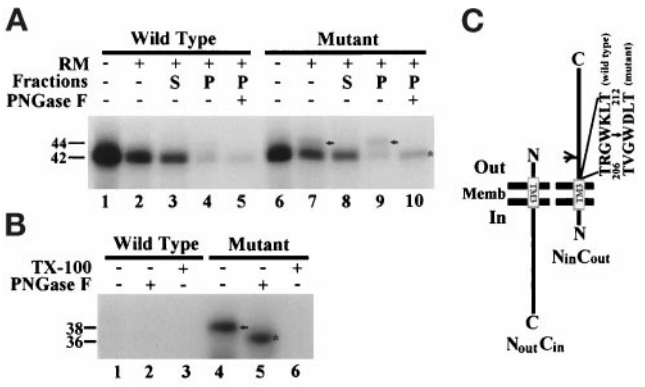

Figure 3. Translation and membrane insertion of Pgp-TM3R. (A) Translation of Pgp-TM3R. Wild-type and mutant Pgp-TM3R RNAs were translated in RRL in the absence (lanes 1 and 6) or presence (lanes 2 and 7) of RM. Lanes 3–4 and 8–9 represent membrane (P) and soluble (S) fractions of wild-type and mutant Pgp-TM3R, respectively. Lanes 5 and 10 are the endoglycosidase-treated wild-type and mutant proteins, respectively. (B) Proteinase K treatment of membrane associated Pgp-TM3R. Proteinase K digestion was performed in the absence (lanes 1 and 4) or presence (lanes 3 and 6) of Triton X-100 or further treated with endoglycosidase PNGase F (lanes 2 and 5). (C) The two orientations of Pgp-TM3R in the membrane. In the mutant Pgp-TM3R, Arg^{207} and Lys^{210} were changed to Val and Asp, respectively. The symbols (←) and (*) indicate glycosylated and deglycosylated proteins respectively.

important in generating the alternative topology of Pgp (Zhang *et al.*, 1993).

De novo N_{in}-C_{out} Insertion of Pgp TM3

In the alternative topology of Pgp (Figure 1A), TM3 was located in cytoplasm. This suggests that TM3 did not initiate the N_{in}-C_{out} insertion in the presence of TM4. To determine the de novo insertion activity of TM3, we engineered a construct (Pgp-TM3R) similar to Pgp-TM4R (see Figure 1B). Translation of Pgp-TM3R in the absence of RM generated a protein of 42 kDa (Figure 3A, lane 1). In the presence of RM, no additional protein was produced (Figure 3A, lane 2). Separation of membrane fractions by centrifugation revealed that most of the 42-kDa protein was in the supernatant and only a small fraction of this protein was associated with membranes (Figure 3A, lanes 3 and 4). Thus, TM3 of Pgp does not have de novo activity to initiate N_{in}-C_{out} membrane insertion as compared with TM4 (compare lanes 3 and 4 in Figures 2A and 3A).

To determine whether the charged amino acids at the C-terminal side of TM3 affect the de novo N_{in}-C_{out} membrane insertion of TM3, we performed an experiment using a mutant Pgp-TM3R that has two positive charges at the C-terminal side of TM3 mutated to neutral and negative charges, respectively (see Figure 3C; see also Zhang *et al.*, 1995). As shown in Figure 3A, separation of membrane fractions by centrifugation (compare lanes 8 and 9) revealed that the amount of membrane-associated mutant proteins was similar to that of the wild-type protein (compare lanes 3–4 with lanes 8–9). However, it should be noted that an additional protein of 44 kDa was observed from the translation of the mutant Pgp-TM3R in the presence of RM (Figure 3A, lane 7). This 44-kDa protein was reduced to 42 kDa by endoglycosidase treatment (Figure 3A, lanes 9 and 10), suggesting that it is glycosylated and likely has an N_{in}-C_{out} orientation (Figure 3C). This is confirmed by proteinase K digestion of the membrane-associated mutant Pgp-TM3R, which revealed that a glycosylated 38-kDa protein was protected from digestion (Figure 3B, lanes 4–6), whereas no detectable fragment was protected from the wild-type protein (Figure 3B, lanes 1–3). These observations indicate that alteration of positive charges at the C-terminal side of TM3 enhances the de novo N_{in}-C_{out} membrane insertion of TM3.

De Novo N_{in}-C_{out} Insertion of Pgp TM4 in the Presence of TM3

To determine the behavior of TM4 in the presence of its preceding TM3, we made a construct consisting of both TM3 and TM4 with a reporter (Figure 1B, Pgp-TM3,4R). As shown in Figure 4A, translation of Pgp-TM3,4R RNA generated a 44-kDa protein (lane 1). In the presence of RM, a protein of 46 kDa (indicated by

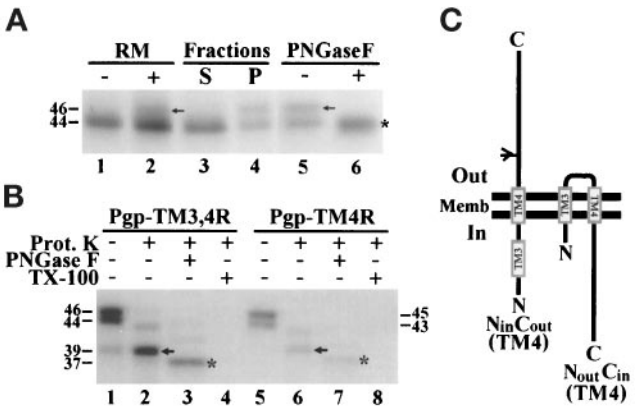

Figure 4. Translation and orientation of Pgp-TM3,4R. (A) Translation of Pgp-TM3,4R. Pgp-TM3,4R RNA was translated in RRL in the absence (lane 1) or presence (lane 2) of RM. Lanes 3–4 and 5–6 represent proteins from separation of membrane (P) and soluble (S) fractions by centrifugation and endoglycosidase treatment, respectively. (B) Proteinase K treatment of membrane-associated Pgp-TM3,4R. Proteinase K digestion of Pgp-TM3,4R and Pgp-TM4R was performed in the absence (lanes 2 and 6) or presence (lanes 4 and 8) of Triton X-100 or further treated with endoglycosidase PNGase F (lanes 3 and 7). Lanes 1 and 5 are untreated control samples. The minor proteolytic fragments with higher molecular weight shown in lanes 2 and 6 probably represent incompletely digested proteins. (C) The two possible orientations of Pgp-TM3,4R in the membrane. The indication (e.g., N_{in}-C_{out}) is for TM4 only. The symbols (←) and (*) indicate glycosylated and deglycosylated proteins, respectively.

an arrow) was also produced (Figure 4A, lane 2). After centrifugation, all of the 46-kDa protein was found in the membrane pellet (Figure 4A, lanes 3 and 4). Endoglycosidase treatment reduced this protein to 44 kDa (Figure 4A, lanes 5 and 6), suggesting that it is a glycosylated form of the 44-kDa protein. Thus, the reporter of Pgp-TM3,4R is located in RM lumen and glycosylated.

To determine whether the lumenal location of the reporter is due to the N_{in}-C_{out} insertion of TM4, we performed proteinase K digestion of membrane-associated Pgp-TM3,4R in comparison with Pgp-TM4R. As shown in Figure 4B, a major 39-kDa peptide was protected from digestion (indicated by an arrow in lane 2) which can be reduced to 37 kDa by endoglycosidase PNGase F (lane 3). The protected 39-kDa fragment has the same size as that protected from the Pgp-TM4R translation products (Figure 4B, compare lanes 2 and 3 with lanes 6 and 7). Thus, the glycosylated 46-kDa Pgp-TM3,4R translation product represents the molecule with a N_{in}-C_{out} orientation for TM4 (see Figure 4C). The above studies demonstrated that the Pgp TM4 initiates a N_{in}-C_{out} insertion into membranes even in the presence of its preceding TM3. Therefore, TM4 has a strong de novo activity to initiate N_{in}-C_{out} insertion and likely leaves TM3 in cytoplasm. This is consistent with our previous observation that TM4 displays a N_{in}-C_{out} orientation in the presence of its all preceding sequences (Zhang *et al.*, 1993).

De novo N_{in}-C_{out} Insertion of TM3 and TM4 of CFTR

Previously, we have shown that, unlike Pgp, CFTR expresses only the predicted topology (Chen and Zhang, 1996). It is interesting to compare the membrane insertion properties of TM3 and TM4 of CFTR with those of Pgp. To this end, we made CF-TM3R, CF-TM4R, and CF-TM3,4R constructs (Figure 1C), similar to those of Pgp (Figure 1B). Translations of these constructs are shown in Figure 5A. Both CF-TM3R and CF-TM4R produced glycosylated proteins (Figure 5A, indicated by arrows in lanes 2 and 5) in the presence of RM as compared with the reaction in the absence of RM (Figure 5A, compare lanes 1 and 4 with lanes 2 and 5, respectively). The glycosylation is confirmed by endoglycosidase treatment (Figure 5A, lanes 3 and 6). However, translation of CF-TM3,4R in the presence of RM did not generate any glycosylated proteins (Figure 5A, lanes 8 and 9).

To confirm that the reporters in the glycosylated CF-TM3R and CF-TM4R proteins are located in the RM lumen, we treated the membrane-associated proteins with proteinase K. As shown in Figure 5B, proteinase K digestion of membrane-associated CF-TM3R and CF-TM4R resulted in peptide fragments of 33 kDa and 42 kDa, respectively (lanes 1 and 4). These peptides were sensitive to PNGase F treatment (Figure 5B, lanes 2 and 5), suggesting that they are glycosylated. Complete removal of these fragments in the presence of Triton X-100 suggests that they are located in the RM lumen. Proteinase K digestion of membrane-associated CF-TM3,4R, on the other hand, did not generate any membrane-protected fragments (Figure 5B, lanes 7–9), consistent with the observation that the reporter in CF-TM3,4R is not glycosylated. Thus, the reporter of CF-TM3,4R is likely located outside of RM.

To test further the de novo membrane insertion activity of TM3 and TM4 of CFTR, the translation of CF-TM3R and CF-TM4R in the presence of RM was separated by centrifugation. As shown in Figure 5C, TM3R and TM4R proteins are present in similar low levels in the membrane pellet. Thus, there is no significant difference between the de novo activity of TM3 and TM4 to initiate N_{in}-C_{out} membrane insertion.

Based on the above observations, we conclude that TM3 and TM4 of CFTR by itself can initiate a similar level of membrane insertion to adopt N_{in}-C_{out} orientation. However, unlike Pgp, the TM4 of CFTR does not initiate the N_{in}-C_{out} insertion in the presence of its preceding TM3. Therefore, CFTR cannot form the alternative topology (Figure 1A, model II).

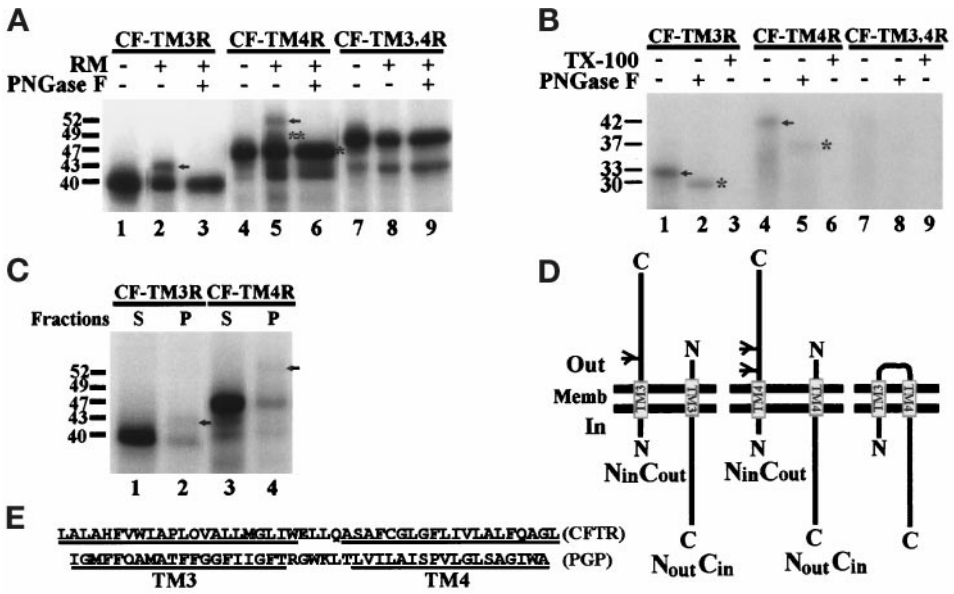

Figure 5. Characterization of TM3 and TM4 of CFTR. (A) Translation of CF-TM3R, CF-TM4R, and CF-TM3,4R. RNAs of CF-TM3R, -TM4R, and -TM3,4R were translated in RRL. Lanes 1, 4, and 7 show the protein products generated in the absence of RM. Lanes 2, 5, and 8 are membrane-associated proteins translated in the presence of RM. Lanes 3, 6, and 9 represent translation products treated by endoglycosidase PNGase F. The symbols (←) and (*) indicate fully glycosylated and deglycosylated proteins, respectively. The symbol (**) indicates the protein with one oligosaccharide chain. (B) Proteinase K digestion of membrane-associated CF-TM3R, -TM4R, and -TM3,4R. Proteinase K digestion was performed in the absence (lanes 1, 3, and 7) or presence (lanes 3, 6, and 9) of Triton X-100, or further treated with endoglycosidase PNGase F (lanes 2, 5, and 8). (C) Separation of membrane-associated from soluble translation products. Translation of CF-TM3R and -TM4R was performed in the presence of RM, and the membrane-associated proteins (P) were separated from soluble proteins (S) by centrifugation. The arrows indicate the glycosylated protein. Note that the glycosylated CF-TM4R proteins contain two oligosaccharide chains. (D) Orientations of CF-TM3R, -TM4R, and -TM3,4R in the membrane. (E) Sequence comparison between TM3-TM4 of Pgp and CFTR. The amino acid sequence of TM3, TM4, and their linking loop from Pgp and CFTR are shown in single letter code. The underlined sequences are TM segments.

De Novo Membrane Insertion of TM3 and TM4 with Shorter Flanking Domains

Previously, it has been shown that the cytoplasmic loops affect the membrane insertion of TM segments (McGovern *et al.*, 1991; Seligman and Manoil, 1994). In the above studies with Pgp-TM4R and CF-TM4R proteins, a loop linking TM2 and TM3 was included at the N-terminal side of TM4 in our TM4R constructs. To determine whether this loop affects the membrane insertion property of TM4, we engineered new constructs named Pgp-TM4R(S) and CF-TM4R(S) (see Figure 6A). In these constructs, only five to seven amino acid residues from the short loop between TM3 and TM4 were included at the N-terminal side of TM4. It has been shown that about 15 amino acids on each side of a TM is important for topogenesis (Hartmann *et al.*, 1989). Thus, to avoid interrupting the integrity of the TM4 topogenesis 19–21 amino acids were retained at the C-terminal side of TM4 in these constructs. As shown in Figure 6B, translation of Pgp-TM4R(S) in the presence of RM generated a protein of a similar size with that in the absence of RM (Figure 6B, lanes 1 and 2). However, more than half of the Pgp-TM4R(S) products translated in the presence of RM were found in the membrane pellet (Figure 6B, lanes 3 and 4), suggesting that the TM4 without the long loop at the N-terminal side can still insert into membranes. When treated with PNGase F, the size of all membrane-associated Pgp-TM4R(S) was reduced (Figure 6C, lanes 1 and 2), suggesting that the reporter of Pgp-TM4R(S) has been translocated into the RM lumen and glycosylated. It should be noted that the deglycosylated Pgp-TM4R(S) is smaller in size than that translated in the absence of RM. This is likely due to the translocation of the C-terminal reporter into the RM lumen and exposure of a cryptic signal sequence cleavage site at the C-terminal side of TM4 (Figure 6D, Pgp-TM4R(S)). The membrane-associated Pgp-TM4R(S) is resistant to proteinase K treatment, confirming its location in the RM lumen (Figure 6C, lanes 3 and 4). The fact that all membrane targeted Pgp-TM4R(S) is glycosylated suggests that all membrane associated Pgp-TM4R(S) has the N_{in}-C_{out} orientation. Therefore, deletion of the domain at the N-terminal side of TM4 did not reduce the de novo N_{in}-C_{out} membrane insertion activity of TM4. However, the deletion revealed a cryptic signal sequence cleavage site located at the C-terminal side of TM4.

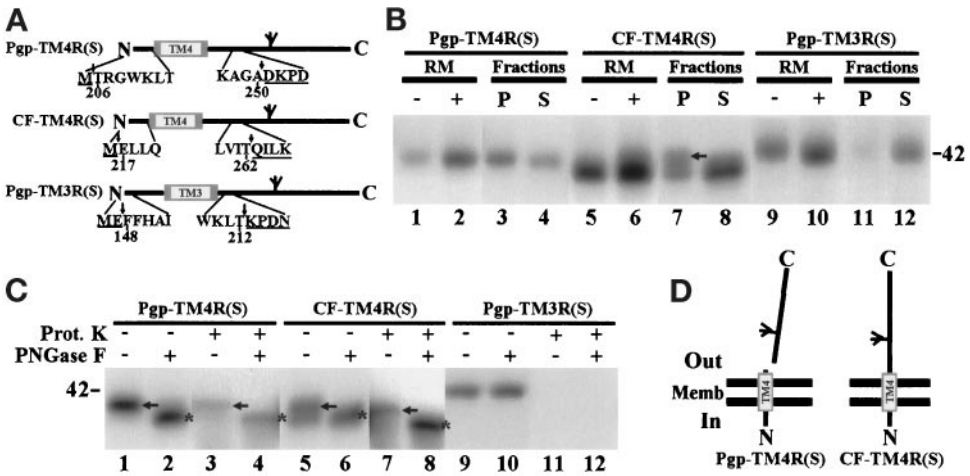

Figure 6. Translation and characterization of Pgp-TM4R(S), CF-TM4R(S), and Pgp-TM3R(S). (A) Linear diagram of Pgp-TM4R(S), CF-TM4R(S), and Pgp-TM3R(S). The fusion site is indicated by an arrow. The potential glycosylation sites are shown as branched symbols. The underlined amino acids are residues from the reporter peptide. (B) Translation and membrane fractionation. RNAs were translated in RRL in the absence (lanes 1, 5, and 9) or presence (lanes 2, 6, and 10) of RM. Lanes 3–4, 7–8, and 11–12 represent proteins after separation of membrane (P) and soluble (S) fractions by centrifugation. (C) PNGase F and proteinase K treatment of membrane-associated proteins. Membranes were pelleted after translation and then treated with PNGase F alone (lanes 2, 6, and 10), proteinase K alone (lanes 3, 7, and 11), or proteinase K followed by PNGase F (lanes 4, 8, and 12). Lanes 1, 5, and 9 are untreated control membrane samples. The symbols (←) and (*) indicate glycosylated and deglycosylated proteins, respectively. (D) Topological model of Pgp-TM4R(S) and CF-TM4R(S). Note that the reporter of Pgp-TM4R(S) is translocated into the RM lumen and cleaved off by signal peptidase whereas that of CF-TM4R(S) remains intact with the TM segment.

Translation of CF-TM4R(S) in the presence of RM generated a glycosylated protein (Figure 6B, indicated by an arrow in lane 7) as shown by PNGase F treatment (Figure 6C, lanes 5 and 6). Proteinase K treatment showed that the glycosylated reporter is in the RM lumen (Figure 6C, lanes 7 and 8). Most of the unglycosylated CF-TM4R(S) were found in the supernatant (Figure 6B, lanes 7 and 8) as the CF-TM4R products. These results suggest that truncating the domains surrounding TM4 did not affect the N_{in}-C_{out} insertion of CFTR TM4 (Figure 6D).

In the studies of Pgp-TM3R shown in Figure 3, Pgp-TM3R contains four amino acids derived from TM4 at the C-terminal side of TM3. These amino acids may affect the membrane insertion property of TM3. To test this possibility, we made a new construct Pgp-TM3R(S) by deleting these four amino acids (see Figure 6A). Translation of Pgp-TM3R(S) did not generate a glycosylated molecule in the presence of RM (Figure 6B, lanes 9 and 10). Most of the Pgp-TM3R(S) products translated in the presence of RM were found in the supernatant and not associated with membranes (Figure 6B, lanes 11 and 12). The Pgp-TM3R(S) protein was not changed by PNGase F digestion, and no protected fragments were found when the membranes were treated with proteinase K (Figure 6C, lanes 9–12), confirming that the reporter of Pgp-TM3R(S) is not exposed to the RM lumen. Thus, removing the four residues of TM4 at the C-terminal side of TM3 did not increase the de novo N_{in}-C_{out} membrane insertion activity of Pgp TM3.

Effects of N-Terminal Membrane Anchorage Sequence on the N_{in}-C_{out} Insertion of Pgp TM4

The above studies suggested that TM4 of Pgp has a strong de novo N_{in}-C_{out} insertion activity, which is responsible for the generation of an alternative topology (Figure 1A, model II). Previously, we have shown that the N_{in}-C_{out} insertion of TM4 occurs even in the presence of the N-terminal membrane-anchorage sequences TM1–TM2 (Zhang *et al.*, 1993; 1995). To determine whether the N-terminal membrane anchorage sequences affect the N_{in}-C_{out} membrane insertion of TM4, we replaced the N-terminal TM1-TM2 of Pgp by the homologous sequence from CFTR. The newly created fusion protein was named CF1,2-P3,4R (Figure 1D). When this fusion protein was translated in the presence of RM, to our surprise, no glycosylated protein was generated (Figure 7A, lanes 1 and 4) although all of the nascent proteins are associated with membranes (Figure 7A, lanes 2 and 3). Thus, the N_{in}-C_{out} insertion of TM4 in CF1,2-P3,4R did not occur. On the other hand, the N_{in}-C_{out} insertion of TM4 was generated in Pgp-N4 with the native TM1-TM2. Using both limited endoglycosidase and proteinase K digestion, we have previously shown that the protein indicated by an arrow in lanes 5 and 7 of Figure 7A represents

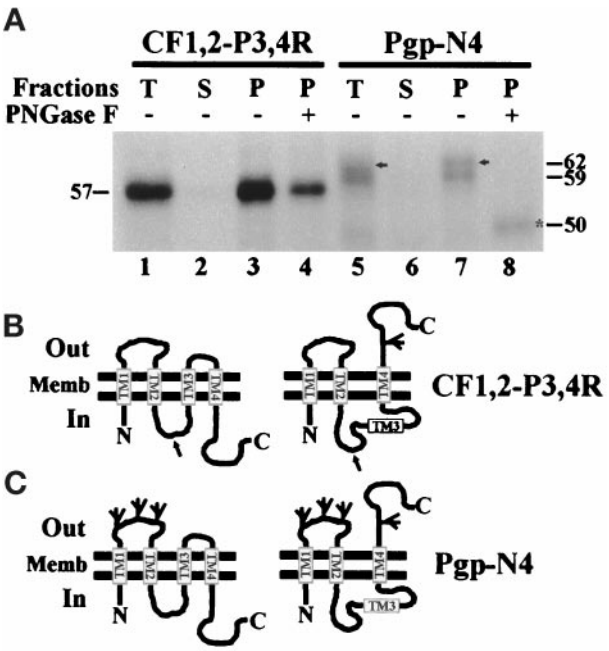

Figure 7. Effects of N-terminal sequence on TM4 membrane insertion. CF1,2-P3,4R and Pgp-N4 RNAs were translated in RRL in the presence of RM, and the membrane-associated proteins (P) (lanes 3 and 7) were separated from soluble fractions (S) (lanes 2 and 6) by centrifugation. Lanes 1 and 5 represent the total (T) reaction before separation by centrifugation. Lanes 4 and 8 are membrane-associated proteins subjected to endoglycosidase treatment. The symbols (←) and (*) indicate glycosylated and deglycosylated proteins, respectively. Panels B and C show the two possible orientations of CF1,2-P3,4R and Pgp-N4 proteins in the membrane, respectively. It should be noted that there are three oligosaccharide chains (represented by the branched symbols) in the first loop linking TM1 and TM2 of Pgp-N4 molecules (panel C). The arrow indicates the site of fusion between CFTR and Pgp.

the model II topology shown in Figure 1A (Zhang *et al.*, 1993). Thus, we conclude that the N_{in}-C_{out} insertion by TM4 of Pgp is affected by the N-terminal membrane-anchorage sequences (i.e., TM1-TM2). However, these sequences in Pgp permit TM4 to have the N_{in}-C_{out} insertion.

Effects of N-Terminal Membrane Anchorage Sequence on the Membrane Insertion of Pgp TM3

Although we have shown above that TM3 of Pgp does not have de novo N_{in}-C_{out} membrane insertion activity, TM3 can initiate N_{in}-C_{out} membrane insertion in a molecule that is preanchored into membranes by TM1 and TM2 (Zhang *et al.*, 1993). We have also shown above that the de novo N_{in}-C_{out} insertion of TM4 can be affected by the replacement of TM1 and TM2 from CFTR. It would be interesting to know whether a similar replacement would affect the TM3 insertion. When the N-terminal membrane anchorage sequences (TM1 and TM2) of Pgp were replaced by the same sequence from CFTR, a glycosylated protein was generated in the presence of RM (Figure 8A, indicated by an arrow in lanes 1 and 4), and it was associated with membranes (Figure 8A, lanes 2 and 3). Proteinase K digestion of membrane-associated proteins also revealed a membrane-protected and PNGase F-sensitive 38-kDa peptide fragment (Figure 8B, lanes 1 and 2), confirming the lumenal location of a glycosylated reporter. Thus, preanchorage of the nascent protein into membranes by TM1 and TM2 from both CFTR and Pgp can help TM3 of Pgp initiate an N_{in}-C_{out} membrane insertion. This further suggests that TM3 and TM4 of Pgp have different membrane insertion properties.

DISCUSSION

In this study, we investigated the de novo membrane insertion of TM3 and TM4 of hamster *pgp1* Pgp in comparison with that of CFTR to elucidate the mechanism of generating alternative topologies of Pgp (model II, Figure 1A). We found that TM3 of Pgp does not have de novo activity to initiate N_{in}-C_{out} membrane insertion whereas TM4 of Pgp does. TM4 of Pgp can initiate an N_{in}-C_{out} membrane insertion even in the presence of its preceding TM3. However, unlike Pgp, TM4 of CFTR does not initiate an N_{in}-C_{out} membrane insertion in the presence of its preceding TM3. Based on this study and our previous findings, we conclude that 1) the membrane insertion of TM3 and TM4 of Pgp does not necessarily follow the sequential model, which predicts that TM3 initiates N_{in}-C_{out} insertion whereas TM4 stops the membrane translocation event and forms a N_{out}-C_{in} orientation; and 2) the "leaving one out" strategy may be universal for polytopic membrane proteins to fold properly when topogenic signals from various TM segments contradict each other (see below), and it is regulated by the N_{in}-C_{out} membrane insertion activities of the TM segments.

In this study, we also showed that the N-terminal membrane-anchorage sequence including TM1 and TM2 of Pgp permits TM4 to have the N_{in}-C_{out} insertion. Replacing the N-terminal membrane-anchorage sequence with an equivalent one from CFTR abolished this insertion. Apparently, the N_{in}-C_{out} membrane insertion by TM4 of Pgp is not independent of the N-terminal membrane anchorage sequences. It is only that the N-terminal membrane anchorage sequences of Pgp allow TM4 to perform the N_{in}-C_{out} membrane insertion. This is consistent with our previous observation that the N-terminal sequence of human *MDR3* Pgp is important for the folding of internal TM segments (Zhang, 1996).

Although we have shown that TM3 of Pgp does not have de novo N_{in}-C_{out} membrane insertion activity, it

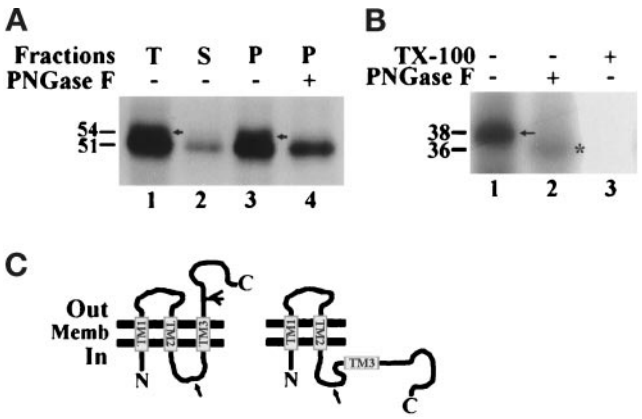

Figure 8. Effects of N-terminal sequence on TM3 membrane insertion. (A) Translation of CF1,2-P3R. CF1,2-P3R RNA was translated in RRL in the presence of RM, and the membrane-associated proteins (P) (lane 3) were separated from soluble fractions (S) (lane 2) by centrifugation. Lane 1 represents the total (T) reaction before separation by centrifugation. Lane 4 is membrane-associated proteins subjected to endoglycosidase treatment. (B) Proteinase K digestion of membrane-associated CF1,2-P3R. Proteinase K digestion was performed in the absence (lanes 1 and 2) or presence (lane 3) of Triton X-100 or further treated with endoglycosidase PNGase F (lane 4). The symbols (←) and (*) indicate glycosylated and deglycosylated proteins, respectively. (C) Two possible orientations of CF1,2-P3R in the membrane. The branched symbol indicates oligosaccharide chains and the arrow indicates the site of fusion between CFTR and Pgp.

can initiate such an insertion if the protein is preanchored in the membrane by its preceding sequence TM1 and TM2 from Pgp (Zhang *et al.*, 1993) or from CFTR (this study). Thus, proteins with the predicted topology (i.e., TM3 in N_{in}-C_{out} and TM4 in N_{out}-C_{in} orientation) was also observed in addition to the alternative topology when all four TM segments are present (Zhang *et al.*, 1993). The de novo N_{in}-C_{out} membrane insertion activity of TM3 can also be increased by mutating the positively charged amino acids at the C-terminal side of TM3 (see Figure 3). Hence, mutations of these positively charged amino acid residues will increase the generation of the predicted topology (i.e., TM3 in N_{in}-C_{out} and TM4 in N_{out}-C_{in} orientation), consistent with our previous observation of mutation analysis (Zhang *et al.*, 1995).

Using human *MDR1* Pgp as a model protein, Skach and Lingappa (1994) showed that TM3 does not have de novo membrane insertion activity. A chimeric protein with TM3 and its following 19 amino acid residues including 17 of 20 residues of TM4 can initiate a de novo membrane insertion. These observations are consistent with this study. However, they also showed that 1) TM4 does not have de novo N_{in}-C_{out} membrane insertion activity and 2) TM3 alone cannot initiate membrane insertion in a molecule preanchored into membranes (Skach and Lingappa, 1994). It is not known what caused the discrepancy between the observations by Skach and Lingappa and our studies. However, it may be due to 1) the sequence diversity between human *MDR1* Pgp used by Skach and Lingappa (1994) and hamster *pgp1* Pgp used in this study, 2) the different reporter peptide sequence used (prolactin vs ATP-binding domain of Pgp), 3) different fusion sites where the reporter gene was engineered (amino acid 276 of human Pgp vs amino acid 249 of hamster Pgp for TM4), 4) different preanchorage sequences used for TM3 (IgM-derived stop-transfer sequence vs TM1 and TM2 of Pgp), and/or 5) different expression systems used (frog oocytes vs cell-free system). We are currently investigating these possibilities.

In the alternative topology of Pgp, TM3 is likely located in the cytoplasm when TM4 is inserted in membranes in an N_{in}-C_{out} orientation. Such a "leaving one out" strategy may be universal and possibly an important folding mechanism when topogenic signals from various TM segments contradict each other. For example, when both TM3 and TM4 of Pgp can initiate the N_{in}-C_{out} insertion, only the one (TM4) with stronger activity can adopt such an orientation and leaves the other (TM3) out of the membrane. By increasing the N_{in}-C_{out} insertion activity of TM3 (e.g., altering the charged amino acids at the C-terminal side of TM3) to compete with TM4, more N_{in}-C_{out} insertion for TM3 will be obtained, resulting in a decreased proportion of the alternative topology (Zhang *et al.*, 1995). Recently, polytopic membrane proteins that were forced to adopt alternative topology by leaving one or more TM segments out of the membrane have been created in both bacteria (Gafvelin and von Heijne, 1994) and mammalian cells (Gafvelin *et al.*, 1997).

Earlier studies on the biogenesis of polytopic membrane proteins (Friedlander and Blobel, 1985; Audigier *et al.*, 1987) suggested that transmembrane segments in polytopic membrane proteins have different functions as signal-anchor and stop-transfer sequences. Presumably, the first TM segment functions as a signal anchor sequence whereas the second one functions as a stop-transfer sequence (Wessels and Spiess, 1988; Lipp *et al.*, 1989; Hartmann *et al.*, 1989; Skach and Lingappa, 1993). This membrane insertion event by signal-anchor and stop-transfer sequences repeat itself until all TM segments are in the membrane. According to the sequential membrane insertion model, TM3 of Pgp initiates N_{in}-C_{out} membrane insertion (signal-anchor) whereas TM4 stops the membrane-translocation event (stop-transfer) and forms a N_{out}-C_{in} orientation. However, our studies indicated that TM4 is a strong signal-anchor sequence and initiates the N_{in}-C_{out} insertion even in the presence of TM3, resulting in the alternative topology. Both our studies on Chinese hamster Pgp and the studies on bacterial leader peptidase by von Heijne's group (Gafvelin and von Heijne, 1994; Gafvelin *et al.*, 1997) suggest that the sequen-

tial signal-anchorage and stop-transfer model for membrane insertion of polytopic membrane proteins is not applicable to every protein. Using Pgp as a model protein, Borel and Simon (1996a,b) have shown that the TM segments of Pgp do not integrate into lipid bilayer until the protein is completely synthesized and released from the ribosome. While the protein is being synthesized, the TM segments are likely accumulating in the putative protein-conducting channel (Simon and Blobel, 1991). This observation may indicate an underlying mechanism for the generation of alternative topologies of Pgp. Thus, we believe that Pgp is an ideal model protein for investigating membrane insertion mechanisms of polytopic membrane proteins alternative to the prevailing sequential membrane insertion mechanism.

It is believed that polytopic membrane proteins acquire their final topology in ER membranes (Goldman and Blobel, 1981; Braell and Lodish, 1982; Brown and Simoni, 1984; Wessels and Spiess, 1988). Although topologies alternative to the predicted one for Pgp were found using cell-free frog oocytes and bacteria expression systems (Zhang and Ling, 1991, Zhang *et al.*, 1993; Zhang, 1996; Skach *et al.*, 1993; Bibi and Béjà, 1994), studies of mutant Pgp by Loo and Clark (1995) and Kast *et al.* (1995) suggested that Pgp has only the predicted topology on cell surface. Yet, using site-specific antibodies and wild-type Pgp in a multidrug-resistant cell line, we were able to show that the alternative topology of Pgp exists on plasma membranes (Zhang *et al.*, 1996). It is currently unknown what the alternative topologies of Pgp mean to its function. However, interchanges between different topological structures of Pgp may be involved in its transport function. Our recent studies suggest that Pgp has a large conformational change during its catalytic cycle, and this conformational change may represent the putative topological conversion of Pgp (Wang *et al.*, 1997).

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