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# Sequence Requirements for Membrane Assembly of Polytopic Membrane Proteins: Molecular Dissection of the Membrane Insertion Process and Topogenesis of the Human *MDR3* P-Glycoprotein

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The biogenesis of membrane proteins with a single transmembrane (TM) segment is well understood. However, understanding the biogenesis and membrane assembly of membrane proteins with multiple TM segments is still incomplete because of the complexity and diversity of polytopic membrane proteins. In an attempt to investigate further the biogenesis of polytopic membrane proteins, I used the human *MDR3* P-glycoprotein (Pgp) as a model polytopic membrane protein and expressed it in a coupled cell-free translation/translocation system. I showed that the topogenesis of the C-terminal half *MDR3* Pgp molecule is different from that of the N-terminal half. This observation is similar to that of the human *MDR1* Pgp. The membrane insertion properties of the TM1 and TM2 in the N-terminal half molecule are different. The proper membrane anchorage of both TM1 and TM2 of the *MDR3* Pgp is affected by their C-terminal amino acid sequences, whereas only the membrane insertion of the TM1 is dependent on the N-terminal amino acid sequences. The efficient membrane insertion of TM3 and TM5 of *MDR3* Pgp, on the other hand, requires the presence of the putative TM4 and TM6, respectively. The TM8 in the C-terminal half does not contain an efficient stop–transfer activity. These observations suggest that the membrane insertion of putative TM segments in the human *MDR3* Pgp does not simply follow the prevailing sequential event of the membrane insertion by signal–anchor and stop–transfer sequences. These results, together with my previous findings, suggest that different isoforms of Pgp can be used in comparison as a model system to understand the molecular mechanism of topogenesis of polytopic membrane proteins.

## INTRODUCTION

The early steps of biogenesis of membrane proteins closely resemble the biogenesis of secretory proteins in the endoplasmic reticulum (ER).[1] A signal sequence in membrane proteins, as in secretory proteins, is responsible for the membrane targeting to the ER, whereas topogenic sequences (including the signal sequence) determine how the protein is folded in the membrane (Blobel, 1980). Additionally, it has been shown that N-linked glycosylation of a glycoprotein is also important for the membrane protein folding in the ER (reviewed, Helenius, 1994). Membrane insertion and translocation of a nascent membrane protein also involves many translocation–machinery proteins on the ER (reviewed, Schekman, 1994).

Most polytopic (spanning the membrane twice or more) transmembrane proteins in eukaryotic cells are thought to acquire their final membrane orientations during or immediately after synthesis on the rough ER, like the monotopic (spanning the membrane once)

[1] Abbreviations used: ER, endoplasmic reticulum; MDR, multidrug resistance; Pgp, P-glycoprotein; PNGase F, peptide N-glycosidase F; RM, rough microsomes; RRL, rabbit reticulocyte lysate; TM, transmembrane.

membrane proteins (Goldman and Blobel, 1981; Braell and Lodish, 1982; Brown and Simoni, 1984; Wessels and Spiess, 1988). Topogenic sequences involved in signal, signal–anchorage, and stop–transfer activities have been identified in eukaryotic polytopic membrane proteins (Friedlander and Blobel, 1985; Audigier *et al.*, 1987; Lipp *et al.*, 1989; Chavez and Hall, 1991; Silve *et al.*, 1991). It is thought that a polytopic topology is generated by the sequential translocation and membrane integration of independent topogenic sequences (Blobel, 1980; Wessels and Spiess, 1988; Hartmann *et al.*, 1989; Lipp *et al.*, 1989; Skach and Lingappa, 1993a). To test this hypothesis, I used the human multidrug resistance (MDR) *MDR3* P-glycoprotein (Pgp) as a model polytopic membrane protein to investigate the topogenesis and membrane assembly process.

The results obtained suggest that the membrane topogenesis of the C-terminal half of the human *MDR3* Pgp is different from the N-terminal half molecule. This observation is similar to studies of the human *MDR1* Pgp (Skach *et al.*, 1993). The proper membrane anchorage of both transmembrane (TM)1 and TM2 of *MDR3* Pgp is affected by their C-terminal amino acid sequences. However, the membrane insertion of the TM1 is also dependent on the N-terminal amino acid sequences. The membrane insertion of TM3 and TM5, on the other hand, requires the presence of the putative TM4 and TM6, respectively. TM8 does not contain an efficient stop–transfer activity. These observations suggest that the membrane insertion of putative TM segments in the human *MDR3* Pgp does not follow the sequential event of the membrane insertion by signal–anchor and stop–transfer sequences.

## MATERIALS AND METHODS

### *Materials*

pGEM-4z plasmid, SP6 and T7 RNA polymerase, RNasin, ribonucleotides, RQ1 DNase, rabbit reticulocyte lysate, and dog pancreatic microsomal membranes 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$^7$G(5')ppp(5')G cap analogue 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. All other chemicals were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

### *Construction of Fusion DNA between Human MDR3 cDNA and the Reporter Gene*

To study the relative orientation of the various predicted TM segments of human *MDR3* Pgp, I linked a cDNA fragment encoding the first ATP-binding domain of the hamster *pgp1* Pgp to different sites of the human *MDR3* Pgp cDNA and used it as a reporter. This reporter has been used successfully to study the membrane orientation of the Chinese hamster *pgp1* Pgp (Zhang *et al.*, 1993) and human CFTR (Chen and Zhang, 1996). It has a potential glycosylation site that can be used as an indicator of the membrane sidedness of the site to which the reporter is attached and does not have any hydrophobic sequences that will potentially act as a TM segment. The fusion between the two genes does not create any potential glycosylation sites.

The reporter cDNA fragment was released by digestion with *Hin*dIII combined with *Eco*RI, *Rsa*I, *Ssp*I, or *Bgl*II for fusion to TM2, TM3, TM4, and TM6 of human *MDR3*, respectively. Different enzymes were chosen to keep sequences in frame in the fusion constructs. The longest reporter gene was generated by digestion with *Rsa*I, and the shortest one was generated by *Bgl*II digestion. There is a difference of 25 amino acids in length between these two reporters, which has been shown not to affect the membrane translocation process of the reporter (Zhang *et al.*, 1993; Chen and Zhang, 1996). The *Eco*RI end was blunted with Klenow polymerase. Fragments of human *MDR3* Pgp cDNA encoding the N-terminal transmembrane domain were released by digesting the cDNA with *Eco*RI combined with *Hin*cII (amino acids 1–143, including TM1–TM2), *Msl*I (amino acids 1–217, including TM1–TM3), *Nco*I (amino acids 1–301, including TM1–TM4), or *Bgl*II (amino acids 1–411, including TM1–TM6). The *Msl*I, *Nco*I, and *Bam*HI ends were blunted by treatment with Klenow polymerase or T4 DNA polymerase. Different restriction endonucleases were used to create a correct reading frame of the fusion cDNAs. All DNA fragments were separated on 1% agarose gel by electrophoresis and purified as described previously (Zhang and Ling, 1991). The cDNA fragments encoding the various N-terminal transmembrane domains of the human *MDR3* Pgp were ligated to the corresponding reporter cDNA of hamster *pgp1* Pgp and cloned into pGEM-4z vector. The resulting DNA constructs were named 3-N2R, 3-N3R, 3-N4R, and 3-N6R, respectively. The 3-N5R construct was made from the 3-N6R DNA by deleting the TM6 segment and its surrounding sequences with *Bam*HI and *Xho*I. The DNA was treated with Klenow polymerase and then self-ligated to produce the 3-N5R DNA. The cDNA inserts in the 3-N2R, 3-N3R, 3-N4R, 3-N5R, and 3-N6R constructs are in the orientation such that the SP6 RNA polymerase can be used to produce sense RNA transcript. For making 3-N2 RNA transcript, the 3-N2R DNA was linearized at *Xmn*I site in the reporter sequence. Thus, the potential glycosylation site in the reporter sequence of 3-N2R was deleted. The 3-C6 DNA was constructed by cloning the 1.55-kb *Acc*I–*Sac*I fragment (encoding amino acids 618–1110) into the pGEM-4z. The orientation of cDNA insert in the 3-C6 construct required the use of T7 RNA polymerase to produce sense RNA transcript. All constructs were confirmed by DNA sequencing.

### *Construction of Mutant DNAs*

To remove the potential N-linked glycosylation sites in the human *MDR3* Pgp, I performed site-directed mutagenesis by polymerase chain reaction (PCR) as previously described (Zhang *et al.*, 1995). The *Eco*RI–*Hin*cII, *Pst*I–*Pst*I, and the *Hin*cII–*Hin*cII fragments containing mutations of glycosylation sites in the N-terminal end (Asn-8→Gln-8), in the loop linking TM2 and TM3 (Thr-170→Ala-170), and in the loop linking TM8 and TM9 (Thr-810→Ala-810), respectively, were cloned and sequenced. These fragments were then used to replace the wild-type cassette in the corresponding DNA constructs. The final mutant DNAs were confirmed by DNA sequencing. The primers carrying mutations were 5'-GTTCCTTGCTTTGCC (Asn-8→Gln-8), 5'-GAGTTCAGATGCATCATTG-3' (Thr-170→Ala-170), and 5'-GCACCAGCACTGTTTTTA-3' (Thr-810→Ala-810). To replace the amino terminus in 3-N2R and 3-N3RTA with the corresponding sequence of the *MDR1* Pgp, I replaced the *Eco*RI–*Bsp* HI cDNA fragment in the 3-N2R and 3-N3RTA DNA by the corresponding *Eco*RI–*Bsp* HI fragment of the human *MDR1* Pgp cDNA to generate 1/3-N2R and 1/3-N3RTA. The 1/3-N3RTA2 was generated by creating a *Kpn*I site at amino acid 53 of the *MDR1* Pgp, and the *Eco*RI–*Kpn*I fragment was used to replace the *Eco*RI–*Kpn*I fragment in the 3-N3RTA.

### *In Vitro Transcription and Translation*

Approximately 6 μg of recombinant DNA linearized with *Hin*dIII was transcribed in the presence of 5 $A_{260}$ units/ml cap analogue $m^7G(5')ppp(5')G$ as described previously (Zhang and Ling, 1991). Removal of DNA templates with RQ1 DNase after transcription and purification of RNA transcripts was performed according to Zhang and Ling (1991). Cell-free translations in rabbit reticulocyte lysate (RRL), proteolysis/membrane protection assay, limited endoglycosidase treatment, isolation of membrane fractions by centrifugation, as well as analysis with SDS-PAGE and fluorography, were performed as previously described (Zhang *et al.*, 1993). At the end of each translation, puromycin was added to a final concentration of 10 μM to terminate the translation reaction.

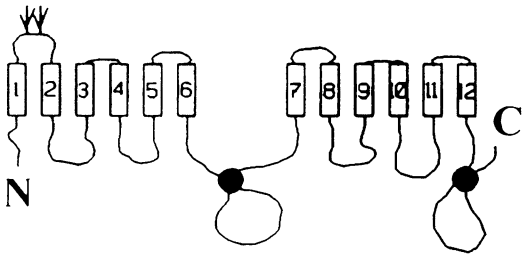



**Figure 1.** Predicted topology of human *MDR3* Pgp. The human *MDR3* Pgp is predicted to have 12 TM segments (indicated by numbered bars), and both N (N) and C termini (C) are located cytoplasmically. The loop linking TM1 and TM2 is predicted to contain two oligosaccharide chains (indicated by branched symbols). The solid circles represent the putative ATP-binding sites.

## RESULTS

### *Membrane Topogenesis of the C-Terminal Half Sequences of Human MDR3 Pgp*

Pgp consists of two homologous halves (Figure 1) and possibly originated from fusion of genes for two related, but independently evolved, proteins (Chen *et al.*, 1990). It has been shown that both the N- and the C-terminal halves contain independent signal sequences to target and insert into microsomal membranes via the SRP-dependent pathway (Zhang and Ling, 1991). To investigate the membrane insertion process and topogenesis of the C-terminal half of the human *MDR3* Pgp, I engineered a cDNA fragment encoding only the C-terminal half Pgp molecule into the pGEM-4z vector (Figure 2E). It has all six C-terminal half TM segments and three potential N-linked glycosylation sites. It was predicted that all of the potential glycosylation sites are in the cytoplasm and are not glycosylated (Figure 2D, model I).

When the 3-C6 in vitro transcript was used to direct translation in RRL in the absence of rough microsomes (RM), a 47-kDa protein was produced (Figure 2A, lane 1). In the presence of RM, an additional protein of 50 kDa was generated (Figure 2A, lane 2). Alkaline treatment and separation of membranes from the soluble fraction suggest that the 50-kDa protein was integrated into the RM membrane, whereas the 47-kDa protein was not (Figure 2A, compare lanes 3 and 5 with lanes 4 and 6, respectively). It is worth noting that, unlike other Pgp molecules (Zhang and Ling, 1991; Zhang *et al.*, 1993), <50% of the *MDR3* translation products was associated with membranes. This is consistent with observation of a previous study on membrane insertion properties of Pgp molecules (Zhang and Ling, 1993). Schinkel *et al.* (1991) also showed that the human *MDR3* Pgp was detected mainly in cytoplasm, whereas the *MDR1* Pgp was found in plasma membranes.

Treatment of membrane-associated proteins with endoglycosidase PNGase F, which removes the N-linked oligosaccharide chains at the innermost residue, reduced the 50-kDa protein to 47 kDa (Figure 2B, compare lanes 1 and 2). This indicates that the 50-kDa protein is a membrane-associated and glycosylated form of the 47-kDa protein. Proteinase K digestion of the membrane-associated proteins generated an 18-kDa protease-resistant fragment (Figure 2C, lane 1). However, no proteinase K-resistant fragment was produced if the RM was permeabilized with Triton X-100 (Figure 2C, lane 3). Further treatment of the protease-resistant 18-kDa fragment with PNGase F reduced its size to ~15 kDa (Figure 2C, lane 2). These results indicate that a fragment containing an oligosaccharide chain is located in the RM lumen. It presumably represents the fragment consisting of TM7-loop-TM8-loop-TM9 with an oligosaccharide chain (see below).

To determine whether the potential glycosylation site between the TM8 and TM9 of the *MDR3* Pgp was used, I silenced this glycosylation site by mutation in the 3-C6 DNA (Figure 2E, 3-C6TA construct). When the mutant transcript was used to direct translation in the presence of RM, only the unglycosylated protein was produced (Figure 2B, lane 3), whereas the glycosylated 50-kDa peptide was produced from the wild-type transcript (Figure 2B, lane 1). Therefore, the loop linking the TM8 and TM9 was located in the RM lumen and was glycosylated (Figure 2D, model II). This is consistent with the study of a truncated 3-C4 transcript, which encodes a peptide that lacks the potential glycosylation site at the C terminus of TM12 (Figure 2E). Proteinase K digestion of the 3-C4 translation product also generated a PNGase F-sensitive and protease-resistant 18-kDa fragment (Figure 2C, lanes 4–6).

### *In Vitro Translation of the N-Terminal Half Sequences of Human MDR3 Pgp*

A 3-N6R DNA, which encodes a protein with all six N-terminal TM segments and a C-terminal reporter, was constructed to study the membrane integration and assembly of the N-terminal half human *MDR3* Pgp (Figure 3C). If the N-terminal half molecules integrate into RM as predicted (Figure 3B), the reporter

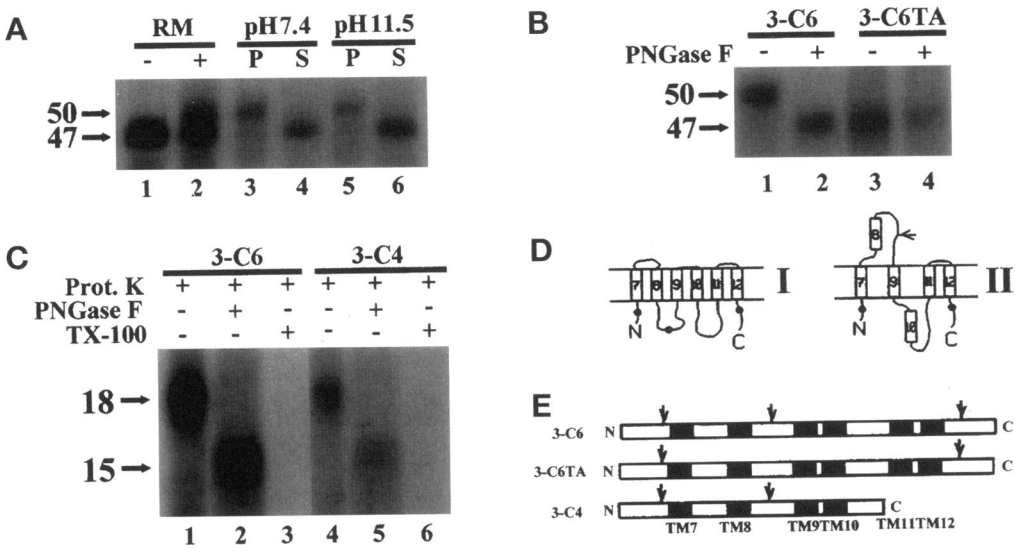



**Figure 2.** In vitro translation and post-translational treatment of wild-type and mutant 3-C6. (A) Translation and post-translational treatment of 3-C6. The translation was performed in the absence (lane 1) or presence (lane 2) of RM. Alkaline treatment with $Na_2CO_3$, pH 11.5, was used to separate the integral membrane proteins (compare lanes 5 and 6). In the control treatment, Tris-buffered saline, pH 7.4, containing 0.25 mM sucrose was used to separate membrane (lane 3) and soluble (lane 4) fractions by centrifugation; p, pellet, S, supernatant. (B) PNGase F treatment of 3-C6 and 3-C6TA. The membrane-associated translation products of 3-C6 and 3-C6TA were digested with (lanes 2 and 4) or without (lanes 1 and 3) PNGase F, and the samples were used for SDS-PAGE analysis. (C) Proteinase K treatment of 3-C6 and 3-C4. The membrane-associated 3-C6 (lanes 1–3) and 3-C4 (lanes 4–6) translation products were digested by proteinase K in the absence (lanes 1 and 4) or presence (lane 3 and 6) of Triton X-100. PNGase F treatment after proteinase K digestion is shown in lanes 2 and 5. (D) Topologies of 3-C6. The numbered bars represent the putative TM segments. The branched symbols are oligosaccharide chains. The unglycosylated potential sites in cytoplasm are indicated by solid circles. (E) Schematic drawing of linear structure of the truncated C-terminal half Pgp. The solid and open bars indicate the TM segments and their linking loops of Pgp, respectively. The branched symbols indicate the potential glycosylation sites.

peptide will have a cytoplasmic location. Thus, the nascent molecules will likely be modified by addition of only two N-linked oligosaccharide chains in the loop linking the TM1 and TM2. As shown in Figure 3A, the 3-N6R in vitro transcript directed translation of a 68-kDa protein in the absence of RM (Figure 3A, lane 1) and an additional protein of 76 kDa in the presence of RM (Figure 3A, lane 2). Limited endoglycosidase digestion of the membrane-associated products shows that the 76-kDa peptide contains two oligosaccharide chains (Figure 3A, lanes 3–5). Proteinase K digestion of the membrane fraction generated a glycosylated peptide of ~18 kDa, similar in size to the fragment from the C-terminal half molecules (Figure 2C), which presumably represents the TM1-loop-TM2 fragments. No other higher molecular weight protease-resistant fragments were observed.

### *The Loop Linking TM1 and TM2 Is Glycosylated*

To investigate whether the two potential glycosylation sites in the loop linking TM1 and TM2 were used, I generated the 3-N2 in vitro transcript and used it to direct translation in RRL. The 3-N2 transcript encodes a peptide that has the first two TM segments, three potential N-linked glycosylation sites (one at the N-terminal end and two in the loop linking the TM1 and TM2), and a shortened C-terminal reporter peptide (Figure 4D). In the absence of RM, a 24-kDa peptide was translated from the 3-N2 transcript (Figure 4A, lane 1). In the presence of RM, an additional membrane-associated product of 32 kDa was produced (Figure 4A, lane 2).

To investigate how many oligosaccharide chains were associated with the nascent 32-kDa peptide, I performed limited endoglycosidase treatment of the 32-kDa peptide. Under this condition, a partially deglycosylated intermediate of 28 kDa was detected (Fig-

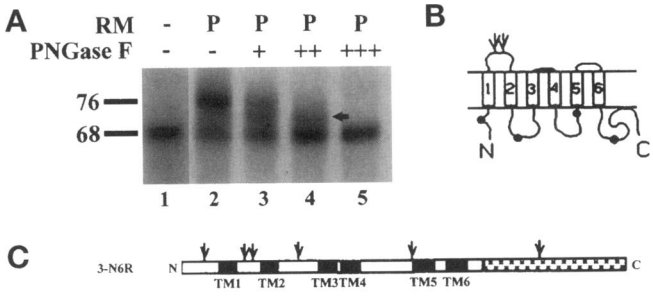



**Figure 3.** In vitro translation and post-translational treatment of 3-N6R. (A) Translation and post-translational treatment of 3-N6R. Membrane-associated translation products (p, pellet) were treated with different amounts of PNGase F (lanes 3–5) or without PNGase F as a control (lane 2). Lane 1 is a translation of 3-N6R in the absence of RM. The arrow in lane 4 indicates the deglycosylated intermediate product. (B) Topologies of 3-N6R. See Figure 2D, legend, for details. (C) Schematic drawing of the 3-N6R linear structure. See Figure 2E, legend, for details. Note that the stippled bars represent reporter peptides.

ure 4B, lane 3), confirming that the 32-kDa peptide has only two oligosaccharide chains. With proteinase K treatment of the membrane-associated 32-kDa peptide, a protease-resistant fragment of ~18 kDa was observed, which was reduced to ~10 kDa by endoglycosidase PNGase F (Figure 4E, lanes 2–3). This result suggests that the protease-resistant 18-kDa peptide contains both oligosaccharide chains and likely represents the fragment of TM1-loop-TM2 with two sugar chains, as predicted (Figure 4C). Analysis of the amino acid sequence suggests that the predicted size of the TM1-loop-TM2 is consistent with the apparent size of the protected fragment, as shown in Figure 4E. Complete removal of these fragments by proteinase K in the presence of Triton X-100 confirms that they were protected by the membrane (Figure 4E, lane 4). Minor fragments with higher molecular weight were also observed after proteinase K digestion (Figure 4E, lane 2). Their origin is not known. However, they probably represent the protected fragments of some peptides that have their C-terminal domain located in the RM lumen (see discussion of Figure 5).

### *The C-Terminal Reporter of the 3-N2R Peptide Is Exposed to the RM Lumen*

According to the "signal–stop transfer" theory (Blobel, 1980), the TM1 of 3-N2 proteins functions as a signal–anchor sequence to initiate membrane targeting and translocation, whereas the TM2 stops the translocation event. To test whether this theory applies to human *MDR3* Pgp, I performed a translation directed by the 3-N2R transcript. The difference between the 3-N2 and the 3-N2R transcripts is that the latter encodes a larger reporter with an additional glycosylation site (compare Figure 4D with Figure 5F). If TM2 stops the membrane translocation event completely, the C-terminal reporter domain will remain in the cytoplasm, and the potential glycosylation site in the reporter domain will not be exposed to the RM lumen. Thus, only two sugar chains will be added to the nascent peptides, as observed with the products of 3-N2 transcript. Surprisingly, two additional peptides of 51 and 55 kDa were generated in the presence of RM (Figure 5A, lane 2), compared with only one translation product of 43 kDa in the absence of RM (Figure 5A, lane 1). These two peptides represent proteins with two and three oligosaccharide chains, as demonstrated by limited endoglycosidase treatment (Figure 5B). The 51-kDa peptide may represent the membrane-integrated peptide with the reporter in the cytoplasm (Figure 5D, model I). The 54-kDa peptide presumably has its C-terminal reporter located in the RM lumen and therefore has an extra oligosaccharide chain attached (Figure 5D, model II, and Table 1).

To determine whether the model II structure exists, I performed a proteolysis/membrane protection as-

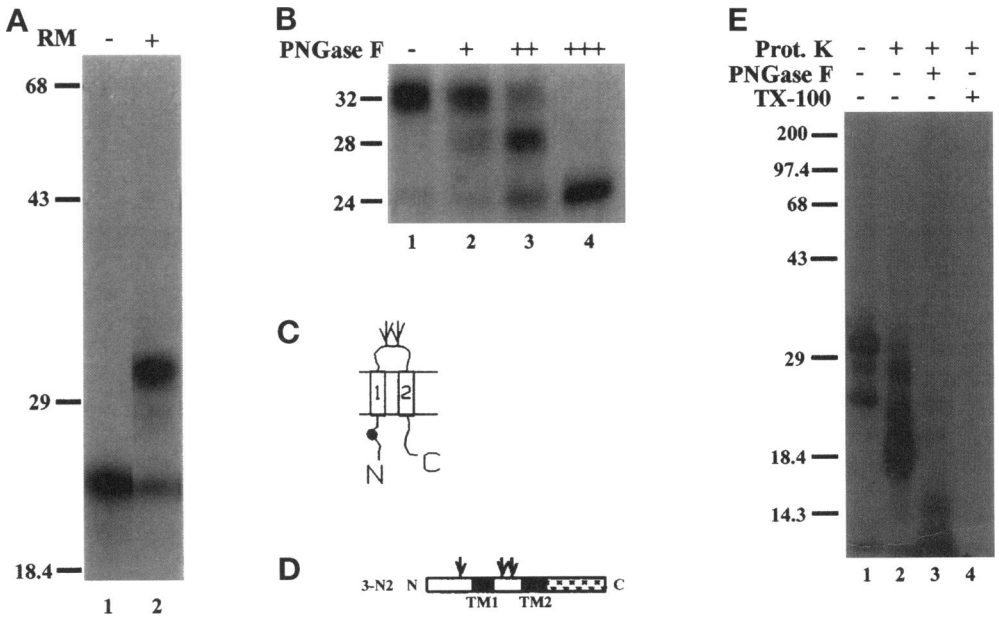



**Figure 4.** In vitro translation and post-translational treatment of 3-N2. (A) Cell-free translation. In vitro 3-N2 transcripts were used to direct translation in the absence (lane 1) or presence (lane 2) of RM. (B) Endoglycosidase PNGase F treatment. The membrane-associated proteins were treated in the absence (lane 1) or presence of increasing amounts (lanes 2–4) of PNGase F. The completely deglycosylated peptides are shown in lane 4. Intermediate products were observed when less PNGase F was used (lanes 2 and 3). (C) Topological model of 3N-2. See Figure 2D, legend, for details. (D) Schematic drawing of the 3-N2 linear structure. See Figure 2E, legend, for details. (E) Proteinase K treatment. The membrane-associated 3-N2 proteins were treated by proteinase K in the absence (lanes 2 and 3) or presence (lane 4) of Triton X-100. PNGase treatment after proteinase K digestion is shown in lane 3. The asterisk in lane 3 indicates the deglycosylated protease-resistant peptide backbone of ~10 kDa.

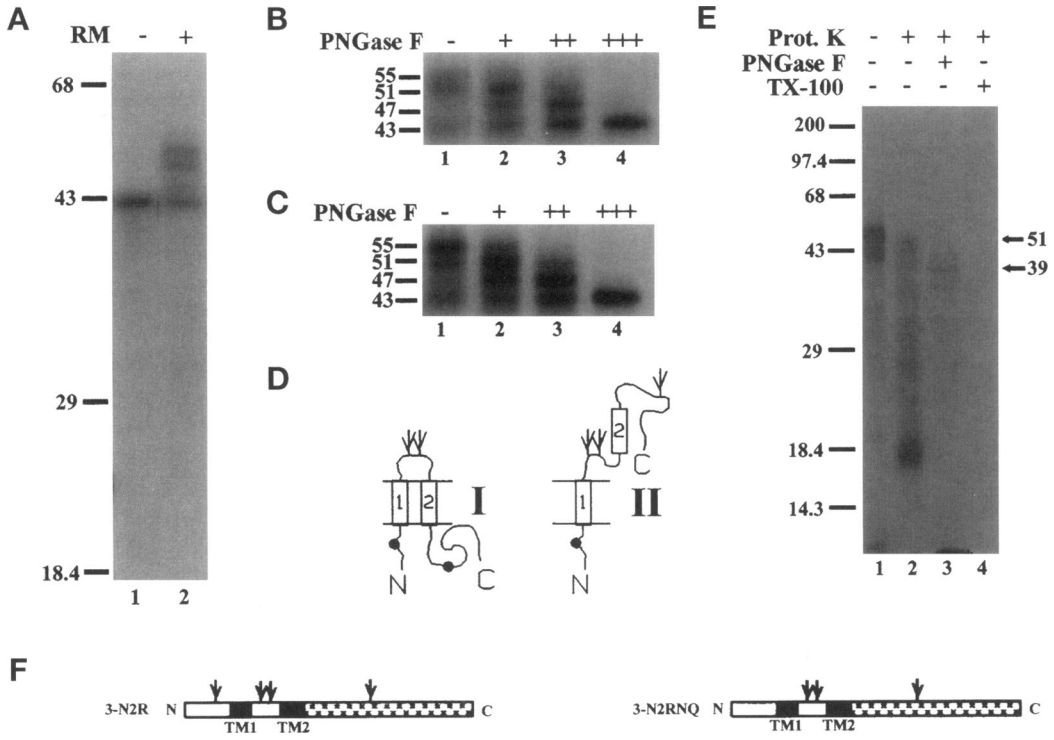



**Figure 5.** In vitro translation and post-translational treatment of 3-N2R. (A) Cell-free translation. In vitro transcripts of 3-N2R were used to direct translation in the absence (lane 1) or presence (lane 2) of RM. (B and C) Endoglycosidase PNGase F treatment. The membrane-associated wild-type 3-N2R (B) or mutant 3-N2RNQ (C) proteins were treated in the absence (lane 1) or presence of increasing amounts (lanes 2–4) of PNGase F. The completely deglycosylated peptides are shown in lane 4 of B and C. Intermediate products were observed when less PNGase F was used (lanes 2 and 3). (D) Topological model of 3-N2R. See Figure 2D, legend, for details. (E) Proteinase K treatment. The membrane-associated wild-type 3-N2R proteins were treated by proteinase K in the absence (lanes 2 and 3) or presence (lane 4) of Triton X-100. PNGase F treatment after proteinase K digestion is shown in lane 3. (F) Schematic drawing of the wild-type 3-N2R and mutant 3-N2RNQ linear structure. See Figure 2E, legend, for details.

say. If the reporter is in the RM lumen and is glycosylated (Figure 5D, model II), a protease-resistant peptide of ~52 kDa (including the three oligosaccharide chains) should be observed with proteinase K treatment of the membrane fraction. As shown in Figure 5E, lane 2, a peptide of ~18 kDa and a doublet of peptides of 49–51 kDa were produced from protease treatment. Further treatment with PNGase F reduced the 18-kDa peptide to ~10 kDa and the 49–51 kDa peptides to 39–41 kDa (Figure 5E, lane 3). The 18-kDa product presumably represents the fragment of TM1-loop-TM2 with two oligosaccharide chains, as shown in the model I structure (Figure 5E). The 49–51 kDa doublet presumably represents the fragment of TM1-loop-TM2-reporter with three oligosaccharide chains, as shown in the model II structure (Figure 5D). The size shift of the protease-resistant fragments induced by PNGase F is consistent with two and three oligosaccharide chains removed (~3 kDa/sugar chain), respectively. It is not known why more than one protected peptide band at 39–41 kDa was observed. This, possibly, is due to protease cleavage at different sites, and the 39-kDa peptide is the major one. All fragments were completely digested if the RM was permeabilized with Triton X-100 (Figure 5E, lane 4), confirming that they were protected from protease digestion by the membrane.

To ascertain that the third glycosylation was not due to the glycosylation of another potential glycosylation site at the N-terminal end, I constructed a mutant cDNA that does not have the potential glycosylation site at the N-terminal end (Figure 5F, 3-N2RNQ). Translation of the 3-N2RNQ transcript produced both the membrane-associated 51- and 55-kDa proteins (Figure 5C, lane 1). Limited endoglycosidase treatment of these peptides showed that the 51-kDa peptides contain two, whereas the 55-kDa peptides contain three, oligosaccharide chains (Figure 5C, lanes 2–4). These results exclude the possibility that the potential glycosylation site at the N-terminal end was used (see Table 1). Together, these results suggest that the efficiency of TM2 to stop the membrane translocation of the C-terminal reporter into the RM lumen is only ~40-70% (derived from the intensity of the two glycosylated products of 55 and 51 kDa, such as the gels shown in Figure 5, A and B). This event resulted

**Table 1.** Summary of glycosylations in the truncated N-terminal half proteins

| | N-Terminal end | First loop[a] | Reporter peptide |
|---|---|---|---|
| 3-N2 | − | + | N/A[b] |
| 3-N2R | − | + | + |
| 3-N3 | + | + | N/A[b] |
| 3-N3R | + | + | − |
| 3-N4R | +[c] | + | − |
| 3-N5R | − | + | + |
| 3-N6R | − | + | − |
| 1/3-N2R | − | + | + |
| 1/3-N3RTA | − | + | − |
| 1/3-N3RTA2 | − | + | − |

[a] The loop linking TM1 and TM2.

[b] The 3-N2 and 3-N3 proteins do not contain reporter peptide with the glycosylation site.

[c] Glycosylation in the N-terminal end of 3-N4R protein is much less than the 3-N3R protein.

in the translocation of the C-terminal reporter into RM lumen (Figure 5D, models I and II). However, it should be noted that the membrane-protected 39-kDa peptides seem to be less than the protected 10-kDa peptides (Figure 5E, lane 3). It is currently not known whether some of the reporter in the model II molecules (Figure 5D) was relocated to the cytoplasm after glycosylation, resulting in the model I structure but with a glycosylated reporter. Thus, only portions of the model II molecules exist after translation is completed. This has been observed with the reporter of another polytopic membrane protein (In-Inc-Inc fusion protein; Lipp *et al.*, 1989).

### *The N-Terminal End of the 3-N3R Peptide Is Translocated into the RM Lumen*

To determine the role of the TM3 in topogenesis of the human *MDR3* Pgp, I made a 3-N3R cDNA construct. This construct encodes the first three TM segments, a reporter peptide, and, in total, five potential glycosylation sites (Figure 6D). Translation of the 3-N3R transcript in RRL generated a 58-kDa peptide (Figure 6A, lane 1). In the presence of RM, additional membrane-associated proteins of 66 and 70 kDa were generated (Figure 6A, lane 2). These proteins are glycosylated versions of the proteins generated in the absence of RM, as shown by complete endoglycosidase treatment (Figure 6A, lane 5). Endoglycosidase digestion under limited conditions showed that the 66-kDa and the 70-kDa proteins contain two and three oligosaccharide chains, respectively (Figure 6A, lanes 3–4).

The 66-kDa peptide presumably represents the nascent 3-N3R protein with both N and C terminus in cytoplasm and the glycosylated loop between TM1 and TM2 in RM lumen (Figure 6C, model II). To determine whether the 70-kDa protein was generated because of glycosylation of the reporter peptide in RM lumen as predicted (Figure 6C, model I), I used a truncated 3-N3 transcript (Figure 6D) that lacks the reporter to direct translation. In the absence of RM, a 22-kDa peptide was generated (Figure 6B, lane 1). In the presence of RM, three additional membrane-associated products of 26, 30, and 34 kDa were produced (Figure 6B, lane 2). Treatment with endoglycosidase PNGase F reduced all three products to 21 kDa (Figure 6B, lane 5). Intermediate products with one and two oligosaccharide chains were observed under limited conditions of PNGase F digestion (Figure 6B, lanes 3 and 4). These results indicate that products with three oligosaccharide chains were also generated from the 3-N3 transcript. Therefore, I conclude that the third glycosylation in the 70-kDa 3-N3R translation products in Figure 6A was not due to the glycosylation of the C-terminal reporter in RM lumen, as shown in the model I structure (Figure 6C and Table 1). This was supported by the observation that no peptide fragments corresponding to the glycosylated reporter

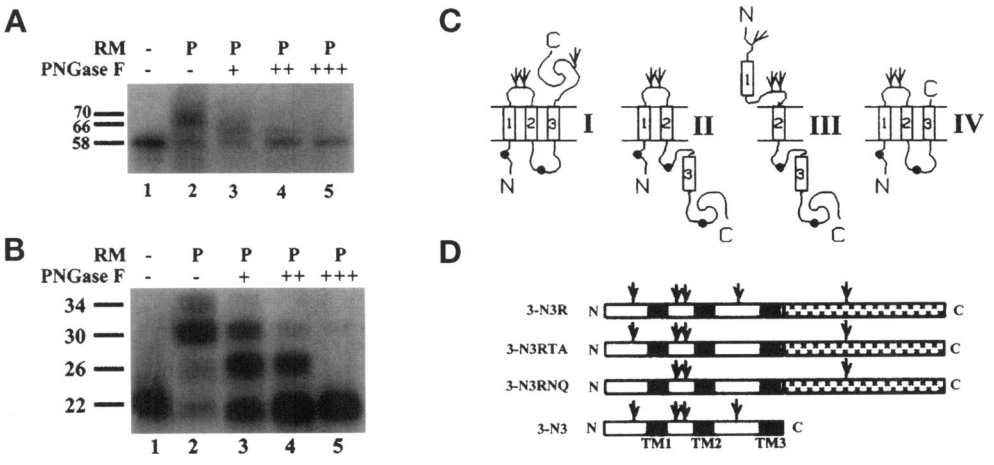



**Figure 6.** In vitro translation and post-translational treatment of 3-N3R and 3-N3. (A and B) Translation and post-translational treatment of 3-N3R and 3-N3. In vitro 3-N3R (A) and 3-N3 (B) transcripts were used to direct translation in RRL in the absence (lane 1) or presence (lane 2) of RM. Limited and complete endoglycosidase (PNGase F) digestions of membrane-associated proteins (p, pellet) are shown in lanes 3–5. (C) Topologies of 3-N3R and 3-N3. See Figure 2D, legend, for detail. (D) Schematic drawing of the wild-type 3-N3R, the mutant 3-N2RTA and 3-N2RNQ, and the truncated 3-N3 linear structure. See Figure 2E, legend, for details.

were protected from the proteinase K digestion of 3-N3R translation products. It is worth noting that the generation of the glycosylated 34 kDa 3-N3 protein is decreased, as compared with the glycosylated 70-kDa 3-N3R protein (compare the ratio of 34- to 30-kDa proteins in Figure 6B, lane 2, with that of 70- to 66-kDa proteins in Figure 6A, lane 2). The reason for this is not known. It is probably due to the truncation of the C-terminal end of the protein in 3-N3, which affects the anchorage of the TM1 and TM2 (see DISCUSSION).

To determine which of the two remaining potential glycosylation sites (one at the N-terminal end and one in the loop linking TM2 and TM3) in 3-N3R protein was used, two glycosylation-mutant 3-N3R DNAs (Figure 6D, 3-N3RTA and 3-N3RNQ) were constructed and used for in vitro transcription and translation. The yield of glycosylated peptides of 66 and 70 kDa was similar between the wild-type 3-N3R and the mutant 3-N3RTA transcript (Figure 7, lanes 1 and 2). However, the 70-kDa glycosylated protein was not produced from the mutant 3-N3RNQ transcript (Figure 7, lane 3). These results indicate that the third glycosylation in 3-N3R was likely at the N-terminal end of the protein and suggest that the N-terminal end of the 70-kDa 3-N3R proteins was translocated into the RM lumen (Figure 6C, model III, and Table 1).

### *Topogenesis of the TM4*

To determine the topogenic role of the TM4, I constructed 3-N4R DNA. This DNA encodes a peptide with four TM segments and a C-terminal reporter peptide (Figure 8C). Protein products of 61 kDa were translated from the 3-N4R in vitro transcripts in the absence of RM (Figure 8A, lane 1). An additional protein of 69 kDa was translated in the presence of RM (Figure 8A, lane 2). Complete endoglycosidase PNGase F treatment reduced the 69-kDa protein to 61 kDa (Figure 8A, lane 5), indicating that it is glycosylated. Under limited conditions of PNGase F treatment, one intermediate product was observed (Figure 8A, lanes 3–4). These results suggest that the 69-kDa protein contain two oligosaccharide chains. A minor diffused band of ~73 kDa (indicated by an arrow in Figure 8A, lane 2) was also generated in the presence of RM and was sensitive to PNGase F digestion. It may be a minor product that has three oligosaccharide chains attached (see discussion below).

The 69-kDa protein with two oligosaccharides represents molecules with the model I structure (Figure 8B). To determine the origin of the third glycosylation in the minor 73-kDa protein, I made two mutant 3-N4RTA and 3-N4RNQ (Figure 8C) constructs. As shown in Figure 7, the 73-kDa product was generated from the 3-N4RTA (Figure 7, lane 5) as from the wild-type sequence (Figure 7, lane 4), but not from the 3-N4RNQ (Figure 7, lane 6). These results suggest that the minor 73-kDa 3-N4R protein, like the 70-kDa 3-N3R protein (Figure 8), has a glycosylated N-terminal end in the RM lumen (Figure 8B, model II, and Table 1).

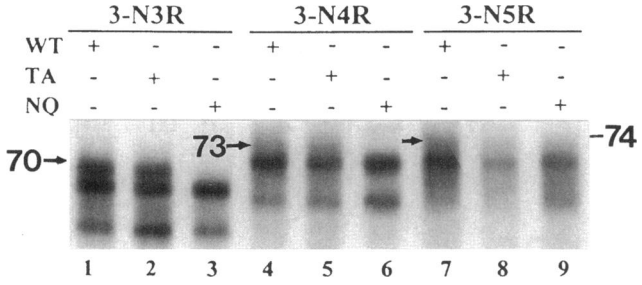



**Figure 7.** In vitro translation of wild-type and mutant 3-N3R, 3-N4R, and 3-N5R. In vitro transcripts of wild-type and mutant 3-N3R, 3-N4R, and 3-N5R were used to direct translation in RRL in the presence of RM; the membrane fractions were isolated and analyzed on SDS-PAGE. The arrows indicate the peptides with three oligosaccharide chains, and their apparent molecular weights are shown. WT, wild type; TA, single mutant; NQ, double mutant.

### *Topogenesis of the TM5*

To determine the topogenic role of the TM5, I constructed 3-N5R DNA. It encodes a peptide with five TM segments and a C-terminal reporter peptide (Figure 9D). A 62-kDa protein was translated from the 3-N5R in vitro transcripts in the absence of RM (Figure 9A, lane 1). In the presence of RM, two membrane-associated proteins of 70 and 74 kDa were generated in addition to the 62-kDa protein (Figure 9A, lane 2). Endoglycosidase PNGase F treatment indicates that the 70- and the 74-kDa proteins contain two and three oligosaccharide chains, respectively (Figure 9A, lanes 3–5).

The 70-kDa protein with only two oligosaccharide chains presumably represents the model II molecules (Figure 9B). To determine the origin of the third glycosylation in the 74-kDa protein, I constructed mutant 3-N5R DNAs to make 3-N5RTA and 3-N5RNQ (Figure 9D). Unlike the mutant 3-N3R and mutant 3-N4R, neither of the mutations in 3-N5R eliminated the generation of the 74-kDa protein (Figure 7, compare lanes 7–9). Therefore, the third glycosylation in 3-N5R protein was not due to the glycosylation at the N-terminal end nor in the loop linking TM2 and TM3 of the protein. Likely, the reporter of the 3-N5R protein was translocated into the RM lumen and glycosylated (Figure 9B, model I, and Table 1). Proteinase K treatment of membrane-associated 3-N5R proteins generated 18- and 34-kDa protease-resistant fragments (Figure 9C, lane 1). Both fragments contain oligosaccharide chains, as demonstrated by their susceptibility to PNGase F treatment (Figure 9C, lane 2). The 18-kDa frag-

ment presumably represents the glycosylated loop linking TM1 and TM2, whereas the 34-kDa fragment is likely the glycosylated reporter peptide in the RM lumen, as shown in the model I structure (Figure 8E).

However, the above experiments did not exclude the possibility that the 74-kDa protein represents a molecule with its TM4 exposed to the RM lumen and TM3 and TM5 in membranes (Figure 9B, model III). In this case, the third glycosylation would be in the loop linking TM4 and TM5. If this is true, proteinase K digestion will generate a glycosylated fragment consisting of TM3-loop-TM4-loop-TM5. According to the amino acid sequence, this fragment has a calculated size of 16 kDa plus a ~3-kDa oligosaccharide chain. However, I did not observe a proteinase K-resistant 19-kDa fragment that was reduced to 16 kDa with endoglycosidase PNGase F treatment (Figure 9C, compare lanes 1 and 2). Furthermore, the glycosylation site in the loop linking TM4 and TM5 is too close to the membrane (at the N-terminal end of TM5) to be accessible to the glycosyltransferase in the RM lumen (Figure 9D). Thus, it is unlikely that the model III structure (Figure 9B) exists.

### *Effects of the N-Terminal Sequence on Membrane Anchorage of the TM1 and TM2*

The above results suggest that the proper membrane anchorage of the TM1 and TM2 of human *MDR3* Pgp was affected by their C-terminal sequence. To determine whether the N-terminal sequence is also important in topogenesis, I substituted the N terminus (amino acids 1 to 75) of the 3-N2R and 3-N3RTA proteins with the corresponding N terminus (69 amino acids) of human *MDR1* Pgp (Figure 10B). In addition to a 6-amino-acid difference in length, 39 of the 75 amino acids of these two proteins are identical (or 52% identity), and the N terminus of both proteins has a potential glycosylation site. The fusion DNAs were used to make transcripts, which, in turn, were used to direct translation in RRL in the presence of RM. As shown in Figure 10A, proteins with two and three oligosaccharide chains were generated from both the wild-type (3-N2R) and the hybrid (1/3-N2R) transcript (Figure 10A, compare lanes 1 and 2). It is possible that the reporter in both 3-N2R and 1/3-N2R protein was glycosylated in the RM lumen. It is unlikely that the N-terminal end of the 1/3-N2R protein was located in the RM lumen, causing the glycosylation of the potential site in the N-terminal end. First, it has been shown previously that the N-terminal end of human *MDR1* Pgp was located in the cytoplasm when the first TM segment was fused to a reporter and expressed in frog oocytes and RRL (Skach and Lingappa, 1993b). Second, the N-terminal end of human *MDR1* Pgp was not glycosylated when it was expressed in RRL in a construct consisting of human *MDR1* TM1–TM2 and a reporter (Skach and Lingappa, 1993b; our unpublished observation). Third, the N-terminal end of human *MDR1* Pgp was not glycosylated when engineered into the 3-N3R protein (see 1/3-N3R below). Thus, replacing the first 75 amino acids of human *MDR3* Pgp with that of the human *MDR1* Pgp may not help the membrane anchorage of the TM2 of human *MDR3* Pgp. However, replacing the first 75 amino acids (including the TM1) of human *MDR3* Pgp with that of human *MDR1* Pgp in the 3-N3RTA construct eliminated the generation of the protein with three oligosaccharide chains (Figure 10A, compare lanes 3 and 4). Therefore, the N-terminal end of the hybrid 1/3-N3RTA protein is likely in the cytoplasm and not translocated into the RM lumen. Replacing the N-terminal 59 amino acids (without the TM1) of 3-N3RTA with the corresponding sequence from the *MDR1* sequence (Figure 10B, 1/3-N3RTA2) also inhibits the generation of the 70-kDa protein with three oligosaccharide chains (Figure 10A, lane 5). Therefore, the membrane anchorage of the TM1 of the *MDR3* Pgp depends on the N-terminal amino acid sequence, whereas the TM2 does not.

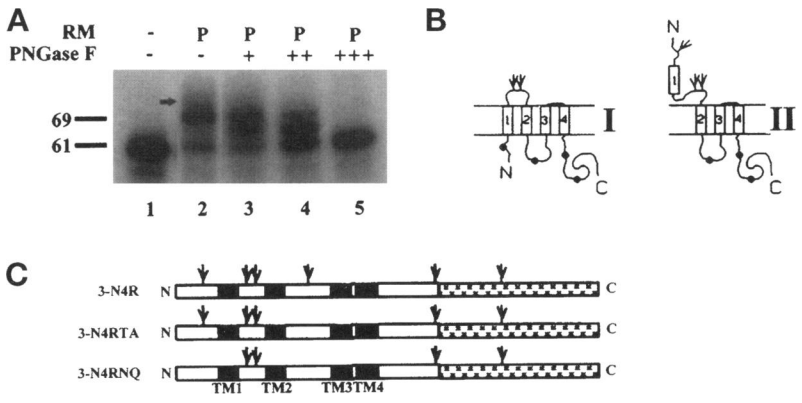
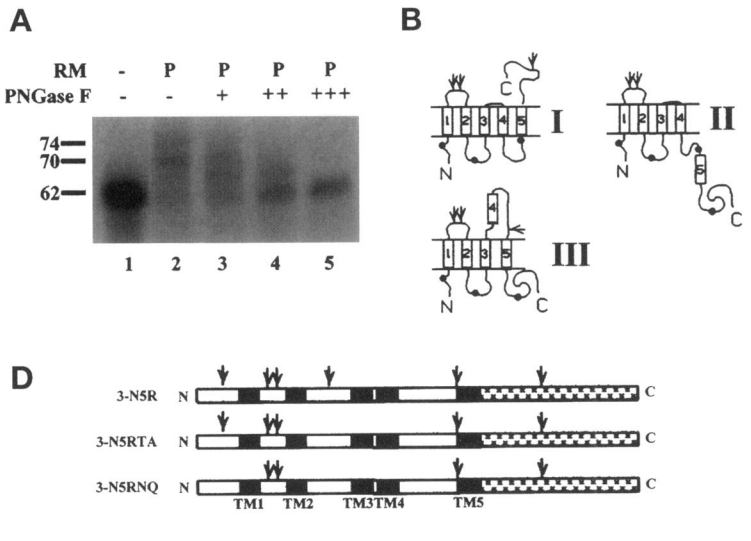



**Figure 8.** In vitro translation and post-translational treatment of 3-N4R. (A) Translation and PNGase F treatment. The translation was performed in the absence (lane 1) or presence (lane 2) of RM. The membrane-associated proteins were treated without (lane 2) or with (lanes 3–5) different amounts of PNGase F. The arrow in lane 2 indicates the minor 73-kDa protein (p, pellet). (B) Topologies of 3-N4R. See Figure 2D, legend, for details. (C) Schematic drawing of the wild-type 3-N4R, the mutant 3-N4RTA, and 3-N4RNQ linear structure. See Figure 2E, legend, for details.



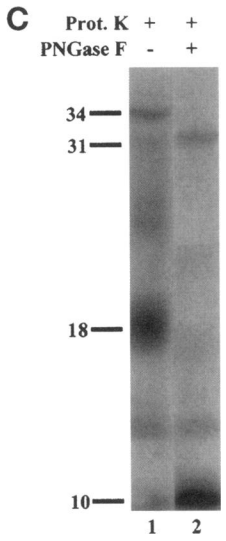




**Figure 9.** In vitro translation and post-translational treatment of 3-N5R. (A) Translation and PNGase F treatment. The translation was performed in the absence (lane 1) or presence (lane 2) of RM. The membrane-associated proteins were treated without (lane 2) or with (lanes 3–5) different amounts of PNGase F (p, pellet). (B) Topologies of 3-N5R. See Figure 2D, legend, for details. (C) Proteinase K digestion. Proteinase K digestion was performed on the membrane pellet fraction (lane 1) or followed by PNGase F digestion (lane 2). (D) Schematic drawing of the wild-type 3-N5R, the mutant 3-N5RTA, and 3-N5RNQ linear structure. See Figure 2E, legend, for details.

## DISCUSSION

In this study, I have investigated the membrane assembly process and the topogenesis of the non-MDR–causing human *MDR3* Pgp by using a cell-free system. The results suggest that all the TM segments in the N-terminal half of *MDR3* Pgp insert into membranes as expected. However, the C-terminal half molecules of *MDR3* Pgp behave differently in RM. The TM8 in the C-terminal half could not stop the membrane translocation event and was translocated into the RM lumen. These observations are similar to the study with the MDR-causing human *MDR1* Pgp (Skach *et al.*, 1993).

It has been suggested that in polytopic membrane proteins the transmembrane segments are topogenic sequences that have different functions as signal and stop–transfer sequence (Friedlander and Blobel, 1985; Audigier *et al.*, 1987; Skach and Lingappa, 1993a). 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; Hartmann *et al.*, 1989; Lipp *et al.*, 1989; Skach and Lingappa, 1993a). In this study, the TM2 of the *MDR3* Pgp was found to stop only ~40-70% of the membrane translocation event when its C-terminal end was linked to a reporter (3-N2R protein). On the other hand, the TM2 of the *MDR1* Pgp has been shown to stop the translocation event up to 90% when linked to a reporter (Skach and Lingappa, 1994). Addition of the TM3 and the linking sequences between TM2 and TM3 assists the stop–transfer activity of TM2 in the *MDR3* Pgp sequence (see results on 3-N3R and 3-N4R proteins). However, in the later context, the TM1 and the N-terminal sequence were released into the RM lumen. Only when all of the amino acid residues up to and including the TM5 were added did both TM1 and TM2 completely anchor in the membrane (see results on 3-N5R and 3-N6R). These observations suggest that the membrane assembly of the TM1 and TM2 of human *MDR3* Pgp is affected by their following amino acid sequences. Replacing the N-terminal sequence with human *MDR1* Pgp did not enhance the membrane anchorage of the TM2 in the truncated protein, suggesting that the N-terminal sequence may not be equally important to the C-terminal sequence. Further work is required to test this hypothesis. However, this replacement restored the membrane anchorage of the TM1. Apparently, the membrane insertion and anchorage of the TM1 of Pgp are affected by the N-terminal amino acid sequence. It is interesting to note that the TM8 in the C-terminal half molecule (see discussion below) did not stop the membrane translocation even in the presence of sequence up to and including the TM12. Thus, the function in membrane assembly of TM2 in the N-terminal half and TM8 in the C-terminal half is different.

It is not known why the TM3 and TM5 in *MDR3* Pgp do not initiate efficient membrane insertion event. Similar observation has been made with TM3 in the human *MDR1* Pgp (Skach and Lingappa, 1994) and with TM3 and TM5 in the CFTR protein (Chen and Zhang, 1996). There are several possible explanations for the behavior of the TM3 and TM5 in these proteins. First, the membrane insertion of TM segments in these proteins may occur by pairs. It has been shown that the TM3 in *MDR1* Pgp does not insert in the membrane unless the sequence from TM4 is present (Skach and Lingappa, 1994). It is, therefore, likely that the membrane insertion of TM3 and TM5 of *MDR3* Pgp requires the TM4 and TM6, respectively. This paired membrane insertion may be more thermodynamically favorable than insertion by a single TM segment. Sec-

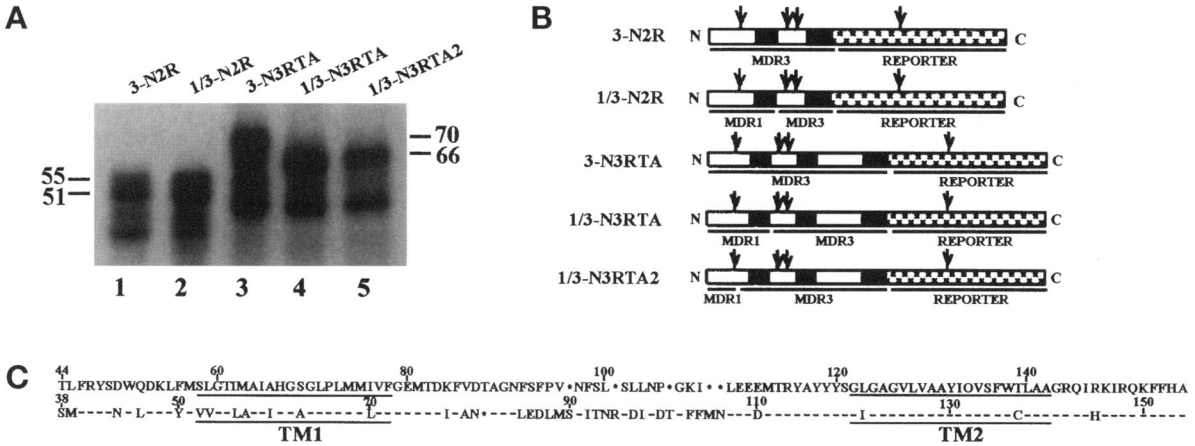



**Figure 10.** In vitro translation of 1/3-N2R and 1/3-N3RTA hybrid constructs. (A) In vitro translation of wild-type and hybrid constructs. The 3-N2R (lane 1), 1/3-N2R (lane 2), 3-N3RTA (lane 3), 1/3-N3RTA (lane 4), and 1/3-N3RTA2 (lane 5) transcripts were used to direct translation in RRL in the presence of RM; the membrane fraction was analyzed by SDS-PAGE. The glycosylated proteins were indicated by molecular weight markers. (B) Schematic linear structure of the MDR1–MDR3 fusion proteins. The first 75 amino acids in 3-N2R and 3-N3RTA were replaced at cDNA level by the corresponding amino acids from human MDR1 Pgp to generate the 1/3-N2R and 1/3-N3RTA constructs. The N-terminal 59 amino acids in 3-N3RTA were replaced by the corresponding amino acid from *MDR1* Pgp to generate the 1/3-N3RTA2 construct. The approximate positions of fusion among MDR1, MDR3, and reporter are underlined. (C) Alignment of the TM1, TM2, and their flanking amino acid sequences between human *MDR1* (bottom) and *MDR3* (top) Pgp. The amino acids are shown in single-letter code, and the TM segments are underlined. The (−) indicates the identical amino acids between the two Pgp isoforms, and an asterisk (*) denotes a gap created to align the two sequences.

ond, the nonmembrane-spanning segments may contain sequences that signal the proper membrane anchorage of the TM segments. Indeed, deletion of C-terminal sequences altered the proper membrane anchorage of TM2, although the TM1 was present to form a pair with TM2 in the 3-N2R protein. It has also been suggested that the loop in the RM lumen of an artificial polytopic membrane protein affects the membrane translocation of TM segment (Lipp *et al.*, 1989). Third, the reporter peptide used in this study may be too bulky and cannot be efficiently translocated into the RM lumen by TM3 and TM5. However, this possibility is unlikely because the similar reporter has been successfully used previously (Zhang *et al.*, 1993), and it can be translocated into RM lumen when present in the 3-N2R protein (Figure 4). Finally, the TM3 and TM5 may not be in the membrane. Their cytoplasmic location cannot be excluded from this study. Further studies are necessary to distinguish among these possibilities and help unveil the membrane insertion and assembly process of mammalian polytopic membrane proteins.

In the C-terminal half of Pgp, the TM7 presumably initiated the membrane targeting and insertion into RM. The TM8 was expected to stop the membrane translocation event to form an extracellular loop. However, in this study the TM8 did not stop the membrane translocation event and was found in the RM lumen. Similar observation has been made with other Pgp isoforms, including the human *MDR1* Pgp (Skach *et al.*, 1993) and mouse *mdr1* Pgp (Zhang and Ling, 1991). It is not known why the TM8 did not stop the membrane translocation event, but possibly there are not enough positive charges after the TM8 that can help to stop the membrane translocation event. It is also possible that the TM8 lacks information that can interact with the TM7 or components in the protein-conducting channel to stop the membrane translocation. Further work is required to resolve this issue. Another possibility is that truncation of the N-terminal half molecule causes the generation of the alternative topology of the C-terminal half molecule. However, I think that this possibility is unlikely. First, it has been shown that the TM8 of human *MDR1* Pgp (Skach *et al.*, 1993), mouse *mdr1* Pgp (Zhang and Ling, 1991), and hamster *pgp1* Pgp (Han and Zhang, unpublished observation) did not stop the membrane translocation event in the presence of their N-terminal half sequences. Second, recent studies with hamster *pgp1* Pgp indicate that addition of positive charges at the C-terminal side of TM8 may help the TM8 to anchor in the membrane, resulting in the predicted topology (Han and Zhang, unpublished observation). Third, the loop linking TM8 and TM9 were detected in Pgp expressed in MDR cells with site-specific antibodies (Zhang *et al.*, 1996).

The results in this study suggest that the membrane insertion event of the internal signal–anchor and stop–transfer sequences in mammalian polytopic membrane proteins such as Pgp and CFTR cannot be easily separated. The process of initiation of membrane translocation and membrane anchorage of a mamma-

lian polytopic membrane protein is likely more complicated than anticipated and may vary with different TM segments in different proteins. This is consistent with the conclusion derived from studies on a yeast polytopic membrane protein, *Saccharomyces cerevisiae* HMG-CoA reductase, (Sengstag *et al.*, 1990). Thus, the membrane insertion of TM segments of polytopic membrane proteins in mammalian systems may not simply follow the sequential event as suggested (Friedlander and Blobel, 1985). Future studies are being directed to investigate the molecular mechanisms underlying the membrane biogenesis of ATP-binding cassette transporters. The specific amino acid residues responsible for the variation in membrane insertion and anchorage of TM segments in mammalian ABC transporters will be identified. Further molecular dissection of the membrane insertion process and comparison between different isoforms of Pgp (e.g., *MDR1* and *MDR3* Pgp) will help understand the molecular mechanism of membrane insertion and assembly of mammalian polytopic membrane proteins.

Finally, different conclusions concerning Pgp topology have been derived from different laboratories. Although alternative topologies of Pgp were observed (Zhang and Ling, 1991; Zhang *et al.*, 1993, 1996; Skach *et al.*, 1993; Bibi and Béjà, 1994; Béjà and Bibi, 1995; this study), Loo and Clarke (1995) and Kast *et al.* (1995, 1996) believe that Pgp has only the predicted topology. The reason for this difference is not known. It may be due to the use of different expression systems or to different approaches such as truncations, addition of reporters or epitopes, and site-directed mutagenesis. It, however, should be noted that the loop linking TM4 and TM5 and the loop linking TM8 and TM9 are very sensitive to mutation. Engineering epitopes or changing amino acids in these loops often caused a nonfunctional protein (Kast *et al.*, 1995, 1996; Loo and Clarke, 1995). Although controversial views on the final Pgp topology still exist, I believe that observation of different Pgp topologies reflects only different faces of the same molecule. It has been shown that two different topological forms of colicin Ia exist in different functional states (Slatin *et al.*, 1995). Interchanges between the different topological structures of Pgp may involve the transport function of Pgp, and this hypothesis makes Pgp a very interesting model also to study the topology–function relationship.

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