Determinant of the extracellular location of the N-terminus of human multidrug-resistance-associated protein

Jian-Ting ZHANG
Department of Pharmacology and Toxicology, IU Cancer Center, R-4 Room 166, Indiana University School of Medicine, 1044 W. Walnut St., Indianapolis, IN 46202, U.S.A.

Multidrug-resistance-associated protein (MRP) is a member of the ATP-binding cassette (ABC) membrane-transport superfamily and is responsible for multidrug resistance in cancer cells. Distinct from other members of the ABC superfamily, MRP has three membrane-spanning domains (MSDs) and the N-terminus is located extracellularly. It has been shown that the first MSD (MSD1) with an extracellular N-terminus is important for MRP function. To address what ensures the generation of this structural organization of MRP and to understand in general the molecular mechanism of membrane folding of polytopic proteins with extracellular N-termini, the biogenesis of MSD1 in human MRP1 was examined using an in vitro expression system. Surprisingly, the second transmembrane segment (TM2) in MSD1 was found to play a critical role in the correct membrane translocation and folding of MSD1 in human MRP1. TM2 not only plays an essential role to ensure the N-terminus-outside/C-terminus-inside orientation of TM1 with an extracellular N-terminus, it can also translocate into membranes post-translationally in a signal-recognition particle and ribosome-dependent manner to provide an additional insurance for correct folding of MSD1 in MRP. These findings suggest that TM2 in a polytopic membrane protein with an extracellular N-terminus may play a critical role in controlling correct membrane translocation and folding of the protein in general.

Key words: membrane targeting, MRP, ribosome, signal sequence, topogenesis.

INTRODUCTION
The early steps in biogenesis of membrane proteins closely resemble the biogenesis of secretory proteins in the endoplasmic reticulum (ER). 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) will determine how the protein is folded in the membrane [1]. Membrane targeting and translocation of a nascent membrane protein also involves many cytoplasmic and translocation-machinery proteins on the ER [2].

Most polytopic (spanning membranes twice or more) and bitopic (spanning membranes once) membrane proteins in eukaryotic cells are thought to acquire their final membrane orientations during or immediately after synthesis on the ER [3–6]. Topogenic sequences involved in signal, signal anchorage and stop-transfer activities have been identified in eukaryotic polytopic membrane proteins [7–11]. A polytopic topology was thought to be generated by a sequential translocation and membrane integration of independent topogenic sequences [1,6,9,12,13]. However, for polytopic proteins with an extracellular N-terminus it remains unknown what determines the generation of the extracellular location of the N-terminus.

Multidrug resistance in cancer cells is a major problem in successful chemotherapy. One of the known causes for multidrug resistance is the elevated expression of plasma-membrane proteins that efflux anti-cancer drugs. Two major proteins have been found which have a drug-efflux function: P-glycoprotein (Pgp) [14,15] and multidrug-resistance-associated protein (MRP) [16,17]. Both Pgp and MRP are polytopic membrane proteins and belong to the same ATP-binding cassette (ABC) transport superfamily [18].

The topological folding of Pgp has been mapped and more than one topology was found [19–22]. Although it remains controversial, the observed alternative topologies may be very important for Pgp-mediated drug transport. Recently, the topology of MRP has also been studied. Surprisingly, MRP was found to have three rather than two membrane-spanning domains (MSDs) and its N-terminus was found in the extracellular space [23–25] (see also Figure 1A). This observation is different from the prediction and also contradicts the traditional view of two MSDs with N-termini located in the cytoplasm for most other ABC transporters. This peculiar feature may underline a unique property in MRP-mediated drug transport. A recent study on the functional importance of the additional MSD (or MSD1) showed that truncation of the extracellular N-terminus with the first transmembrane (TM) segment, TM1, eliminated MRP function [26], although more recent studies showed that the loop linking MSD1 and MSD2 was essential for MRP function [27,28].

In this study, I determined the biogenesis of MSD1 of human MRP1 and examined what controls the generation of the extracellular N-terminus of MRP in the biogenesis process. I found that (i) both TM1 and the second TM segment, TM2, had de novo activity to initiate signal-recognition-particle (SRP)-dependent membrane translocation; (ii) the orientation of TM1 was determined by charged amino acids flanking TM1 and by the strong N-terminus-inside/C-terminus-outside (N\textsubscript{in}/C\textsubscript{out}) membrane-translocation activity of TM2; (iii) TM2 could initiate

Abbreviations used: ABC, ATP-binding cassette; MRP, multidrug-resistance-associated protein; MSD, membrane-spanning domain; ER, endoplasmic reticulum; Pgp, P-glycoprotein; TM, transmembrane; RRL, rabbit reticulocyte lysate; WGE, wheat germ extract; WGR, wheat germ ribosome; SRP, signal-recognition particle; RM, microsome membranes; KRM, SRP-striped RM; NEM, N-ethylmaleimide; NRM, NEM-treated RM; PNGase F, peptide N-glycosidase F; N\textsubscript{in}/C\textsubscript{out}, N-terminus inside/C-terminus outside; CFTR, cystic fibrosis transmembrane conductance regulator.

1 E-mail jianzhan@iupui.edu.
post-translational membrane translocation to ensure correct membrane folding of MSD1; (iv) the membrane translocation of TM2, TM3 and TM4 appeared to follow the sequential membrane-translocation model independent of TM1; and (v) truncation of the extracellular N-terminal sequence including TM1 did not affect the membrane translocation and folding of MSD1.

EXPERIMENTAL PROCEDURES

Materials

Human MRP1 cDNA [29] was obtained from Dr Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). pGEM-4z plasmid, SP6 RNA polymerase, RNasin, ribonucleotides, RQ1 DNaSe, rabbit reticulocyte lysate (RRL) and pGEM-T Easy vector were obtained from Promega. Dog pancreatic microsomal membranes were prepared as described previously [30]. [32P]Methionine and Amplify were purchased from New England Nuclear and Amersham, respectively. 

m7G(5')ppp(5')G cap analogue (diguanosine triphosphate) was obtained from Pharmacia. Peptide N-glycosidase F (PNGase F) and restriction enzymes were obtained from Boehringer Mannheim, New England Biolabs or Promega. All other chemicals were obtained from Sigma or Fisher Scientific.

Construction of human MRP1 cDNA with a glycosylation reporter gene and site-directed mutagenesis

PCR was used to engineer fusion constructs and mutations as described previously [31]. Briefly, cDNAs encoding TM1, TM1–TM2, TM1–TM3 and TM1–TM4 of human MRP1 were amplified by PCR using a sense primer 5'-TGCAGGTGACGTGGGGG-3' (for all constructs), and antisense primers 5'-CTTCAAGATCTGTGTTGAGAGG-3' (for TM1), 5'-CTTCAAGATCTGGGCACGCTTCTCT-3' (for TM1–TM2), 5'-CTTCAAGATCTGTCCCTCCTCTCCTCCT-3' (for TM1–TM3), and 5'-CTTCAAGATCTGGGCTACAGGTACCC-3' (for TM1–TM4). The sense primer contained a Kozak initiation codon ATG (underlined) and antisense primers contained a BglII
The fluorograph images were digitized using a HP ScanJet the presence of wheat germ ribosome (WGR) and purification of performed as described previously [38]. Translation in RRL in the presence of wheat germ ribosome (WGR) and purification of performed as described previously by Gilmore et al. [36] and Walter and Blobel [37], respectively. For the post-translational performed as described previously [19]. Treatment of microsome translation using pre-mixed RRL and WGE were performed as described previously [38]. Translation in RRL in the presence of wheat germ ribosome (WGR) and purification of WGR were done in the same way as described by Wang et al. [39]. The fluorograph images were digitized using a HP ScanJet 6100C and Adobe Photoshop 4.0.

RESULTS

Topology of MSD1 in ER membranes

MRP differs from other ABC transporters by having three MSDs with an extracellular N-terminus (Figure 1A). To understand the importance of the additional MSD (MSD1) in biogenesis of human MRP1, the TM segments in MSD1 were first tested for their membrane-translocation properties. To achieve this goal, a glycosylation reporter was engineered at the C-terminal side of each of the four TM segments of MSD1. These constructs (named MRP-N1R, MRP-N2R, MRP-N3R and MRP-N4R) are shown schematically in Figure 1(B). Figure 1(C) shows the predicted topology of these constructs. Note that the two potential glycosylation sites at the N-terminus are attached with oligosaccharides in all constructs whereas the potential site in the C-terminal glycosylation reporter is glycosylated only in MRP-N2R and MRP-N4R (Figure 1C).

As shown in Figure 1(D), translation of MRP-N1R, MRP-N2R, MRP-N3R and MRP-N4R transcripts in RRL in the absence of dog pancreatic RM generated products with incremental increases in size, as expected (Figure 1D, lanes 1, 3, 5 and 7). In the presence of RM, products with higher molecular mass were generated from all constructs (as indicated by asterisks in Figure 1D, lanes 2, 4, 6 and 8). The increased size in the presence of RM was due to glycosylation, as demonstrated by endoglycosidase (PNGase F) treatment, which reduced their sizes to the same level as the ones produced in the absence of RM (see Figure 1E). Therefore, the nascent protein containing one, two, three and four TM segments of MSD1 actively translocated into membranes and were modified by glycosylation. However, it is noteworthy that the membrane translocation by MRP-N1R was less than that by other constructs (compare lane 2 with lanes 4, 6 and 8, Figure 1D).

To determine the membrane orientation of the N- and C-terminals of these proteins, the glycosylation status was examined. As indicated by the number of asterisks in Figure 1(D), MRP-N1R and MRP-N3R both had a maximum of two high-mannose chains whereas MRP-N2R and MRP-N4R had a maximum of three high-mannose chains attached. The glycosylation status was also independently demonstrated using limited deglycosylation studies (J.-T. Zhang, unpublished work). Thus, MRP-N1R, MRP-N2R, MRP-N3R and MRP-N4R all had their N-termini located in the RM lumen and glycosylated with a maximum of two high-mannose chains (Figure 1C). MRP-N2R and MRP-N4R proteins, in addition, also had their C-terminal glycosylation reporter located in the RM lumen and attached with an additional oligosaccharide chain (Figure 1C). The orientation of the C-terminal glycosylation reporter in these proteins was demonstrated further by proteolysis/membrane-protection assay. As shown in Figure 1(F), no protease-resistant glycosylation reporter was observed from proteinase K digestion of membrane-associated MRP-N1R (Figure 1F, lanes 1–3) or MRP-N3R (Figure 1F, lanes 7–9), suggesting the cytoplasmic location of the C-terminal glycosylation reporter of these proteins. On the other hand, peptide fragments corresponding to the glycosylation reporter were produced from proteinase K digestion of MRP-N2R (Figure 1F, lane 4) and MRP-N4R (Figure 1F, lane 10). The protected fragments from MRP-N2R and MRP-N4R were also sensitive to PNGase F treatment (Figure 1F, lanes 5 and 11). The protected fragments were digested away if membranes were first permeabilized with Triton X-100 (Figure 1F, lanes 6 and 12). These results indicate that the glycosylation reporters of MRP-N2R and MRP-N4R were glycosylated and located in the RM lumen. Therefore, the N-terminus of human MRP1 was in the extracellular space and the

Transcription and translation in vitro

Transcription in vitro was performed as described previously [19]. Cell-free translations in RRL or wheat germ extract (WGE), 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 described previously [19]. Treatment of microsome membranes (RM) with N-ethylmaleimide (NEM) and salt were performed as described previously by Gilmore et al. [36] and Walter and Blobel [37], respectively. For the post-translational membrane-translocation experiment, translation was performed without RM for 1 h and terminated by addition of puromycin to a final concentration of 20 μM and incubation for 20 min, then followed by incubation with RM for 30 min. All incubations were at 30 °C. Translation using pre-mixed RRL and WGE were performed as described previously [38]. Translation in RRL in the presence of wheat germ ribosome (WGR) and purification of WGR were done in the same way as described by Wang et al. [39]. The fluorograph images were digitized using a HP ScanJet 6100C and Adobe Photoshop 4.0.

© 2000 Biochemical Society
topology of MSD1 of human MRP1 in ER membranes was the same as in plasma membranes, as shown previously [23–25].

Effect of N-terminal truncation on the orientation of MSD1

Previously, it was shown that the N-terminal sequence may be important for MRP function and that deletion of TM1 inhibited MRP function [26]. It has also been suggested in previous studies that the N-terminal sequence is important for the biogenesis of polytopic membrane proteins [34,40]. To determine whether truncation of the N-terminal sequence affects the topogenesis of MSD1, the first 17 amino acids at the N-terminal end of MRP-glycosylation reporter fusion proteins were deleted and a new start codon was engineered (see Figure 2A). The two potential N-linked glycosylation sites at the N-terminus, however, remain intact. The effect of this deletion on TM1 and TM2 biogenesis was tested. As shown in Figure 2(C), translation of the N-terminally truncated MRP-N1R and MRP-N2R (named MRP-N1\_DNE and MRP-N2\_DNE, respectively) generated proteins of predicted size in the absence of RM (Figure 2C, lanes 1 and 3). In the presence of RM, proteins with maximum of two and three oligosaccharide chains were produced for MRP-N1\_DNE and MRP-N2\_DNE, respectively (indicated by asterisks, Figure 2C, lanes 2 and 4), suggesting that the N-termini of both MRP-N1\_DNE and MRP-N2\_DNE were glycosylated whereas only the C-terminus of MRP-N2\_DNE was glycosylated. The glycosylation status of these two proteins was confirmed by endoglycosidase treatment (Figure 2D, lanes 1–4) and the membrane orientation of the glycosylation reporter was confirmed by proteinase K digestion (Figure 2E, lanes 1–6). Thus the truncated MRP-N1\_DNE and MRP-N2\_DNE proteins had the same topology as their corresponding wild-type proteins (compare Figure 2B with Figure 1B).

The above study suggested that removal of the N-terminal 17 amino acids did not affect either TM1 or TM2 membrane translocation and orientation. However, in the previous functional study by Gao et al. [26], the N-terminal 66 amino acids of MRP1 were deleted to create an inactive protein. To determine whether a large truncation affects the membrane orientation of MSD1, the N-terminal 61 amino acids were deleted to create MRP-N2\_DNTM1 and MRP-N3\_DNTM1. Translation of MRP-N2\_DNTM1 in the presence of RM generated a protein with a single oligosaccharide chain compared with the translation in the absence of RM (Figure 2C, compare lanes 5 and 6), suggesting the lumenal location of the glycosylation reporter (Figure 2B). This was confirmed by a reduction of \( \approx 2-3 \) kDa in size with endoglycosidase treatment (Figure 2D, lanes 5 and 6) and by protection of the glycosylated reporter from proteinase K digestion (Figure 2E, lanes 7–9). Translation of MRP-N3\_DNTM1 generated proteins of the same sizes in the absence and presence of RM (compare lanes 7 and 8, Figure 2C), suggesting that no oligosaccharide was added to the nascent protein. This observation was further confirmed by endoglycosidase treatment that did not change the size of the protein (Figure 2D, lanes 7 and 8). Thus the glycosylation reporter of MRP-N3\_DNTM1 was located in the cytoplasm (Figure 2B).
Biogenesis of human multidrug-resistance-associated protein

Figure 3 SRP and docking-protein dependence of the membrane translocation of MRP TM1 and TM2

MRP-N1R and MRP-N2ΔNTM1 were translated in RRL (lanes 1–4 and 7–10) or WGE (lanes 5, 6, 11 and 12) supplemented with normal RM (lanes 1, 5, 7 and 11), NEM-treated RM (NRM; lanes 3 and 9), control RM treated without NEM (cNRM; lanes 2 and 8) and SRP-stripped RM (KRM; lanes 4, 6, 10 and 12). The translocated and glycosylated proteins are indicated by asterisks.

conclusion was supported by proteinase K digestion, which did not generate any membrane-protected glycosylation reporter from MRP-N3ΔNTM1 (Figure 2E, lanes 10–12). Therefore, truncation of N-terminal sequences up to 61 amino acids did not affect the biogenesis of MSD1 of human MRP1.

SRP and SRP-receptor-dependent membrane translocation of MSD1

The above studies also suggested that TM2, in the absence of TM1 (MRP-N2ΔNTM1 construct), could initiate de novo membrane targeting and translocation while TM1 initiated N-terminus-outside/C-terminus-inside (N_out-C_in) membrane translocation. To determine whether the membrane targeting and translocation of TM1 and TM2 in MSD1 is dependent on SRPs, translations of MRP-N1R and MRP-N2ΔNTM1 were performed in WGE in conjunction with normal microsomes (RM) and SRP-stripped RM (KRM), depleted of SRP by high-salt wash [37]. In contrast to RRL, WGE does not contain endogenous functional SRP [30]. As shown in Figure 3, fully glycosylated MRP-N1R and MRP-N2ΔNTM1 were generated in the presence of RM both in RRL (Figure 3, lanes 1 and 7) and WGE (Figure 3, lanes 5 and 11), although the production of these proteins in WGE was lower than in RRL because of the existence of a lower level of SRP in WGE brought in by RM. In the presence of KRM the glycosylated proteins were not generated in WGE (Figure 3, lanes 6 and 12), whereas they were still produced in RRL (Figure 3, lanes 4 and 10). Mock treatment of RM without high salt did not affect membrane translocation in WGE (results not shown). Thus the de novo membrane targeting and translocation of TM1 (in MRP-N1R) and TM2 (in MRP-N2ΔNTM1) is SRP-dependent.

The dependency of membrane translocation on SRP receptor was demonstrated by producing no glycosylated proteins in RRL supplemented with microsomes (NEM-treated RM, NRM) that had been treated with 2 mM NEM to inactivate SRP receptors (Figure 3, lanes 3 and 9), as demonstrated previously [36].

Determinant of the extracellular location of the N-terminus of MSD1

The amino acid sequence surrounding TM1 was examined in order to investigate what determines the N_out-C_in membrane orientation of TM1, which results in an extracellular N-terminus. The result showed that the domain at the N-terminal side of TM1 was clearly less positive (with a total net charge of \(-2\)) than the domain at the C-terminal side of TM1 (with a total net charge of \(+3\); Figure 4A). Thus it is possible that the membrane orientation of TM1 is determined by the ‘positive-inside’ rule [41]. To test this hypothesis, three negative charges (Asp\(^{10}\), Asp\(^{13}\) and Asp\(^{17}\)) at the N-terminus of TM1 were mutated to positive charges (Arg\(^{10}\), Arg\(^{13}\) and Arg\(^{17}\), respectively; Figure 4B). The
mutant MRP-TM1R was named MRP-N1RNmut and had a net charge of +4 at the N-terminal domain of TM1. When MRP-N1RNmut was translated in the presence of RM, a protein with one oligosaccharide chain was produced in comparison with the translation in the absence of RM (compare lanes 1 and 2, Figure 4C). This observation was confirmed by endoglycosidase treatment that reduced ≈2–3 kDa of the protein to the size of the protein translated in the absence of RM (compare lanes 3 and 4, Figure 4C). Proteinase K digestion of the membrane-associated MRP-N1RNmut generated a protected glycosylation reporter which was glycosylated (compare lanes 5 and 6, Figure 4C). The protected fragments were digested away when membranes were first permeabilized with Triton X-100 (Figure 4C, lane 7). However, in our control digestion using wild-type MRP-N1R, no protected glycosylation reporter was found (results not shown, but see Figure 1F). These results suggest that the glycosylation reporter of MRP-N1RNmut was located in RM lumen, an orientation opposite to the wild-type MRP-N1R (Figure 4D). Thus, N1Rmut C-term orientation of TM1 is determined by the charged amino acids flanking TM1 and mutation of these charges can invert the membrane orientation of TM1 in MRP-N1R (Figure 1D). It should be noted that the mutations at the N-terminus completely inverted the orientation of TM1 in the presence of the glycosylation reporter, indicating that the reporter domain did not affect the membrane translocation and orientation of TM1.

To determine if the same mutations would still affect the membrane topology of TM1 in the presence of other TM segments, TM2–TM4, Asp110 and Asp157 were all mutated to Arg to create MRP-N2RNmut, MRP-N3RNmut and MRP-N4RNmut, respectively. The membrane orientations of these mutant proteins were then determined. As shown in Figure 5A, translation of MRP-N2RNmut, MRP-N3RNmut and MRP-N4RNmut in the presence of RM generated proteins with a maximum of three, two and three oligosaccharide chains attached (as indicated by the numbers of asterisks), respectively. This was demonstrated by comparison of the products generated in the absence and presence of RM (compare lanes 1, 4 and 7 with lanes 2, 5 and 8, respectively, Figure 5A) and by endoglycosidase treatment (lanes 3, 6 and 9, Figure 5A). Proteinase K digestion in combination with PNGase F treatment of the membrane-associated products showed that both MRP-N2RNmut and MRP-N4RNmut proteins had their C-terminal glycosylation reporters protected by RM (Figure 5B, lanes 1, 2, 7 and 8), indicating the RM-lumen location of the glycosylation reporter. MRP-N3RNmut, on the other hand, did not have its C-terminal glycosylation reporter protected by RM (Figure 5B, lanes 4–6), indicating a cytoplasmic location of the glycosylation reporter. These results suggest that the N-terminus be glycosylated and located in the RM lumen for all MRP-N2RNmut, MRP-N3RNmut and MRP-N4RNmut and that the C-terminal glycosylation reporter be located in the RM lumen only for MRP-N2RNmut and MRP-N4RNmut. Thus, the topology of MRP-N2RNmut, MRP-N3RNmut and MRP-N4RNmut in RM was the same as that of the wild-type MRP-N2R, MRP-N3R and MRP-N4R, respectively (see Figure 1B). That is, the mutations did not invert the orientation of TM1 in MRP-N2RNmut, MRP-N3RNmut and MRP-N4RNmut. This observation is surprisingly interesting because mutations of Asp110, Asp13 and Asp157 to Arg could invert the TM1 orientation in the absence of other TMs following TM1 (MRP-N1RNmut) but not in the presence of these TMs (MRP-N2RNmut, MRP-N3RNmut and MRP-N4RNmut). The inverted orientation of TM1 by mutations had apparently been corrected by the existence of a minimum sequence of TM2 plus its flanking residues. Therefore, TM2 appeared to play a very important role in helping MSD1 of human MRP1 fold correctly and could correct the mutation-caused inversion of the TM1 orientation in membranes. However, it should be noted that replacing the glycosylation reporter domain of MRP-N1R with TM2 might have also altered the charges at the C-terminal side of TM1, which in turn affected the folding of TM1. Nevertheless, replacing the glycosylation reporter of MRP-N1R with TM2 (MRP-N2R) in the wild-type sequence did not affect the membrane orientation of TM1 and it only affected the mutated sequence. This observation suggests that TM2 is very important for TM1 membrane translocation and orientation.

Post-translational membrane translocation of MSD1

In a previous study on cystic fibrosis transmembrane conductance regulator (CFTR), Lu et al. [42] found that TM2 of CFTR has an ability to translocate into membranes post-translationally as a redundant mechanism to ensure correct membrane insertion of CFTR. In the current study, TM2 of human MRP1 was found to have an ability to initiate membrane translocation in an SRP-dependent manner and that TM2 could correct the mutation-induced inversion of TM1 orientation. This suggests that TM2 plays a very important role in regulating the topogenesis of MSD1. To determine whether TM2 of human MRP1 also has post-translational membrane targeting and translocation activity to provide an additional insurance for correct folding of MSD1 in case TM1 fails to initiate membrane translocation correctly, a
post-translational membrane-translocation experiment was performed. RM was added to a translation of MRP-N2NTM1 after the translation was completed and terminated by addition of puromycin and then membrane fractions were isolated by centrifugation for analysis by SDS/PAGE. Extended incubation in the presence of puromycin did not generate more translation products, indicating that the translation was fully stopped (results not shown). As shown in Figure 6(A), a glycosylated protein (indicated by an asterisk) was generated from MRP-N2NTM1 and it was associated with the membrane pellet (compare lanes 3 and 4, Figure 6A), whereas no glycosylated protein was found from MRP-N1R (compare lanes 1 and 2, Figure 6A). This observation suggests that TM2 in MRP-N2NTM1 can target and translocate into membranes after the translation is terminated, whereas TM1 in MRP-N1R cannot target and translocate into membranes post-translationally.

To confirm the post-translational membrane translocation of MRP-N2NTM1, membrane-associated nascent MRP-N2NTM1 was treated with proteinase K. As shown in Figure 6(B), the glycosylated protein was resistant to protease digestion (Figure 6B, lane 2) and its size was reduced by endoglycosidase treatment (Figure 6B, lane 3). This observation is the same as that for co-translationally translocated MRP-N2NTM1 (Figure 6B, lanes 6 and 7). Thus the post-translationally translocated MRP-N2NTM1 had the same orientation as the co-translationally translocated MRP-N2NTM1. Note that the only difference between the co-translational and post-translational targeting and translocation of MRP-N2NTM1 was that the efficiency of this process was lower by post-translational than co-translational mechanism (compare the amount of glycosylated protein indicated by an asterisk in Figure 6B, lanes 1 and 5).

To determine whether the post-translational membrane targeting and translocation of TM2 is also SRP- and SRP-receptor-dependent, NRM was added to the translation after terminating the translation with puromycin. As shown in Figure 6(C), the glycosylated protein in the membrane pellet was not observed in the absence of NRM (compare lanes 5 and 6, Figure 6C), whereas it was produced in the presence of control RM treated without NEM (compare lanes 3 and 4, Figure 6C). This observation was confirmed by proteinase K digestion that did not produce membrane-protected glycosylation reporter from the translation in the presence of NRM (results not shown). Thus the post-translational membrane targeting and translocation of TM2 was also by the SRP and its receptor-dependent mechanism. Note that the unglycosylated band found in the pellet appears to represent background since it was also observed when no membranes were added to the translation (Figure 6C, lane 2).

Ribosomal role in membrane translocation of MSD1

In previous studies, it has been reported that ribosomes are important for the membrane targeting and translocation of polytopic membrane proteins [39]. Because TM2 of human MRP1 is a very important element in the topogenesis of MSD1, MRP-N2R was translated in both RRL or WGE to determine whether the membrane translocation and orientation of MRP-N2R is different between these two different systems. Surprisingly, MRP-N2R did not generate the fully glycosylated protein in WGE as compared with the translation in RRL (compare lanes 1 and 2, Figure 7A). Only proteins with two oligosaccharide chains and the unglycosylated peptide backbone were produced in WGE. This suggests that, in WGE, TM1 of MRP-N1R may initiate the membrane targeting and translocation with the N<sub>ε</sub>C<sub>ε</sub> orientation. However, TM2 of MRP-N2R may be unable to initiate the N<sub>ε</sub>C<sub>ε</sub> membrane translocation. Thus only the two potential sites at the N-terminus were glycosylated while the C-terminal glycosylation reporter was located in cytoplasm. This was supported by proteinase K treatment of MRP-N2R translated in WGE that did not generate any glycosylated or unglycosylated and membrane-protected glycosylation reporter fragments (compare lanes 1–4 with lanes 5–8, Figure 7C).

A lack of SRP in WGE was unlikely to be responsible for the failure of membrane translocation of TM2 in MRP-N2R, because supplementation of RM would provide enough SRP to support the membrane translocation of TM2 in WGE (see Figure 3). To support this argument, translations of MRP-N2R in pre-mixed RRL and WGE were performed. As shown in Figure 7(D), MRP-N2R containing three oligosaccharide chains (indicated by three asterisks) was produced in RRL (Figure 7D, lane 1) but not in WGE (Figure 7D, lane 9). In the mixture of different ratios of RRL and WGE, production of fully glycosylated MRP-N2R (with three oligosaccharide chains) in the presence of up to 50% RRL (Figure 7B, lane 5) was not recovered to the same extent as in RRL alone (Figure 7B, lane 1). Thus lacking SRP in WGE was not responsible for the failure of TM2 membrane trans-
location in WGE because addition of a small fraction of RRL should supply enough SRP to support fully the membrane translocation of TM2. Therefore, factors other than lacking SRP in WGE may be responsible for the failure of membrane translocation of TM2 of MRP-N2R.

One possible cause for generating the fully glycosylated MRP-N2R in WGE is that WGR may function differently in folding the nascent protein than RRL ribosome and that TM2 translated by WGR may not re-initiate membrane translocation after TM1 was translocated into membranes. To test this hypothesis, WGR was supplemented into the RRL translation system and production of the glycosylated MRP-N2ANTM1 was compared with that generated in the RRL alone. As shown in Figure 7(B), addition of WGR did not increase or decrease significantly the generation of the glycosylated MRP-N2ANTM1 proteins (compare lanes 3 and 4, Figure 7B). Thus membrane targeting and translocation of TM2 in the absence of TM1 was not ribosome-dependent. The lower level of membrane targeting and translocation of TM2 in MRP-N2ANTM1 (in the absence of TM1) in WGE as compared with that in RRL (Figure 7A, lane 3) was due to the low level of SRP associated with RM in WGE (see Figure 3 and the Discussion).

DISCUSSION

The domain structure of MRP is different from other ABC transporters such as Pgp. While Pgp consists of two domains with each containing a MSD followed by an ATP-binding site, MRP has an additional MSD (MSD1). This additional MSD is at the N-terminal end of the protein and has an extracellular N-terminus. In this study, the controlling elements for the extracellular location of the N-terminus and the biogenesis of MSD1 were investigated. Both the membrane-targeting and -translocation properties of TM2 and the charged residues flanking TM1 were found to be important factors in generating the unique structure of human MRP1. The MSD1 of human MRP1 was also used as a model to investigate how the sequential membrane-translocation theory applies to a polytopic protein that has an extracellular N-terminus. The results showed that except TM1 the membrane translocation of MSD1 was sequential and independent of TM1. That is, TM2 initiated a N\textsubscript{in}-C\textsubscript{out} membrane-translocation event that was stopped by TM3. TM4 initiated the second event to be stopped by TM5.

TM1 alone could also function as a signal-anchor sequence in an SRP-dependent manner and resulted in an N\textsubscript{in}-C\textsubscript{out} membrane-translocation event that was stopped by TM3. TM4 initiated the second event to be stopped by TM5.

TM2 of MRP-N2R did not translocate into membranes efficiently in the presence of WGR. Thus the difference in ribosomes between RRL and WGE translation systems may be the cause for the difference in topogenesis of TM2 of MRP-N2R. WGR could probably not help TM2 of MRP-N2R target and translocate into membranes. These observations indicate that the membrane targeting and translocation of MRP TM2 in the presence of TM1 was regulated by ribosomes that may involve a re-targeting process (see the Discussion).
by the N$_{\text{in}}$-C$_{\text{out}}$ membrane translocation of TM2. Thus the extracellular location of the N-terminus was secured by two factors: charged amino acids flanking TM1 and N$_{\text{in}}$-C$_{\text{out}}$ membrane translocation of TM2. Interestingly, a recent study on a polytopic membrane protein with an extracellular N-terminus also showed that TM2 was required for the extracellular location of the N-terminus when mutation was engineered into the protein [43]. The fact that TM2 determined the N$_{\text{in}}$-C$_{\text{in}}$ orientation of TM1 in MRP and could correct mutation-induced inversion of TM1 orientation may be a general mechanism for generating a polytopic topology with an extracellular N-terminus. Thus, the ‘positive-inside’ rule may not apply to the first TM segment with an extracellular N-terminus in a polytopic membrane protein. It is not known how TM2 forced TM1 of MRP to translocate into membranes in an N$_{\text{in}}$-C$_{\text{in}}$ orientation, notwithstanding that the mutated charges flanking TM1 favoured the opposite TM1 orientation. It became even more difficult to comprehend whether the membrane targeting and translocation of TM1 and TM2 involve two separate ribosome-targeting processes (see below). Clearly, further studies are needed to determine the molecular mechanism of TM2 control of TM1 biogenesis in MRP.

TM2 not only can correct mutation-induced inversion in TM1 orientation, it may also play an important role in MSD1 biogenesis. First, TM2 has a strong de novo activity to initiate N$_{\text{in}}$-C$_{\text{out}}$ membrane targeting and translocation in an SRP-dependent manner. The membrane targeting and translocation of TM2 does not need its preceding or following TMs (TM1 and TM3). It should be noted that the membrane translocation of TM2 might not need SRP if TM1 translocates into membranes correctly as reported previously for an artificial protein [6]. Should TM1 fail to correctly target and translocate into membranes, the SRP-dependent strong de novo membrane-insertion activity of TM2 may come into effect. Secondly, TM2 has an ability to target and translocate into membranes post-translationally. This ability of TM2 may render an additional mechanism to ensure the correct membrane folding of MRP. Should TM2 fail to translocate into membranes co-translationally, it may initiate N$_{\text{in}}$-C$_{\text{in}}$ membrane targeting and translocation post-translationally. Therefore, TM2 appears to play a critical role in correct membrane targeting and translocation of MRP MSD1.

A recent study [42] showed that post-translational translocation of TM2 of human CFTR provided a redundant mechanism to ensure correct membrane folding for CFTR. If TM1 of CFTR fails to translocate into membranes, topogenic information encoded in TM2 provides a second chance for the nascent chain to acquire its correct topology. It is interesting to note that TM1 of CFTR had insufficient activity to initiate membrane translocation, the same as TM1 of human MRP1. This observation argues for the importance of the alternative mechanism provided by TM2 to ensure correct topogenesis of the nascent CFTR and MRP. Based on the observation with TM2 of human CFTR and MRP1, a tentative speculation would be that post-translational membrane targeting and translocation by TM2 provide a general redundant mechanism to ensure correct membrane translocation and folding for polytopic proteins in eukaryotes.

Post-translational membrane translocation has been reported for other eukaryotic membrane proteins such as connexin 26 [44], yeast prepro-$\alpha$-factor [37,45] and glucose transporter [46]. The post-translational translocation of these proteins has been shown to be dependent on ATP binding [44] and hydrolysis [47,48] and on nascent-chain-attached ribosomes [49]. Currently, it is not clear whether the post-translational membrane translocation of MRP-N2$\Delta$NTM1 also requires ATP binding and hydrolysis. Although the post-translational translocation of MRP-N2$\Delta$NTM1 was performed after puromycin pre-treatment, which presumably released ribosome from nascent chains, it cannot be ruled out that nascent-chain-attached ribosomes were still involved in the post-translational translocation of MRP-N2$\Delta$NTM1.

The results from the comparison of translations in RRL and WGE systems suggested that the membrane targeting and translocation of TM2 in the presence of TM1 depended on the types of ribosome used. WGR appeared unable to help TM2 target and translocate into membranes once TM1 was membrane translocated. This observation suggests that there may be a ribosome re-targeting process involved in TM2 membrane translocation following TM1 membrane translocation. A similar observation has been made about the role of ribosomes in the membrane translocation of Pgp sequences [39,50,51]. It is therefore possible that following the membrane translocation of TM1, ribosomes release from the protein-conducting channel and re-dock back when TM2 emerges from the ribosome. However, WGR may lack proteins that help them to re-dock on to dog pancreatic RM. Thus TM2 failed to translocate into membranes co-translationally and remained in the cytoplasm of WGE. It is unknown why TM2 did not use its post-translational activity to initiate the membrane translocation after failing to translocate into membranes co-translationally in WGE. However, it is possible that the post-translational membrane targeting and translocation of TM2 requires SRP (Figure 6B), and WGE does not have SRP. Thus WGE could not support post-translational membrane translocation of TM2. This was supported by an observation that no post-translationally translocated MRP-N2$\Delta$NTM1 was found in WGE (J.-T. Zhang, unpublished work).

Although, in this study, TM2 was shown to play a very important role in ensuring the correct folding of MSD1 in vitro, it cannot be ruled out that TM2 may not do so in vivo. However, the topology of MSD1 obtained in this study is the same as the one generated using the full-length molecule in vivo systems. This suggests that the in vitro system faithfully translated and folded truncated MRP1 and it is probably in accord with the in vivo system. Furthermore, the study by Li et al. [42] showed both in vitro and in vivo that isolated TM2 of CFTR provides a redundant mechanism for correct folding of CFTR. Thus the use of truncated molecules in an in vitro system in this study should reflect the natural process of human MRP1 biogenesis in vivo. Moreover, because of the limitation of available techniques it is almost necessary to dissect and examine each domain separately to understand the detailed biogenesis process of a large and complex membrane protein such as human MRP1.

I thank Dr Piet Borst for the generous gift of human MRP cDNA, Dr Ernest Han and Ms Roxana Pincheira for their valuable suggestions during the course of this study, and Dr David Ohannesian for his critical comments on this manuscript. This work was supported by the National Institutes of Health grants CA64539 and GM56475. J.-T. Z. is a recipient of a Career Investigator Award from the American Lung Association.

REFERENCES


Received 8 December 1999/2 March 2000; accepted 29 March 2000

© 2000 Biochemical Society