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

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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 underlie 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_{in} - C_{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-stripped RM; NEM, *N*-ethylmaleimide; NRM, NEM-treated RM; PNGase F, peptide N-glycosidase F; N_{in} - C_{out} , N-terminus inside/C-terminus outside; N_{out} - C_{in} , N-terminus outside/C-terminus inside; CFTR, cystic fibrosis transmembrane conductance regulator.

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Figure 1 Translation and topology of MRP-N1R, MRP-N2R, MRP-N3R and MRP-N4R

(A) Schematic diagram of human MRP topology. The topological structure shown here is from Bakos et al. [23]. CYT, cytoplasm; EXT, extracellular; MEM, membrane. TM segments are denoted by open boxes. (B) Schematic diagram of MRP-N1R–MRP-N4R constructs. TM segments are denoted by numbered boxes. The glycosylation reporter is shown as a black ellipse. The potential glycosylation sites are indicated by branched symbols. The amino acid sequence of the fusion site is shown with the glycosylation reporter sequence underlined. (C) Predicted membrane orientation of MRP-N1R–MRP-N4R proteins. Glycosylations are indicated by branched symbols. Note the membrane orientation and glycosylation status of the glycosylation reporter in each construct. (D) Fluorography of truncated MRP translation products. Translations were performed in RRL in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of RM. The number of asterisks denotes the number of oligosaccharide chains attached to the protein product. (E) Endoglycosidase PNGase F (PNG) treatment of truncated MRP translation products. Translation products produced in the presence of RM were treated by PNGase F (lanes 2, 4, 6 and 8). Samples in lanes 1, 3, 5 and 7 are control reactions without PNGase F. ← and * indicate deglycosylated and glycosylated proteins respectively. (F) Proteinase K digestion of truncated MRP translation products. Translation products generated in the presence of RM were treated by proteinase K in the absence (lanes 1, 2, 4, 5, 7, 8, 10 and 11) or presence of Triton X-100 (TX-100; lanes 3, 6, 9 and 12). Samples in lanes 2, 5, 8 and 11 were further treated with PNGase F.

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]. [³⁵S]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'-GTCGTGATGGCGC-TCCGGGG-3' (for all constructs), and antisense primers 5'-CTTCAAGATCTGTTTGTGAGAGG-3' (for TM1), 5'-CTTCAAGATCTGGCCCCGACTTCT-3' (for TM1–TM2), 5'-CTTCAAGATCTGTCCCTTCCTCCT-3' (for TM1–TM3), and 5'-CTTCAAGATCTGAAACAGGTCCAC-3' (for TM1–TM4). The sense primer contained a Kozak initiation codon ATG (underlined) and antisense primers contained a Bg/II

digestion sequence AGATCT (underlined). The PCR products were cloned into pGEM-T Easy vectors. The cDNA inserts in pGEM-T Easy vectors were then released by *EcoRI* and *BglII* digestion and ligated into an *EcoRI/BglII*-linearized pGEM 4z vector containing a glycosylation reporter gene. The glycosylation reporter was derived from an ATP-binding domain of Pgp and has been used successfully in our previous studies [32–35]. These fusion DNA constructs were named MRP-N1R, MRP-N2R, MRP-N3R and MRP-N4R, respectively.

Truncations at the N-terminus of MRP1 were engineered using MRP-N1R, MRP-N2R and MRP-N3R DNA as templates for PCR. To remove the N-terminal sequence, a sense primer, 5'-GAATACGAATTCGTCGTGATGTGGAATGTCACGTGG-3' (MRPTNE) or 5'-GAATACGAATTCGTCGTGATGCGAGGCTACATTCAG-3' (MRPTTM1), and a T7 vector primer were used. Both MRPTNE and MRPTTM1 primers contained an *EcoRI* site (GAATTC) and a translation-initiation codon (ATG; both underlined). The PCR products were digested with *EcoRI* and *HindIII* and cloned into an *EcoRI/HindIII*-linearized pGEM-4z vector. The truncated DNA constructs were named MRP-N1ΔNE, MRP-N2ΔNE, MRP-N2ΔNTM1 and MRP-N3ΔNTM1.

To mutate Asp¹⁰, Asp¹³ and Asp¹⁷ to Arg, an antisense oligonucleotide containing these mutations 5'-GACATTCCA-GCGCCAGAGCGGGCGGGAGCCCCGGGCGCTGCA-3' (MRPN_{mut}) was synthesized. The three codons underlined are the mutated sequences. The first PCR reaction was performed using MRPN_{mut} and SP6 vector primers on MRP-N1R DNA template. The PCR products were then isolated and used as a primer, together with T7 vector primer, to perform PCR on the remaining sequence of MRP-N1R. The final PCR products were digested with *EcoRI* and *BglII* and cloned into the *EcoRI/BglII*-linearized pGEM-4z vector containing a glycosylation reporter gene as described above. The mutant DNA construct was named MRP-N1RN_{mut}. To engineer these mutations into MRP-N2R, MRP-N3R and MRP-N4R, a cassette containing the mutations was released from MRP-N1RN_{mut} using *EcoRI* and *DraIII* digestion and then used to replace the wild-type sequence in MRP-N2R, MRP-N3R and MRP-N4R DNA. The mutant DNAs were named MRP-N2RN_{mut}, MRP-N3RN_{mut} and MRP-N4RN_{mut}. All DNA constructs were sequenced to confirm the correct fusion, truncation and mutation.

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.

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 oligosaccharide chains 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 masses 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-termini 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

Figure 2 Translation and topology of deletion mutants MRP-N1ΔNE, MRP-N2ΔNE, MRP-N2ΔNTM1 and MRP-N3ΔNTM1

(A) Schematic diagram of MRP-N1ΔNE, MRP-N2ΔNE, MRP-N2ΔNTM1 and MRP-N3ΔNTM1 deletion constructs. The symbols used are the same as in Figure 1(B). (B) Membrane orientation of deletion-mutant proteins. The symbols are the same as in Figure 1(C). (C) Fluorography of translation products of deletion mutants. Translations were performed in RRL in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of RM. The number of asterisks indicates the number of oligosaccharide chains attached with the translation product. (D) Endoglycosidase PNGase F (PNG) treatment of translation products. Deletion-mutant products translated in the presence of RM were treated by PNGase F (lanes 2, 4, 6 and 8). Samples in lanes 1, 3, 5 and 7 are control reactions without PNGase F. ← and * indicate deglycosylated and glycosylated proteins respectively. (E) Proteinase K digestion of translation products. Deletion-mutant products translated in the presence of RM were treated by proteinase K in the absence (lanes 1, 2, 4, 5, 7, 8, 10 and 11) or presence of Triton X-100 (lanes 3, 6, 9 and 12). Samples in lanes 2, 5, 8 and 11 were further treated with PNGase F.

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-N1ΔNE and MRP-N2ΔNE, 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-N1ΔNE and MRP-N2ΔNE, respectively (indicated by asterisks, Figure 2C, lanes 2 and 4), suggesting that the N-termini of both MRP-N1ΔNE and MRP-N2ΔNE were glycosylated whereas only the C-terminus of MRP-N2ΔNE 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-N1ΔNE and MRP-N2ΔNE 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-N2ΔNTM1 and MRP-N3ΔNTM1. Translation of MRP-N2ΔNTM1 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 luminal location of the glycosylation reporter (Figure 2B). This was confirmed by a reduction of ≈ 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-N3ΔNTM1 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-N3ΔNTM1 was located in the cytoplasm (Figure 2B). This

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* N_{in}-C_{out} 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 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 (Asp¹⁰, Asp¹³ and Asp¹⁷) at the N-terminus of TM1 were mutated to positive charges (Arg¹⁰, Arg¹³ and Arg¹⁷, respectively; Figure 4B). The

Figure 4 Translation and topology of MRP-N1RN_{mut}

(A) Charge distribution surrounding TM1. The symbols used are the same as in Figure 1(B). Charged amino acids are underlined. (B) Mutations at the N-terminus of TM1. Aspartic acids at positions 10, 13 and 17 were changed to arginine in MRP-N1R by site-directed mutagenesis. (C) Translation of MRP-N1RN_{mut}. Translations were performed in RRL in the absence (lane 1) or presence (lanes 2–7) of RM. The products translated in the presence of RM were further treated by PNGase F (PNG; lane 4) or by proteinase K (Prot. K; lane 5). Lane 3 is a control reaction for PNGase F digestion. Lanes 6 and 7 are proteinase K digestion followed by PNGase F treatment and in combination with Triton X-100 (TX-100), respectively. ← and * indicate deglycosylated and glycosylated proteins, respectively. (D) Membrane orientation of mutant and wild-type MRP-N1R.

mutant MRP-TM1R was named MRP-N1RN_{mut} and had a net charge of +4 at the N-terminal domain of TM1. When MRP-N1RN_{mut} 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 $\approx 2\text{--}3\text{ kDa}$ 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-N1RN_{mut} 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-N1RN_{mut} was located in RM lumen, an orientation opposite to the wild-type MRP-N1R (Figure 4D). Thus, N_{out}-C_{in} 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, Asp¹⁰, Asp¹³ and Asp¹⁷ were all mutated to Arg to create MRP-N2RN_{mut}, MRP-N3RN_{mut} and MRP-N4RN_{mut}, respectively. The membrane orientations of these mutant proteins were then determined. As shown in Figure 5(A), translation of MRP-N2RN_{mut}, MRP-N3RN_{mut} and MRP-N4RN_{mut} 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-N2RN_{mut} and MRP-N4RN_{mut} 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-N3RN_{mut}, 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-N2RN_{mut}, MRP-N3RN_{mut} and MRP-N4RN_{mut} and that the C-terminal glycosylation reporter be located in the RM lumen only for MRP-N2RN_{mut} and MRP-N4RN_{mut}. Thus, the topology of MRP-N2RN_{mut}, MRP-N3RN_{mut} and MRP-N4RN_{mut} 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-N2RN_{mut}, MRP-N3RN_{mut} and MRP-N4RN_{mut}. This observation is surprisingly interesting because mutations of Asp¹⁰, Asp¹³ and Asp¹⁷ to Arg could invert the TM1 orientation in the absence of other TMs following TM1 (MRP-N1RN_{mut}) but not in the presence of these TMs (MRP-N2RN_{mut}, MRP-N3RN_{mut} and MRP-N4RN_{mut}). 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

Figure 5 Translation and topology of MRP-N2RN_{mut}, MRP-N3RN_{mut} and MRP-N4RN_{mut}

(A) Translation and endoglycosidase treatment of MRP-N2RN_{mut}–MRP-N4RN_{mut}. Translations were performed in RRL in the absence (lanes 1, 4 and 7) or presence (lanes 2, 3, 5, 6, 8 and 9) of RM. Endoglycosidase PNGase F (PNG) treatment was performed with the products translated in the presence of RM (lanes 3, 6 and 9). The number of asterisks denotes the number of oligosaccharide chains attached to the protein product. ← indicates deglycosylated proteins. (B) Proteinase K digestion of MRP-N2RN_{mut}–MRP-N4RN_{mut}. Membrane-associated MRP-N2RN_{mut}–MRP-N4RN_{mut} were treated with proteinase K (Prot. K), followed by PNGase F digestion (lanes 2, 5 and 8), or in combination with Triton X-100 (TX-100; lanes 3, 6 and 9). ← and * indicate deglycosylated and glycosylated fragments, respectively.

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

Figure 6 Post-translational membrane translocation of MRP TM2

(A) Post-translational membrane translocation. MRP-N1R and MRP-N2ΔNTM1 were translated in RRL in the absence of RM and terminated by addition of puromycin followed by addition of RM and incubation. Membranes (P, lanes 2 and 4) were then separated from soluble (S, lanes 1 and 3) fractions by centrifugation and analysed by SDS/PAGE. The glycosylated protein is indicated by asterisks. (B) Proteinase K (Prot. K) digestion of post-translationally translocated MRP-N2ΔNTM1. Membrane-associated MRP-N2ΔNTM1 from post-translational translocation (lanes 1–4) or co-translational translocation (lanes 5–8) were treated with proteinase K (lanes 2–4 and 6–8), followed by PNGase F (PNG) digestion (lanes 3 and 7), or in combination with Triton X-100 (TX-100; lanes 4 and 8). ◀ and * indicate deglycosylated and glycosylated fragments, respectively; ← indicates the protein resistant to proteinase K digestion. (C) SRP-dependence of post-translational translocation. MRP-N2ΔNTM1 was translated in RRL in the absence (lanes 1 and 2) or presence of control NRM (cNRM; lanes 3 and 4) and NRM (lanes 5 and 6). The insoluble fraction was then isolated by centrifugation and analysed by SDS/PAGE. The glycosylated protein is indicated by the asterisk.

post-translational membrane-translocation experiment was performed. RM was added to a translation of MRP-N2ΔNTM1 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-N2ΔNTM1 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-N2ΔNTM1 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-N2ΔNTM1, membrane-associated nascent MRP-N2ΔNTM1 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-N2ΔNTM1 (Figure 6B, lanes 6 and 7). Thus the post-translationally translocated MRP-N2ΔNTM1 had the same orientation as the co-translationally translocated MRP-N2ΔNTM1. Note that the only difference between the co-translational and post-translational targeting and translocation of MRP-N2ΔNTM1 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 presence 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-N2R may initiate the membrane targeting and translocation with the N_{out} - C_{in} orientation. However, TM2 of MRP-N2R may be unable to initiate the N_{in} - C_{out} 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-

Figure 7 Ribosomal dependence of the membrane translocation of MRP TM2

(A) MRP-N2R and MRP-N2ΔNTM1 were translated in RRL or WGE and membrane fractions were isolated and analysed by SDS/PAGE. (B) Translation of MRP-N2R in RRL and WGE translation systems that were pre-mixed prior to translation. (C) Proteinase K (Prot. K) digestion of MRP-N2R translated in WGE. Membrane-associated MRP-N2R translated in RRL (lanes 1–4) or WGE (lanes 5–8) were treated with proteinase K (lanes 2–4 and 6–8), followed by PNGase F (PNG) digestion (lanes 3 and 7), or in combination with Triton X-100 (TX-100; lanes 4 and 8). The arrowhead indicates the membrane-protected glycosylation reporter. (D) Effect of WGR on membrane translocation of TM2 in MRP-N2R and MRP-N2ΔNTM1. MRP-N2R and MRP-N2ΔNTM1 in RRL were translated in the absence or presence of WGR and membrane fractions were then isolated and analysed by SDS/PAGE. The numbers below the panel denote the percentages (in volume) of RRL and WGE. The glycosylated proteins are indicated by the asterisks. The arrow indicates the unglycosylated protein.

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 were isolated from WGE and added to the RRL translation system to determine if WGR could compete with RRL ribosomes to increase the production of MRP-N2R with two oligosaccharide chains. As shown in Figure 7(B), the amount of proteins with two oligosaccharide chains (indicated by two asterisks) was clearly increased by addition of WGR (compare lanes 1 and 2, Figure 7B). This observation indicates that the

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).

It should be noted, however, that a glycosylated protein was generated from MRP-N2ΔNTM1 (lacking TM1) in WGE (Figure 7A, lane 4). This observation suggests that TM2 in the absence of TM1 can target and translocate into RM with a N_{in} - C_{out} orientation in WGE, albeit at a much lower level than that in RRL (compare lanes 3 and 4, Figure 7A). This is in contrast to the observation that TM2 of MRP-N2R (in the presence of TM1) in WGE could not target and translocate into membranes with a N_{in} - C_{out} orientation (Figure 7A, lane 2). This difference may have been due to the possibility that the TM2-initiated membrane targeting and translocation in the absence of TM1 was ribosome-independent whereas in the presence of TM1 it was ribosome-dependent. To test this hypothesis, WGR was supplemented into the RRL translation system and production of the glycosylated MRP-N2ΔNTM1 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-N2ΔNTM1 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-N2ΔNTM1 (in the absence of TM1) in WGE as compared with that in RRL (Figure 7A, lanes 3 and 4) 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_{in} - C_{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_{out} - C_{in} orientation. The N_{out} - C_{in} orientation of TM1 appeared to be determined by charged amino acids surrounding TM1 and this observation was consistent with the 'positive-inside' rule [41]. However, the membrane translocation and orientation of TM2 also determined the membrane orientation of TM1. In the presence of TM2, the effect of charges on the membrane orientation of TM1 became obsolete. That is, the inversion of TM1 orientation induced by charge mutations could be corrected

by the N_{in} - C_{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_{in} - C_{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_{out} - C_{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_{out} - C_{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_{in} - C_{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_{in} - C_{out} 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- α -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 Δ NTM1 also requires ATP binding and

hydrolysis. Although the post-translational translocation of MRP-N2 Δ 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 Δ 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 Δ 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 *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 Lu 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.

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REFERENCES

- 1 Blobel, G. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 1496–1500
- 2 Corsi, A. K. and Schekman, R. (1996) J. Biol. Chem. **271**, 30299–30302
- 3 Goldman, B. M. and Blobel, G. (1981) J. Cell Biol. **90**, 236–242
- 4 Braell, W. A. and Lodish, H. F. (1982) Cell **28**, 23–31
- 5 Brown, D. A. and Simoni, R. D. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 1674–1678
- 6 Wessels, H. P. and Spiess, M. (1988) Cell **55**, 61–70
- 7 Friedlander, M. and Blobel, G. (1985) Nature (London) **318**, 338–343
- 8 Audigier, Y., Friedlander, M. and Blobel, G. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 5783–5787
- 9 Lipp, J., Flint, N., Haeuptle, M. T. and Dobberstein, B. (1989) J. Cell Biol. **109**, 2013–2022

- 10 Chavez, R. A. and Hall, Z. W. (1991) *J. Biol. Chem.* **266**, 15532–15538
- 11 Silve, S., Volland, C., Garnier, C., Jund, R., Chevallier, M. R. and Haguénauer-Tsapis, R. (1991) *Mol. Cell. Biol.* **11**, 1114–1124
- 12 Hartmann, E., Rapoport, T. A. and Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5786–5790
- 13 Skach, W. R. and Lingappa, V. R. (1993) in *Mechanisms of Intracellular Trafficking and Processing of Proproteins* (Peng Loh, Y., ed.), pp. 19–77, CRC Press, Boca Raton
- 14 Gottesman, M. M., Hrycyna, C. A., Schoenlein, P. V., Germann, U. A. and Pastan, I. (1995) *Annu. Rev. Genet.* **29**, 607–649
- 15 Ling, V. (1997) *Cancer Chemother. Pharmacol.* **40**, S3–S8
- 16 Cole, S. P., Bhardwaj, G., Gerlach, J., Mackie, J., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. and Deeley, R. G. (1992) *Science* **258**, 1650–1654
- 17 Borst, P., Kool, M. and Evers, R. (1997) *Semin. Cancer Biol.* **8**, 205–213
- 18 Higgins, C. F. (1992) *Annu. Rev. Cell Biol.* **8**, 67–113
- 19 Zhang, J. T. and Ling, V. (1991) *J. Biol. Chem.* **266**, 18224–18232
- 20 Bibi, E. and Beja, O. (1994) *J. Biol. Chem.* **269**, 19910–19915
- 21 Skach, W. R., Calayag, M. C. and Lingappa, V. R. (1993) *J. Biol. Chem.* **268**, 6903–6908
- 22 Zhang, M., Wang, G., Shapiro, A. and Zhang, J. T. (1996) *Biochemistry* **35**, 9728–9736
- 23 Bakos, E., Hegedus, T., Hollo, Z., Welker, E., Tusnady, G. E., Zaman, G. J., Flens, M. J., Varadi, A. and Sarkadi, B. (1996) *J. Biol. Chem.* **271**, 12322–12326
- 24 Hipfner, D. R., Almquist, K. C., Leslie, E. M., Gerlach, J. H., Grant, C. E., Deeley, R. G. and Cole, S. P. (1997) *J. Biol. Chem.* **272**, 23623–23630
- 25 Kast, C. and Gros, P. (1997) *J. Biol. Chem.* **272**, 26479–26487
- 26 Gao, M., Yamazaki, M., Loe, D. W., Westlake, C. J., Grant, C. E., Cole, S. P. C. and Deeley, R. G. (1998) *J. Biol. Chem.* **273**, 10733–10740
- 27 Bakos, E., Evers, R., Szakacs, G., Tusnady, G., Welker, E., Szabo, K., de Haas, M., van Deemter, L., Borst, P., Varadi, A. and Sarkadi, B. (1998) *J. Biol. Chem.* **273**, 32167–32175
- 28 Hipfner, D. R., Deeley, R. G. and Cole, S. P. C. (1999) *Biochim. Biophys. Acta* **1461**, 359–376
- 29 Zaman, G. J., Flens, M. J., van Leusden, M. R., de Haas, M., Mulder, H. S., Lankelma, J., Pinedo, H. M., Scheper, R. J., Baas, F., Broxterman, H. J. et al. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8822–8826
- 30 Walter, P. and Blobel, G. (1983) *Methods Enzymol.* **96**, 84–93
- 31 Zhang, J. T., Lee, C. H., Duthie, M. and Ling, V. (1995) *J. Biol. Chem.* **270**, 1742–1746
- 32 Zhang, J. T., Duthie, M. and Ling, V. (1993) *J. Biol. Chem.* **268**, 15101–15110
- 33 Chen, M. and Zhang, J. T. (1996) *Mol. Membr. Biol.* **13**, 33–40
- 34 Zhang, J. T. (1996) *Mol. Biol. Cell* **7**, 1709–1721
- 35 Zhang, J. T., Chen, M., Han, E. and Wang, C. (1998) *Mol. Biol. Cell* **9**, 853–863
- 36 Gilmore, R., Blobel, G. and Walter, P. (1982) *J. Cell Biol.* **95**, 463–469
- 37 Walter, P. and Blobel, G. (1981) *J. Cell Biol.* **91**, 551–556
- 38 Zhang, J. T. and Ling, V. (1995) *Biochemistry* **34**, 9159–9165
- 39 Wang, C., Chen, M., Han, E. and Zhang, J. T. (1997) *Biochemistry* **36**, 11437–11443
- 40 Chen, M. and Zhang, J. T. (1999) *Biochemistry* **38**, 5471–5477
- 41 von Heijne, G. (1986) *EMBO J.* **5**, 3021–3027
- 42 Lu, Y., Xiong, X., Helm, A., Kimani, K., Bragin, A. and Skach, W. R. (1998) *J. Biol. Chem.* **273**, 568–576
- 43 Monne, M., Gafvelin, G., Nilsson, R. and von Heijne, G. (1999) *Eur. J. Biochem.* **263**, 264–269
- 44 Zhang, J. T., Chen, M., Foote, C. and Nicholson, B. J. (1996) *Mol. Biol. Cell* **7**, 471–482
- 45 Rothblatt, J. A. and Meyer, D. I. (1986) *Cell* **44**, 619–628
- 46 Mueckler, M. and Lodish, H. F. (1986) *Cell* **44**, 629–637
- 47 Mueckler, M. and Lodish, H. F. (1986) *Nature (London)* **322**, 549–552
- 48 Hansen, W., Garcia, P. D. and Walter, P. (1986) *Cell* **45**, 397–406
- 49 Garcia, P. D. and Walter, P. (1988) *J. Cell Biol.* **106**, 1043–1048
- 50 Borel, A. C. and Simon, S. M. (1996) *Biochemistry* **35**, 10587–10594
- 51 Borel, A. C. and Simon, S. M. (1996) *Cell* **85**, 379–389

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