Translocation of a long amino-terminal domain through ER membrane by following signal-anchor sequence

Yuichiro Kida^{1,2}, Katsuyoshi Mihara³
and Masao Sakaguchi^{1,2,*}

¹CREST of the Japan Science and Technology Agency, University of Hyogo, Ako, Hyogo, Japan, ²Graduate School of Life Science, University of Hyogo, Ako, Hyogo, Japan and ³Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan

Type I signal-anchor sequences mediate translocation of the N-terminal domain (N-domain) across the endoplasmic reticulum (ER) membrane. To examine the translocation in detail, dihydrofolate reductase (DHFR) was fused to the N-terminus of synaptotagmin II as a long N-domain. Translocation was arrested by the DHFR ligand methotrexate, which stabilizes the folding of the DHFR domain, and resumed after depletion of methotrexate. The targeting of the ribosome–nascent chain complex to the ER requires GTP, whereas N-domain translocation does not require any nucleotide triphosphates. Significant translocation was observed even in the absence of a luminal hsp70 (BiP). When the nascent polypeptide was released from the ribosomes after the membrane targeting, the N-domain translocation was suppressed and the nascent chain was released from the translocon. Ribosomes have a crucial role in maintaining the translocation-intermediate state. The translocation of the DHFR domain was greatly impaired when it was separated from the signal-anchor sequence. Unfolding and translocation of the DHFR domain must be driven by the stroke of the signal-anchor sequence into translocon.

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Introduction

Most membrane proteins in the secretory pathway are synthesized by membrane-bound ribosomes and are cotranslationally integrated into the endoplasmic reticulum (ER) membrane through the protein-conducting channel, the so-called translocon. The channel consists of the heterotrimeric Sec61 complex and is also involved in the translocation of soluble proteins (Rapoport *et al*, 1996; Johnson and van Waes, 1999). Cotranslational integration is initiated by signal sequences. The signal sequences are recognized by the

signal recognition particle (SRP), which arrests polypeptide chain elongation on the ribosome and targets the ribosome–nascent chain complex (RNC) to the ER (Walter and Johnson, 1994). On the ER membrane, the SRP is released from the RNC by the SRP receptor, protein synthesis resumes, and the signal sequence enters the translocon pore.

The core functions of signal sequences, targeting and triggering insertion into the translocon, are defined by their hydrophobic region (H-region) and either the N- or C-terminal side of the H-region is translocated through the membrane. If the N-terminal portion is translocated, the signal sequence forms an N_{lum}/C_{cyt} topology, where the N-terminus is on the luminal side and the C-terminus is on the cytoplasmic side (termed type I signal-anchor, SA-I). When the C-terminal portion is translocated, it forms an N_{cyt}/C_{lum} topology; in this type of signal sequence, the signal peptide is cleaved off by the signal peptidase in the lumen, while the signal-anchor sequence remains to become the transmembrane (TM) segment (termed type II signal-anchor, SA-II). The H-regions of signal peptides (6–15 residues) are shorter than those of signal-anchor sequences (17–27 residues), and the longer H-region tends to mediate the translocation of the N-domain (Sakaguchi *et al*, 1992b; Goder and Spiess, 2001). Charge characteristics of both flanking regions of the H-region (Hartmann *et al*, 1989; Sakaguchi *et al*, 1992b; Goder and Spiess, 2001) and folding properties of the N-domain (Denzer *et al*, 1995) affect the orientation of the H-region. Regions rich in positive charges tend to be retained on the cytoplasmic side. SA-I sequences have been identified in the microsomal cytochrome P450 family (Sakaguchi *et al*, 1987), synaptotagmin family (Kida *et al*, 2000), and NADPH-cytochrome P450 reductase (Kida *et al*, 1998), etc. The first TM segments of most members of the G-protein-coupled receptor family, which have seven TM segments, are also SA-I sequences (Wallin and von Heijne, 1995). Internal SA-I sequences in multispansing membrane proteins can mediate integration of the preceding segment with little hydrophobicity (Ota *et al*, 1998a, b).

In topogenesis of the SA-I sequence, N-domain translocation occurs after the H-region emerges from the ribosome (Heinrich *et al*, 2000; Kida *et al*, 2000). The N-domain is thus translocated after being synthesized, in contrast to the translocation mediated by other signal sequences, in which the following passenger polypeptide is directly inserted into the translocon from the exit site of the ribosomes. In this context, N-domain translocation somewhat resembles post-translational translocation, while the translocation proceeds from the C-terminus to the N-terminus (C-to-N translocation). The ER targeting and the N-domain translocation of synaptotagmin II (SytlII) occur at different chain lengths (Kida *et al*, 2000). A nascent chain of 125 residues bound to the ribosome is targeted to the translocon, but the N-domain remains on the cytoplasmic side, whereas the N-domain of 130-residue nascent chain is translocated though the membrane (Kida

*Corresponding author. Graduate School of Life Science, University of Hyogo, Ako, Hyogo 678-1297, Japan. Tel.: +81 791 58 0206; Fax: +81 791 58 0132; E-mail: sakag@sci.u-hyogo.ac.jp

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et al, 2000). The SA-I sequence transiently contacts the translocon subunits and then dissociates from the translocon to the lipid environment (Heinrich *et al*, 2000; Kida *et al*, 2000). The translocon mediates partitioning of the H-regions into a lipid hydrophobic environment and the less hydrophobic TM segment tends to be accommodated inside the channel for longer periods (Heinrich *et al*, 2000; McCormick *et al*, 2003).

Post-translational protein translocation across the ER membrane has been extensively studied with *Saccharomyces cerevisiae* (Rapoport *et al*, 1996). It is mediated by the so-called Sec complex, which includes seven heterosubunits including Sec62, 63, 71, 72, and the heterotrimeric Sec61 complex. In the lumen, the hsp70 homolog, BiP, binds the polypeptide chain emerging from the translocon channel to retain the chain. Sec63, which is a partner of BiP, modulates BiP function (Misselwitz *et al*, 1998, 1999). ATP is consumed for tight binding of the polypeptide chain substrates (Matlack *et al*, 1999). Only BiP binding and Brownian movement of polypeptide chains through the translocon explain the vectorial movement of post-translational protein translocation (Matlack *et al*, 1999). In the post-translational system, GTP is not required, consistent with the fact that SRP is not involved in the targeting process. A similar system specialized for post-translational translocation has not yet been characterized in the mammalian ER system and almost all mammalian translocation is believed to proceed via a cotranslational pathway, which is mediated by the heterotrimeric Sec61 complex. On the other hand, small secretory proteins, such as prepromelittin and preprocecropin A, are post-translationally translocated through the rough microsomal membrane (RM) in a cell-free system (Muller and Zimmermann, 1988; Klappa *et al*, 1991). Even the larger chimera proteins of preprocecropin A and dihydrofolate reductase (DHFR) can be translocated across the ER in an ATP-dependent manner (Schlenstedt *et al*, 1994). These observations indicate that post-translational translocation can occur in the mammalian system. On the other hand, the overall process of translocation of relatively short N-domains by SA-I requires GTP, but not ATP (High *et al*, 1991; Kanner *et al*, 2003). To investigate N-domain translocation in detail, we utilized a chimera protein in which mouse DHFR was fused to the N-terminus of the SA-I sequence of SytII. The DHFR domain is useful because its folding state can be controlled by a specific ligand, methotrexate (MTX). The DHFR domain has been used as a passenger protein of import into mitochondria and other organelles (Eilers and Schatz, 1986; Arkowitz *et al*, 1993; Endo *et al*, 1994; Walton *et al*, 1995; Salvador *et al*, 2000). Eilers and Schatz (1986) used a cell-free system to demonstrate that mitochondrial import of the DHFR domain following a matrix targeting signal is blocked by MTX and then resumes after MTX is removed. They demonstrated that mitochondrial import requires unfolding of the polypeptide chain. In the mammalian ER system, signal peptide-mediated post-translational translocation across the rough ER membrane of the DHFR domain following preprocecropin A was suppressed by MTX (Schlenstedt *et al*, 1994).

In this paper, we established an experimental system using the DHFR-SytII fusion proteins, in which translocation of a long N-domain is arrested by MTX and resumes after the

removal of MTX. In this system, targeting and translocation can be analyzed separately. Although GTP is involved in the ER targeting of RNC, neither nucleotide triphosphates (NTPs) nor the luminal hsp70 (BiP) are essential for the translocation. Ribosomes also have a critical function in the subsequent translocation, even after correct targeting to the ER membrane. The ribosomes seem to maintain correct positioning of the nascent chain in the translocon. Furthermore, we suggest that the driving force for the N-domain translocation at the initial step is larger than that for the following continuous translocation.

Results

Translocation of a large N-domain by the SA-I sequence

SytII is type I signal-anchor protein that consists of an N-terminal hydrophilic domain (1–60), a hydrophobic TM segment (61–87), and a cytoplasmic domain (88–422) (Figure 1A). When synthesized in a cell-free system in the presence of RM, the N-domain was glycosylated by more than 70% (Figure 1A, lane 2), as previously reported (Kida *et al*, 2000). To examine the N-domain translocation in detail, mouse DHFR was fused to the N-terminus of SytII (D-S; Figure 1A). The fusion protein included an N-linked glycosylation site near the N-terminus of the DHFR domain in addition to the SytII endogenous site. When D-S was synthesized in the absence of RM, a 70 kDa band was detected (Figure 1B, lane 3, arrowhead). Upon synthesis in the presence of RM, two larger bands were observed (Figure 1B, lane 4). These two bands were shifted down by endoglycosidase H (EndoH) treatment (data not shown), and the lower and upper bands correspond to mono- and diglycosylated forms, respectively. The mutant, in which three potential glycosylation sites in the cytoplasmic domain of SytII were silenced, gave the same glycosylation results, confirming that the glycosylation occurred in the N-domain (data not shown). Thus, the long N-domain was translocated into the luminal space and glycosylated. When a termination codon was introduced after Arg²⁰⁰ (D-S200-ter), glycosylation of the N-domain was decreased (Figure 1B, lane 6). In contrast, translocation of the short N-domain of the original SytII was still greater than 70%, even when the termination codon was created after the Leu¹⁵⁰ (data not shown).

To examine the effects of ribosomes bound to the nascent chain, we used truncated mRNAs encoding up to Arg²⁰⁰ of SytII (D-S200; Figure 1C). The truncated mRNAs possess no in-frame termination codon, so that the nascent chains are retained on the ribosomes as peptidyl-tRNA, even after translation to the 3'-end of the RNA. When translated for 1 h, the glycosylation efficiency of D-S200 was higher than that of D-S200-ter (compare lane 6 in Figure 1B and lane 2 in Figure 1C). After additional incubation for 1 h in the presence of cycloheximide (CHX), which inhibits chain elongation but does not release the nascent chain from the ribosome, glycosylation was further improved and the diglycosylated form became the major form (Figure 1C, lane 3). Glycosylation of the larger bands was confirmed by EndoH treatment (Figure 1C, lane 4). These findings strongly suggested that translocation of the larger N-domain can be improved by prolonged binding to the ribosome.

Figure 1 Translocation of the N-terminal DHFR domain across the ER membrane. (A) Mouse SytII consists of a 60-residue N-terminal hydrophilic domain, a 27-residue H-region (polka-dotted box), and a 335-residue cytoplasmic domain. DHFR was fused to SytII (D-S). Glycosylation sites exist in the DHFR domain and the N-terminal region of SytII (open circles). Truncated mRNAs were translated to make RNC up to Arg²⁰⁰ (D-S200). Numbers in the figures indicate residue numbers of SytII or numbers of residues within the indicated regions. (B) Constructs were translated in a cell-free system in the absence (–) or presence (+) of RM. In D-S200-ter, a stop codon was created after the Arg²⁰⁰. Arrowhead, single open circles, and double open circles indicate unglycosylated, monoglycosylated, and diglycosylated forms, respectively. (C) mRNA truncated at Arg²⁰⁰ was translated for 1 h and further incubated in the presence of CHX for 1 h (CHX chase + lanes). After the reaction, an aliquot was treated with EndoH (+ lanes).

Translocation of the DHFR N-domain can be controlled by MTX

To examine the effect of the folding state on translocation, MTX was included in the cell-free translation system. When truncated mRNA encoding D-S200 was translated in the presence of 100 nM MTX, glycosylation was abolished (Figure 2A, lane 2). On the other hand, a mutated DHFR domain (Dmut), in which three residues were changed (C7S, S42C, and N49C) to destabilize the folding state (Vestweber and Schatz, 1988), was translocated even in the presence of MTX (Figure 2A, lane 4). When the MTX concentration was titrated, there was a clear effective concentration threshold (Figure 2B).

When the D-S and Dmut-S constructs were expressed in COS-7 cells and pulse-labeled, they were glycosylated with similar low efficiency as when synthesized in an *in vitro* system (Figure 2C, lanes 1 and 5). Translocation of the DHFR domain was blocked when MTX was added to the culture medium, whereas translocation of the Dmut domain was not affected (Figure 2C, lanes 3 and 7). Glycosylation of the upper bands was confirmed by EndoH treatment (Figure 2C, lanes 2, 6, and 8). The same glycosylation was observed with a mutant construct, in which three potential glycosylation sites in the cytoplasmic domain were silenced (data not shown). These findings indicated that the N-terminal DHFR domain is folded before translocation, both in the cell-free system and in living cells, to be bound to MTX and then must be unfolded to be translocated.

To remove MTX, membranes were sedimented by ultracentrifugation through a sucrose cushion at 4°C and resuspended (Figure 3A). Upon shifting the temperature to 25°C,

the glycosylated form appeared, and glycosylation reached 70% after 3 h incubation (Figure 3B and C), indicating that the arrested translocation of long N-domain had resumed. At each time point, the translocation was confirmed by proteinase K (ProK) treatment. Initially, only the 23 kDa fragment (Figure 3B, lane 2), which corresponds to the ProK-resistant core of the DHFR domain, was observed. As glycosylated form increased, the larger ProK-resistant fragment of 42 kDa increased. The 42 kDa fragment was degraded in the presence of detergent (data not shown), indicating that the long N-domain is translocated and protected from ProK by the membrane. Thus, N-domain translocation of D-S200 can be controlled by MTX and translocation of the long N-domain can be assessed separately from the ER targeting.

While translocation occurred even after depletion of soluble fractions by centrifugation, some factor(s), such as the nascent chain-associating complex, might remain bound to the RNC. The nascent chain-associating complex is removed from the RNC by washing with 0.5 M potassium acetate (KOAc) (Wiedmann *et al*, 1994). To examine the contribution of such salt-extractable factor(s), the RNC and membrane vesicles were treated under various salt conditions and isolated by centrifugation (Figure 3D), and then translocation was chased by shifting up the temperature. Significant translocation occurred (Figure 3D, lanes 3, 6, and 9), suggesting that salt-extractable factors, which might interact with the ribosome D-S200 nascent chain complex and/or membrane, are not involved in N-domain translocation.

These results also demonstrated that the RNC is associated with the membrane in a high salt-resistant manner. To

Figure 2 Translocation of the DHFR domain is blocked by MTX. **(A)** Translocation of wild-type and mutated DHFR (Dmut) was examined. Dmut possesses three point mutations (C7S, S42C, and N49C). mRNAs truncated at Arg²⁰⁰ were translated in the absence (–) or presence (+) of MTX, and further incubated in the presence of CHX. **(B)** Translation was performed in the presence of indicated concentrations of MTX and the glycosylation efficiencies were quantitated. **(C)** Full-length fusion proteins (D-S and Dmut-S) were expressed in COS-7 cells. Proteins were pulse-labeled and immunoprecipitated with anti-SytII antibody. Aliquots of the immunoprecipitants were treated with EndoH. **(D)** Membrane topology of D-S200 and its mutant. The DHFR domain can be translocated into the ER lumen and glycosylated (open circle) in the absence of MTX, but not in the presence of MTX (M). The Dmut domain is translocated even in the presence of MTX.

address membrane anchoring state, we performed alkaline extraction experiments. Most nascent chain was recovered with the membrane under the high-salt conditions, whereas 54% of the nascent chain was extracted in the soluble fraction under alkaline conditions (0.1 M Na₂CO₃, pH 12.5) (data not shown). The RNC was correctly targeted to the translocon in the salt-resistant state, while it was in the partially hydrophilic environment.

GTP and ATP are not required for DHFR domain translocation

Next we examined the NTP requirement. First, the RNC of D-S200 assembled in the absence of the RM was passed through a spin column to remove small molecules, and then incubated with the RM under various conditions supplemented with NTPs and/or nonhydrolyzable analogs (Figure 4A). Translocation of the N-domain was not detected in the absence of NTPs, even after 3 h incubation (Figure 4A, column 2). GTP, ATP, or UTP, however, mediated the translocation (Figure 4A, columns 3–5). GMPPNP, a nonhydrolyzable analog of GTP, improved translocation weakly but significantly (Figure 4A, column 6), while AMPPNP had a weaker effect (column 7). Moreover, translocation in the presence of 0.5 mM UTP was partially inhibited by excess GMPPNP, to a level similar to that of GMPPNP alone (Figure 4A, column 9). AMPPNP did not have this inhibitory

effect (Figure 4A, column 10). Similar results were obtained with ATP (data not shown). These data indicated that GTP is essential for overall translocation of the N-domain, consistent with a previous report that GTP is required for the N-domain translocation of opsin, while UTP and ATP serve to recycle the remnant GTP after depletion (Kanner *et al*, 2003).

Second, to examine the effect on post-targeting events, a translation mixture of D-S200 obtained in the presence of RM and MTX was passed through a spin column (Figure 4B). MTX was removed from the reaction mixture as other small molecules. Translocation occurred efficiently, even in the absence of NTPs, but the translocation yield was slightly increased by the addition of NTPs (Figure 4B, columns 2–5). Translocation was not affected by AMPPNP (Figure 4B, column 7), indicating that ATP hydrolysis is not involved in driving the translocation. As described in the next section, the AMPPNP was permeable through the RM membrane and effective to BiP. Taken together, no NTP is required for N-domain translocation after the targeting step, whereas NTPs, highly likely GTP, is required only in the targeting.

Luminal BiP is not required for DHFR domain translocation

To examine the contribution of alkali-extractable factors in the lumen, especially BiP, RM vesicles were treated under various alkaline conditions in which soluble and peripheral

proteins both on the luminal and cytoplasmic sides could be extracted. Unexpectedly, 20% of the BiP remained in the membrane fraction even after the alkali extractions (Figure 5A, lanes 7, 9, and 11), whereas 90 and 50 kDa proteins that reacted with anti-KDEL antibody were depleted. Because Hsp70s stably bind substrates in an ADP form but not in an ATP form (Schmid *et al*, 1994; Zhu *et al*, 1996), we attempted preincubation of RM with AMPPNP before alkaline extraction to fix the BiP molecule in an ATP form (see Materials and methods). After incubation with AMPPNP,

alkali extraction depleted more than 95% of the BiP (Figure 5A, lanes 8, 10, and 12). All the membranes showed significant translocation activity for the long N-domain,

Figure 4 NTPs are not required for translocation of the DHFR domain. (A) Effects of NTPs on targeting and translocation. After translation of D-S200 in the absence of RM, the translation mixture was depleted of small molecules using a desalting spin column. The eluate was supplemented with RM. An aliquot was directly subjected to SDS-PAGE (column 1), and other aliquots were incubated for 3 h at 25°C with indicated NTPs of indicated concentrations. The glycosylation efficiencies were quantitated. (B) The requirement for NTPs for translocation of the DHFR domain after the targeting step. Translation was performed in the presence of RM and MTX, and subjected to a desalting column. The eluate was incubated in the presence of indicated NTPs.

Figure 3 Arrested translocation is resumed after removal of MTX. (A) Truncated mRNA (D-S200) was translated in the presence of RM and MTX. Membrane vesicles were precipitated through a sucrose cushion at 4°C and resuspended with PSB containing 1 mM CHX. (B) The membrane vesicles were incubated at 25°C to resume the N-domain translocation. At each indicated time, an aliquot (10 µl) was directly subjected to SDS-PAGE (ProK - lanes). An aliquot was treated with ProK for 30 min on ice (ProK + lanes). Asterisk and double asterisk indicate the ProK-resistant fragment protected by membrane and the ProK-resistant core of DHFR domain, respectively. (C) The results shown in panel B were quantitated. Open circle and square indicate glycosylation (%) and intensity of membrane-protected form, respectively. (D) During membrane isolation, membrane vesicles were extracted with the indicated concentration of KOAc. The resuspended membrane pellet (P) was incubated for 1 h at 25°C (chase + lanes). Other aliquots of precipitates (chase - lanes) and supernatant fractions (S) were directly analyzed by SDS-PAGE.

Figure 5 BiP in the lumen is not required for translocation of the N-terminal DHFR domain. (A) Western blot analysis of alkaline-extracted membranes. RM was incubated in the presence (+) or absence (–) of 2 mM AMPPNP for 20 min at 25°C, and then extracted with the alkaline buffer of indicated pH. The extracted membranes (lanes 7–12), input RM (lane 1), and serially diluted RM as references (lanes 2–6) were subjected to SDS-PAGE and immunoblotting analysis using anti-BiP, anti-KDEL sequence, and anti-Sec61α antibodies. (B) D-S200 mRNA was translated for 1 h in the presence of RM that had been treated under the indicated conditions, and then further incubated for 1 h in the presence of CHX. Where indicated, the translation and subsequent incubations were performed in the presence of MTX (MTX + lanes).

although extraction under the higher pH conditions led to lower efficiency (Figure 5B). Importantly, the efficiencies were almost indistinguishable whether or not there was preincubation with AMPPNP (Figure 5B, compare lanes 3, 7, and 11 with lanes 5, 9, and 13), indicating that translocation is not affected by the residual amount of BiP. Apparent translocation defects should be caused by other factor(s), for example, inactivation of the translocon and/or glycosylation enzyme by alkaline buffer. The DHFR domain was not glycosylated in the presence of MTX (Figure 5B, lanes 4, 6, 8, 10, 12, and 14), demonstrating that glycosylation depended on N-domain translocation and that the glycosylation enzyme was not accessible from the cytoplasmic side even after the alkaline treatment. These data indicate that BiP has little effect on DHFR domain translocation. The effect of preincubation with AMPPNP on BiP depletion confirmed that AMPPNP is membrane permeable.

Translocation intermediates are maintained by ribosomes

To further examine the contribution of ribosomes, translocation of the nascent chain after the targeting was chased in the presence of puromycin, which induces the release of the nascent chain from ribosomes and inhibits protein synthesis. The level of DHFR domain translocation was considerably lower than in the presence of CHX (Figure 6), indicating that the ribosomes have a critical role in translocation, even after the targeting step.

Figure 6 Translocation of the DHFR domain was inhibited by puromycin even after targeting. (A) RM vesicles were obtained as in Figure 3B. Translocation was observed in the presence of CHX or puromycin (Puro) for the indicated time. (B) The results were quantitated.

The effect of ribosomes on the proximity of the nascent chain to the translocon was examined using a site-specific chemical crosslinking procedure. Two or three neighboring hydrophobic residues at each position were substituted with Cys and all other endogenous cysteines except Cys⁷ in the DHFR domain were changed to Ala (Figure 7A, bold-faced characters). After synthesis of the Cys mutants in the presence of RM and MTX, crosslinking with the bifunctional crosslinker bis-maleimido-hexane (BMH) and immunoprecipitation with anti-Sec61α antibodies were performed. Immunoreactive crosslinked bands were observed with all constructs (Figure 7B, lanes 3, 6, 9, 12, 15, 18, 21, and 24); position one had weaker reactivity (lane 3) and the original construct without the Cys residues showed no crosslinking band (data not shown). These findings indicated that the region between the DHFR domain and the H-region is adjacent to Sec61α. Crosslinking at positions 2 and 8 was also observed, even after incubation in the presence of CHX for 30 min (Figure 7C, lanes 3, 6, 15, and 18) and 2 h (data not shown). When puromycin was added, however, crosslinking at both positions was abolished (Figure 7C, lanes 12 and 24); crosslinking at position 8 disappeared immediately after the addition of puromycin (Figure 7C, lane 21), whereas crosslinking at position 2 initially remained (Figure 7C, lane 9) and then disappeared 30 min after puromycin treatment (Figure 7C, lane 12). This indicates that the nascent chain was in the proximity of the translocon as long as it was bound to the ribosome. After release from the ribosome, the nascent chain was released from translocon, even though the N-domain was still on the cytoplasmic side. We thus concluded that ribosomes are involved in maintaining the translocation-intermediate state of the nascent chain and the translocon channel after the targeting process and that the H-region is

Figure 7 Proximity of the translocation intermediate to the translocon. (A) For site-specific chemical crosslinking, two or three hydrophobic residues at the indicated positions were substituted with Cys (bold-faced characters) using the Cys-deleted mutant of D-S200 (D-S200 Δ 7C) in which seven cysteines were changed to alanines (bold-italic-faced characters). Numbers below the residues correspond to the residue numbers of the original SytII. (B) The Cys mutants were synthesized in the presence of RM and MTX, and then subjected to crosslinking using the homo-bifunctional crosslinker BMH (BMH + lanes). Proteins crosslinked with the Sec61 α subunit were immunoprecipitated. Arrow and arrowhead indicate products crosslinked with Sec61 α and a free probe, respectively. (C) The Cys mutants (positions 2 and 8) were translated and further incubated in the presence of CHX or puromycin (Puro) at 25°C. Crosslinking and immunoprecipitation were then performed. (D) The nascent chain is in the proximity of the translocon as long as the ribosome binds the nascent polypeptide chain. It bound to membrane in high-salt-resistant state but was partially alkali-sensitive. The H-region is more readily released from the translocon than the preceding hydrophilic regions.

released from the translocon faster than the former hydrophilic chain (Figure 7D).

Unfolding of the DHFR domain must be coupled with insertion of the H-region

To determine the relationship between the translocation phase and unfolding of the DHFR domain, the N-domain was separated from the SA-I sequence by a sequence from the luminal loop of anion exchanger-1 (Figure 8A, residues labeled X). The sequence has been often used as a spacer between TM segments (e.g. Ota *et al.*, 2000). When the constructs were translated in the absence of MTX and incubated for 1 h, the diglycosylated form was decreased and instead a monoglycosylated form appeared according to the insertion (Figure 8B, lanes 1, 3, 5, 7, and 9). In the presence of MTX, constructs with an insertion (X) of more than 30 residues gave the monoglycosylated form instead of the nonglycosylated form (Figure 8B, lanes 6, 8, and 10). We estimated glycosylation of the a-site and b-site using constructs in which one of these sites was destroyed (Figure 8C), and found that an insertion of 30 residues allowed the second site (b-site) to be glycosylated even though translocation of

the DHFR domain was impaired (Figure 8E). When a spacer of less than 30 residues was used, the monoglycosylated form was only slightly observed in the presence of MTX. These data indicated that with a spacer sequence of more than 30 residues, the H-region acquired a TM topology and the second glycosylation site (b-site) entered the lumen whereas the DHFR domain remained on the cytoplasmic side. Once the H-region formed a TM helix, DHFR domain translocation hardly proceeds even in the absence of MTX. It is likely that folding of the DHFR domain prevents further translocation.

To change the folding property of the DHFR domain, the C-terminal sequence was shortened by 40 or 60 residues ($\Delta 40$ and $\Delta 60$; Figure 8A and D). From the view of 3D structure of human DHFR, the C-terminal 40 residues form C-terminal module and are located on the surface of the molecule and do not firmly interact with internal sequences, while the C-terminal 60 residues include additional 20 residues that directly interact with the N-terminal segment forming parallel β -strand (PDB ID, 1U72). Thus, deletion of the 60 residues should have a more serious effect on the folding state of DHFR domain than that of the 40 residues. As expected, the diglycosylation was greatly improved by $\Delta 60$ (Figure 8D,

Figure 8 Unfolding of the DHFR domain is coupled with insertion of the H-region. (A) The DHFR domain was separated by the insertion of X residues (X) from the H-region and the C-terminal region of DHFR was shortened by 40 ($\Delta 40$) or 60 ($\Delta 60$) residues. Glycosylation sites in the DHFR domain and the following SytII sequence were termed a-site and b-site, respectively. (B) Constructs were translated in the presence of RM for 1 h and further incubated in the presence of CHX for 1 h. For MTX + lanes, MTX was added throughout the incubations. Unglycosylated, diglycosylated, and monoglycosylated forms are indicated by upward, downward, and open arrowheads, respectively. (C) The glycosylation efficiency of the a-site or b-site under the same conditions as for panel B was estimated using different constructs lacking the b-site and a-site, respectively. (D) Effect of partial deletions of the DHFR domain on translocation efficiency. The indicated constructs were translated in the presence of RM and further incubated in the presence of CHX. The glycosylation efficiency of the a-site (%) is indicated. (E) Working model of N-terminal DHFR domain translocation. Nascent chain on the ribosome is targeted to the ER by SRP. The DHFR domain is folded and can bind MTX. After RNC is transferred to the translocon, the H-region enters the translocon. TM helix formation of the H-region triggers unfolding and translocation of the DHFR domain. Release of the nascent chain from ribosome by puromycin affects the translocation. When the DHFR domain is separated from the H-region by more than 30 residues, stroke of the H-region into translocon is no longer coupled with unfolding of the DHFR domain and cannot induce translocation of the DHFR domain.

lanes 5 and 6). The effect of $\Delta 60$ was greater than that of $\Delta 40$. It is thus indicated that the spacer sequence (X) did not block the translocation and that destroying the folding domain of the DHFR can compensate the apparent effect of the spacer. In the presence of the spacer, even the DHFR domain alone is toxic for the N-domain translocation. When the spacer was absent, the DHFR domain was translocated despite the folding property and the translocation was suppressed only by MTX, inducing more stable conformation. These data strongly suggested that once the H-region was inserted as a TM helix, the DHFR domain was hardly unfolded to be translocated through the translocon, even without MTX. When the DHFR domain is near the SA-I sequence, unfolding of the DHFR domain was achieved by coupling with the insertion of the H-region into the translocon.

Discussion

In the present study, we established an experimental system in which translocation of the long N-domain before the signal-anchor sequence can be regulated and can be observed separately from the targeting process. Despite the strict requirement of GTP for targeting to the ER, translocation of the long N-domain does not require either NTPs or luminal hsp70 (BiP). Ribosomes have a crucial role in mediating polypeptide chain movement through the translocon even after the RNC is already targeted to the membrane. Unfolding of the DHFR domain should be coupled with the initial insertion of the hydrophobic segment into the translocon.

Inhibition of translocation of the DHFR domain by the MTX moiety provides clear evidence that the DHFR domain is once folded and then should be unfolded on the cytoplasmic side before being translocated by the following SA-I sequence. The MTX inhibitory effect was also observed in living cells. Various cytoplasmic chaperones are postulated to maintain the translocation-competent conformation before translocation through the mitochondria, peroxisomes, and ER membranes. The DHFR domain was, thus, postulated to be in a somewhat unfolded conformation. The domain, however, is folded into a functional conformation before translocation that can bind the ligand. The fact that the N-terminal DHFR domain on the elongating polypeptide chain achieves an active structure is clear evidence of cotranslational domain folding in eukaryotic cells (Netzer and Hartl, 1997).

Although MTX inhibits N-domain translocation, it does not inhibit the targeting process. The subsequent translocation is efficiently resumed by removing MTX. GTP is required only for ER targeting and no NTPs are involved in the translocation of the long N-domain of DHFR. These observations are consistent with previous reports that the overall process of translocation of the N-terminal domain of opsin requires GTP but not ATP (High *et al*, 1991; Kanner *et al*, 2003). It is highly likely that GTP functions in the SRP-SRP receptor system during targeting. The nonhydrolyzable GTP analog, GMPPNP, also mediated translocation of the N-domain with 40% of the efficiency of GTP. This is consistent with the notion that hydrolysis itself is not required for targeting but for dissociation of the SRP-SRP receptor complex (Connolly *et al*, 1991).

ATP hydrolysis is not involved in driving the translocation of such a long N-domain. Furthermore, translocation occurred in BiP-depleted membranes. In the post-transla-

tional protein translocation mediated by signal peptides in *Saccharomyces cerevisiae*, BiP, Sec63, and ATP have essential roles by retaining the chain on the luminal side (Matlack *et al*, 1999). ATP hydrolysis results in tight binding of BiP to the substrate polypeptide chain. The tight binding of the ADP form of BiP to the polypeptide chain biases Brownian movement of the chain to drive the translocation. BiP function is regulated by a J-domain protein, Sec63, and other factors to exchange ADP with ATP for recycling the BiP. In contrast to the yeast system, the function of Sec63 protein in the cotranslational translocation system remains unclear in the mammalian ER system, although both Sec62 and Sec63 homologous proteins have been identified (Tyedmers *et al*, 2000). In the mammalian ER system, chimeric proteins of preprocecropin A and DHFR are translocated through the RM after protein synthesis in an ATP-dependent manner (Schlenstedt *et al*, 1994) and BiP functions as a ratchet in the lumen for post-translational translocation (Tyedmers *et al*, 2003). In contrast to these previous observations, the long N-domain translocation required no such factors. In the above reports, constructs possessed a signal peptide that is cleaved off by a signal peptidase on the luminal side, while in our case the translocation was mediated by a signal-anchor sequence. Signal-anchor sequences possess longer H-regions than signal peptides, and the longer segments are positioned differently in the translocon pore (Nilsson *et al*, 1994). The uncleavable H-region in TM topology might function as a ratchet for C-to-N translocation. The translocon might drive the C-to-N translocation more easily than the N-to-C translocation.

Translocation of the short (60 residues) N-domain of the original SytII molecule was very efficient. The structural requirement of the H-region for the long N-domain translocation is almost the same as that for the short N-domain (data not shown). It is reasonable to assume that TM-helix formation of the H-region triggers translocation of the N-domain and provides a major driving force for polypeptide chain movement in the initial phase of translocation. This tendency of the TM-helix formation pulls the former polypeptide chain. In the construct (D-S200) where the DHFR domain is located 60 residues from the H-region, the stroke can directly drive the unfolding of the DHFR domain. In this situation, the pulling force should overcome the stabilizing energy of the DHFR domain folding. The initial force can be considered as a 'power stroke' that triggers polypeptide chain movement. In contrast, when spacers of more than 30 residues were inserted between the DHFR domain and the H-region, the DHFR domain translocation was greatly impaired even though the following portion was in the lumen (Figure 8). Once the TM formed, the initial stroke does not couple with unfolding process of the DHFR domain. Our data demonstrated that the driving force in the initial phase is sufficient to unfold the DHFR domain but that in the latter phase is not. The completed TM helix should be a ratchet for the translocated chain, but should no longer provide any power other than Brownian movement. It is unknown whether the stroke of the TM-helix formation alone can explain translocation of such a long N-domain, even after the unfolding of the DHFR domain. It is possible that Brownian movement of the polypeptide chain achieves the overall translocation committed by the TM segment. Alternatively, once TM topology of the signal-anchor sequence is formed, lateral movement of the

TM segment in the lipid environment might drive the translocation. It might also be that local folding of the DHFR domain in the lumen and/or binding of unknown factor(s) within the membrane are involved in driving the movement.

Retaining the nascent chain in the ribosome is essential for efficient translocation of the N-domain. In the case of the D-S200 construct synthesized in the presence of MTX, H-region remains inside the translocon, although the H-region is 113 residues away from the ribosome. The length should be sufficient to allow the H-region to be released from the translocon. Indeed, the translocation intermediate of the original SytII truncated at Thr¹²⁵ is crosslinked with Sec61 α as long as it is retained in the ribosome, while the longer nascent chain truncated at Arg²⁰⁰ is not crosslinked with Sec61 α even when it is bound to the ribosome (Kida *et al*, 2000). Even under these circumstances, the nascent chain of D-S200 was maintained in the translocon as long as it was retained in ribosome. In the SytII signal-anchor, there is a cluster of eight Lys residues, which strongly fix the C-terminus of the H-region on the cytoplasmic side. Thus, both ends of the translocation intermediate are on the cytoplasmic side. Although a vacant ribosome induces a ring structure of the translocon (Hanein *et al*, 1996), ribosomes bound to the nascent chain might have additional allosteric effects on the translocon. A possible communication between the ribosome and translocon was previously proposed by Liao *et al* (1997).

The bulk translocation rate of the long N-domain is considerably slow. There was, however, no significant accumulation of the monoglycosylated intermediate (Figure 3B). Translocation, once started, is relatively fast, so that the two glycosylation sites appeared to be modified simultaneously. The frequency of translocation-competent conformation, which is in an activated state and partially unfolded for translocation, should be low. Association of the nascent chain with the ribosome maintains the translocation intermediate, prolongs the time span of N-domain translocation, and thus improves the yield of translocation of the DHFR domain. Single molecule observation of the DHFR domain translocation would provide information on the frequency and rate of the dynamic event in the translocon.

N-terminal domain sequence with such a low efficiency for translocation should be excluded during evolution. Alternatively, N-terminal signal peptide should be added to secure translocation as seen in rare but significant cases of G-protein-coupled receptor family (Kochl *et al*, 2002). Only a few examples of naturally occurring large N-domain that are translocated by the following SA-I (e.g. neuregulin; Goder and Spiess, 2001) should give little translocation inhibitory effect.

Translocation of the long N-domain is summarized as follows (Figure 8E). RNC is targeted to the ER by the SRP-SRP receptor system, which is dependent on GTP. At this stage, the DHFR domain folds into a functional conformation. The RNC then binds to the translocon in a high-salt-resistant state. The H-region enters the translocon and triggers translocation of the N-domain. The membrane insertion of the H-region is coupled to the unfolding of the DHFR domain. The following chain is translocated through the translocon independent of the luminal BiP and NTPs. Ribosomes have a critical role in maintaining the translocation intermediate to prolong the time span for translocation. When the DHFR domain is separated from the H-region, the pulling force induced by stroke of the H-region does not couple with

unfolding of the DHFR domain. In this situation, the DHFR domain remains on the cytoplasmic side, although the following region is in the lumen.

Materials and methods

Materials

Reagents and materials were obtained essentially as previously described (Kida *et al*, 2000). Antiserum for rat BiP was prepared by immunizing rat BiP that had been purified from rat microsomes by ATP-affinity chromatography.

Constructs

To construct the DHFR and SytII fusion, the DNA fragment encoding mouse DHFR was isolated from the plasmid pSD(69) (Ungermann *et al*, 1994), by polymerase chain reaction (PCR) using the primers 5'-TTCCAAGCTTCCACCATTGTCGAGATCCGGCATCATG-3', which contained a *Hind*III site, a Kozak sequence, and an extension sequence from the plasmid (underlined), and 5'-GGAA GAATTCGTCTTTCTTCTCTAGACTT-3' (*Eco*RI site is underlined), and then digested with *Hind*III and *Eco*RI. The SytII DNA was isolated from pSytII-03 (Kida *et al*, 2000) by PCR using the following primers: 5'-TCCGAATTCAGAAACATCTTCAAGAGGAAC-3' (*Eco*RI site is underlined) and 5'-TAGCTCTAGATCACGTCAGTGTCC-3' (*Xba*I site is underlined), and digested with *Eco*RI and *Xba*I. The two DNA fragments were subcloned between the *Hind*III and *Xba*I sites of pRc/CMV to obtain pD-S. The mutation (I8T) for the N-glycosylation site in the DHFR domain, three-point mutations (C7S, S42C, N49C) for DHFR destabilization, the *Afl*III site following Arg²⁰⁰ of SytII, the mutation for the stop codon following Arg²⁰⁰ of SytII, the mutations for cysteine substitutions, and the mutation for V74R of SytII were generated by site-directed mutagenesis. For constructs with insertions shown in Figure 8, PCR fragments encoding N-terminal 20 and 30 residues, full-length, and tandem repeats of the sequence from Thr⁶²⁷ to Trp⁶⁶² of human anion exchanger 1 (TYTQKLSVPDGFVNSARGWVIHPLGLRSEFPW; underlined Ala residue was altered from the original Ser to disrupt the N-glycosylation site) were inserted into the *Eco*RI site between the DHFR and SytII domains (Figure 8). Deletions of the C-terminus of DHFR were performed using PCR. All the mutants were confirmed by DNA sequencing. The sequences of the oligonucleotides used and the construction details for each plasmid are available from the authors.

In vitro transcription, translation, and enzyme treatment

For truncated mRNA, plasmids were linearized by *Afl*III. For mRNA with a termination codon, plasmids were linearized by *Xho*I. The templates were transcribed with T7 RNA polymerase as previously described (Sakaguchi *et al*, 1992a). The obtained mRNAs were translated in the reticulocyte lysate cell-free system for 1 h at 25°C. The translation reaction contained 100 mM KOAc, 1.0 mM magnesium acetate (Mg(OAc)₂), 32% reticulocyte lysate, 15.5 kBq/ml EXPRESS protein-labeling mix (Perkin-Elmer), and 100 nM MTX (Sigma) as indicated in the figures. After translation was terminated by 2 mM CHX, the mixture was incubated further for 1 h to allow for completion of the translocation and glycosylation. Aliquots of the translation mixture were treated with EndoH at 37°C for 1 h under denaturing conditions. Radiolabeled proteins were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and visualized on image analyzer (Fuji). All the quantitation experiments were carried out at least two times and the results were reproducible.

Washing membranes and translocation resumption

Translation reactions containing RM and MTX (100 μ l aliquots) were diluted with 400 μ l of physiologic salt buffer (PSB; 150 mM KOAc, 2.5 mM Mg(OAc)₂, 30 mM HEPES-KOH (pH 7.4), and 1 mM DTT). The mixture was then layered onto a 500 μ l of 0.5 M sucrose in PSB. Where indicated, the concentration of KOAc in the dilution buffer and cushion were changed. After centrifugation for 10 min at 100 000 g (Hitachi S120AT2 rotor), the membrane pellet was resuspended in 100 μ l PSB containing 1 mM CHX, and incubated at 25°C to resume translocation. Alternatively, 2 mM puromycin and 0.5 mM GTP, instead of CHX, were added to release nascent chain from ribosome. Where indicated, another 10 μ l aliquot was treated with 0.2 mg/ml ProK for 30 min on ice.

Desalting the translation mixture

After translation, the reaction mixtures were passed through a gel filtration spin column. A Centriscip spin column (Applied Biosystems) was reconstituted with PSB and centrifuged for 2 min at 2500 r.p.m. The column was equilibrated twice with 100 μ l PSB. Translation mixtures were applied onto the columns and centrifuged under the same conditions. The eluted fractions were supplied with various additives and further incubated for 3 h at 25°C.

Alkaline extraction of RM

RM (100 equivalents) was precipitated and resuspended with 100 μ l PSB without DTT. The RM suspension (100 μ l) was incubated for 20 min at 25°C either in the absence (–) or presence (+) of 2 mM AMPPNP. A 200 μ l portion of alkaline buffer containing 500 mM HEPES and 500 mM CAPS (adjusted to the indicated pH) was added and diluted with 1700 μ l water. After incubation for 20 min on ice, mixtures were layered onto a 0.5 M sucrose cushion (50 mM HEPES pH 7.5, 40 mM KOAc pH 7.5, and 5 mM $MgCl_2$), and centrifuged for 20 min at 135 000 g (S100AT4 rotor, Hitachi). The membrane pellet was resuspended in 100 μ l storage buffer (50 mM HEPES–KOH pH 7.4, 200 mM sucrose, and 1 mM DTT). A 10 μ l aliquot was subjected to SDS–PAGE and subsequent immunoblotting analysis using antibodies against BiP, the KDEL (StressGen), and Sec61 α . Protein bands were visualized by ECL reagent and chemiluminescent image (LAS1000mini, Fuji film).

Chemical crosslinking and immunoprecipitation

After Cys-substituted mutants were synthesized in the presence of RM and MTX, translation mixtures were transferred to ice and CHX was added. Other aliquots were further incubated at 25°C for 30 min

in the presence of 2 mM CHX or puromycin. The mixtures were treated with 5 mM BMH (Pierce Chemical) on ice for 1 h and then quenched by 20 mM 2-mercaptoethanol on ice for 20 min. The reaction mixtures were treated with 1% SDS for 5 min at 95°C and then diluted with a 10-fold volume of immunoprecipitation buffer (1% Triton X-100, 50 mM Tris–HCl pH 7.5, 150 mM NaCl, and 1% Trasyol). After insoluble materials were removed by centrifugation, the solutions were incubated for 30 min with protein A-Sepharose (Amersham) alone, and the unbound fraction was incubated with anti-Sec61 α antiserum and protein A-Sepharose at 4°C for 3 h. The resin was washed once with 1 ml of immunoprecipitation buffer and then extracted with sample buffer for SDS–PAGE.

Expression in COS-7 cells

The expression plasmids were transfected into COS-7 cells with FuGene 6 (Roche Chemical). After culturing for 24 h, the cells were pulse-labeled with EXPRESS protein labeling mix for 30 min and then lysed with 2% SDS. Where indicated, 100 μ M MTX was added to culture medium at 8 h before pulse-labeling. The labeling was performed in the presence of MTX. D-S molecules were immunoprecipitated with anti-Sytl1 antibody as previously described (Anderson and Blobel, 1983). Aliquots of the immunisolated proteins were treated with EndoH.

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