# Two translocating hydrophilic segments of a nascent chain span the ER membrane during multispanning protein topogenesis

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During protein integration into the endoplasmic reticulum, the N-terminal domain preceding the type I signal-anchor sequence is translocated through a translocon. By fusing a streptavidin-binding peptide tag to the N terminus, we created integration intermediates of multispanning membrane proteins. In a cell-free system, N-terminal domain (N-domain) translocation was arrested by streptavidin and resumed by biotin. Even when N-domain translocation was arrested, the second hydrophobic segment mediated translocation of the downstream hydrophilic segment. In one of the defined intermediates, two hydrophilic segments and two hydrophobic segments formed a transmembrane disposition in a productive state. Both of the translocating hydrophilic segments were crosslinked with a translocon subunit, Sec61α. We conclude that two translocating hydrophilic segment in a single membrane protein can span the membrane during multispanning topogenesis flanking the translocon. Furthermore, even after six successive hydrophobic segments entered the translocon, N-domain translocation could be induced to restart from an arrested state. These observations indicate the remarkably flexible nature of the translocon.

## Introduction

Many membrane proteins are integrated into the endoplasmic reticulum (ER) membrane of eukaryotic cells and the plasma membrane of prokaryotic cells via an evolutionarily conserved machinery, the so-called translocon (Walter and Lingappa, 1986; Hartmann et al., 1994). The functional translocon of eukaryotes is composed of multiple copies of the Sec61 complex, which corresponds to the SecY complex of bacteria (Osborne et al., 2005). The complex consists of three heterologous subunits: Sec61α, β, and γ in mammals; SecY, E, and G in *Escherichia coli*; and Sec61p, Sbh1p, and Sss1p in budding yeast. In addition to the core complex, there are several accessory factors. Translocating chain-associating membrane protein (TRAM) is responsible for stabilizing the integration intermediate of less hydrophobic transmembrane (TM) segments at the lateral exit site of the translocon (Do et al., 1996; Heinrich et al., 2000). The translocon-associated protein complex associates with the Sec61 complex (Morgan et al., 2002; Menetret et al., 2005) and is suggested to be involved in substrate-specific translocon function (Fons et al., 2003).

Polypeptide segments on the trans-side are translocated through the membrane, and TM segments are inserted into the membrane via the translocon (Alder and Johnson, 2004; Osborne et al., 2005). Polypeptide chains traverse the hydrophilic environment of the translocon (Gilmore and Blobel, 1985). In mammalian ER, the size of the water environment is estimated to be between 9 and 60 Å (Alder and Johnson, 2004). Four copies of the Sec61 complex associate to form a multimeric complex, which, in electron microscopy, is observed as a ring structure (Menetret et al., 2005). The multimeric form is induced by the ribosome, and the center of the ring was thought to be a pore for polypeptide chains (Hanein et al., 1996). A significant portion of Sec61 complexes exists as monomers on rough ER membrane (Schaletzky and Rapoport, 2006). In bacterial membranes, a dimeric form of the SecY complex is often observed (Manting et al., 2000; Mori et al., 2003). Recent cryo-electron microscopy observations of the SecY complex engaged with the

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Abbreviations used in this paper: BMB, 1,4-bismaleimidobutane; BMH, 1,6-bismaleimidohexane; BMOE, bismaleimidoethane; $BM(PEO)_2$, N,N-(methylene-4-1-phenylene)bismaleimide; CHX, cycloheximide; EndoH, endoglycosidase H; H-segment, hydrophobic segment; N-domain, N-terminal domain; Puro, puromycin; RM, rough microsomal membrane; SA-I, type I signal-anchor sequence; SAv, streptavidin; SytII, synaptotagmin II; TM, transmembrane; TRAM, translocating chain-associating membrane protein.

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translating ribosome indicated that SecY forms a front-to-front dimer (Mitra et al., 2005), although the previously reported dimer organization of the bacterial SecY complex is back-to-back (Manting et al., 2000; Breyton et al., 2002; Mori et al., 2003). The crystal structure of the archaean SecY complex indicates that a single SecY subunit possesses 10 TM segments and can form a pore for polypeptide chain translocation (Van den Berg et al., 2004). SecY is pseudo-symmetrical and the TM1-5 and TM6-10 halves are connected by a loop between TM5 and TM6 to form a clamshell-like structure. The mouth of the clamshell between TM2b and TM7 is thought to open laterally to the lipid environment. The central portion of the pore narrows, creating an hourglass shape, and possesses a pore ring structure and plug, which are responsible for maintaining the membrane seal (Li et al., 2007; Saparov et al., 2007). The signal acceptor site is thought to be formed by TM2b and TM7 near the exit site of the Sec61 complex. Cross-link experiments indicate that translocating polypeptide chains pass through the center of the SecY molecule (Cannon et al., 2005). Both the signal sequence and translocating polypeptide chain are contained within the same SecY molecule during SecA-dependent translocation (Osborne and Rapoport, 2007). Only one SecY subunit in the SecY dimer mediates translocation. SecY is demonstrated to be involved in integration and folding of multispanning membrane proteins (Shimohata et al., 2007). The dynamic nature of the SecY/61 channels among the multimeric complexes during integration of the multispanning membrane protein has yet to be clarified.

Hydrophobic segments (H-segments) of signal sequences emerging from the ribosome are recognized by the signal recognition particle and the ribosome-nascent chain complex is targeted to the translocon. The signal recognition particle receptor on the ER membrane induces transfer of the H-segment from the particle to the translocon. Either side of the H-segment can then be translocated through the translocon (Sakaguchi, 1997; Goder and Spiess, 2001). When the N-terminal flanking region possesses fewer positive charges and the H-segment is relatively longer, the H-segment tends to form an $N_{lum}/C_{cyt}$ orientation, in which the N-terminal domain (N-domain) is in the lumen and the C terminus is in the cytoplasm (type I signal-anchor, SA-I). In contrast, when the N-terminal flanking segment possesses more positive charges and the C-terminal flanking segment has fewer positive charges, the H-segment forms the opposite orientation ($N_{cyt}/C_{lum}$; type II signal-anchor) (Sakaguchi et al., 1992b). The Sec61 complex is essential for partitioning the TM segment of SA-I into the lipid environment (Heinrich et al., 2000). The translocon mediates the insertion of multiple TM segments; hydrophobic TM segments are sequentially inserted from the N terminus to the C terminus into the translocon and released to the lipid phase (Sadlish et al., 2005). The translocon can support the insertion of less hydrophobic segments into the membrane depending on their context; weakly hydrophobic TM segments can be integrated into the lipid environment by interacting with other TM segments (Ota et al., 2000; Heinrich and Rapoport, 2003), and even a hydrophilic TM segment can be forced to form a TM disposition by its downstream SA-I sequence (Ota et al., 1998).

In the SA-I sequence, ER-targeting and N-domain translocation proceed immediately after the H-segment emerges from the ribosome (Heinrich et al., 2000; Kida et al., 2000). Positive charges after the H-segment have a critical role in N-domain translocation (Kida et al., 2006). A long N-domain of more than 200 residues, including a fused dihydrofolate reductase domain, can be translocated through the translocon (Kida et al., 2005). Stabilization of the domain by a dihydrofolate reductase-specific ligand (methotrexate) arrests translocation and the release of the ligand induces the resumption of translocation (Kida et al., 2005). Translocation of the long N-domain does not require nucleotide triphosphate, whereas the ER-targeting process has a strict requirement for GTP. The ER luminal hsp70, BiP, is also not required. The ribosome has a critical function in N-domain translocation, even after membrane targeting. Methotrexate can inhibit translocation of the dihydrofolate reductase domain even in cultured cells (Ikeda et al., 2005).

In this paper, we report the development of a new cell-free system in which N-domain translocation can be arrested and then induced to resume. A streptavidin-binding peptide tag (SBP-tag) (Keefe et al., 2001) was fused to the N-domain. N-domain translocation was arrested by streptavidin (SAv) and resumed after biotin-induced release of SAv from the SBP-tag. We show that the arrest of N-domain translocation does not influence subsequent insertions of polypeptide chains and that two translocating hydrophilic polypeptide segments in a single molecule can span the membrane in the translocation-competent state. Furthermore, the N-domain translocation intermediate can be maintained in a productive state, even after multiple downstream hydrophobic TM segments enter the translocon pore.

## Results

### Translocation of the SBP-tagged N-domain is arrested by SAv

To arrest N-domain translocation, a 38-residue SBP-tag was fused to the N terminus of mouse synaptotagmin II (SytII) (Fig. 1 A, S-I). The model protein construct consisted of a 38-residue N-terminal SBP-tag, a 70-residue hydrophilic sequence (7-residue glycosylation probe sequence, 4 residues encoded by restriction enzyme sites, and a 59-residue SytII N-terminal domain; for details see Materials and methods), the 27-residue H-segment of the SA-I sequence, and the cytoplasmic domain of SytII (Fig. 1 A). The two glycosylation sites in the N-terminal hydrophilic domain were used as an indicator of translocation because glycosylation occurs only in the ER lumen. To produce defined translocation intermediates, we used truncated mRNAs for cell-free synthesis. Because they did not possess an in-frame termination codon, the synthesized nascent chain remained on the ribosome as a peptidyl tRNA to form the ribosome-nascent chain complex. When mRNA, truncated at $Arg^{200}$ of SytII, was translated for 60 min, the S-I protein product was efficiently diglycosylated in the presence of rough microsomal membrane (RM) (Fig. 1 B, lane 2). There was little of the monoglycosylated form. Endoglycosidase H (EndoH) treatment caused a downward shift of the top band, confirming that the top band is glycosylated (lane 3). When translated in the presence of

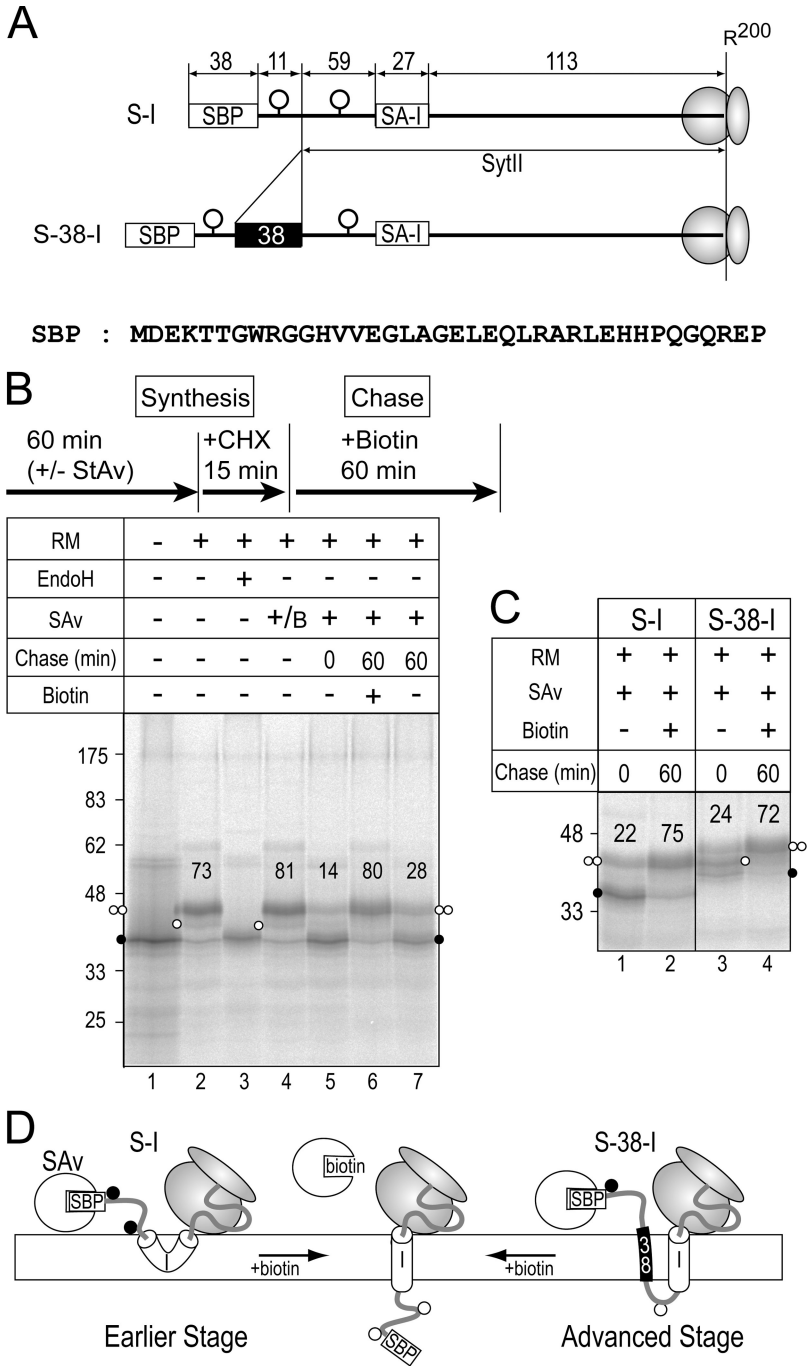


Figure 1. **Two intermediates of N-domain translocation generated by SBP-tag trapping.** (A) The SBP-tag (SBP) and the glycosylation sequence were fused to the N terminus of SytII (S-I). A 38-residue spacer sequence was inserted between the glycosylation probe sequence and SytII (S-38-I). Glycosylation sites are indicated by open circles. Truncated mRNAs encoding to $Arg^{200}$ of SytII in the fusion proteins were used for in vitro translation. Numbers indicate the amino acid residues within the indicated regions. The amino acid sequence of the SBP-tag is indicated. (B) The truncated mRNA for the S-I fusion protein was translated in the cell-free system in the absence (−) or presence (+) of RM, SAv, and biotin for 60 min, and then further incubated for 15 min in the presence of CHX. Some aliquots translated in the presence of SAv were further incubated in the presence (+) or absence (−) of biotin for 60 min. Lane 4 shows the translation products in the presence of SAv and biotin (SAv +/B). An aliquot was treated with EndoH (EndoH + lane). Filled circles indicate the nonglycosylated form. Single and double open circles indicate monoglycosylated and diglycosylated forms, respectively. Diglycosylation efficiencies (%) are indicated. (C) The fusion proteins were synthesized in the presence of RM and SAv. After CHX treatment, N-domain translocation was chased for the indicated time in the presence of biotin. Diglycosylation efficiencies (%) are indicated in the panel. (D) N-domain translocations of S-I and S-38-I proteins were arrested at different stages by SAv. Neither glycosylation site of S-I was glycosylated, whereas the second site of S-38-I was glycosylated. In both cases, N-domain translocation resumed during the biotin chase. In the S-38-I intermediate, the SA-I sequence and the preceding hydrophilic segment spanned the membrane (advanced stage). Insertion of the S-I protein was arrested at an earlier stage than S-38-I protein (earlier stage). Open and filled circles indicate the glycosylated and nonglycosylated potential sites, respectively.

SAv, glycosylation was suppressed (lane 5). In contrast, SAv did not affect glycosylation in the presence of biotin (lane 4), indicating that N-domain translocation was arrested by a specific interaction of SBP-tag with SAv. To examine whether the arrested translocation can be resumed, biotin was added after translation was performed in the presence of SAv and terminated with cycloheximide (CHX). Upon addition of biotin, glycosylation efficiently resumed (lane 6). In the absence of biotin, little glycosylation occurred, even after a 60-min chase (lane 7). These results indicate that N-domain translocation via the SA-I sequence was arrested by SAv and could be chased by biotin.

To insert the polypeptide chain further into the translocon, a 38-residue sequence was inserted as a spacer between the SBP-tag and SA-I sequence (Fig. 1 A, S-38-I). This 38-residue sequence was derived from the lumenal loop of human anion exchanger 1, which is often used as a passenger sequence for translocation experiments (Sato et al., 2002; Kida et al., 2005). When translated in the presence of RM and SAv, the monoglycosylated form was predominantly observed (Fig. 1 C, lane 3). When the reaction was chased in the presence of biotin, both the nonglycosylated and monoglycosylated forms were converted to the diglycosylated form (lane 4). When the former glycosylation site was silenced, the monoglycosylated intermediate form was not

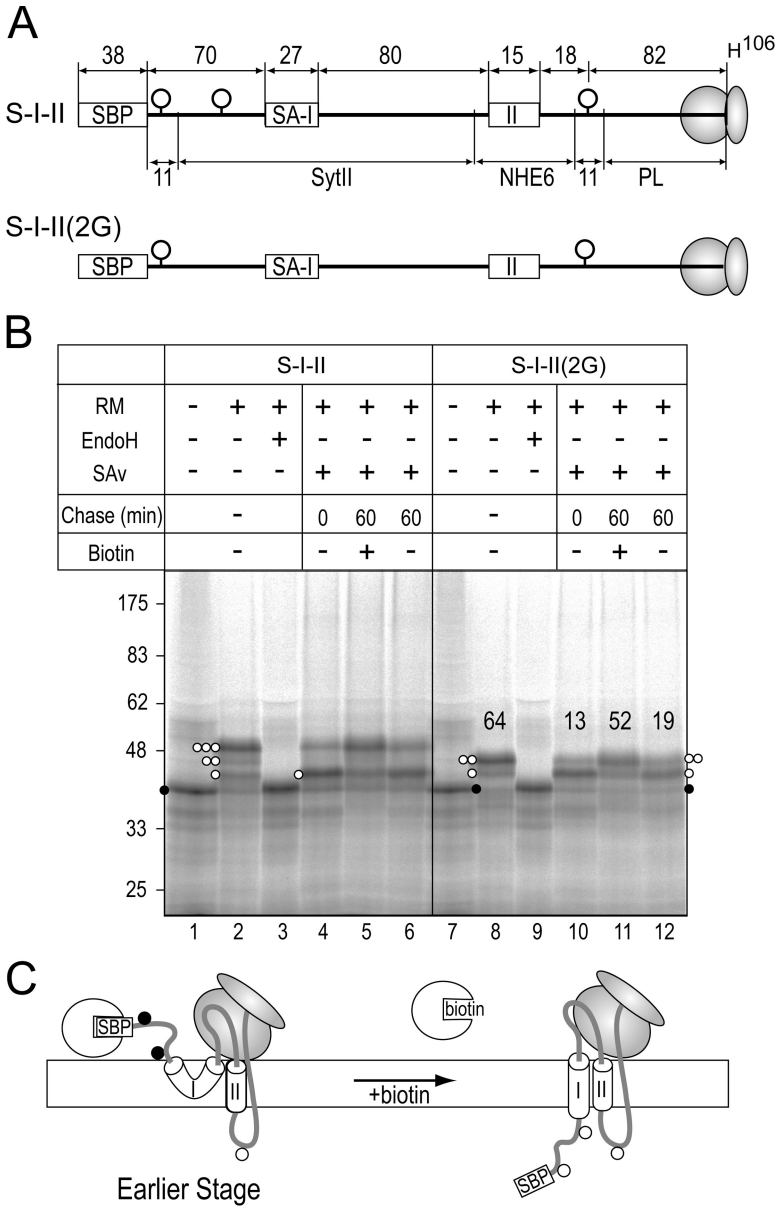


Figure 2. **Earlier intermediate does not influence the next insertion.** (A) The SA-I sequence in the S-I protein was followed by the second H-segment (II) of the TM3 in human $Na^+/H^+$ exchanger isoform 6, the glycosylation probe sequence (third open circle), and a hydrophilic sequence from bovine prolactin (PL) (S-I-II). The numbers of residues within the indicated regions are shown. In the S-I-II(2G) protein, the second glycosylation site was silenced. (B) The truncated RNA was translated and N-domain translocation was chased as described in Fig. 1. Filled circles indicate nonglycosylated forms. Single, double, and triple open circles indicate mono-, di-, and triglycosylated forms, respectively. The diglycosylation efficiencies of S-I-II(2G) are indicated in the panel. (C) While the N-domain translocation was in the earlier stage, the second H-segment (II) and the following hydrophilic sequence spanned the membrane. The N-domain translocation was chased despite the presence of the downstream translocating polypeptide chain.

affected (unpublished data), indicating that the first glycosylation site of the intermediate was still on the cytoplasmic side and the latter glycosylation site was in the lumen (Fig. 1 D). The 38-residue spacer resulted in the hydrophilic sequence forming a TM disposition (Fig. 1 D). Collectively, these findings indicate that we created two different intermediates of N-domain translocation: in one intermediate, the SA-I sequence and hydrophilic segment spanned the membrane (termed "advanced stage"); whereas in the other intermediate, the SA-I sequence was at an earlier stage of translocation (termed "earlier stage") (Fig. 1 D).

### N-domain translocation resumes even in the presence of a second translocating segment

We then examined whether insertion of a downstream polypeptide chain is influenced by the N-domain translocation intermediates. The TM3 segment of human $Na^+/H^+$ exchanger isoform 6, which mediates membrane insertion of its following portion (Miyazaki et al., 2001), was positioned as the second insertion sequence (Fig. 2 A, II). A third glycosylation site and a prolactin sequence were fused as a reporter. When the S-I-II protein was synthesized in the presence of RM, the triglycosylated form was mainly observed (Fig. 2 B, lane 2). EndoH treatment confirmed glycosylation of the top bands (lane 3). When synthesized in the presence of SAv, triglycosylation was suppressed and the monoglycosylated form was observed as the main product (lane 4). When chased in the presence of biotin, the triglycosylated form became the major product (lane 5). In the absence of biotin, 60-min incubation induced little glycosylation (lane 6).

To confirm the identity of the glycosylated sites, the second glycosylation site was silenced by a single point mutation (Fig. 2 A, S-I-II(2G)). The diglycosylated form was observed as the major product instead of the triglycosylated form (lanes 8 and 11), whereas the monoglycosylated form observed in the presence of

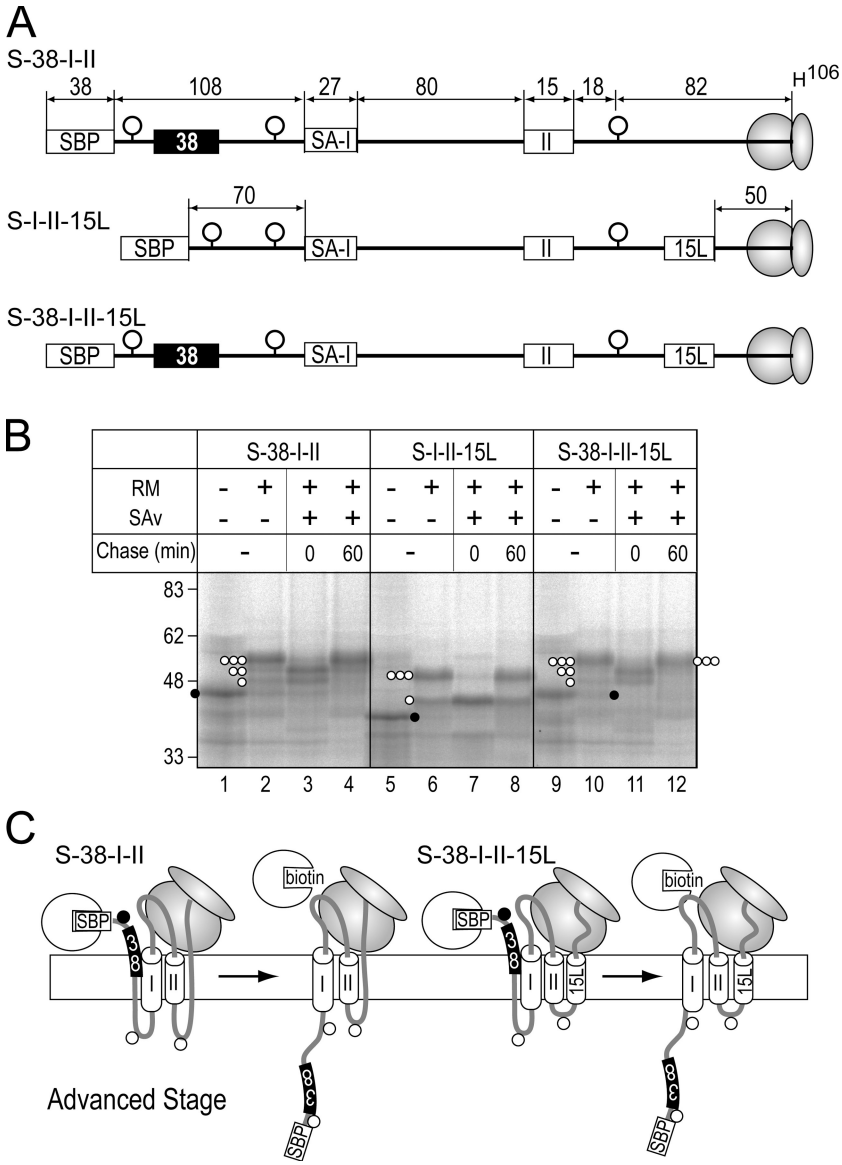


Figure 3. **Two translocating hydrophilic segments span the membrane.** (A) The SBP-tag of S-I-II was separated from SA-I by a 38-residue spacer (S-38-I-II). As a third H-segment, 15 residues in the indicated region were replaced with 15 leucine residues (S-I-II-15L). Because the 15-leucine segment is 50 residues away from the truncation site, it should be in the translocon. (B) Translation and the translocation chase were performed as described in Figs. 1 and 2. (C) In the presence of SAv, two hydrophilic segments of S-38-I-II protein spanned the membrane and the two glycosylation sites, other than the N-terminal site, were glycosylated (open circles). Insertion of the hydrophobic 15-leucine segment did not interfere with the resumption of N-domain translocation.

SAv was not affected (lane 10). Only the third glycosylation site was thus glycosylated in the presence of SAv (Fig. 2 C).

Collectively, when N-domain translocation was arrested by SAv, the third glycosylation site after the second H-segment was translocated into the lumenal side of the RM. In this situation, the SA-I sequence should occupy the signal recognition site of the Sec61 complex, while the second H-segment could be inserted and mediate the following translocation. It should be noted that the N-domain polypeptide chain could move across the membrane, even when the second translocating hydrophilic segment spanned the membrane.

### Two translocating segments can span the membrane

To form the advanced intermediate stage of the S-I-II protein, the 38-residue spacer sequence was inserted between the SBP-tag and SA-I sequences (Fig. 3 A, S-38-I-II). When synthesized in the absence of SAv, the triglycosylated form was observed depending on the RM (Fig. 3 B, lane 2). When translated in the presence of SAv, the diglycosylated form was mainly observed (lane 3), indicating that the second and third positions of the main product were in the lumen and only the N-terminal position was on the cytoplasmic side (Fig. 3 C). The glycosylation of the N-terminal site was chased by the post-translational addition of biotin (Fig. 3 B, lane 4). This finding demonstrates that the N-domain of the S-38-I-II protein was in the advanced intermediate stage (Fig. 3 C). As shown in the next section, the C-terminus of this intermediate was in the ribosome. The two hydrophilic segments were simultaneously in the TM disposition (Fig. 3 C). The former translocating segment did not interfere with the insertion of the second polypeptide chain and the second segment did not affect the resumption of N-domain translocation.

To examine the effect of an H-segment in the C-terminal chain on the translocation intermediate stage, 15 amino acid residues in the second translocating segment were exchanged with 15 leucine residues (Fig. 3 A, 15L constructs). The 15-leucine sequence was followed by 50 residues, so that the segment should exit the ribosome and enter the translocon. Even in the presence

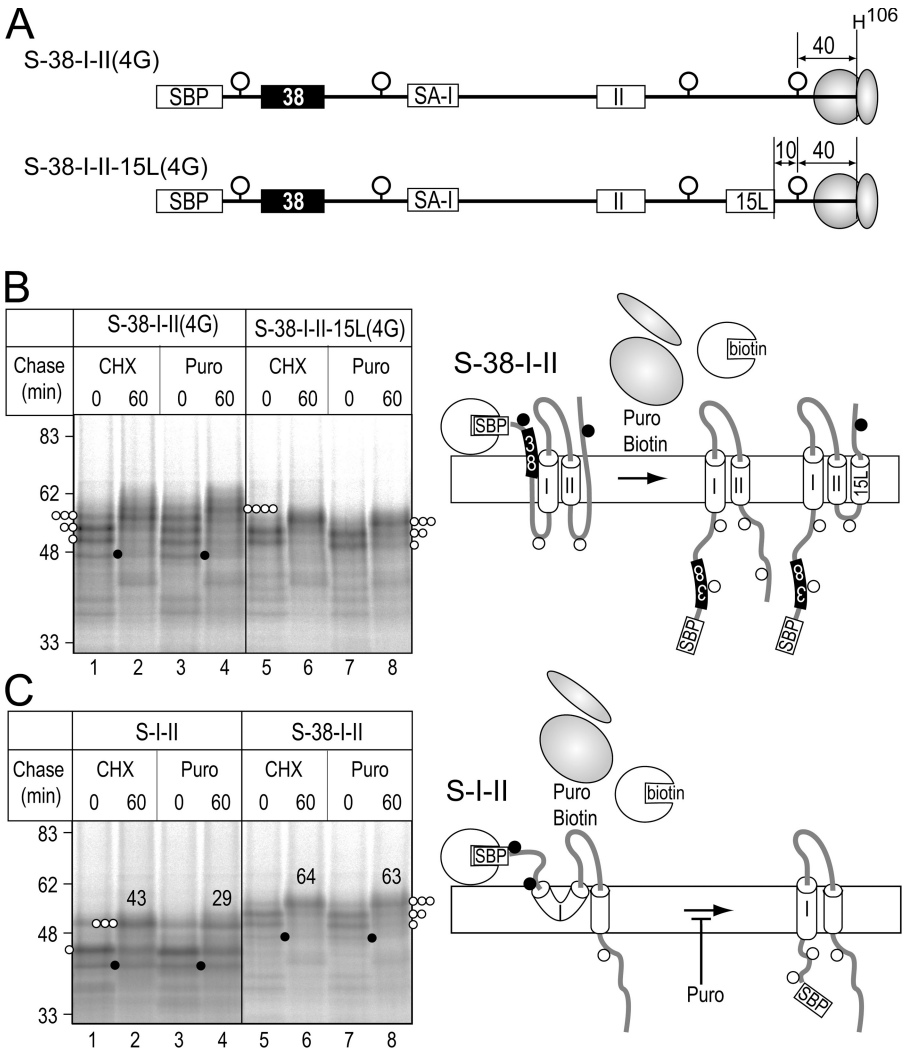


Figure 4. **Productivity of the translocation intermediates.** (A) The fourth glycosylation site was generated 40 residues away from the C-terminal truncation site of (4G) constructs. (B) After translation was performed for 60 min, the reaction was terminated by CHX or Puro and further incubated for 10 min. A biotin chase was then performed in the presence of CHX or Puro for 60 min. Translocations of both the N-domain and C-terminal segments resumed after release from the ribosome. The tetraglycosylated form was observed (four open circles) after Puro treatment. The third H-segment (15L) interrupts the translocation of the C-terminal segment, so that the fourth site is not accessible to the glycosylation enzyme. (C) S-I-II and S-38-I-II proteins were synthesized in the presence of SAv, and the N-domain translocation was chased in the presence of CHX or Puro. Triglycosylation efficiency (%) after the chase reaction is indicated. The translocation chase of the N-domain of the S-I-II protein was influenced by Puro treatment, whereas that of the S-38-I-II protein was not.

of the 15-leucine segment, essentially the same results were obtained as with S-I-II and S-38-I-II (Fig. 3 B, lanes 5–12). When synthesized in the absence of SAv, the triglycosylated form was mainly observed (lanes 6 and 10). In the presence of SAv, N-domain translocation was suppressed and the monoglycosylated form of S-I-II-15L (lane 7) and the diglycosylated form of S-38-I-II-15L (lane 11) were the main products. Triglycosylation was observed after a 60-min chase in the presence of biotin (lanes 8 and 12), indicating that N-domain translocation was chased in both cases. The third H-segment (15L) did not the affect resumption of N-domain translocation (Fig. 3 C).

### Productivity of translocation intermediates

To ascertain the fate of the C-terminal segment, a fourth glycosylation site was created 40 residues from the truncation site (Fig. 4 A). This glycosylation site should be accessible only when the C-terminal segment is released from the ribosome and translocated through the membrane. In the presence of CHX, the diglycosylated form was converted to the triglycosylated form by biotin (Fig. 4 B, lane 2). In contrast, when treated with puromycin (Puro), there was a significant increase in the triglycosylated form before the biotin chase (Fig. 4 B, compare lanes 1 and 3). After a 60-min biotin chase, the intermediate forms were converted to the tetraglycosylated form (lane 4). The fourth glycosylation site became accessible to the glycosylation enzyme in the presence of Puro (Fig. 4 B). When the 15-leucine segment was present, tetraglycosylation was suppressed (Fig. 4 B, compare lanes 4 and 8), indicating that the 15-leucine segment stopped translocation of the C-terminal chain. The C terminus of the S-38-I-II protein was trapped in the ribosome and C-terminal translocation can be chased as efficiently as N-terminal translocation. The resumption of N-domain translocation was also not affected by C-terminal release from the ribosome.

In contrast to the results with the advanced intermediate of the S-38-I-II protein, the resumption of N-domain translocation in an earlier stage of the S-I-II protein was significantly affected by Puro treatment (Fig. 4 C, compare lanes 2 and 4). Even in the earlier intermediate stage, the SA-I and N-terminal domain remain actively engaged with the translocon, depending on the ribosome, during the translocation arrest. The differential influence of Puro indicates that SA-I sequences in the earlier and advanced stages are in different states.

Using the S-38-I-II(4G) model protein, we confirmed membrane topology of the two TM segments. The loop between

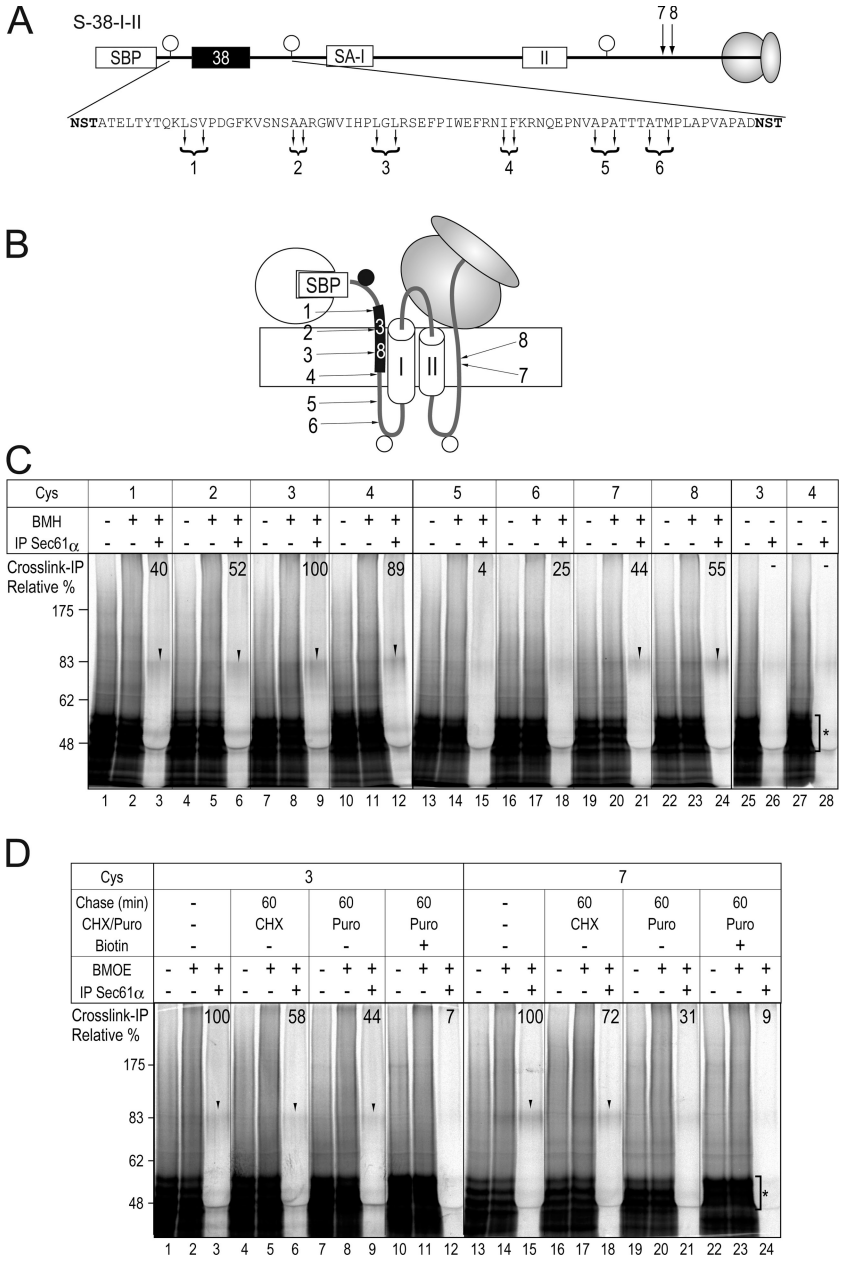


Figure 5. **Two translocating polypeptides flank Sec61α.** (A and B) For site-specific chemical cross-linking, two Cys residues were created by point mutations at the indicated positions (numbered) using a Cys-less mutant of S-38-I-II. (C) The Cys mutants were synthesized in the presence of RM and SAv, and then subjected to chemical cross-linking using homobifunctional cross-linkers, BMH (+ lanes), whose cross-linking distances are 16.1 Å. Proteins cross-linked with the Sec61α were immunoprecipitated (IP Sec61α + lanes). Downward arrowheads indicate the products cross-linked with Sec61α. Asterisk indicates free probe products. Relative cross-linking efficiency was calculated by the formula: (immunoprecipitated band) × 100/(translation product). The efficiencies were normalized with that of position 3 and indicated in the panel. (D) Effects of Puro treatment and biotin chase on the cross-linking reactions were examined with positions-3 and -7 Cys mutants. After CHX, Puro, and/or biotin treatment, a cross-linking reaction was performed with BMOE. Immunoprecipitated bands were quantitated and normalized values were indicated in the panel.

two TM segments is on the cytoplasmic side and accessible to the externally added proteinase K (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200707050/DC1).

### Each translocating hydrophilic segment flanks Sec61α

To probe proteins adjacent to the translocating polypeptides, we performed chemical cross-linking experiments. Two cysteine residues were created in either the first or second translocating hydrophilic segment using a Cys-less mutant (see Materials and methods) (Fig. 5, A and B). The Cys mutants were synthesized in the presence of RM and SAv, and the cross-linking reaction was performed with a homobifunctional cross-linker BMH, whose spacer is 16.1 Å. Cross-linked products were subjected to immunoprecipitation with anti-Sec61α antibody (Fig. 5 C). A Cys-residue at positions-1, -2, -3, -4, -7, or -8 gave a significant cross-linked band of 90 kD that was immunoreactive with anti-Sec61α antibody. Given that the probe is ∼50 kD (Fig. 5 C, asterisk), the size of the cross-linking partner is consistent with that of Sec61α. The negligible and weak cross-linking of Cys-residues at positions-5 and -6 indicate the position specificity of the cross-linking reaction. The immunoreactive cross-linked products were not observed when incubated in the absence of a cross-linker (Fig. 5 C, lanes 26 and 28). Essentially the same results were obtained using other cross-linkers with shorter spacers, bismaleimidoethane (BMOE; 8.0 Å), 1,4-bismaleimidobutane (BMB; 10.9 Å), and N,N-(methylene-4-1-phenylene)bismaleimide [$BM(PEO)_2$; 14.7 Å] (Fig. S2). The immunoreactive band of the position-4 Cys mutant was not observed with unrelated antibodies, anti-Sec63 and anti-SAv (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200707050/DC1). These results indicate that both translocating

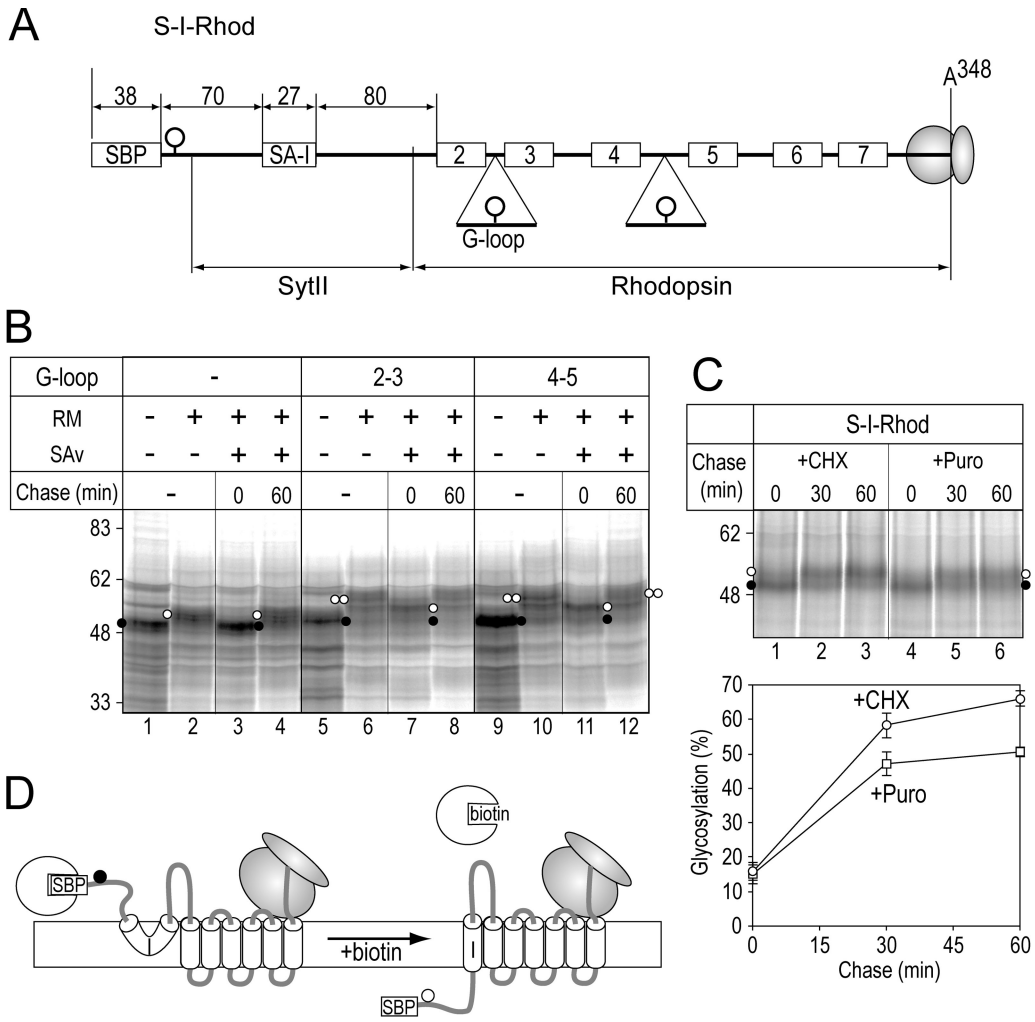


Figure 6. **Insertion of six TM segments did not compete with N-domain translocation.** (A) Six TM segments of rhodopsin were fused after the SA-I (S-I-Rhod). The endogenous glycosylation site in SytII was silenced. In the G-loop constructions, the glycosylation sequence (G-loop) was inserted either between TM2 and TM3 or TM4 and TM5 of rhodopsin. (B) The mRNAs truncated at the C-terminal residue ($Ala^{348}$) of rhodopsin were translated in the presence of RM and SAv. The biotin chase was then performed. (C) Effect of Puro on the N-domain translocation resumption. Puro was used to terminate translation instead of CHX and then the biotin chase was performed. The glycosylated and nonglycosylated forms were quantitated and the glycosylation efficiencies were calculated. The experiments were performed more than three times, and the average and standard deviations are indicated in the figure. (D) Schematic of translocation intermediates. The arrest of N-domain translocation did not affect insertion of the following TM segments and insertion of six TM segments did not influence the resumption of N-domain translocation.

hydrophilic chains flanked the Sec61 channel. It is likely that the nascent chain existed within Sec61 pore. The polypeptide might be outside the small Sec61 pore and still adjacent to Sec61 subunit. We performed the same cross-linking experiment using the construct in which Cys-residues were included in both translocating hydrophilic segments and found a faint but significant super-shifted band in addition to the band cross-linked with one Sec61α (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200707050/DC1), suggesting that two Sec61α can be cross-linked with the single nascent polypeptide chain. The exact nature of the super-shifted molecule, however, remains to be examined.

We then examined the effect of biotin or Puro treatments on the environment of the integration intermediates. Cross-linking in the position-3 Cys mutant was little affected by Puro, but was abolished by biotin (Fig. 5 D, lanes 9 and 12). Cross-linking with the position-7 Cys residues in the presence of Puro was more diminished than that with position-3 (Fig. 5 D, lanes 9 and 21). However, the residual cross-linking of position-7 in the presence of Puro was further decreased after biotin treatment (lane 24). On the other hand, position-7 cross-linking was not affected by biotin chase in the presence of CHX (unpublished data). These results indicate that cross-linking with Sec61α reflects the productive and specific intermediate and that the upstream translocating segment in the advanced intermediate stage continued to flank the Sec61 channel even after Puro treatment. The residual cross-linking of position-7 after Puro treatment in the absence of biotin might suggest that upstream translocating chain affects the downstream translocation.

### Multiple TM insertions do not affect N-domain translocation

To examine the effect of the insertion of multiple H-segments on N-domain translocation, the TM segments (TM2–TM7) of

bovine rhodopsin were attached downstream of the SA-I sequence (Fig. 6 A). When synthesized in the presence of RM, the N-terminal site was glycosylated (Fig. 6 B, lane 2). The glycosylation was arrested in the presence of SAv, but resumed after the biotin chase (lanes 3 and 4). To confirm multiple insertions of the following TM segments, a glycosylation loop sequence was inserted into the lumenal loop between either TM2 and TM3 or TM4 and TM5 (Fig. 6 A). In both cases, the products were diglycosylated in the presence of RM (Fig. 6 B, lanes 6 and 10). When translated in the presence of SAv, the monoglycosylated forms were the major products (lanes 7 and 11). These were converted to the diglycosylated forms after the biotin chase (lanes 8 and 12). For unknown reasons, the diglycosylated form of these constructs was occasionally smeared or split (e.g., lanes 6, 8, and 12).

When the intermediate was treated with Puro instead of CHX, glycosylation after the chase reaction was significantly affected (Fig. 6 C), indicating that the ribosome is actively involved in maintaining the productive state of the earlier intermediate of N-domain translocation, even after the insertion of six successive TM segments. The insertion of multiple H-segments did not affect the resumption of arrested N-domain translocation. An insertion intermediate of the N-terminal SA-I sequence did not affect the insertion of the following multiple TM segments.

## Discussion

Translocation of the SBP-tagged N-domain via the SA-I sequence was arrested by SAv but then resumed after biotin treatment. We constructed two different N-domain translocation intermediates (Fig. 1 D). In the earlier stage, in which the SBP-tag was proximal to the SA-I sequence, neither of the two glycosylation sites in the N-domain was glycosylated. In the advanced stage, in which the N-terminal SBP-tag was separated from the SA-I sequence by the 38-residue spacer, the hydrophilic and hydrophobic segments spanned the membrane and the second site was glycosylated. In both intermediates, the two glycosylation sites in the N-domain were eventually glycosylated after chasing with biotin.

Using these intermediates, we demonstrated that two hydrophilic translocating segments of the single membrane protein can simultaneously span the membrane. Even when the N-domain translocation was arrested, the second H-segment initiated membrane insertion of the downstream hydrophilic segment. In the earlier intermediate of N-domain translocation, the SA-I sequence orients in the membrane after the biotin chase, while both the second H-segment and the downstream segment span the membrane (Fig. 2). In the advanced intermediate, two H-segments and two translocating hydrophilic segments span the membrane (Fig. 3). The two intermediates reflect different stages of signal function. Release of the ribosome significantly affected the translocation chase of the earlier intermediate, whereas it had little effect on the chase of the advanced intermediate (Fig. 4). At the earlier intermediate stage, maintenance of the active engagement of SA-I and the N-terminal domain with the translocon during translocation arrest was dependent on the ribosome. On the other hand, the upstream translocating chain in the advanced stage was still in an active state in the translocon, even after Puro treatment. This post-translational movement of polypeptide chain in a ribosome independent manner is reminiscent of the ribosome-independent reorientation of TM segments observed with some membrane proteins (Lu et al., 2000).

The SA-I sequence emerging from the ribosome is initially recognized by SRP, transferred from SRP to the signal pocket of Sec61α on the ER, forms the TM helix, and is then released to the lipid environment (Heinrich et al., 2000; Kida et al., 2000). Insertion of the TM segment into the Sec61 complex and interactions with lipids might provide the driving force for N-terminal translocation (Heinrich et al., 2000; Kida et al., 2005). A signal pocket exists between TM2b and TM7, which are located at the lateral exit side of Sec61α (Van den Berg et al., 2004; Osborne et al., 2005). The Sec61 complex is involved in cotranslational sequential insertion of TM segments of multispanning membrane proteins; TM segments interact with the Sec61α subunit in an ordered and sequential manner (Sadlish et al., 2005). The TM segment flanking the initial site of translocon is replaced by the next TM segment and moves to the second site. The hydrophobic TM segments readily move from the pore to the lipid phase (Osborne et al., 2005). Although overall integration of the H-segment of the SA-I sequence into lipid requires the Sec61 complex, once inserted in the Sec61 pore, the H-segment readily exits to the lipid phase (Heinrich et al., 2000). The weakly hydrophobic segment stays longer flanking the Sec61 complex and TRAM. The translocon provides the environment for TM segments to interact with each other. In some cases, less-hydrophobic segments are inserted into the translocon and then exit the translocon depending on their interaction with other TM segments (Ota et al., 2000; Ott and Lingappa, 2002; Heinrich and Rapoport, 2003). In the stop transfer context, ongoing translocation of polypeptide through Sec61 pore is stopped by hydrophobic TM segment, which is cotranslationally and stepwise exit from the pore to the lipid phase via TRAM protein (Do et al., 1996). The cotranslational integration process is an ordered and regulated multistep process. Based on these lines of evidence of the cotranslational integration of membrane proteins, it is reasonable to assume that the SA-I and N-domains of the model constructs used in this study are inserted into the Sec61 pore and that the second hydrophobic insertion signal and the downstream hydrophilic chain are also inserted via the Sec61 pore. SAv arrested the sequential process of translocation of the SBP-tagged N-domain. The SA-I sequence in the earlier intermediate was targeted to the translocon and was likely on the way to being inserted into the Sec61 pore. The hydrophilic polypeptide chain spanning the membrane in the advanced stage should be in the Sec61 pore. Even under these conditions, the second H-segment mediated the following translocation.

The preexisting translocating segment did not compete with the downstream stop-transfer process. In the case of S-38-I-II-15L (Fig. 3), the 15-leucine segment is recognized by the translocon as a third TM segment, which stops the translocation of the downstream portions of the molecule. The stop-transfer process is mediated via hydrophobic interaction with lipid

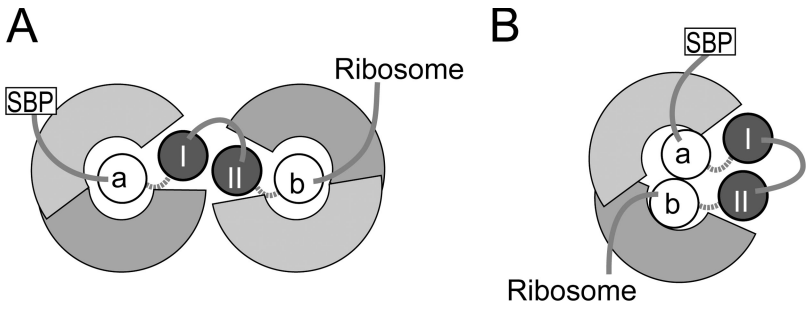


Figure 7. **Working models of possible arrangements of two translocating hydrophilic segments in the translocon.** Vertical views of the translocon pore from the cytoplasmic side are shown. (A) Two Sec61 pores cooperate to accommodate polypeptide chains. Translocating polypeptide chains (a and b) are in different Sec61 pores. The two insertion signals (I and II) are recognized by different sites and the translocating chains do not compete with each other. Although the front-to-front dimer model is represented in the figure, the back-to-back configuration is equally possible. (B) A single Sec61 pore accommodates two polypeptide chains (a and b). The hydrophilic environment is enlarged.

(Hessa et al., 2005). It is highly likely that the second H-segment and the 15-leucine segment interact with lipids. The upstream insertion intermediate that should be at the exit site toward the lipid environment did not affect the interaction between the downstream H-segments and lipids. In the case of S-I-Rhod (Fig. 6), six TM segments of the rhodopsin were inserted into the translocon pore, maintaining the productive states of the first SA-I sequence. The rhodopsin TM segments are likely to be stepwise released from the Sec61 pore depending on the nature of the TM segments. Some of them might still be adjacent to the Sec61 molecule as observed with aquaporin 4 (Sadlish et al., 2005), or to TRAM protein as observed with type-I membrane protein (Do et al., 1996). It should be noted that the translocating N-domain does not jam multiple insertion of TM segments, and vice versa. Whereas the last H-segment (TM7) of rhodopsin is just under the ribosome and the N-terminal SA-I should be farthest from the ribosome exit site, the N-domain insertion intermediate is maintained depending on the ribosome. The translocon possesses extreme flexibility.

How are the two translocating hydrophilic segments accommodated in the membrane? A hypothetical large pore composed of multimeric Sec61 complexes might account for the intermediates observed in this study. There is, however, no hydrophilic surface around the archaean Sec61 complex in a resting state (Van den Berg et al., 2004). Amino acid residues at the narrow ring of *E. coli* SecY are cross-linked with the translocating polypeptide (Cannon et al., 2005). Both TM2b and TM7, located at the mouth of the Sec61α clamshell, are cross-linked with the signal peptide (Plath et al., 1998). These observations support the idea that a single Sec61α molecule provides the translocation pore. Although the precise location of the translocating chains remains to be determined, we propose the following working hypotheses. The simplest interpretation of the unexpected intermediates is that there are two Sec61 pores actively inserting the single membrane protein. The first Sec61 pore maintains the productive intermediate of N-domain translocation and the second Sec61 complex accommodates the second translocating segment (Fig. 7 A). In this model, one Sec61 complex is involved in the sequential insertion of multiple TM segments and release into the membrane, maintaining the productive state of the N-domain translocation intermediate. An alternative hypothesis is that the hydrophilic environment of the pore is enlarged via an unknown mechanism (Fig. 7 B). TRAM and translocon-associated protein complex might cause such an enlargement of the pore during multispanning insertion. Both translocating segments might be accommodated in the same Sec61 pore; or the two segments alternate being in the channel and some other factors stabilize the polypeptide chain outside of the Sec61 pore. As recently demonstrated, large hydrophilic segments of a C-terminally anchored protein can be translocated through protein-free lipid vesicles (Brambillasca et al., 2006). We cannot exclude a possibility that the one of the two hydrophilic segments spanning membrane might largely be in the lipid bilayer despite the large energetic cost. The two-pore model assumes the existence of two signal pockets and two translocation pores. There is no drastic conformational change of the Sec61 pore. This would, furthermore, explain the insertion of a complex multispanning protein that possesses weakly hydrophobic TM segments; even if they occupy a translocon pore, the second translocon pore participates in other TM insertion and TM segments are allowed to be folded into mature conformation stable in membrane lipid.

## Materials and methods

**Constructs**

In the following DNA manipulation procedures, the desired DNA fragments were obtained by chemical synthesis or polymerase chain reaction. The DNA fragments were designed to possess the appropriate restriction enzyme sites at both ends; these are described in parentheses. The DNA fragments were ligated into plasmid vectors that had been digested with the indicated restriction enzymes. At each junction, six bases of the restriction enzyme site encoded two codons. These amino acid residues were taken into account when numbering the residues indicated in the figures. For the S-I protein (Fig. 1), DNA fragments encoding the SBP-tag, MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP (Wilson et al., 2001; Terpe, 2003) ($Met^{1}$-$Pro^{38}$; HindIII–EcoRI), glycosylation probe sequence (KLNSTAT, MfeI–EcoRI), and mouse SytII ($Arg^{2}$-$Lys^{422}$; EcoRI–XbaI) were inserted between the HindIII and XbaI sites of pRc/CMV to obtain pSBP-Syt-full. The SA-I sequence of SytII is $K^{60}$IPLPPWALIAMAVVAGLLLLTCCFCICK$^{88}$. For the S-38-I protein (Fig. 1), a 38-residue spacer sequence from human anion exchanger 1 ($Thr^{627}$-$Trp^{662}$; MfeI–EcoRI) was inserted between the glycosylation probe sequence and SytII. An endogenous glycosylation site in the 38-residue spacer sequence was silenced by a point mutation (Ser-644Ala). The sequence was $T^{627}$YTQKLSVPDGFKVSNSAARGWVIHPLGLRSEFPIW$^{662}$EF (the last two residues were derived from the restriction enzyme site). To make truncated mRNAs, an AflII site was generated immediately after the $Arg^{200}$ of SytII in pSBP-SytII-full by site-directed mutagenesis (Kida et al., 2000). For the S-I-II protein (Fig. 2), DNA fragments encoding the SBP-SytII fusion (from $Met^{1}$ of SBP to $Thr^{160}$ of SytII; HindIII–NheI), TM3, and the flanking regions of human $Na^{+}/H^{+}$ exchanger isoform 6 ($R^{97}$FLHETGLAMIYGLLVGLVLRYGIHVPSDVNNV$^{129}$, SpeI–NheI), the glycosylation probe sequence (KLNSTAT; NheI–EcoRI), and bovine prolactin ($Thr^{31}$-$Cys^{229}$; EcoRI–ApaI) were ligated into pRc/CMV (HindIII–ApaI). For the S-I-II(2G) protein, the endogenous glycosylation site in SytII was silenced by a point mutation (Thr34Ala). For the S-38-I-II protein (Fig. 3), the 38-spacer of anion exchanger 1 was inserted between the glycosylation probe sequence and SytII of the S-I-II protein. For S-I-II-15L and S-38-I-II-15L (Fig. 3),

the Gln$^{42}$-Ser$^{56}$ segment of the prolactin sequences in the S-I-II and S-38-I-II proteins was substituted by 15-leucine residues using site-directed mutagenesis. For (4G) constructs (Fig. 4), the Glu$^{65}$-Asp$^{71}$ segment of prolactin in the S-38-I-II and S-38-I-II-15L proteins was substituted with the glycosylation probe sequence (KLNSTAT) using site-directed mutagenesis. For Cys mutants (Fig. 5), a Cys-less mutant of the SBP-38-I-II protein was used. In this mutant, the following seven Cys residues of SytII (Cys$^{82}$, Cys$^{83}$, Cys$^{85}$, Cys$^{87}$, Cys$^{90}$, Cys$^{91}$, and Cys$^{92}$) and three Cys residues of prolactin (Cys$^{34}$, Cys$^{41}$, and Cys$^{88}$) were replaced by Ala residues. In addition, the following residues were replaced with Cys: L$^{632}$ and V$^{634}$ (position-1), A$^{644}$ and A$^{645}$ (position-2), Leu$^{653}$ and Leu$^{655}$ (position-3) of human anion exchanger 1 in the 38-spacer; Ile$^{4}$ and Phe$^{5}$ (position-4), A$^{14}$ and A$^{16}$ (position-5), and A$^{20}$ and M$^{22}$ (position-6) of SytII; Leu$^{48}$ and Phe$^{49}$ of prolactin (position 7); and Val$^{53}$ and Met$^{54}$ of prolactin (position 8). For the rhodopsin fusion protein (Fig. 6), DNA fragments encoding the SBP-SytII fusion (from Met$^{1}$ of SBP to Thr$^{160}$ of SytII; HindIII–NheI) and the TM2-TM7 of bovine rhodopsin (Arg$^{69}$-Ala$^{348}$; NheI–XbaI) were subcloned between the HindIII and XbaI sites of pRc/CMV. For the truncated RNA, the AflII site was inserted between the C-terminal residue, Ala$^{348}$, and the stop codon. To insert the glycosylation sequence into the lumenal loops of rhodopsin, codons for the Phe$^{103}$-Val$^{104}$ region between TM2 and TM3 and the Gly$^{188}$-Ile$^{189}$ region between TM4 and TM5 were exchanged with the six bases of the PmaCI site. The glycosylation loop segment of human anion exchanger 1 (T$^{627}$YTQKLSVPDGFKVSNSSARGWVIHPLGLRSEFPIW$^{662}$; ScaI–ScaI; referred to as the G-loop) was inserted into the PmaCI site. All the mutants were screened by restriction enzyme mapping and confirmed by DNA sequencing. The sequences of the oligonucleotides used and construction details are available from the authors.

**In vitro transcription, translation, enzyme treatment, and cross-linking**

For the truncated mRNAs shown in Fig. 1, plasmids were linearized by AflII at Arg$^{200}$ of SytII. For the truncated mRNAs shown in Figs. 2–5, plasmids were linearized by BspHI at His$^{106}$ of prolactin. For truncation of the rhodopsin fusion constructs shown in Fig. 6, the plasmids were linearized by AflII at Ala$^{348}$ of rhodopsin. The templates were transcribed with T7 RNA polymerase (Takara), as previously described (Sakaguchi et al., 1992a). The obtained mRNAs were translated in a reticulocyte lysate cell-free system for 1 h at 25°C in either the absence or presence of RM. Preparation of RM (Walter and Blobel, 1983) and rabbit reticulocyte lysate (Jackson and Hunt, 1983) was performed as previously described. RM was extracted with EDTA and treated with *Staphylococcus aureus* nuclease (Roche Chemical) as described previously (Walter and Blobel, 1983). The translation reaction contained 100 mM potassium acetate (KOAc), 1.0 mM magnesium acetate ($Mg(OAc)_2$), 32% reticulocyte lysate, and 15.5 kBq/μl EXPRESS protein-labeling mix (PerkinElmer). Where indicated, 0.2 mg/ml SAv (Wako) was included in the translation reaction. For the translocation chase in the presence of biotin (Sigma-Aldrich), translation was terminated by incubation in the presence of 2 mM CHX (Sigma-Aldrich) or Puro (Sigma-Aldrich) for 10 min at 25°C, and then further incubated at 25°C for 1 h in the presence of 50 μM biotin. Aliquots of the translation mixture were treated with EndoH (New England Biolabs, Inc.) at 37°C for 1 h under denaturing conditions in accordance with the manufacturer's instructions. In the experiments illustrated in Fig. 1, the reactions were terminated with 5% trichloroacetic acid and protein precipitates were solubilized with SDS-PAGE sample buffer. In the experiments illustrated in Figs. 2, 3, 4, and 6, reaction mixtures translated in the presence of RM were diluted with a 10-fold volume of high salt buffer (0.5 M KOAc, 2.5 mM $Mg(OAc)_2$, 30 mM Hepes, pH 7.4) and centrifuged at 100,000 *g* for 10 min at 4°C and the membrane precipitates were subjected to SDS-PAGE. Radiolabeled proteins analyzed by SDS-PAGE were visualized on a BioImage analyzer BAS-1800 (Fuji Film). Quantification was performed using Image Gauge software (v4.0; Fuji Film).

For chemical cross-linking, Cys mutants were synthesized in the presence of RM and SAv. Aliquots were incubated at 25°C for 60 min with CHX, Puro, and/or biotin (Fig. 5 C). The mixtures were then diluted with a fourfold volume of physiologic salt buffer (PSB; 30 mM Hepes/KOH, pH 7.4, 120 mM KOAc, 2 mM $Mg(OAc)_2$, 0.5 mM CHX, 0.2 mg/ml SAv) and centrifuged at 100,000 *g* for 10 min. Membrane pellets were resuspended with PSB and treated with 2 mM BMH, BMOE, BMB, $BM(PEO)_2$ (Thermo Fisher Scientific), or dimethyl sulfoxide only (where indicated as *minus*-cross-linker) on ice for 60 min. Cross-linking reactions were quenched with 10 mM DTT for 15 min. For immunoprecipitation, the reaction mixtures were solubilized with 1% SDS for 5 min at 95°C and then diluted with more than 10-fold volume of immunoprecipitation buffer (1% Triton X-100, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM PMSF). After insoluble materials were removed by centrifugation, the solutions were incubated for 30 min with protein A–Sepharose (GE Healthcare) alone to remove materials nonspecifically bound to the resin. The unbound fractions were incubated for 2 h with anti-Sec61α antiserum, anti-Sec63 antibody (raised against 15 amino acid residues [MRIAKAYAALTDEES] in the J-domain of Sec63), or anti-SAv antibody (Abcam), and further for 1 h with protein A–Sepharose. The resin was washed once with 1 ml of immunoprecipitation buffer and then extracted with sample buffer for SDS-PAGE.

**Online supplemental material**

Fig. S1 shows that the loop between the two TM segments of S-38-I-II(4G) is on the cytoplasmic side of the membrane and accessible to the externally added proteinase K. Fig. S2 indicates essentially the same results using other cross-linkers with shorter spacers as Fig. 5 C. Fig. S3 indicates that the model proteins including Cys residues in both translocating hydrophilic segments gave a faint but significant super-shifted band. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200707050/DC1.

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