Stability and flexibility of marginally hydrophobic–segment stalling at the endoplasmic reticulum translocon

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ABSTRACT **Many membrane proteins are integrated into the endoplasmic reticulum membrane through the protein-conducting channel, the translocon. Transmembrane segments with insufficient hydrophobicity for membrane integration are frequently found in multispanning membrane proteins, and such marginally hydrophobic (mH) segments should be accommodated, at least transiently, at the membrane. Here we investigated how mH-segments stall at the membrane and their stability. Our findings show that mH-segments can be retained at the membrane without moving into the lipid phase and that such segments flank Sec61α, the core channel of the translocon, in the translational intermediate state. The mH-segments are gradually transferred from the Sec61 channel to the lipid environment in a hydrophobicity-dependent manner, and this lateral movement may be affected by the ribosome. In addition, stalling mH-segments allow for insertion of the following transmembrane segment, forming an $N_{cytosol}/C_{lumen}$ orientation, suggesting that mH-segments can move laterally to accommodate the next transmembrane segment. These findings suggest that mH-segments may be accommodated at the ER membrane with lateral fluctuation between the Sec61 channel and the lipid phase.**

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INTRODUCTION

In eukaryotic cells, most integral membrane proteins in the secretory pathway are integrated into the endoplasmic reticulum (ER) membrane. At the rough ER, ribosomes are directly attached to protein-conducting channels, so-called translocons, and membrane translocation and integration of proteins are cotranslationally accomplished at the ribosome-translocon complex (Walter and Lingappa, 1986; Alder and Johnson, 2004; Skach, 2009; Shao and Hegde, 2011; Park and Rapoport, 2012; Cymer *et al.*, 2015). The Sec61 complex, comprising three subunits (α, β, and γ), is the core channel of the ER translocon and is highly conserved from bacteria to mammals and plants. Structural analyses of archaeal and bacterial SecY (Van den Berg *et al.*, 2004; Tsukazaki *et al.*, 2008), which are homologues of the Sec61 complex, and mammalian Sec61 (Voorhees *et al.*, 2014) complexes revealed that the Sec61α/SecY protein forms an hourglass-like pathway with 10 transmembrane (TM) segments. TM1–5 and TM6–10 are pseudosymmetric halves that form a crack between TM2b and TM7, called the lateral gate. Site-specific cross-linking and some structural data suggest that TM segments are partitioned from the Sec61/SecY channel to the lipid bilayer (Martoglio *et al.*, 1995; Do *et al.*, 1996; Mothes *et al.*, 1997; Heinrich *et al.*, 2000) via the lateral gate (Plath *et al.*, 1998; Egea and Stroud, 2010; Gogala *et al.*, 2014; Mackinnon *et al.*, 2014; Park *et al.*, 2014).

Integration of membrane proteins starts by ER targeting caused by signal sequences within nascent polypeptides (Akopian *et al.*, 2013). When a hydrophobic signal sequence emerges from a ribosome, it is recognized by a signal-recognition particle, and translation is interrupted. The ribosome–nascent chain complex (RNC) is transferred to the ER translocon via an interaction between the signal-recognition particle and its receptor on the ER. Translation then resumes, and membrane integration starts simultaneously by

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Abbreviations used: ER, endoplasmic reticulum; H-segment, hydrophobic segment; mH, marginally hydrophobic; NEM, *N*-ethylmaleimide; PEGmal, polyethyleneglycol-maleimide; RM, rough microsomal membrane; RNC, ribosome–nascent chain complex; RSA, rat serum albumin; SA, signal anchor; TM, transmembrane.

insertion of the signal sequence via the translocon. Among signal sequences to the ER, cleavable signal peptides and type II signal anchor (SA) sequences are inserted with a type II ($N_{cytosol}/C_{lumen}$) orientation and begin translocation of the following portions. On the other hand, type I SA sequences are integrated with an $N_{lumen}/C_{cytosol}$ orientation (Kida *et al.*, 2000, 2009). The topology of SA sequences is determined by their hydrophobicity and net charge between the ends (Sakaguchi, 1997; Goder and Spiess, 2001; Kida *et al.*, 2006).

After insertion of the signal sequences, the following TM segments are inserted into the membrane by several modes. In the authentic mode, a start-transfer TM segment forming a type II orientation induces translocation of the following part, and the next TM segment is arrested at the translocon and integrated into the membrane. Although the majority of TM segments possess sufficient hydrophobicity for membrane integration, TM segments with lower hydrophobicity and/or topogenic function are also found within multispanning membrane proteins, and ~30% of TM segments are predicted to have difficulty moving into the membrane by themselves (Ojemalm *et al.*, 2012). Previous reports provided a model in which membrane insertion of such marginally hydrophobic (mH) segments can be assisted by the topogenesis of neighboring TM segments (Lu *et al.*, 1998; Ota *et al.*, 1998b; Ojemalm *et al.*, 2012). Our previous data also strongly suggest that type I SA-like TM segments in human erythrocyte band 3 protein pull upstream TM segments with an $N_{cytosol}/C_{lumen}$ orientation into the membrane (Yabuki *et al.*, 2013). In the case of mH-segments in the stop-transfer context, at least temporary stalling might be required for membrane integration by interaction and/or shedding from the hydrophobic environment with other TM segments. Our previous studies elucidated the translocation arrest caused by mH-segments (Onishi *et al.*, 2013), positively charged residues (Fujita *et al.*, 2011; Yamagishi *et al.*, 2014), and their cooperative actions (Fujita *et al.*, 2010). Our findings also demonstrated that mH-segments arrested at the membrane can be translocated into the lumen as long as the polypeptide chains are in the ribosome-bound state (Onishi *et al.*, 2013), suggesting that such mH-segments are arrested at, but not fully integrated into, the membrane.

In the present study, we focused on how the mH-segments stall at the membrane and their stability. Our findings revealed that mH-segments can be retained at the membrane without moving into the lipid phase. Because such mH-segments were flanking Sec61α in the translational intermediate state, they may be accommodated around the Sec61 channel. mH-segments were gradually transferred from the Sec61 channel to the lipid environment in a hydrophobicity-dependently way, whereas the segment with considerably high hydrophobicity in the ribosome-bound chain remained in an aqueous–lipidic boundary location. This indicates that lateral movement of hydrophobic segments (H-segments) is affected by the ribosome. Moreover, mH-segments stalling at the membrane assist in the insertion of the following TM segment forming an $N_{cytosol}/C_{lumen}$ orientation, suggesting that mH-segments can move laterally for accommodation of the next TM segment. These findings suggest that mH-segments are accommodated in the ER membrane with lateral fluctuation between the Sec61 channel and lipid phase and that the ribosome may play a role in lateral sorting of H-segments.

RESULTS

Translocation arrest caused by mH-segments

To explore the details of the arrested state of mH-segments at the ER membrane, we first determined the role that hydrophobicity plays in the arrest. Various 20-residue H-segments based on a sequence comprising 19 Ala and 1 Cys (19A1C; shown in Figure 1A) were inserted into a rat serum albumin (RSA)-derived protein species, which terminates at 320 residues and possesses an original signal peptide and two exogenously introduced N-glycosylation sites. When these proteins were synthesized in a reticulocyte lysate system in the presence of rough microsomal membranes (RM), both monoglycosylated and diglycosylated forms, arrested and fully translocated protein species, respectively, were detected (Figure 1B). Both forms of each protein were quantified, and the percentages of the arrested species were calculated. Approximately 60% of the protein species containing the 19A1C segment stalled. Arrest efficiency was gradually reduced by increasing the number of Ser residues substituted for Ala in the 19A1C segment, whereas the efficiency was gradually increased by increasing the number of Leu residues within the segment. Fragments shaved by proteinase K on the cytoplasmic side were increased by increasing hydrophobicity of H-segments, also indicating the increase of arrested species (Figure 1C). In the presence of 6 Lys (6K) residues just downstream of the segments, however, even a 2S segment (here we refer to each segment by a number and the one-letter abbreviation of the introduced amino acid) was efficient for the arrest because the 6K promoted translocation arrest of the preceding H-segments, presumably by interaction within the ribosome (Lu and Deutsch, 2008) and/or any factors around the ribosome–translocon junction (Fujita *et al.*, 2011; Yamagishi *et al.*, 2014).

We then examined whether the arrested segments were partitioned into the lipid bilayer (Figure 1D). To this end, we checked the reactivity of the Cys at the center of each segment to *N*-ethylmaleimide (NEM), which is conjugative to SH groups only in an aqueous environment. Because NEM modification causes little mobility shift on SDS–PAGE gel, after solubilization with SDS, free Cys residues in the proteins were labeled with polyethyleneglycol-maleimide (PEGmal). PEGmal-modified products have their mH-segments in a hydrophobic environment, probably the lipid phase. Surprisingly, we found that only the 4L segment was obviously partitioned to the hydrophobic phase, and the other mH-segments were still in an aqueous environment while arrested at the membrane.

These observations indicate that mH-segments could cause translocation arrest at the membrane while remaining in an aqueous site. In addition, positively charged residues following mH-segments enhanced the arrest but rarely affected their membrane integration.

mH-segments stall around the Sec61 channel in the translational intermediate state

The foregoing data suggested that mH-segments stalling at the membrane might be harbored at the translocon. To examine this possibility, after performing translation in the presence of RM, we performed cross-linking at the Cys residue in each segment by a homo-bifunctional cross-linker, 1,2-bis(maleimido)ethane (BME), and immune-purified adducts with Sec61α, the central channel of the translocon, with anti-Sec61α antibody were analyzed by SDS–PAGE (Figure 2). In the case of protein species with the following 6K, the 19A1C and 2S segments were meaningfully cross-linked with Sec61α, whereas other segments showed only faint cross-linked products. In addition, mH-segments without the following 6K showed little cross-linking with Sec61α.

We next examined the association between mH-segments and Sec61α in the translational intermediate state. An RNC can be formed by translation of a truncated mRNA without an in-frame stop codon. We checked cross-linking of 19A1C segments in

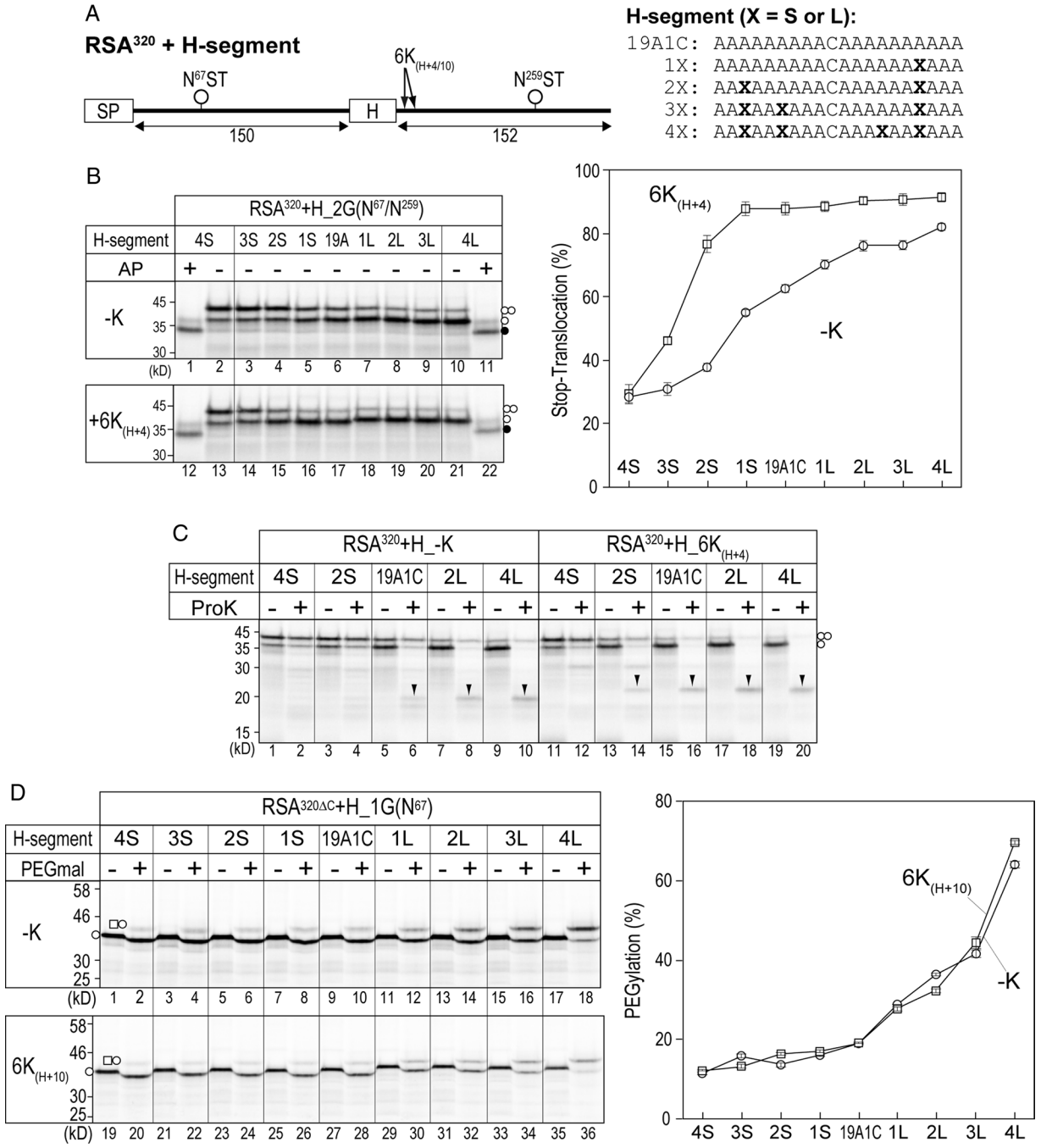

FIGURE 1: Stop transfer and membrane integration of model H-segments. (A) Constructions used here. Two N-glycosylation sites ($N^{67}ST$ and $N^{259}ST$; superscript numbers indicate the residue number of RSA), H-segments (H), and six Lys residues (6K) were introduced into the N-terminal 320 residues of RSA (RSA^{320}) or its Cys-less mutant ($RSA^{320\Delta C}$). 6K stretches at both positions, H+4 and H+10, show the same effect on translocation arrest (unpublished data). From one to four Ala residues in the basic segment comprising 19 Ala and 1 Cys (19A1C) were substituted with Ser or Leu residues as shown. Here each H-segment is named using the one-letter code of the introduced residue and the number of residues. (B) Translocation arrest by H-segments. Protein species were synthesized in a cell-free system including the RM and/or an N-glycosylation acceptor peptide (AP). The monoglycosylated and diglycosylated forms (one and two open circles, respectively), representing arrested and translocated molecules, were quantified, and the percentage of arrested forms was calculated (right). Plots of protein species with and without 6K are shown with square and circle symbols, respectively. Mean values with SEM ($n = 4$). (C) After translation in the presence of RM, an aliquot was treated with proteinase K (ProK). Fragments shaved at just downstream regions of H-segments are shown with arrowheads. (D) Partition of H-segments to the lipid phase. After synthesis of the protein species possessing only one Cys residue in H-segments, the environment of each H-segment was assessed by reactivity to NEM. Then free Cys residues were modified with PEGmal. Percentage of PEGmal-modified forms (left, squares), representing Cys residues partitioned into a hydrophobic environment, were quantified (right). Mean values with SEM ($n = 3$).

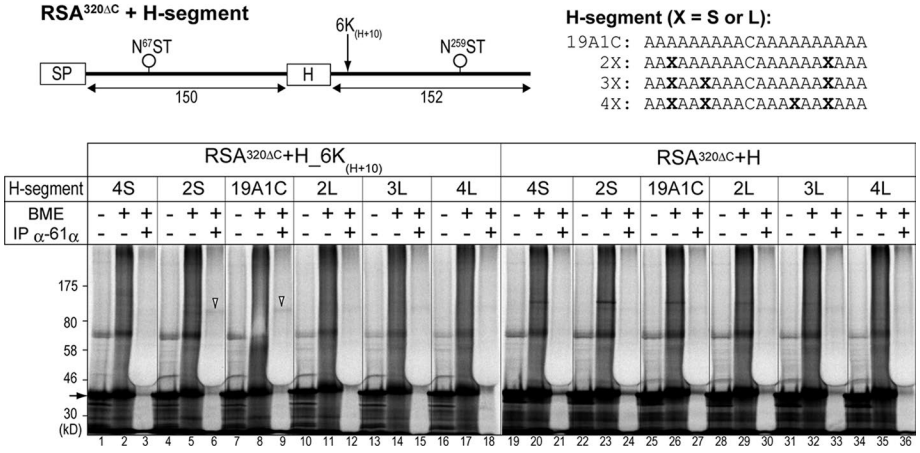

FIGURE 2: Proximity of arrested H-segments to the Sec61 channel. After in vitro synthesis of protein species containing one Cys within H-segments, chemical cross-linking was performed with BME, and cross-linked products with Sec61α were isolated by immunoprecipitation with anti-Sec61α serum. In the gel image, synthesized proteins and their adducts with Sec61α are shown with arrows and open triangles. In the species with 6K, mH-segments 19A1C and 2S were cross-linked with Sec61α, whereas cross-linking was scarcely detected in the case of no Lys cluster following the H-segments.

intermediates of several lengths (Figure 3). Here the Cys residue was placed at the C-terminus of the segment because this position was more suitable for cross-linking (unpublished data). From 35 to 40 residues from the P site in the ribosome are within the ribosomal tunnel (Bhushan *et al.*, 2010), and thus a 19A1C segment 38 or 42 residues from the P site is presumably clear of the ribosome, but they were not cross-linked with Sec61α. The cross-linking became detectable in 19A1C segments that were >60 residues from the P site, and it was maintained on longer chains. Of note, in the case of protein species without a 6K cluster, cross-linking was most efficient when 19A1C was 82 residues away from the P site, whereas cross-linking efficiencies of the 19A1C segment followed by the 6K cluster changed little among several intermediates. The cross-linking largely disappeared after translation termination, suggesting that

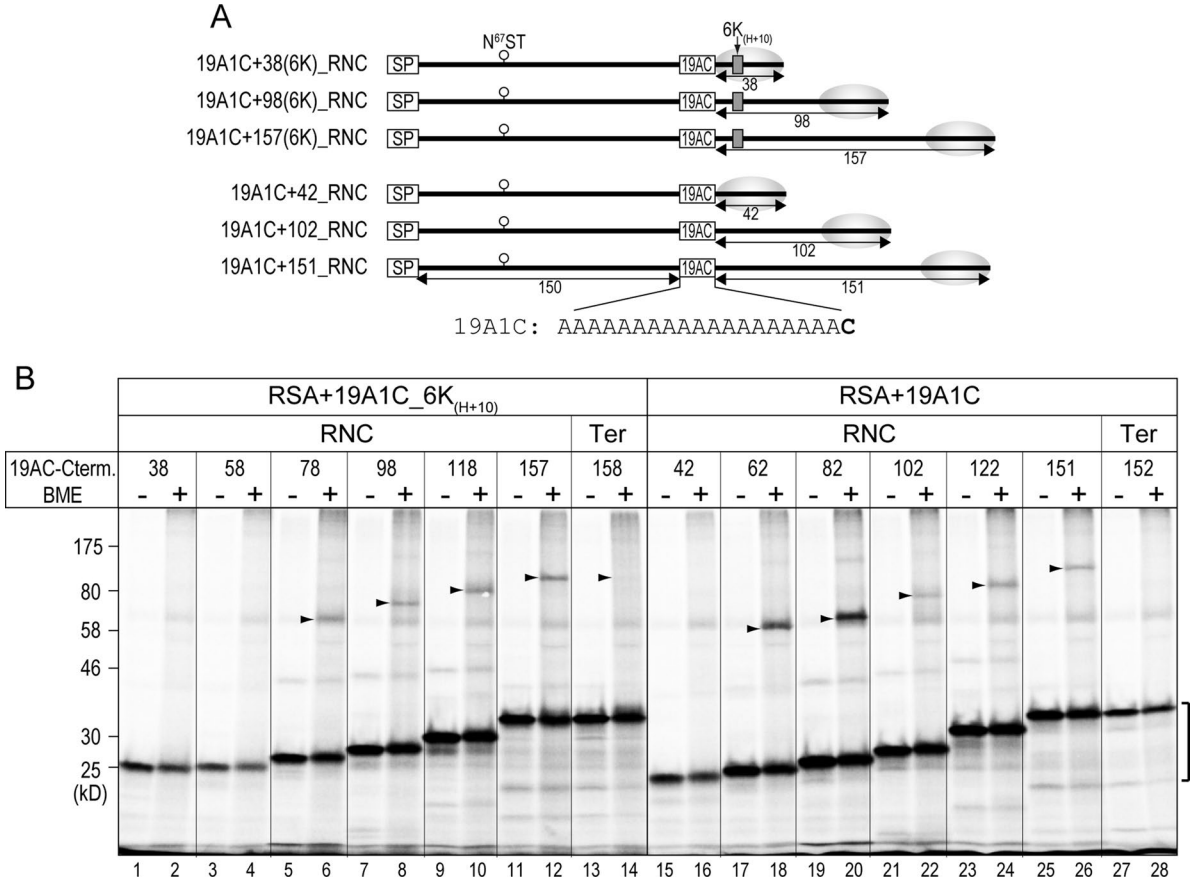

FIGURE 3: The 19A1C segment in ribosome-bound polypeptide chains is efficiently cross-linked with the Sec61 channel. (A) The typical polypeptide chains used here are picked up. The C-terminal regions (~35 residues) within the ribosomal tunnel are indicated by the gray ovals. (B) Truncated and full-length mRNAs were in vitro translated in the presence of RM, and cross-linking was performed as in Figure 2. Translational intermediates (RNC) can be formed by translation of truncated mRNAs without the termination codon. Adducts with Sec61α (arrowheads) of synthesized polypeptides (brackets) were confirmed by immunoprecipitation with anti-Sec61α (unpublished data). Irrespective of the presence or absence of the following 6K, the cross-linking was clearly observed in the translational intermediate state.

mH-segments are more efficiently associated with Sec61α in the translational intermediate state, and after termination of translation, mH-segments might move to a different but aqueous position, possibly around the translocon.

In our previous study, mH-segments, once arrested at the membrane, could be translocated into the lumen as long as they were in the translational intermediate state (Onishi *et al.*, 2013). We thus checked whether mH-segments are stably settled around Sec61α. When an additional potential N-glycosylation site was introduced downstream of the 19A1C segment, the 19A1C+82 polypeptide was partially glycosylated at that site (Figure 4, A and B). The Asn residue at the second glycosylation site in the 19A1C+82 was 70 residues from the P site, consistent with the previous data regarding the distance between the active center of the oligosaccharyltransferase and the P site of the ribosome (Whitley *et al.*, 1996). Polypeptide chains longer than 19A1C+82 were efficiently diglycosylated, whereas their cross-linking with Sec61α disappeared. This indicates that the 19A1C segment and the following glycosylation site were transiently translocated into the lumen and the return of the 19A1C segment to the translocon was hindered by the attached sugar chain.

We assessed the hydrophobicity required for the cross-linking in the RNC state by introducing Leu, Ser, or Asp into the 19A1C segment (Figure 4D). In the H+82 polypeptide, all segments except the 4L segment, even a 10D segment that must be highly hydrophilic, were considerably cross-linked with Sec61α. These cross-linked products were reduced by releasing polypeptide chains from ribosomes with puromycin. Combined with the glycosylation at the second N-glycosylation site shown in Figure 4B, the Cys in this situation should be settled around the translocon, whereas the lateral movement of the 4L segment is not restricted. On the other hand, segments from 10S to 2L in the H+102 polypeptide chain were cross-linked with Sec61α, and the 4S segment had greater efficiency than the other segments. Because the 19A1C segment at this position can move into the lumen (Figure 4B), these segments may be retained in the translocon at least temporarily by their weak but meaningful hydrophobicity.

We further analyzed the environment of H-segments in ribosome-bound polypeptide chains by NEM-PEGmal sequential alkylation (Figure 4E). Although H-segments were gradually transferred to the lipid environment upon increase of their hydrophobicity, even the 4L segment in the ribosome-bound chain remained in an aqueous–lipidic boundary, whereas the same segment in the terminated chain efficiently moved into the lipid phase. This suggests that mH-segments in ribosome-bound chains are more accessible to an aqueous environment and thus may be mobile to the luminal space after they are arrested at the membrane (Onishi *et al.*, 2013). In addition, because the environment of H-segments, even the 4L segment, was rarely affected by release of polypeptide chains with puromycin, polypeptide chain release from the ribosome itself may have little effect on lateral movement of H-segments.

These findings suggest that mH-segments and even the segments with very low hydrophobicity can be associated with Sec61α in the translational intermediate state. They also show that H-segments are accommodated around the Sec61 channel and transferred to the lipid environment in a manner that depends on their hydrophobicity. This implies that mH-segments stalling at the membrane may be in equilibrium between the channel and the lipid phase. Further, the 4L segment within the ribosome-bound chain was retained in an aqueous–lipidic boundary location, and this balance may be biased toward the channel by the ribosome.

mH-segments can induce ordinary topogenesis of the following translocation-start TM segment

To examine further the stalling state of mH-segments, we introduced the third TM segment of human Na^+/H^+ exchanger isoform 6, which is a start-transfer TM segment with an $N_{cytosol}/C_{lumen}$ orientation (Miyazaki *et al.*, 2001), and the following N-glycosylation consensus site downstream of the mH-segments (Figure 5A). In the case of protein species containing a 4S segment, most of the products in the presence of RM were monoglycosylated. In addition, after proteinase K digestion, we observed ~26-kDa fragments protected by the membrane. These observations indicate that the 4S segment was translocated into the lumen, and the following TM segment was arrested and inserted with an $N_{lumen}/C_{cytosol}$ orientation. On the other hand, the protein species containing the 4L segment was diglycosylated, indicating that the following TM segment was inserted with its innate $N_{cytosol}/C_{lumen}$ orientation. Because the diglycosylated form was almost protected from proteinase K, the loop between the 4L segment and the following TM segment may not be accessible to proteinase K in the cytosol. Surprisingly, protein species with mH-segments with lower hydrophobicity than the 4L segment, even a 1S segment, were similarly diglycosylated and protected from proteinase K. Next, to check the topology of the region between two segments, an additional N-glycosylation site was introduced beside H-segments (Figure 5, A and C). Then protein species with 4S and 3S segments were efficiently diglycosylated, and proteinase K–shaved fragments were also shifted by the additional sugar chain, also indicating the translocation of these segments followed by the N-glycosylation site. In contrast, diglycosylation efficiencies of protein species with 1S and more hydrophobic segments rarely changed. In addition, after proteinase K digestion, two fragments of ~18 and ~22 kDa were detected, presumably because the loop between 2 H-segments was lengthened and became accessible to proteinase K. These findings suggested that these mH-segments can stall at the membrane and induce the correct topogenesis of the following TM segment in this situation.

Furthermore, we also assessed the environment of mH-segments in the presence of the following TM segment by NEM-PEG-mal sequential alkylation (Figure 6). Although the 4L segment in the ribosome-bound chain was not fully transferred to the lipid phase (Figure 4E), it efficiently moved into the lipid environment in the presence of the following TM segment, suggesting that insertion of the next TM segment causes relocation of the upstream H-segment, and consequently its movement into the lipid bilayer may be enhanced. However, mH-segments like 1S and 19A1C were still in an aqueous environment, even after insertion of the following TM segment. These data suggest that mH-segments can move laterally and assist in the insertion of the following TM segment in the membrane without their integration into the membrane.

In addition, we subjected TM2, TM3, and TM4 of an authentic multispanning membrane protein, human erythrocyte band 3, to the same analyses (Figure 5). TM2 and TM3 possess very low hydrophobicity for membrane integration, whereas TM4 is highly hydrophobic and can translocate the upstream portion by its SA-I–like topogenic property (Ota *et al.*, 1998a; Yabuki *et al.*, 2013). Among these TM segments, TM3 showed little ability to correctly insert the following TM segment (Figure 5B) and translocation arrest (Figure 5C), whereas TM4 had high ability for both. In contrast, TM2 exhibited efficiencies for both, similar to the 1S segment. These findings suggest that TM2 also assists in the insertion of the following TM segment without moving into the lipid phase.

DISCUSSION

In the present study, we examined the details of mH-segment stalling at the ER membrane. mH-segments insufficient for integration into the membrane are accommodated at the membrane without moving into the lipid phase (Figure 1), and such segments can be associated with the Sec61 channel, especially in the translational intermediate state (Figures 2 and 3). H-segments are gradually transferred from the Sec61 channel to the lipid phase via a boundary site in a hydrophobicity-dependent manner, and this lateral movement may be affected by translating ribosomes (Figures 4). Moreover, mH-segments stalling at the membrane can assist in the insertion of the following TM segment in its innate $N_{cytosol}/C_{lumen}$ orientation irrespective of movement into the lipid phase (Figures 5 and 6).

We previously reported that mH-segments are involved in translocation arrest at the membrane (Fujita *et al.*, 2010; Onishi *et al.*, 2013). Here we investigated the environment of mH-segments around the Sec61 translocon by site-specific cross-linking and chemical modification. Although some mH-segments in terminated chains could be cross-linked with Sec61α in a manner dependent on the following 6K cluster, the cross-linking was largely improved in the translational intermediate state. In this state, even segments with considerably lower hydrophobicity were considerably cross-linked with Sec61α, and thus such segments may also be temporarily associated with the Sec61 channel during cotranslational translocation. In addition, in the absence of the 6K cluster, the cross-linking of mH-segments fluctuated and was detected most efficiently in the 19A1C+82 polypeptide chain (Figure 3). In the presence of the 6K, however, 19A1C segments in 19A1C+78 and all longer chains were uniformly cross-linked with the Sec61 channel, suggesting that they may be fixed at a specific position by the following positive charges. Therefore we hypothesize that the Sec61 channel possesses a site for contacting H-segments. According to recent studies, the lateral gates in ribosome-bound Sec61 channels are partially opened at the cytoplasmic side (Voorhees *et al.*, 2014), and TM segments can be cross-linked with a similar region (Mackinnon *et al.*, 2014). Further, mutations around the constriction ring of Sec61α modulate the recognition of hydrophobic segments (Trueman *et al.*, 2012). Such sites might be associated with a broad range of H-segments. Although comprehensive analyses of the translocation arrest of H-segments indicate that direct interaction with the lipid bilayer via the lateral gate of the Sec61 channel must be basically involved in membrane insertion (Hessa *et al.*, 2005, 2007), not only does the Sec61 channel function as a platform for the lipid bilayer, but it might also be actively associated with H-segments and involved in their transient stalling at the membrane.

The present findings also revealed that H-segments gradually left the Sec61 channel and moved to the lipid phase in a manner that depends on their hydrophobicity. Further, assistance of insertion of the following TM segment by mH-segments indicated that they should laterally move to accommodate the next TM segment, whereas some of them remain in an aqueous environment after insertion of the following TM segment. These results imply that mH-segments can be accommodated at the membrane with the equilibrium between the Sec61 channel and the lipid bilayer (Figure 6C). In addition, the 4L segment in the ribosome-bound chain showed little cross-linking with Sec61α but was still in an aqueous–lipidic boundary situation, and it was relocated to the lipid phase by termination of translation or insertion of the following TM segment. Therefore mH-segments may be transiently accommodated at such a boundary site. How can the boundary site be formed? TM segments, once removed from the Sec61 channel, can become associated with the channel again (Heinrich and Rapoport, 2003; Sadlish *et al.*, 2005; Ismail *et al.*, 2006). Such a lateral exterior position might be provided by Sec61α itself or contact with flanking proteins, including another Sec61 complex, although recent structural studies of the ribosome-Sec61 complex indicate that one ribosome firmly binds only one Sec61 complex (Menetret *et al.*, 2008; Gogala *et al.*, 2014; Voorhees *et al.*, 2014). Translocating chain-associating membrane protein interacts with signal sequences and TM segments with slightly lower hydrophobicity (Do *et al.*, 1996; Voigt *et al.*, 1996; Heinrich *et al.*, 2000). Further, RAMP4 protein (Pool, 2009; Lin *et al.*, 2011) and the Sec62/63 complex (Conti *et al.*, 2015) are involved in membrane translocation in a substrate-dependent manner. Oligosaccharyltransferase and the translocon-associated protein complex also flank the Sec61 channel in the native ER membrane (Pfeffer *et al.*, 2014). These factors are candidates for contact partner to form such a site.

Our previous data showed that mH-segments can move into the lumen as long as they are in the translational intermediate state (Onishi *et al.*, 2013). In the present study, the 4L segment in the ribosome-bound chain remained in a moderately hydrophobic site, whereas the same segment in the terminated chain efficiently moved to the lipid phase. These observations suggest that mH-segments are retained around the Sec61 channel and allowed to fluctuate among the channel and flanking regions in the translational intermediate state. In addition, polypeptide chains release from the ribosome by puromycin rarely affect the lateral movement of the 4L segment, indicating that polypeptide release itself shows only a slight effect on lateral partitioning. Therefore our findings indicate the possibility that the state of the ribosome is transmitted to the translocon and that ribosomes synthesizing polypeptide chains enhance the accommodation of mH-segments around the translocon. One recent structural study indicated that the lateral gate of the Sec61 channel in the membrane is opened by association with the ribosome (Pfeffer *et al.*, 2015). In addition, ribosomes containing a TM segment within the tunnel interact with RAMP4 protein (Pool, 2009; Lin *et al.*, 2011). Members of HSP40 protein family in the ER membrane, the Sec62/63 complex, and ERj1/DNAJC1 can be also associated with ribosomes (Muller *et al.*, 2010) and may modify the function of the translocon. Nonetheless, cooperativity between the ribosome and the translocon remains to be clarified.

Furthermore, our results revealed that isolated TM2 of the band 3 protein exhibited a stalling property like the 1S segment. This suggests that TM2 in the original condition cannot be integrated immediately upon translocation via the translocon but can assist in the insertion of the following TM3 and TM4 by stalling at the membrane. After that, TM2 may be integrated into the membrane by interaction with other TM segments. Such a semistable stalling state of mH-segments might function in the topogenesis of multispanning membrane proteins.

MATERIALS AND METHODS

Constructs

Constructs were based on RSA as described previously (Fujita *et al.*, 2010). In brief, the cDNA fragment encoding Met^{1}–His^{319} of RSA, Val as the last residue (last His-Val residues encode a *Pma*CI restriction site), and the termination codon TAA was subcloned into pRcCMV (*Xba*I/*Apa*I). For site-specific Cys alkylation and cross-linking, all Cys residues in Met^{1}–His^{319} of RSA were substituted for Ala, as shown by ΔC in the figures. The superscript numbers used here correspond to the residue number of the RSA polypeptide. The cloned RSA was further modified by substituting Asn-Ser-Thr at Val^{67}-Glu^{69} and

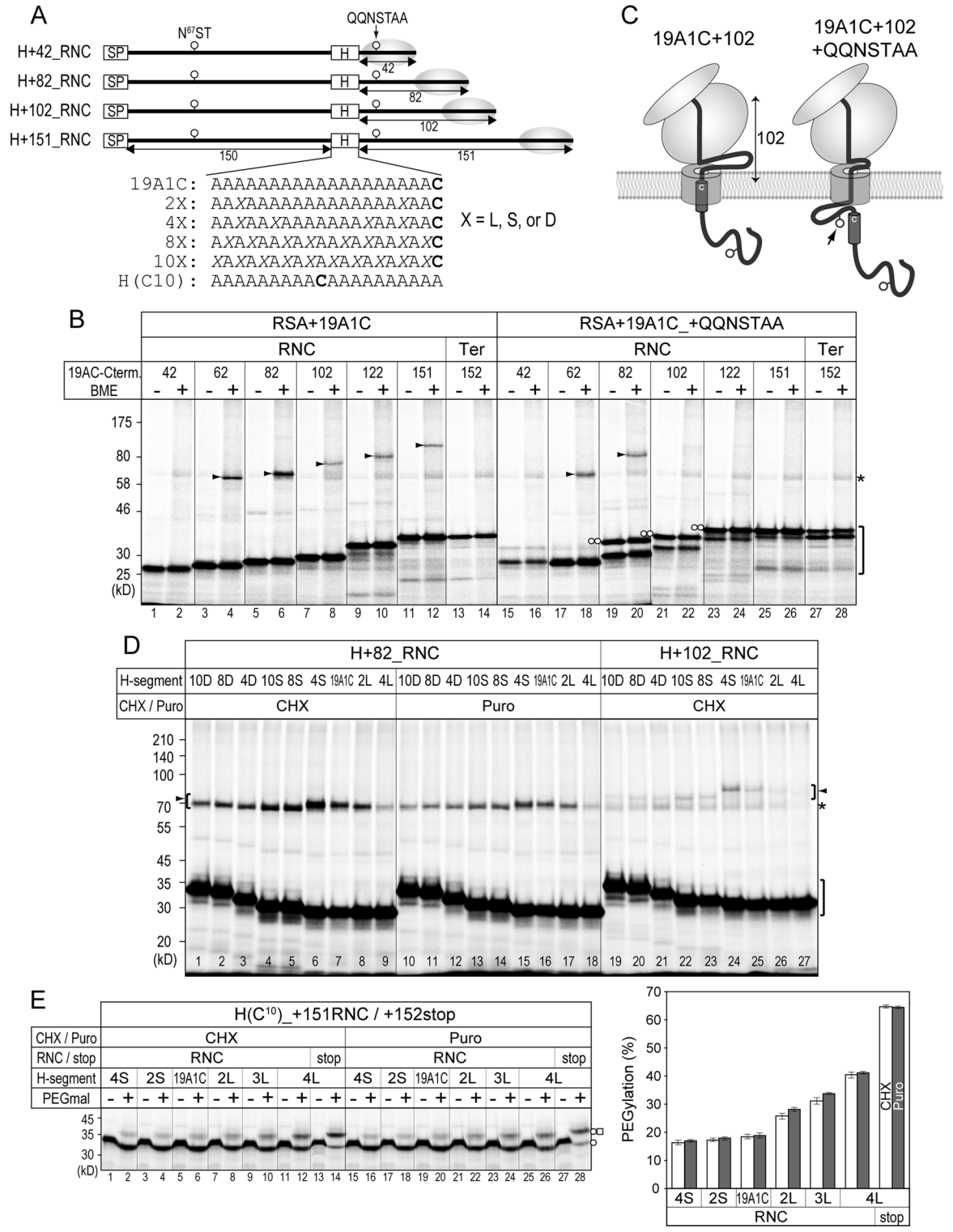

FIGURE 4: Environment and fluctuation of H-segments at the membrane in the translational intermediate state. (A) Structures of polypeptide chains used here. (B) When an additional N-glycosylation site (QQNSTAA) was introduced just downstream of the 19A1C segment, it was glycosylated (two circles), but cross-linking of the same chains with Sec61α disappeared. Unknown products are shown with an asterisk. (C) In 19A1C+102 and longer chains, the 19A1C segment and the following portion can fluctuate between the Sec61 channel and the luminal space. When the N-glycosylation site following the 19A1C segment is translocated and glycosylated, the 19A1C segment cannot be retrieved back into the channel due to hindrance by the attached sugar chain. (D) To determine the hydrophobicity requirement of H-segments for cross-linking, Ala residues of the 19A1C segment in H+82 and H+102 polypeptide chains were exchanged with Leu, Ser, or Asp. In the 19A1C+102 polypeptide chain, the hydrophobic dependence on the cross-linking is shown, whereas even a 10D segment was cross-linked in an H+82 chain. (E) After translation was

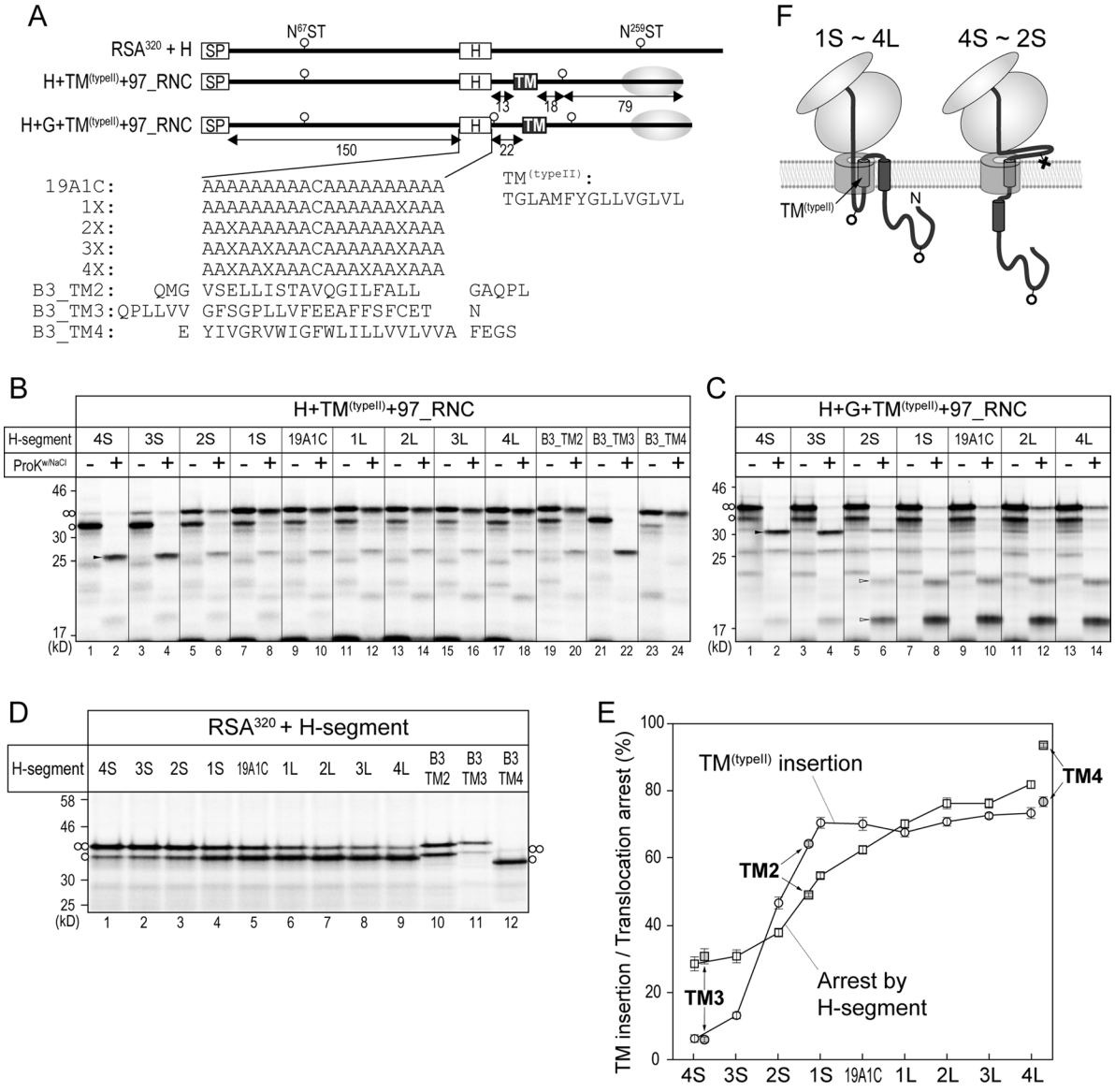

FIGURE 5: Assistance for insertion of the following type II TM segment by mH-segments. (A) An additional TM segment forming a type II orientation ($N_{cytosol}/C_{lumen}$) and an N-glycosylation site were inserted in RSA_H+89 chains as shown. Model H-segments and 26-residue segments containing TM2, TM3, or TM4 from human band 3 protein were inserted as indicated. To check the topology of the region between two H-segments, an additional N-glycosylation site was introduced just downstream of the H-segments. (B) After translation in the presence of RM, an aliquot was treated with ProK. Diglycosylation indicates that the following TM segment is inserted with its innate type II orientation. (C) The additional N-glycosylation site following the 4S or 3S segment was glycosylated, indicating the translocation of these segments. However, the sites following 1S and more hydrophobic segments were little translocated into the lumen, and the loops inserted the N-glycosylation site became accessible to ProK. Fragments cleaved at the loop are shown with open triangles. (D) Translocation arrest analyzed as in Figure 1B. (E) Percentages of diglycosylated forms in B (open circles) and arrested forms in D (open squares; same data as in Figure 1B). Percentages of TM segments from band 3 are shown with filled squares and circles. Mean values with SEM ($n \geq 3$). (F) Summary. Lower hydrophobic segments (4S~2S) are translocated, and the next TM segment is inserted as a stop-transfer segment. Meanwhile, 1S and more-hydrophobic segments can be arrested so stably that they can assist in the correct insertion of the next TM segment.

terminated with cycloheximide (CHX) or puromycin (Puro), the environment of H-segments in H+151 ribosome–bound chains and an H+152– terminated chain was assessed as in Figure 1D. Mean values of PEGylation efficiencies with SEM ($n = 3$; right). The 4L segment in the ribosome-bound chain was still in a moderately hydrophobic situation, whereas the same segment moved to the lipid phase after termination of translation.

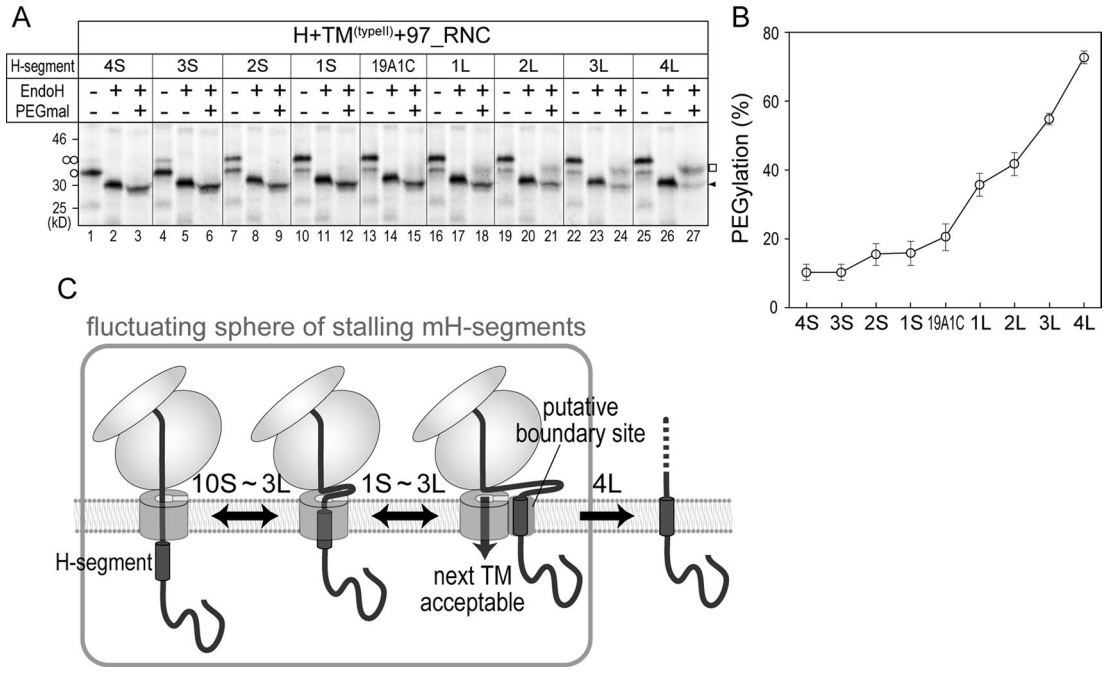

FIGURE 6: Some mH-segments remain in an aqueous environment after insertion of the following type II TM segment. (A) After synthesis in the presence of RM, sequential Cys alkylation was performed as in Figure 1D. To simplify the band pattern to NEM- or PEGmal-modified forms only, after PEGmal reaction, N-glycans attached to proteins were removed with endoglycosidase H (EndoH). (B) Percentages of PEGmal-modified forms were quantified. Mean values with SEM ($n = 3$). (C) Taken together, this and Figures 4 and 5 show that 10S and more-hydrophobic segments can be transiently associated with the Sec61 channel. Among them, 1S and more-hydrophobic segments may laterally fluctuate from the channel to the lipid phase via a boundary site, and thus the channel becomes able to accommodate the next TM segment.

Ala^{259}-Asp^{261} (first and second N-glycosylation sites) and Ala-Ser-Ser-Ala-Asp-Asp at Ala^{167}-Tyr^{172} (Ala-Ser and Ser-Ala encode *Nhe*I and *Aor*51HI sites, respectively). Various 20-residue H-segments were inserted between Ala^{167}-Ser^{168} (*Nhe*I) and Ser^{169}-Ala^{170} (*Aor*51HI) as shown in the figures. Six Lys residues were inserted just upstream of Phe^{173} (H+4) or between Leu^{178} and Leu^{179} (H+10) as indicated. To generate truncated mRNAs of indicated lengths (Figure 3), *Bam*HI sites (GGATCC: last codon encoding Asp of each nascent chain is underlined) were introduced at the indicated positions. To detect the translocation of H-segments, Leu^{178}-Lys^{184} residues in nascent chains were exchanged with a potential N-glycosylation sequence Gln-Gln-Asn-Ser-Thr-Ala-Ala, as shown in Figure 4. In Figure 5, Leu-Lys residues encoding an *Afl*II site and Arg^{93}-Pro^{123} containing TM3 of human Na^+/H^+ exchanger isoform 6 were inserted between Phe^{174} and Ala^{175} of RSA, and a potential N-glycosylation sequence Lys-Leu-Asn-Ser-Thr-Ala-Thr was inserted between Ala^{182} and Glu^{183} in the H+89 construct. Moreover, to introduce an additional N-glycosylation site (Figure 5, A and C), a sequence encoding Ala-Gln-Gln-Asn-Ser-Thr-Ala-Ala-Ser was inserted between Ser^{169} and Ala^{170} (*Aor*51HI site) just following H-segments. Site-directed mutagenesis was performed by a Kunkel (Kunkel, 1985), QuikChange (Agilent, Santa Clara, CA)–like, or inverse PCR–related method. All of the constructed DNAs were confirmed by DNA sequencing.

In vitro transcription, translation, proteinase K treatment, and SDS–PAGE

For synthesis of full-length mRNAs coding RSA^{320}+H and RSA$^{320}\Delta^C$+H proteins, plasmids were linearized by *Bam*HI in pRcCMV. For synthesis of truncated mRNAs, plasmids were linearized by *Bam*HI at several positions or *Pma*CI just upstream of the termination codon. Template DNAs were transcribed with T7 RNA polymerase (TakaraBio, Kusatsu, Japan) as previously described (Takahara *et al.*, 2013). mRNAs were translated in a reticulocyte lysate cell–free system for 1 h at 30°C in the absence or presence of dog pancreatic RM (Sakaguchi *et al.*, 1992) and an N-glycosylation acceptor peptide (*N*-benzoyl-Asn-Leu-Thr-*N*-methylamide). Preparation of rabbit reticulocyte lysate was performed as previously described (Jackson and Hunt, 1983). RM was prepared, extracted with EDTA, and treated with *Staphylococcus aureus* nuclease (Roche, Basel, Switzerland) as previously described (Walter and Blobel, 1983). The translation reaction contained 32% reticulocyte lysate, 80 mM potassium acetate, 1.0 mM magnesium acetate, 20 kBq/μl EXPRESS protein-labeling mix (PerkinElmer, Waltham, MA), and 20 μg/ml castanospermine (Merck, Darmstadt, Germany) to simplify the mobility of N-glycosylated proteins on SDS–PAGE by inhibition of trimming of N-glycans in the ER lumen. After translation, samples were diluted with a 10-fold volume of dilution buffer (30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]/KOH, pH 7.4, 150 mM potassium acetate, 2 mM magnesium acetate) and centrifuged at 100,000 × *g* for 5 min at 4°C. In Figures 1 and 5, after the translation, the mixtures were treated in the presence of 0.1 mg/ml proteinase K and 250 mM sodium chloride on ice for 1 h. After the proteinase K digestion, the mixtures were diluted with a 20-fold volume of dilution buffer containing 1 mM phenylmethanesulfonylfluoride, and membrane fractions were isolated by centrifugation at 100,000 × *g* for 5 min and solubilized with sample buffer for SDS–PAGE. Radiolabeled proteins synthesized in vitro were analyzed by SDS–PAGE and visualized on a Bioimage Analyzer Typhoon FLA 7000 (GE Healthcare, Uppsala, Sweden). Quantification of gel images was performed using ImageQuant TL software, version 7.0 (GE Healthcare).

Two-step Cys alkylation of one-Cys proteins

For modification of Cys residues in H-segments with NEM, translation mixtures were incubated with 1 mM cycloheximide or puromycin at 30°C for 10 min and further incubated in the presence of 10 mM NEM at 15°C for 1 h. NEM treatment was quenched by adding a 10-fold volume of dilution buffer containing 5 mM dithiothreitol (DTT) and further incubation for 10 min. To detect whether each Cys residue was blocked with NEM, membrane fractions were sedimented by centrifugation at 100,000 × *g* for 5 min at 4°C, solubilized with lysis buffer (50 mM Tris/HCl, pH 8.5, 2% SDS, 2 mM Tris[2-carboxyethyl]phosphine hydrochloride), and incubated in the absence or presence of 10 mM mPEGmal, 2 kDa (Creative PEGWorks, Chapel Hill, NC) at 37°C for 1 h. For quenching the PEGmal modification, the mixtures were incubated with 30 mM DTT. To remove N-glycans from the proteins, mixtures were treated with endoglycosidase H_f (New England Biolabs, Ipswich, MA) at 37°C for 1 h in accordance with the manufacturer's instructions.

Chemical cross-linking and immunoprecipitation

After translation, the mixtures were treated with 10 mM BME (Tokyo Chemical Industry, Tokyo, Japan) on ice for 60 min. Cross-linking reactions were quenched by dilution with a 10-fold volume of dilution buffer containing 10 mM DTT and incubated on ice for 15 min. Membrane fractions were sedimented by centrifugation at 100,000 × *g* for 5 min. For immunoprecipitation, isolated membranes were solubilized with 2% SDS at room temperature and then diluted with a 20-fold volume of immunoprecipitation buffer (1% Triton X-100, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl). The solutions were incubated for 1 h with protein G–Sepharose (GE Healthcare) to remove materials nonspecifically bound to the resin. The unbound fractions were incubated for 1 h with anti-Sec61α antiserum and overnight with protein G–Sepharose. The resin was washed once with immunoprecipitation buffer and then extracted with the SDS–PAGE sample buffer.

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