Signal Anchor Sequence Provides Motive Force for Polypeptide Chain Translocation through the Endoplasmic Reticulum Membrane

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Many proteins are translocated across and integrated into the endoplasmic reticulum membrane. The type I signal anchor sequence mediates the translocation of its preceding region through the endoplasmic reticulum membrane, but the source of the motive force has been unclear. Here, we characterized the motive force for N-terminal domain translocation using two probes. First, an Ig-like domain of the muscle protein titin (I27 domain) or its mutants were fused to the N termini, and translocation was examined in a cell-free translation system supplemented with rough microsomal membrane. The N-terminal translocation efficiencies correlated with the mechanical instabilities of the I27 mutants. When the I27 domain was separated from the signal anchor sequence by inserting a spacer, even the most unstable mutant stalled on the cytoplasmic side, whereas its downstream portion spanned the membrane. Proline insertion into the signal anchor sequence also caused a large translocation defect. Second, a streptavidin-binding peptide tag was fused to the N terminus. Titration of streptavidin in the translocation system allowed us to estimate the translocation motive force operative on the tag. The motive force was decreased by the proline insertion into the signal anchor sequence as well as by separation from the signal anchor sequence. When the streptavidin-binding peptide tag was separated from the signal anchor, the proline insertion did not induce further deficits in the motive force for the tag. On the basis of the findings obtained by using these two independent techniques, we conclude that the signal sequence itself provides the motive force for N-terminal domain translocation within a limited upstream region.

The majority of membrane proteins and luminal soluble proteins in the secretory pathway are synthesized on the rough endoplasmic reticulum and cotranslationally inserted into and translocated across the membrane. Ribosomes synthesizing polypeptide chains with a signal sequence are targeted to the endoplasmic reticulum via the signal recognition particle and its receptor system (1). On the endoplasmic reticulum membrane, the signal sequence is released from the particle and then recognized by the protein translocation machinery, the so-called translocon (2, 3). The translocon is composed of several copies of Sec61 complexes (in eukaryotes) or SecY complexes (in bacteria). The crystal structure of the archaeal SecY complex indicates that the 10 transmembrane (TM) segments of a single SecY molecule form a narrow pore through the membrane, and the pore allows for a lateral exit toward the lipid environment (4). The signal recognition pocket is located at the lateral exit site. The polypeptide chain of the secretory protein is translocated through the pore (5). On the other hand, a large aqueous environment of the translocon has been observed (6). Moreover, the translocon possesses such remarkable flexibility that two translocating hydrophilic polypeptide chains can be simultaneously accommodated (7). The dynamics of the Sec61/SecY channel and cooperation of the channels within the oligomeric form, however, remain to be solved (8).

Signal sequences trigger translocation of either the N-terminal or C-terminal flanking portion through the channel. The type II signal anchor sequence and signal peptide mediate translocation of their downstream portion, whereas the type I signal anchor (SA-I) sequence mediates translocation of the N-terminal domain (N-domain) (9). Translocation of the portion downstream from the type II signal anchor sequence and signal peptide is simply explained as the ongoing protein elongation pushing the polypeptide chain through continuous ribosome and translocon tunnels. In contrast, translocation of the upstream portion of SA-I sequences cannot be driven by polypeptide chain elongation, and therefore another mechanism must pull the upstream portion. Previous analyses of the translocation of the large N-domain, including the dihydrofolate reductase domain, indicated that the SA-I sequence mediates translocation, even of large N-terminal domains that are greater than 200 residues (10). Translocation is blocked by a specific ligand (methotrexate), indicating that the N-domain is significantly folded before translocation. The N-domain translocation requires neither nucleotide triphosphates nor the luminal chaperone BiP. Despite this lack of requirements, long domain translocation occurs only while the nascent chain is maintained in the ribosome. The ribosome maintains a productive state of nascent chain and translocon. These results led us to the idea that motive force is provided by the SA-I sequence

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‡The abbreviations used are: TM, transmembrane; H-segment, hydrophobic segment; N-domain, N-terminal domain; RM, rough microsomal membrane; SA-I, type I signal anchor; SBP, streptavidin-binding peptide; SAw, streptavidin; SytII, synaptotagmin II.
Analysis of the energetics of N-domain translocation by the SA-I sequence will provide insight into the function of the signal sequence during protein translocation, i.e. whether the signal sequences only trigger targeting and translocation of the polypeptide chain or whether they also contribute to generate the motive force required for the translocation. If it is the latter case, motive force should be dependent on the signal sequence itself and on the distance between the signal sequence and the passenger domain. To address these questions, quantitative analysis of the translocation motive force is essential.

Here, we examined the motive force for N-domain translocation using the N-terminally fused titin 27th immunoglobulin domain (I27 domain) and streptavidin (SAv)-binding peptide (SBP) tag as passenger probes. The I27 domain consists of 89 amino acid residues and is a constituent of the huge elastic protein cardiac muscle titin. Wild-type and mutant I27 domains have been extensively characterized with regard to their mechanical stability, and their unfolding forces have been estimated by atomic force microscopy (11). Their mechanical unfolding requires a force of more than 200 piconewtons. The two potential sites (filled circles) are glycosylated (open circles) in the lumen of the endoplasmic reticulum membrane. The SA-I sequence strokes into translocon at the initiation stage, and after that the following polypeptide chain slides forward at the progressive stage. The truncated mRNAs were translated in a cell-free system in the presence of RM for 1 h, and then the reaction was chased for the indicated time period in the presence of cycloheximide. Diglycosylated (double-open circles) forms, monoglycosylated (single-open circles) forms, and non-glycosylated (arrowheads) forms are observed. Each form was quantitated, and the diglycosylation efficiency was calculated. Unfolding forces (piconewtons (pN)) of the I27 domain estimated by atomic-force microscopy are indicated. WT, wild-type.

For I27-Syt200 series constructs (see Fig. 1), the DNA fragments encoding Met plus the I27 domain (HindIII/EcoRI), the glycosylation sequence (KLNSTAT; MfeI/EcoRI), synaptotagmin II (SytII) (EcoRI/XbaI), and pRcCMV vector (HindIII/XbaI) were ligated. For I27(V13P)-X-Syt200 series model proteins (see Fig. 2), a DNA fragment (MfeI/EcoRI) encoding the 38-residue spacer sequence was derived from 627–662 of human anion exchanger 1 (ELT627YTQKLSVPDGFKVSNSAARGWVIHPLGLRSEFPIW662). The junction sequence of the SA-I sequence. We concluded that the signal sequence provides the motive force for protein translocation as well as targets the nascent chain to the translocon and that the motive force is effective within a limited region.

**EXPERIMENTAL PROCEDURES**

**DNA Manipulation**—The I27 domain of human cardiac muscle titin and its mutants were described previously (11). The plasmids were kindly provided by Dr. Satô (13), with permission from Dr. Fernandez. DNA fragments encoding the I27 domains (Leu1–Leu89; HindIII/EcoRI) were obtained by polymerase chain reaction with the following primers: CCCCCAGCTTGGCCACC-ACTGTAATAGAAGTGGAAA-GCCTC (with the HindIII site, Kozak sequence, and initiation Met underlined) and CCGGAAT-TCCACATTCTTTACCTTCAGATTGG (with the EcoRI site underlined). In the following DNA construction procedures, the indicated DNA fragments were taken into account when numbering the residues indicated in the figures.

For I27-Syt200 series constructs (see Fig. 1), the DNA fragments encoding Met plus the I27 domain (HindIII/EcoRI), the glycosylation sequence (KLNSTAT; MfeI/EcoRI), synaptotagmin II (SytII) (EcoRI/XbaI), and pRcCMV vector (HindIII/XbaI) were ligated. For I27(V13P)-X-Syt200 series model proteins (see Fig. 2), a DNA fragment (MfeI/EcoRI) encoding the 38-residue spacer sequence was derived from 627–662 of human anion exchanger 1 (ELT627YTQKLSVPDGFKVSNSAARGWVIHPLGLRSEFPIW662). The junction sequence of EcoRI and MfeI is underlined, and the silenced endogenous glycosylation site is double-underlined. Its derivative sequence (X = 10, ELT627YTQKLSVPDGFKVSNSAARGWVIHPLGLRSEFPIW662) was inserted between the glycosylation sequence and SytII. For monoglycosylation constructs (see Fig. 2), either glycosylation site (a or b) was silenced by a point mutation using the QuickChange method and the method of KunVel (15). For proline insertion mutations (see Fig. 3), the proline codon was inserted after residues Ala67, Leu86, Phe84, and Cys85 of the SytII sequence. The SBP tag-SytII fusion constructs (see Fig. 4) were described previously (7). Pro insertions...
of the SBP-fusion model proteins (see Fig. 4) were made by point mutations.3

In Vitro Translation and Translocation—In vitro protein synthesis and translocation experiments were performed essentially as described previously (10). A truncated RNA encoding protein sequence up to Arg200 of SytII was obtained by in vitro transcription using plasmids linearized with AflII. The mRNAs were translated with a reticulocyte lysate cell-free system in the presence of rough microsomal membrane (RM) for 60 min at 25 °C. After translation was terminated by 2 mM cycloheximide, the reaction mixture was further incubated in the presence of cycloheximide for the indicated periods. Monoglycosylated (single-open circles) forms, diglycosylated (double-open circles) forms, and non-glycosylated (arrowheads) forms are observed. The diglycosylation efficiency was calculated. C, similar experiments were performed using model proteins in which either potential a-site or b-site was silenced. The available glycosylation site of each construct is indicated in the panel. ao, amino acid.

detected with an image analyzer (Fuji Bioimage analyzer, BAS1800). Diglycosylation (percent) was calculated using the following formula: (diglycosylated form) × 100/[(non-glycosylated) + (monoglycosylated) + (diglycosylated)].

RESULTS AND DISCUSSION

Translocation Efficiency and Mechanical Instability of the I27 Domain—To evaluate the motive force for N-domain translocation, the titin I27 domain or its mutant in which a single amino acid residue in the N-terminal portion was exchanged with Pro was fused to the N terminus of the SA-I sequence derived from SytII (Fig. 1). In the model protein, the hydrophilic N-domain consisted of the 90-residue I27 domain and a 70-residue hydrophilic segment (under “Experimental Procedures”). The hydrophilic segment between the I27 domain and the SA-I sequence contained two glycosylation sites, which are indicators of N-domain translocation. N-domain translocation was assessed in a cell-free translation system of reticulocyte lysate supplemented with RM. For the analysis, we used truncated mRNA encoding up to Arg200 of SytII (Fig. 1). When the mRNA was translated, the nascent polypeptide chain emerging from the ribosome was targeted to the membrane and was retained in the ribosome as a peptidyl-tRNA to form a ribosome nascent chain complex even after elongation was completed. After translation in the presence of RM for 1 h, the glycosylation level of the nascent polypeptide chain was not very high (Fig. 1C, lanes 1, 5, 9, 13, and 17). Further incubation in the presence of cycloheximide, a polypeptide chain elongation inhibitor, shifted the products up on the SDS-PAGE, indicating that the products were gradually diglycosylated during the chase period (Fig. 1C). The nascent chain is maintained in a translocation-competent state as long as it is retained in the ribosome (10). When the chain was released from the ribosome by a termination codon or with puromycin, the I27 domain translocation was not chased (data not shown). The translocation efficiency correlated with the mechanical instability of the I27 mutations that were reported by Li et al. (11). Two mutants with a lower unfolding force (V11P and V13P) were efficiently diglycosylated. In these cases, few monoglycosylated intermediates were observed, indicating that the two sites were simultaneously exposed to the oligosaccharyltransferase in the lumen. It is also indicated that both glycosylation sites were

3 The primer sequences are available upon request from the author.
Signal Sequence-mediated Protein Translocation

A

Pro-1
Pro-2
Pro-3
Pro-4

NEINKELPFPWAMAYVAGLLLTCYICICKC

Hydrophobic Segment of Syt II

B

WT
Pro-1
Pro-2
Pro-3
Pro-4

Chase (h)
0
0.5
1
1.5
2

Figure 3. Proline insertion into the H-segment decreases translocation of the I27 domain. A, a proline residue was inserted into the indicated position of the SA-I sequence of the I27-Syt200 construct (Pro-1, Pro-2, Pro-3, and Pro-4). B, the Pro-inserted mutants were translated as described in the legend for Fig. 1. Monoglycosylated (single-open circles) forms, diglycosylated (double-open circles) forms, and non-glycosylated (arrowheads) forms are observed. The diglycosylation efficiencies were calculated. C, the I27 domain of each construct was exchanged with the SBP tag sequence. WT, wild-type.

Efficient substrates of the oligosaccharyltransferase in the lumen. On the other hand, the Y9P mutant and wild-type I27 showed lower translocation efficiencies and gave a small but significant amount of monoglycosylated intermediates during the chase, indicating that the movement of the polypeptide chain was slower than that of the other mutants.

The titin I27 domain has seven β-strands, and the first and last ones interact with each other to form a parallel pair. In unfolding analysis by atomic force microscopy, each point mutation in the first strand (V11P, V13P, and V15P) led to unfolding with a lower mechanical force, indicating that dissociation of the interaction between the first and the last strands is the rate-limiting step of its mechanical unfolding. The translocation efficiencies of I27 domain mutants correlated with their mechanical unfolding properties, suggesting that breaking the β-strand pair is the rate-limiting step of the global unfolding and the following translocation. N-domain translocation starts with entry of the SA-I sequence into the translocon. In the case of longer N-domains, the hydrophobic segment (H-segment) of the SA-I sequence acquires the TM orientation before the N-domain is completely translocated (7, 10). It is also possible that the stroke of the N terminus of the SA-I sequence across the membrane traps the last β-strand detached from the first strand in the translocon and leads to unfolding of the N-domain. After the initiation stage, further movement of the polypeptide chain should be possibly driven by lumenal chaperones and/or diffusion of TM segment in the lipid environment during the progression stage. These findings indicate that the folded N-terminal I27 domain functions as a resistor against polypeptide chain movement and that the unfolding of the I27 domain is the rate-limiting step of N-domain translocation.

In the presence of the spacer sequence, the second glycosylation site (b-site) was initially glycosylated. The b-site glycosylation may prevent the polypeptide chain from sliding back so that it eventually stimulates translocation as a molecular ratchet. To examine the contribution of b-site glycosylation to I27 domain translocation, the b-site was silenced by a point mutation (Fig. 2C). The a-site glycosylation of the X = 0 molecule occurred with similar efficiency as the diglycosylation. The a-site glycosylation of the X = 20 molecule also occurred with essentially the same time course as diglycosylation of the original molecule, indicating that the intermediate monoglycosylated form of the X = 20 molecule was due to glycosylation of the b-site. In both cases, silencing of the b-site had little effect on the N-domain translocation (a-site glycosylation) rate. Thus, the ratchet function of the b-site glycosylation is not a major determinant of N-domain translocation.

In the absence of the second site (b-site), X = 30 and X = 38 molecules were little glycosylated (Fig. 2C, lanes 12 and 16), confirming that only the second glycosylation site (b-site) is accessible to the oligosaccharyltransferase and that the I27 domains are on the cytoplasmic side of the membrane. Actually, the b-site of the X = 38 molecule was quite efficiently glycosylated (Fig. 2C, lanes 17–20). Thus, in the presence of longer spacers (X = 30 and 38), the b-site was rapidly translocated to the lumen of the RM, whereas the a-site was not. In these cases, polypeptide chain movement was blocked by the I27 domain. The hydrophilic segment between the I27 domain and the SA-I sequence spanned the membrane; the I27 domain could be unfolded and translocated through the translocon only when it was placed near the SA-I sequence.
Even the most unstable V13P mutant cannot be translocated in the presence of a long spacer. The N-domain translocation of the $X = 38$ molecule was initiated more rapidly than the molecule without a spacer. Thus, translocation is stalled at the I27 domain, and only the monoglycosylated form was observed. In this case, the unfolding of the I27 domain was not the rate-limiting step of the initiation stage, but it readily arrested the progression stage. On the other hand, in the absence of a spacer, it is likely that the unfolding of the I27 domain was a limiting step of the translocation initiation. Once the translocation was triggered, however, the polypeptide chain movement readily progressed up to the N terminus so that the monoglycosylated intermediate was not detected (Fig. 2B).

We conclude that the motive force for the polypeptide movement is not uniform during translocation; the motive force for the initiation stage is greater than that for the progression stage. When the SA-I sequence enters into the membrane and forms a TM helix (initiation stage), it generates a motive force sufficient for the unfolding of the I27 domain. Thereafter, once it forms the TM helix, the motive force operative on the I27 domain decreases (progression stage). The stroke of the H-segment into the translocon and the partition of the H-segment into the hydrophobic environment of lipid likely provide free energy for the motive force. The unfolding of the tightly folded I27 domain can be achieved only by coupling with the TM helix formation at the initiation stage.

Proline Insertion into the Hydrophobic Segment Decreases the Translocation Motive Force—To evaluate the possibility that the signal sequence provides the motive force for N-domain unfolding and translocation, we next examined the effect of altering the SA-I sequence on the motive force. To perturb the helicity and hydrophobicity of the SA-I sequence, a single proline residue was inserted into various sites of the H-segment (Fig. 3A). The Pro insertions induced a large decrease in the I27(V13P) domain translocation (Fig. 3B). We then examined the N-domain translocation in the absence of the resistor domain. For the purpose, the I27(V13P) domain was replaced with SBP tag of 38 residues, which gave no affect on the N-domain translocation of the SytII in the absence of SAv (7). The Pro insertions, except Pro-2 mutant, did not induce a significant decrease of the SBP-tagged N-domain translocation (Fig. 3C). The Pro insertion does not dramatically affect the targeting function of hydrophobic domain either. The N-domain translocation was affected by the Pro insertion only in the presence of the I27 resistor domain, strongly suggesting that the Pro insertions into the H-segment partially decreased the translocation motive force, whereas the Pro-inserted SA-I can still drive the SBP-tagged N-domain. The signal sequence is involved not only in targeting to the translocon but also in generating the translocation motive force.

Motive Force for Translocation Estimated by SBP Tag—To more quantitatively assess the motive force, we took advantage of affinity tag trapping of the N-domain translocation (Fig. 4). The 38-amino acid SBP tag was fused to the N terminus of the wild-type (WT), and Pro-3 mutants. The constructs were translated, and diglycosylation efficiencies were calculated (Fig. 4A). The truncated mRNA of the SBP tag fusions were translated as described in the legend for Fig. 1 in the presence of the indicated concentrations of SAv. The diglycosylation efficiencies were calculated. C, a spacer sequence of 38 residues was inserted into the wild-type (WT), and Pro-3 mutants. The constructs were translated, and diglycosylation efficiencies were calculated.
Pro-4 mutant was inhibited by a 10-fold lower concentration of SAv compared with the original construct. Moreover, the Pro-1 and Pro-3 mutants of the SA-I sequence were more sensitive than the Pro-4 mutant. This finding clearly demonstrated that the Pro-inserted H-segment was correctly targeted and that the SA-I sequence, N-domain translocation became more sensitive to the SAv concentration, and the translocation intermediate was monoglycosylated (Fig. 4C). Translocation was stalled by the SBP tag bound to SAv, and the downstream portion spanned the membrane through the translocon channel as observed for the I27 domain with the 38-residue spacer (Fig. 4A). Interestingly, the Pro insertion caused no additional defect in the spacer construct. It was therefore demonstrated that polypeptide chain movement in the progressive stage does not depend on the SA-I sequence. The motive force induced by the SA-I sequence is not operative on the SBP tag portion in the distal position. We conclude that the motive force caused by the SA-I sequence can be effective only within a limited portion.

Conclusion—Using two independent probes, the I27 domain mutants and SBP tag, we evaluated motive force for N-domain translocation. When the probes were proximal to the SA-I sequence, their translocation was more strongly driven than when they were distal to the SA-I sequence. Insertion of a Pro residue into the SA-I sequence decreased the motive force. When the SBP tag probe was separated from the SA-I sequence, Pro insertion no longer affected translocation of the probe. These observations indicate that the SA-I sequence not only targets the ribosome-nascent chain complex to the translocon but also provides the motive force for the polypeptide chain movement. The SA-I sequence-mediated motive force is operative only within a limited region. Furthermore, it generates the motive force only during the initiation stage.

At the initiation stage, the SA-I sequence is recognized by the translocon and triggers translocation of the N-domain. It is possible that the motive force for the movement is generated by the stroke of the TM segment into the translocon and decreased after it forms the TM topology. The stroke of the H-segment into the translocon or membrane might drive the polypeptide chain movement as a power stroke. Alternatively, the movement might be driven by Brownian movement of the polypeptide chain, and the TM segment formation of the SA-I sequence might be a ratchet. In both hypotheses, the motive force is likely dependent on the free energy of the hydrogen bonds of the TM helix formation and the hydrophobic interaction between the signal sequence and the lipid environment. Some C-terminal tail anchor sequences can mediate translocation of downstream sequences of more than 80 residues through the liposome membrane, even in the absence of the translocon (14), suggesting that partition of the TM sequence into a lipid environment may generate the free energy for the motive force of the 80-residue hydrophilic domain. Because the SA-I sequence of SytII is more hydrophobic than the tail anchor segments, it may be sufficient for translocation of N-terminal probes. Insertion of the Pro residue decreased hydrophobicity and/or α-helicity and led to a weaker interaction between the H-segment and the lipid. It may cause a defect in the exit from the translocon to the lipid environment. Furthermore, the motive force might be dependent on the kinetics of particular processes: TM helix formation, partition of the TM segment into membrane, and lateral gating of the translocon pore. Although further physicochemical studies are required to address these possibilities, the experimental system developed here would provide a powerful system for these analyses.

After the TM conformation is achieved, the helix might function as a ratchet to fix the translocating polypeptide chain, and then the ongoing translocation occurring far from the signal sequence would not be affected by the Pro insertion. After that, several candidate energy-driving sources might be supposed in the progression stage, such as the ratchet and/or power stroke of the luminal chaperone molecules and lateral diffusion of the TM segment into the lipid environment. In any case, those driving-force sources are clearly lower than that of the initiation stage, at which the SA-I sequence generates the motive force.

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