Membrane Topogenesis of a Type I Signal-Anchor Protein, Mouse Synaptotagmin II, on the Endoplasmic Reticulum

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Abstract. Synaptotagmin II is a type I signal-anchor protein, in which the NH_2-terminal domain of 60 residues (N-domain) is located within the lumenal space of the membrane and the following hydrophobic region (H-region) shows transmembrane topology. We explored the early steps of cotranslational integration of this molecule on the endoplasmic reticulum membrane and demonstrated the following: (a) The translocation of the N-domain occurs immediately after the H-region and the successive positively charged residues emerge from the ribosome. (b) Positively charged residues that follow the H-region are essential for maintaining the correct topology. (c) It is possible to dissect the lengths of the nascent polypeptide chains which are required for ER targeting of the ribosome and for translocation of the N-domain, thereby demonstrating that different nascent polypeptide chain lengths are required for membrane targeting and N-domain translocation. (d) The H-region is sufficiently long for membrane integration. (e) Proline residues preceding H-region are critical for N-domain translocation, but not for ER targeting. The proline can be replaced with amino acid with low helical propensity.

Key words: membrane topology • membrane protein • signal-anchor sequence • protein translocation • translocon

Introduction

Synaptotagmin II (Syt II)[1] is an integral membrane protein which exists mainly on the synaptic vesicles in brain and is thought to be involved in Ca^{2+} regulated exocytosis (Sudhof and Rizo, 1996; Fukuda and Mikoshiba, 1997; Schiavo et al., 1998; Osborne et al., 1999). It is composed of three domains: the NH_2-terminal hydrophilic domain of 60 amino acid residues (N-domain), the hydrophobic transmembrane region (H-region), and the following COOH-terminal domain containing two C2-domains (C-domain). Based on its similarity with Syt I, Syt II has been assumed to show N_{exo}/C_{cyt} topology, in which the N-domain is in the lumenal space of the membrane and the following C-domain is on the cytoplasmic side (Geppert et al., 1991; Fukuda et al., 1994; Perin et al., 1991).

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[1]*Abbreviations used in this paper*: C-domain, COOH-terminal domain; EndoH, endoglycosidase H; H-region, hydrophobic region; MBS, m-maleimidobenzoyl-N-hydroxysuccinimide ester; N-domain, NH_2-terminal domain; RM, rough microsomal membrane; SA-I, type I signal-anchor; SA-II, type II signal-anchor; SP, signal peptide; SRP, signal recognition particle; Syt, synaptotagmin.

Almost all the membrane proteins on the secretory pathway are cotranslationally integrated into the ER membrane and are sorted to final destinations via vesicular transport. As the Syt II molecule is transported from ER to the synaptic vesicles, it acquires various modifications; e.g., multiple-palmitoylation (Chapman et al., 1996; Veit et al., 1996), the conversion of the N-linked sugar chain to a complex form, and O-linked glycosylation (Kida, Y., manuscript in preparation; Fukuda, M., manuscript in preparation). The integration of membrane proteins into the ER membrane is initiated by the signal sequences of nascent polypeptides. The signal sequence is recognized by the signal recognition particle (SRP), which targets the ribosomes to the ER membrane. It is released from the SRP by the function of the SRP receptor on the ER membrane, then the sequence enters into the membrane (Walter and Johnson, 1994). The membrane integration processes proceed via the protein translocation channel (so-called translocon), whose minimum components have been elucidated to be the Sec61p-complex and the translocating chain-associating membrane protein (Matlack et al., 1998).

The signal sequences have been classified into three categories: signal peptide (SP), and type I and type II signal-

anchor sequences (SA-I and SA-II sequences, respectively). SP and the SA-II sequence mediate the translocation of their COOH-terminal side resulting in N_{cyt}/C_{exo} orientation, while the SA-I sequence mediates the translocation of its NH_2-terminal portion resulting in N_{exo}/C_{cyt} orientation (High and Laird, 1997; Sakaguchi, 1997). SP is cleaved off by the signal peptidase in the lumenal side, whereas the SA sequences are not cleaved off, and remain responsible for membrane anchoring. The unique topogenic function of the SA-I sequence has been demonstrated in various membrane proteins: in the superfamily of microsomal cytochrome P450, in the NADPH cytochrome P450 reductase (Kida et al., 1998), in various virus proteins (Parks and Lamb, 1993), and in the NH_2-terminal portions of G-protein–coupled receptor proteins (Wallin and von Heijne, 1995). Furthermore, internal SA-I sequences take part in the various situations of integration of multispanning membrane proteins (Ota et al., 1998a,b).

Signal sequences basically consist of three distinct regions: the NH_2-terminal hydrophilic region, the central hydrophobic region (H-region), and the COOH-terminal polar region (von Heijne, 1990). The ER targeting function of the signal sequences is primarily determined by the H-region, and the orientation is affected by the flanking regions. Statistically, the H-regions of signal-anchor sequences (17–27 residues) are longer than those of signal peptides (7–14 residues; Nilsson et al., 1994). The long H-region tends to pull the NH_2-terminal portion. Positively charged residues in the NH_2-terminal flanking region and tight folding of the region prevent the translocation of the NH_2-terminal side, so that the following COOH-terminal side of the H-region should be translocated through the membrane (Sakaguchi et al., 1992b; Sakaguchi, 1997; Wahlberg and Spiess, 1997). Growing polypeptide chains following the SP and SA-II sequences enter into the translocon directly from the ribosomes. In contrast, the NH_2-terminal region of the SA-I sequence is translocated after completion of the synthesis. We herein focus on the unique process of posttranslational translocation of the NH_2-terminal region during integration of the SA-I sequence.

In this study, using the mouse Syt II as a model of SA-I proteins, we explored the early process of membrane integration and estimated the minimum length of the nascent polypeptide chain whose N-domain is translocated through the membrane. We demonstrated that the membrane targeting of the ribosome and translocation of the N-domain proceed at somewhat different chain lengths and that positive charges at the end of the H-region play a critical role in the translocation. We further suggest that the proline residues between the N-domain and the H-region play a key role in the translocation.

Materials and Methods

Materials

The preparations of rough microsomal membrane (RM; Walter and Blobel, 1983) and rabbit reticulocyte lysate (Jackson and Hunt, 1983) were carried out as previously described. RM was extracted with EDTA and treated with *S. aureus* nuclease (Roche) as described (Walter and Blobel, 1983). The plasmid DNAs were prepared using Wizard plus mini-preps (Promega) from 2 ml overnight cultures of *E. coli.* Cycloheximide and puromycin were purchased from Sigma.

Rabbit antiserum specific for the C-domain (anti–C-domain antibody) was raised against the C2A-domain (amino acids 139–267) as previously described (Fukuda et al., 1994). Antibody specific for N-domain (anti–N-domain antibody) was raised against synthetic peptide as follows: a peptide corresponding to the NH_2-terminal 20 amino acids of mouse Syt II (MRNIFKRNQEPNVAPATTTAC) was coupled to keyhole limpet hemocyanin using the COOH-terminal artificial cysteine residue. Rabbit antiserum to this peptide were prepared at Quality Controlled Biochemicals, Inc. The antibody was partially purified by ammonium sulfate fractionation as described previously (Fukuda and Mikoshiba, 1999) and then affinity-purified by exposure to antigenic peptide bound to FMP activated-cellulofine beads according to the manufacturer's instructions (Seikagaku Co.). Antiserum for dog Sec61α was raised against the peptide corresponding to the NH_2-terminal 15 amino acid residues.

Construction of Expression Plasmid

cDNA coding for mouse Syt II was isolated from the plasmid, pBluescript-Syt II (Fukuda et al., 1994) by polymerase chain reaction using the following primers: 5′-TCGGAAGCTTCCTCTGCCACCATGAGAA-3′ (initiation codon of Syt II is underlined) and 5′-TAGCTCTAGATCACGTCAGTGTCC-3′. The obtained DNA fragment was digested with XbaI and HindIII, and was then subcloned between the HindIII and XbaI sites of pRc/CMV (Invitrogen) to obtain pSytII-03.

The N-glycosylation consensus site in the N-domain was disrupted as shown in Fig. 1 to obtain pSytII-NG. AflII sites were generated at various positions as indicated in Fig. 2. Positively charged residues flanking the COOH terminus of the H-region were changed as shown in Fig. 3. Deletions and mutations of the H-regions were made as shown in Fig. 4. Proline residues between the N-domain and the H-region were changed as indicated in Figs. 5 and 6. Deletion mutants within the N-domain were made as shown in Fig. 6. To obtain Δ1-21, a DNA fragment encoding the sequence from Met22 to the termination codon (HindIII/XbaI) of Syt II was amplified by PCR, and subcloned into pRc/CMV (HindIII/XbaI). Other site-directed mutagenesis was performed according to the method of Kunkel (Kunkel, 1985). All the mutants were screened by restriction enzyme mapping and confirmed by DNA sequencing. The sequences of oligonucleotides used and full construction details for each plasmid are available from the authors.

In Vitro Transcription, Translation, Enzyme Treatments, and Immunoprecipitation

Plasmids encoding the wild-type and mutated Syt II molecules were linearized by EcoRI and transcribed with T7-RNA polymerase (Toyobo/Takara) as previously described (Sakaguchi et al., 1992a). The obtained mRNAs were translated in the reticulocyte lysate cell-free system either in the absence or presence of RM. The translation reaction contained 100 mM KOAc, 1.0 mM $Mg(OAc)_2$, 32% reticulocyte lysate, and 15.5 kBq/μl EXPRESS protein-labeling mix (NEN). Aliquots of the translation mixture were treated by endoglycosidase H (EndoH; NEB) at 37°C for 60 min under denaturing conditions according to the manufacturer's procedures. Other aliquots were treated by 0.2 mg/ml proteinase K (Merck) in the presence or absence of 1% Triton X-100 for 40 min at 10°C. After the incubation, the reactions were terminated with 5% trichloroacetic acid.

For immunoprecipitation, the proteinase K reaction was terminated with 20 mM phenylmethylsulfonylfluoride for 10 min at 10°C. Then the reaction mixture (20 μl) was treated with 1% SDS at 96°C for 5 min and then diluted to 1 ml with IP buffer (1% Triton X-100, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 2 mM phenylmethylsulfonylfluoride). After insoluble materials were removed by centrifugation, the solution was incubated once for 30 min with protein A–Sepharose (Amersham-Pharmacia) alone, and then unbound fraction was incubated with domain specific antibodies and protein A–Sepharose at 4°C for 120 min. After the supernatant was removed, the protein A–Sepharose was washed twice with 1 ml of IP-buffer and then extracted with sample buffer for SDS-PAGE.

Plasmids for the truncated mRNAs were linearized by AflII and were then subjected to in vitro transcription. The obtained mRNAs were translated in the reticulocyte lysate cell-free system either in the absence or presence of RM for 20–30 min according to the length. To stabilize the translation intermediate of nascent peptidyl-tRNA on ribosomes, the reaction was performed at 22°C as previously described (Borel and Simon, 1996). After the reaction was terminated by 2 mM puromycin or 2 mM cy-

cloheximide, the mixture was incubated further for 30 min to allow the membrane integration and glycosylation to be completed.

High Salt Extraction of RM

15-μl aliquots of translation reactions were diluted to 50 μl, which was finally adjusted to 500 mM KOAc, 5 mM $Mg(OAc)_2$, and 30 mM Hepes (pH 7.4). The mixture was then layered onto a cushion of 100 μl containing 0.5 M sucrose in the same buffer. After centrifugation for 5 min at 50,000 rpm (Hitachi RP100AT2 rotor), the membrane pellet (P) was dissolved directly in SDS-PAGE sample buffer. Proteins in the supernatant (S) were precipitated with 5% trichloroacetic acid.

Cross-linking and Immunoprecipitation

Cross-linking reaction with the hetero-bifunctional cross-linker, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce) was performed as follows: 1.3 μl of 100 mM MBS was added to a 25-μl translation mixture and incubated on ice for 2 h. The reaction was quenched by 0.1 M glycine and 5 mM 2-mercaptoethanol on ice for 10 min. The mixture was diluted to 500 μl with IP-buffer. After incubation at 4°C for 30 min, insoluble materials were removed by centrifugation. The following procedure was performed as described above section.

Alkaline Floatation

Membrane anchoring was examined by the alkaline floatation method essentially as described (Ota et al., 1998b). In brief, a 25-μl aliquot of translation mixture was added to 25 μl of 0.2 M Na_2CO_3 and incubated for 30 min on ice. This alkaline mixture (50 μl) was mixed with 90 μl 2.5 M sucrose and placed in a 500-μl ultracentrifugation tube. Then two sucrose layers, 150 μl 1.25 M and 150 μl 0.25 M were overlaid on the alkaline mixture. Both these sucrose cushions contained 0.1 M Na_2CO_3. After the tube was centrifuged at 355,000 *g* for 90 min (Hitachi RP100AT2 rotor), the layers of 0.25 M sucrose and 1.25 M sucrose were recovered as a membrane-bound fraction (M) and the bottom layer of 1.6 M sucrose was recovered as a soluble fraction (S). Proteins in each fraction were precipitated with trichloroacetic acid.

Expression of Mutated Syt II in COS7 Cells

The expression plasmids, pSytII-03, pSytII-NG, and pSyt-AAA were transfected into COS7 cells with Lipofect-AMINE (GIBCO BRL) according to the manual supplied by the manufacturer. In brief, 2 ml Opti-MEM (GIBCO BRL) containing plasmid DNA (4 μg) and Lipofect-AMINE (12 μl) were added to COS7 cells in a 60-mm well. After incubation for 5 h under 10% CO_2, the transfection mixture was removed and 4 ml DME (10% fetal calf serum) was added. After being cultured for 40–48 h, the cells were pulse-labeled with EXPRESS protein labeling mix (NEN) for 30 min and were then lysed with 1.5% SDS. Syt II protein was immunoprecipitated with anti–C-domain antibody as previously described (Anderson and Blobel, 1983). Aliquots of the immuno-isolated proteins were treated with EndoH.

Image Analysis

Proteins were analyzed by SDS-PAGE and visualized on a BAS-2000 or FLA-2000 PhosphorImager (Fuji). Quantification was performed using MacBAS software (Ver. 2.5.2, Fuji).

Results

N-Glycosylation of Mouse Syt II

There are four potential asparagine-linked glycosylation sites (-N-X-S/T-) in the mouse Syt II molecule. To confirm the lumenal location of the N-domain, the potential acceptor site in the N-domain was disrupted by a single amino acid substitution of T34A (Fig. 1 A). The mutated and wild-type Syt II molecules were expressed in the reticulocyte lysate cell-free system (Fig. 1 B). When the wild-type was synthesized in the absence of RM, a major band of 52 kD was detected (lane 1), whereas upon synthesis in the presence of RM it gave a larger band of 55 kD in addition to the 52 kD band (lane 2). The larger form disappeared by the treatment with EndoH (lane 3), indicating that the larger form is a glycosylated molecule. In contrast, the T34A mutant did not give a larger form (lanes 4 and 5), indicating that the point mutation causes a defect in glycosylation.

The wild-type and mutant forms were expressed in COS7 cells and pulse-labeled for 30 min, before being immunoprecipitated (Fig. 1 C). The wild-type molecule of 55 kD (lane 1) was shifted to 52 kD by the EndoH treatment (lane 2). In contrast, the 52 kD band of the T34A mutant was not affected by the treatment (lanes 3 and 4), indicating that the mutant was not glycosylated in the cultured cells, either. Under this labeling condition, only the core-glycosylated form was detected as a major molecular species and further modifications of Syt II required prolonged chase (data not shown). These data directly demonstrate that Asn32 is the sole glycosylation site within mouse Syt II and that the N-domain of 60 amino acid residues is located within the ER lumen.

These data are consistent with the report that Syt I, which shares >80% sequence identity with Syt II, is glycosylated within the N-domain (Perin et al., 1991). It has also been suggested from the sequence alignment that rat Syt II is glycosylated within the N-domain (Geppert et al.,

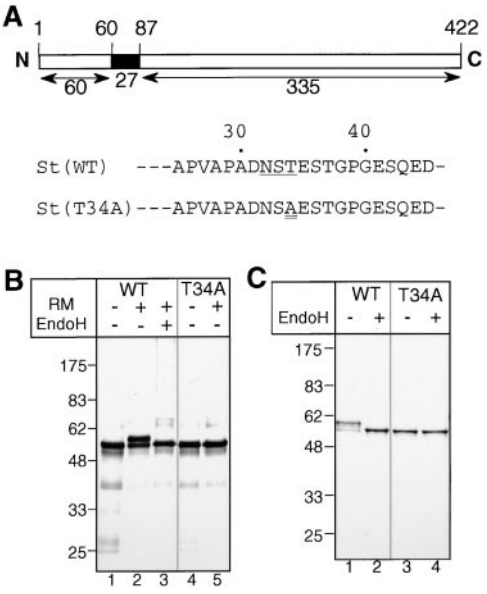

Figure 1. N-glycosylation of mouse Syt II. (A) Domain structure of mouse Syt II and amino acid sequences around the potential glycosylation site within the N-domain. The closed box indicates the H-region. The numbers indicate those of the amino acid residues. The glycosylation site (underlined) was disrupted by a single mutation of T34A (double underlined). (B) The wild-type and mutated molecules were expressed in the reticulocyte lysate cell-free system in the absence (−) or presence (+) of RM. After the translation reaction, an aliquot was treated with EndoH under the denaturing condition (+ lane). (C) Both molecules were expressed in COS7 cells. The cells were pulse-labeled and then the Syt II molecules were immunoprecipitated with rabbit antibody against the C-domain of Syt II. Aliquots of the immunoprecipitants were treated with EndoH (+ lanes).

1991). We used the mouse Syt II as a model of SA-I protein in the following experiments, since the N-domain is so long that the nascent polypeptide being inserted into the membrane can be readily detected.

Timing of Translocation of the N-Domain

To dissect the processes of the membrane targeting of ribosomes and the translocation of the N-domain, nascent Syt II-polypeptides of various lengths were synthesized in the cell-free system by translating truncated mRNAs (Fig. 2 A). In this system, the RNAs transcribed from truncated cDNA possess no in-frame termination codon, so that the nascent polypeptides are retained on ribosomes as peptidyl-tRNA. Each truncated molecule is named according to the number of amino acid residues (e.g., Syt-105). The truncated Syt-molecules gave major single bands (Fig. 2 B, lanes 1, 4, 7, and 10, upward arrowhead). When they were expressed in the presence of RM, the translation reaction was terminated with either cycloheximide or puromycin. Cycloheximide stabilizes the nascent polypeptide bound on the ribosome as peptidyl-tRNA, but puromycin mediates the release of the nascent polypeptide from the ribosome. Upon treatment with cycloheximide, Syt-105 was not glycosylated and Syt-120 was only slightly glycosylated (lane 5, downward arrowhead). In contrast, their glycosylation efficiencies were improved after being released from the ribosome by puromycin treatment (lanes 3 and 6). Syt-135 and Syt-200 were efficiently glycosylated even when translation reactions were terminated with cycloheximide (lanes 8 and 11). Puromycin treatment caused slight mobility shift only in the case of Syt-135, but this was for unknown reasons (lane 9). The results of quantification of the same experiments indicate that Syt-120 and Syt-125 released from the ribosomes were glycosylated as efficiently as the Syt-200 molecule (Fig. 2 C). These data indicate that nascent Syt-120 and Syt-125 molecules on the ribosomes are not integrated into the membrane until they are released by puromycin.

Since the glycosylation site is separated by 29 amino acid residues from the H-region, the Syt II molecule showing the transmembrane topology is expected to be efficiently glycosylated (Nilsson and von Heijne, 1993; Popov et al., 1997). Thus, we assumed the population of the glycosylated form to correlate with the amounts of membrane-integrated molecules. The glycosylation efficiency of all the truncated constructs in the presence of cycloheximide was quantified (Fig. 2 D). There is a threshold of the

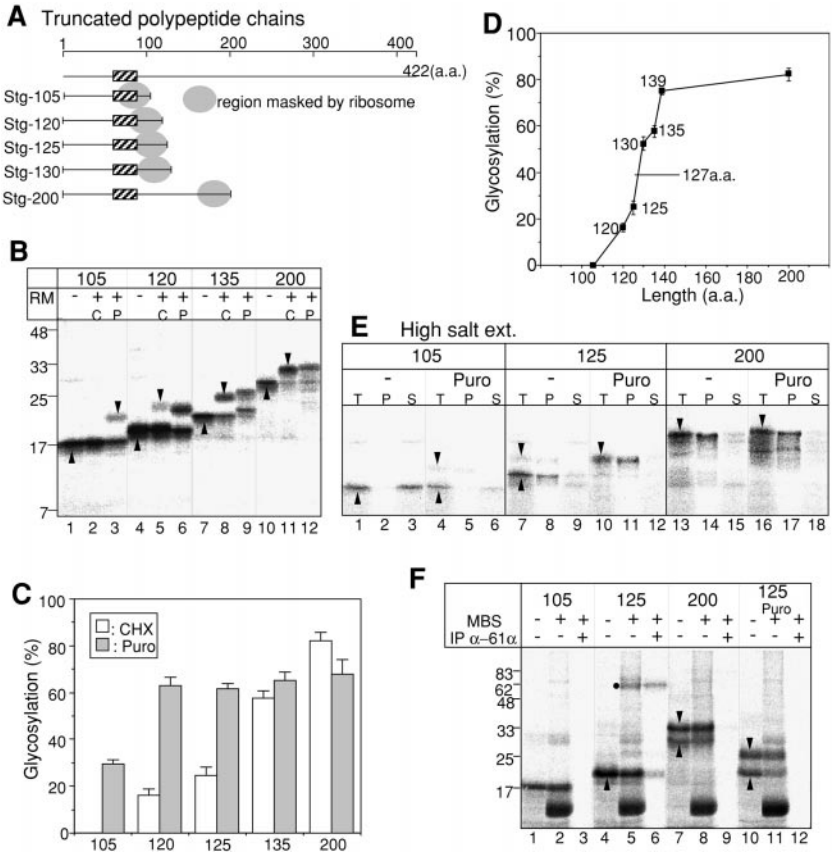

Figure 2. Timing of cotranslational membrane targeting and N-domain translocation. (A) Template DNAs linearized at various sites were transcribed in vitro. The synthesized mRNAs do not possess a termination codon so that the nascent polypeptides remain as peptidyl-tRNA and are not released from the ribosomes. The polypeptide segments within the ribosomes are indicated by closed ovals. The numbers in the names of the constructs correspond to their length. The hatched box indicates the H-region. (B) Glycosylation of the truncated polypeptides in vitro. The mRNAs were translated in vitro in the absence (−) or presence (+) of RM. The translation reaction was terminated by cycloheximide (C) or puromycin (P). Throughout all of the figures, upward and downward arrowheads indicate the unglycosylated and glycosylated polypeptides, respectively. (C) Effect of puromycin on N-domain translocation. After treatment with either cycloheximide (CHX) or puromycin (Puro), glycosylated and unglycosylated forms were quantified by image analysis and the efficiency (%) was calculated by the formula: [glycosylated-form] × 100/[glycosylated-form + unglycosylated forms]. The experiments for each construct were carried out more than three times and the standard deviation is indicated by error bars. (D) Titration of glycosylation efficiency as a function of polypeptide length. The threshold length for glycosylation is estimated to be 127 residues. (E) Syt-125 is bound to membrane in a high-salt–resistant fashion. Translation mixtures were adjusted to a high-salt condition, and then membrane precipitates (P) and supernatants (S) were separated by ultracentrifugation. The total translation products before separation are also shown (T). Where indicated (Puro), polypeptides were released by puromycin from ribosomes before high-salt treatment. (F) Syt-125 is cross-linked with Sec61α. Translation mixtures were treated with heterobifunctional cross-linker (MBS +). After the reactions, aliquots were immunoprecipitated with anti-Sec61α antibody (IP +). Dot indicates the cross-linked polypeptide (lane 5).

length for the efficient N-domain translocation and the value is 127 amino acid residues. Thus, it is concluded that the nascent polypeptide takes transmembrane topology in the very early phase of its protein synthesis; that is just after the H-region emerges from the ribosome, as shown in Fig. 2 A.

To investigate the membrane targeting of the nascent polypeptides, we conducted high-salt extraction and cross-linking experiments. After the translation, the membranes were treated with the high-salt buffer (500 mM KOAc), and separated into a membrane pellet and supernatant (Fig. 2 E). Almost all the Syt-105 molecules were recovered within the soluble fraction irrespective of the puromycin treatment (Fig. 2 E, lanes 3 and 6). However, the unglycosylated form of Syt-125 was largely recovered within the membrane fraction (Fig. 2 E, lane 8). Upon puromycin treatment, it was efficiently glycosylated, and the glycosylated Syt-125 was also recovered within the membrane fraction (Fig. 2 E, lane 11). Syt-200 was glycosylated and membrane-bound, irrespective of puromycin release (Fig. 2 E, lanes 14 and 17). When the translation product of Syt-125 was treated with heterobifunctional cross-linker (MBS), a 60-kD band was newly observed (Fig. 2 F, lane 5, dot). This band was immunoprecipitated with anti-Sec61α antiserum (Fig. 2 F, lane 6), but not with anti-TRAMp antiserum (data not shown). The 60-kD band corresponds to the summation of 20 kD of Syt-125 and 39 kD of Sec61α. Even the uncross-linked Syt-125 was coimmunoprecipitated (Fig. 2 F, lane 6). These immunoprecipitated bands were not observed after the puromycin treatment (Fig. 2 F, lanes 11 and 12), or when Syt-105 and Syt-200 were used either (lanes 3 and 9).

These results clearly indicate that the nascent polypeptide of Syt-125 on the ribosome is targeted to the membrane, whereas its N-domain is not translocated through the membrane (see Fig. 7) until it is released from the ribosome by puromycin. Thus, it is suggested that N-domain translocation requires a longer H-region emerging from the ribosome than ER targeting does. On the other hand, the Syt-105 molecule on the ribosome is not targeted to the membrane, so that its N-domain would not be efficiently translocated through the membrane, even when it is released from the ribosome.

Requirement of the Lysine Cluster Following the H-Region

There is a lysine-rich region just after the H-region of mouse Syt II (Fig. 3 A). To check the possibility that these positive charges determine the orientation of the H-region, we made a mutant in which the eight lysine residues of this region were replaced by seven asparagine and one serine residues. The mutant as well as wild-type molecules were expressed in vitro in the presence or absence of RM and their membrane topologies were examined with proteinase K and endoglycosidase H (Fig. 3).

Upon proteinase K treatment of the product synthesized in the presence of RM, the wild-type molecule gave four major proteinase K–resistant bands (Fig. 3 B, lane 4). When the membrane was solubilized with mild detergent, only the 25-kD band (indicated by closed arrowhead) among them was degraded but the other three polypeptides were not affected (lane 5). The 25-kD band was immunoprecipitated with anti–N-domain antibody (Fig. 3 C, lane 2) and not with anti–C-domain antibody (Fig. 3 C, lane 3), whereas the other three bands were reacted only with the anti–C-domain antibody (Fig. 3 C, lanes 3 and 6). Upon the EndoH treatment after the proteinase K treatment, only the 25 kD band was shifted into a smaller one

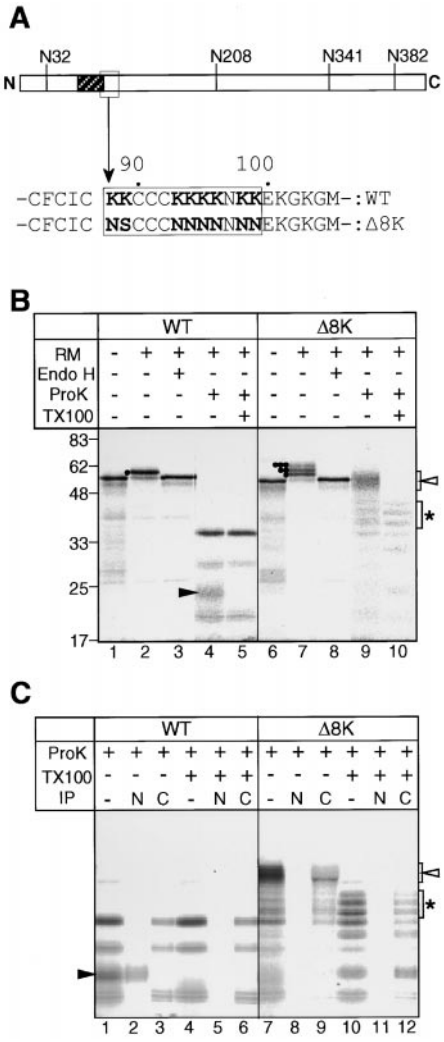

Figure 3. Positive charges following the H-region are essential for SA-I topogenesis. (A) Construct in which eight lysine residues were mutated to neutral ones. Potential glycosylation sites are indicated; one within the N-domain and three within the cytoplasmic domain. (B) The mutant was expressed in vitro. After the translation reaction, aliquots were treated with EndoH, and with ProK in the presence (+) or absence (−) of detergent (Triton X-100). One, two, and three dots indicate mono-, di-, and triglycosylated polypeptides. Closed and open arrowheads indicate membrane-protected N-domain and C-domain, respectively. An asterisk indicates glycosylated ProK-resistant core fragment derived from C-domain. (C) Immunoprecipitation of proteinase K fragments with site-specific antibodies. After the proteinase K treatment in the presence (+) or absence (−) of the detergent, peptide fragments were immunoprecipitated with anti–N-domain (IP, N) or anti–C-domain (IP, C) antibody. The total (IP, −) and the immunoprecipitated polypeptides were subjected for SDS-PAGE.

(data not shown). It is, thus, indicated that the 25-kD band is a membrane protected fragment containing the translocated and glycosylated N-domain, and the other bands are proteinase K–resistant core fragments derived from the C-domain.

In contrast to the wild-type, when the positive charge mutant was synthesized in the presence of RM, it gave three glycosylated bands which were sensitive to EndoH (Fig. 3 B, lanes 7 and 8). Upon proteinase K treatment, smeared band(s) slightly smaller than the three glycosylated bands were newly observed (Fig. 3 B, lane 9, open arrowhead). This broad band(s) was immunoprecipitated with anti–C-domain antibody and not with anti–N-domain antibody (Fig. 3 C, lanes 8 and 9). It should be noted that there is no substantial amount of the 25-kD band in the fraction immunoprecipitated with anti–N-domain antibody (Fig. 3 C, lane 8). Upon proteinase K treatment in the presence of Triton X-100, the broad band(s) disappeared and several proteinase K–resistant fragments were newly observed (Fig. 3 C, lane 10). All of these bands were immunoprecipitated with anti–C-domain antibody but not with anti–N-domain antibody (Fig. 3 C, lane 11 and 12). The EndoH treatment after the proteinase K treatment in the presence of detergent resulted in the completely identical pattern as that obtained with wild-type molecule (data not shown). Since Syt II has three glycosylation potential sites within the C-domain, it is indicated that the higher molecular weight bands in the lane 10 were glycosylated and proteinase K–resistant core fragments derived from the C-domain (Fig. 3 B, lane 10, asterisk). Taken together, these results demonstrated that the C-domain of the Δ8K mutant was translocated across the membrane and glycosylated, and that its N-domain was on the cytoplasmic side. We concluded that the charge-mutation caused complete inversion of the membrane topology.

We further examined effects of the N-domain lengths on the requirement of the positive charges: neither deletions of Met1-Thr21 nor Met46-Lys60 from the N-domain resulted in any improvement of N-domain translocation of Δ8K mutant (data not shown). In contrast, when the N-domain was replaced with the extracellular loop (of 39 residues long) of anion exchanger 1 (Ota et al., 1998a), even the Δ8K mutant showed mainly the SA-I orientation (data not shown). Thus, it is suggested that some unknown characters (not solely the length) of the N-domain cause the requirement of the positive charges following the H-region.

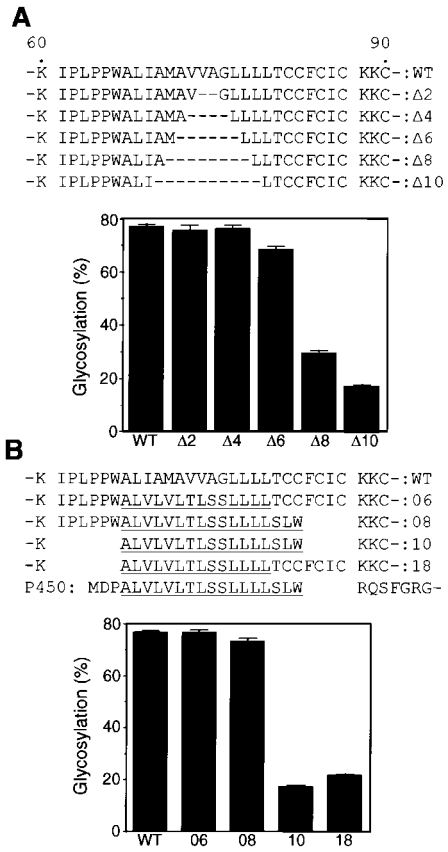

Figure 4. Structural requirements of the H-region for SA-I topogenesis. (A) Effect of serial deletions of the H-region. The glycosylation efficiencies were determined as described in the legend to Fig. 2. (B) The effect of shuffling of the H-region of Syt II with that of the rat cytochrome P450(2C11). The sequences of cytochrome P450(2C11) are underlined.

Structural Requirements of the H-Region

To elucidate the length requirements of the H-region, we constructed a series of mutants in which the H-region was systematically shortened (Fig. 4 A). The constructs were expressed in the cell-free translation system and their glycosylation efficiencies were estimated. The mutants with deletion of less than six residues were glycosylated as efficiently as the wild-type molecule. Di- and tri-glycosylated forms were not observed with any of these constructs (data not shown). Thus, we concluded that the H-region of Syt II possesses sufficient length for the SA-I topogenesis.

To further explore the structural features of the SA-I sequence of Syt II, we made a series of mutants in which the amino acids in the H-region were systematically replaced by those of rat cytochrome P450(2C11) (Fig. 4 B). Microsomal cytochrome P450s are SA-I proteins and form large gene families (Omura et al., 1993). The H-region from Met1 to Trp20 of cytochrome P450(2C11) has been demonstrated to be sufficient for the function of SA-I (Sakaguchi et al., 1987; Sato et al., 1990). Syt-06 with a nonpolar segment of the same residue number as the wild-type Syt II was integrated with the same efficiency, whereas Syt-18 lacking the former sequence (IPLPPW) could not be integrated. Similarly, Syt-08 which includes the entire H-region of P450 was efficiently glycosylated, whereas Syt-10 was hardly glycosylated at all. From these observations, we realized that the H-region of the P450 is not sufficient for the translocation of the Syt II N-domain and that the stretch of 6 amino acid residues (IPLPPW) is critical. Interestingly, in many Syt isoforms, the proline residues are highly conserved between the N-domain and the H-region (Fukuda et al., 1999; Li et al., 1995).

Critical Role of the Proline Residues

To elucidate the importance of the proline residues between the H-region and the N-domain, these prolines were replaced to alanine residues (Fig. 5 A). The AAA mutant

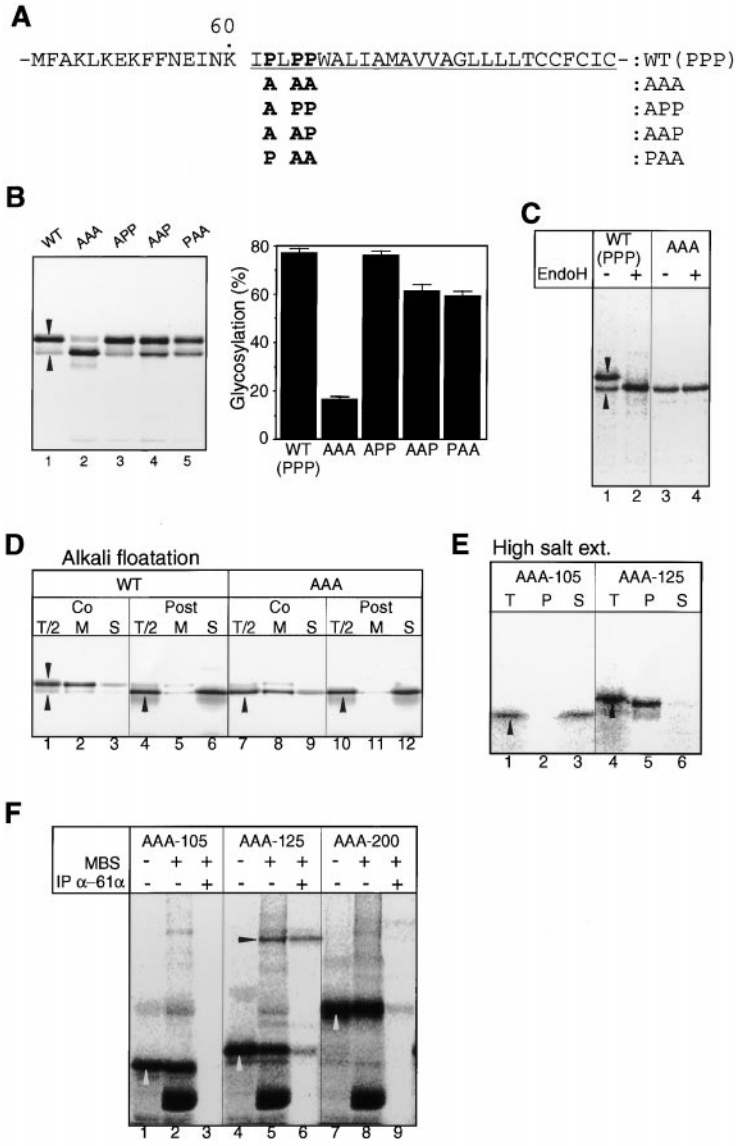

Figure 5. Proline residues between the N-domain and the H-region are critical for SA-I topogenesis. (A) Proline residues were mutated into alanine residues as indicated. (B) The mutants were expressed in a cell-free system in the presence of RM. The upward and downward arrowheads indicate the unglycosylated and glycosylated polypeptides, respectively. In the right panel, the glycosylation efficiency of each construct was determined as described in the legend to Fig. 2. (C) The wild-type and AAA mutant were expressed in the cultured cells. The cells were pulse-labeled and Syt II molecules were immunoprecipitated. Aliquots were further treated by EndoH. (D) Membrane anchoring of the AAA mutant. RM was added during translation reaction (Co) or after the termination of the translation with puromycin (Post). In the latter case (Post), the reaction was continued for a further 30 min at 30°C. The translation products were extracted with 0.1 M Na_2CO_3, and then membrane-bound (M) and soluble (S) fractions were separated by the alkaline floatation procedure. Half equivalents of the total reactions are also shown (T/2). (E) Membrane targeting of AAA mutant of 125 residues. The truncated mRNAs encoding 105 and 125 residues of the AAA mutant were translated in the presence of RM and separated into membrane-bound (P) and supernatant (S) fractions under the high-salt condition. (F) Nascent polypeptide of 125 residues of AAA mutant was cross-linked with Sec61α. After each of the truncated mRNA was translated in the presence of RM, cross-linking (MBS) and immunoprecipitation (IP) were performed as in the legend to Fig. 2.

in which all three proline residues were replaced by alanines was not glycosylated (Fig. 5 B, lane 2), whereas mutants having at least one proline residue were efficiently glycosylated (lanes 3–5). The AAA mutant was not glycosylated in the cultured cells, either (Fig. 5 C).

The membrane anchoring of the in vitro synthesized AAA mutant was examined by extraction under the alkaline condition (pH 11.5), which extracts almost all the peripheral membrane proteins (Fujiki et al., 1982). When the wild-type molecule was incubated with RM after the translation termination, it was no longer glycosylated and little was in the membrane fraction (Fig. 5 D, lane 5), indicating that it was integrated into the membrane only in a cotranslational fashion. When synthesized in the presence of RM, a substantial amount of the AAA mutant was bound to the membrane, although it was not glycosylated (Fig. 5 D, lane 8). The membrane binding of the mutant was not observed when it was incubated posttranslationally with RM (Fig. 5 D, lane 11). The AAA mutant was integrated into the membrane via hydrophobic interaction, probably forming a loop structure.

The truncated form of the AAA mutant of NH_2-terminal 125 residues was recovered in a membrane pellet even after the high-salt extraction, as observed with the wild-type molecule (Fig. 5 E, lane 5). Furthermore, the truncated form of the AAA mutant was cross-linked with Sec61α (Fig. 5 F, lane 5). This cross-linking was stage specific and not observed with the truncated polypeptides of 105 and 200 residues (Fig. 5 F, lanes 3 and 9). Interestingly, coimmunoprecipitation with Sec61α was also noted with this mutant (Fig. 5 F, lane 6), indicating that the integration intermediate of Syt-125 mutant forms a stable complex with the translocon. Taken together, we concluded that the AAA mutant was as efficiently targeted to the ER membrane as the wild-type and that at least one of the proline residues is essential for translocation of the N-domain after the targeting process.

Since the regions flanking the proline residues were predicted to form helix by the method of Chou and Fasman (Fig 6, A and B), there is the possibility that the proline requirement may be important to disrupt a continuous helix from the N-domain into the H-region. To examine

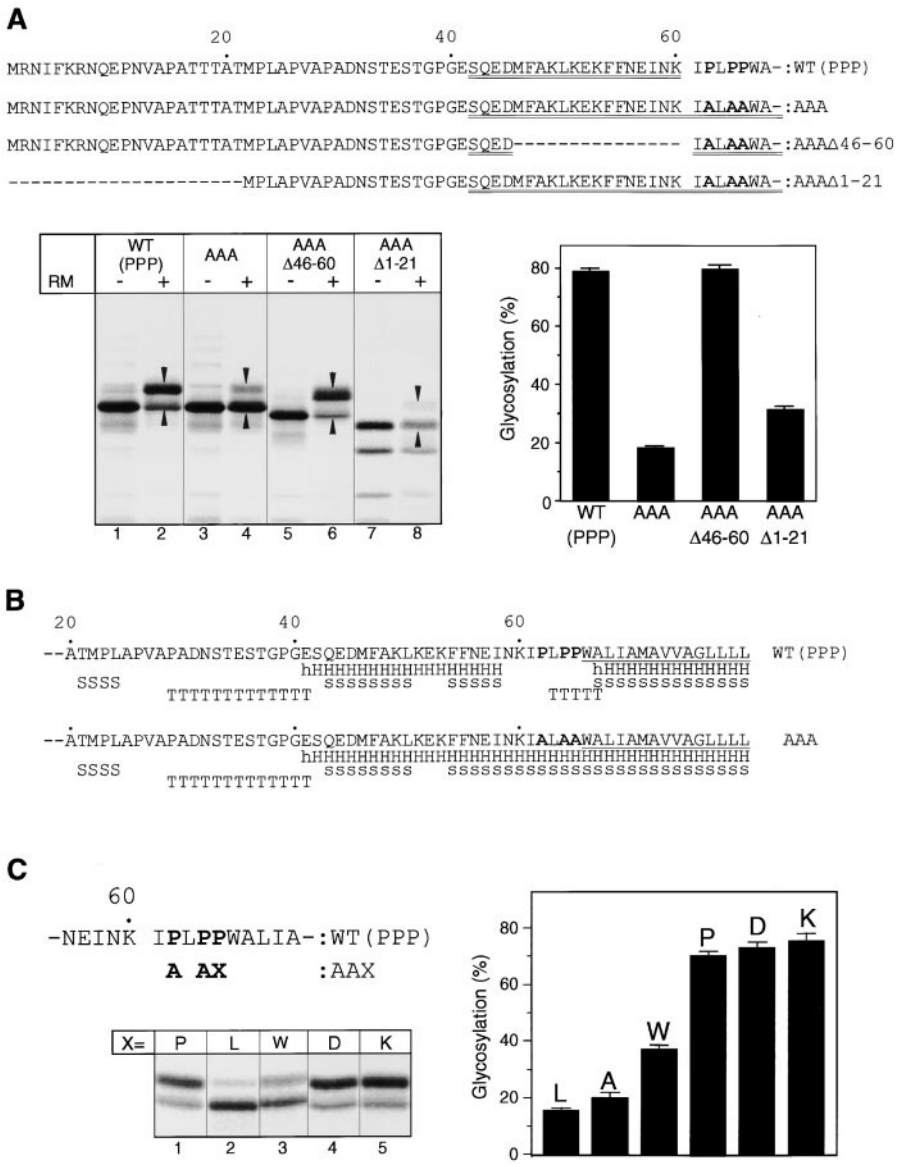

Figure 6. Effect of partial deletions of the N-domain and replacement of the third proline on N-domain translocation of the AAA mutant. (A) N-domain of the proline mutant (AAA) was partially deleted as indicated. The mutated proline residues and deleted amino acid residues are indicated by a bold letter and a dashed line, respectively. The sequence predicted to form helix is indicated by double underlining. The mutants were expressed in a cell-free system in the presence (+) or absence (−) of RM. The upward and downward arrowheads indicate the unglycosylated and glycosylated polypeptides, respectively. Glycosylation efficiencies were determined as described in the legend to Fig. 2. (B) Prediction of secondary structure of the boundary between N-domain and H-region. H, S, and T correspond to the regions predicted as helix, sheet, and turn, respectively. H-region is indicated by underlines. (C) The third proline residue was exchanged with amino acids with various helical propensities. The glycosylation efficiency was determined as described in the legend to Fig. 2.

this possibility, we deleted most of the preceding region which predicted to form helix (AAAΔ46-60). As expected, this mutant was glycosylated as efficiently as the wild-type, whereas the another mutant, AAAΔ1-21, in which the distal region had been deleted, was hardly glycosylated at all, similar to the AAA mutant. Thus, it is suggested that the proline residues are responsible for disrupting helix encompassing the H-region and the preceding 20 residues.

To directly examine the effect of the helical propensities, we introduced several kinds of amino acid residues with different helical propensities into the third position (Monne et al., 1999; Fig. 6 C). Asp and Lys residues with low helical propensity in the third position could result the efficient N-domain translocation as Pro residue. In contrast, Leu residue with high helical propensity could not mediate efficient glycosylation as Ala residue, and Trp residue with moderate helical propensity resulted in moderate glycosylation. These results demonstrated that the helical propensity of those amino acid residues are crucial for determination of the fate of the N-domain. Together, it is highly likely that the proline residing between the N-domain and H-region is critical for breaking helix and for inducing turn structure to mediate translocation of the N-domain. Interestingly, it should be noted that it can be replaced with Lys and Asp residues, despite their charges.

Discussion

The threshold value of the length of the Syt II nascent polypeptide for the N-domain translocation is 127 residues (Fig. 2). The positively charged residues just after the H-region play an essential role in N-domain translocation (Fig. 3). Since the 30–40 residues of the nascent polypeptide chain should be buried within the ribosome (Matlack and Walter, 1995), the positively charged residues of the nascent chain of 127 residues lie just at the exit site of the ribosome upon translocation of the N-domain (Fig. 7). The nascent polypeptides of Syt-120 and Syt-125 shorter than the threshold are targeted to the ER membrane, so that they are bound to the membrane in a salt-resistant

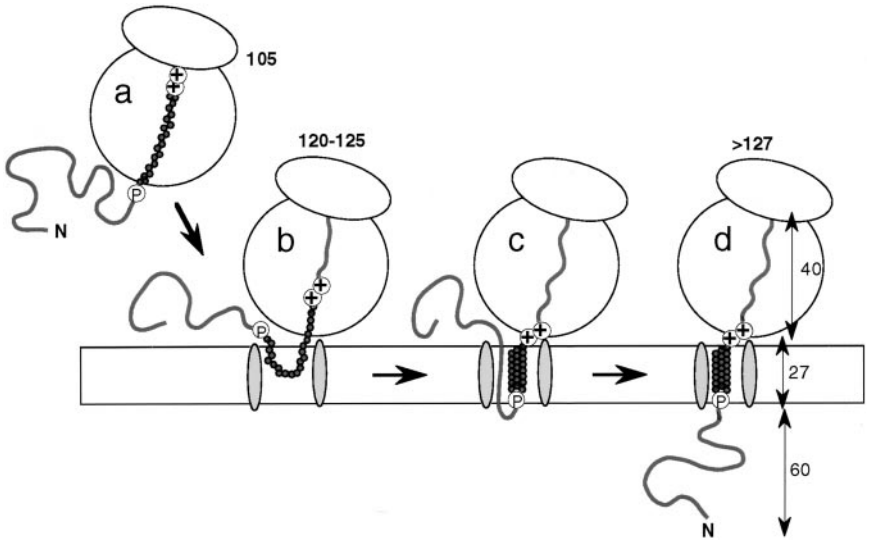

Figure 7. Working model for topogenesis of the Syt II molecule. Syt-105 is not targeted to the membrane (stage a). Syt-120 and Syt-125 are targeted to the membrane but are not sufficient in length for N-domain translocation (stage b). These are efficiently chased with puromycin to be glycosylated. For completion of the N-domain translocation, further chain elongation is essential so that the positively charged residues beyond the H-region emerge from the ribosome (stage c). Proline residues between the N-domain and the H-region are critical between stage b and stage d to break continuous helix. The threshold of the length for correct integration is 127 residues. The numbers indicate the numbers of amino acid residues of the nascent polypeptide chains.

fashion and in the close proximity of Sec61α (Fig. 2). These findings demonstrate that the polypeptide chain length required for translocation of the N-domain and that required for the ER targeting are different. The correct integration occurs only after the H-region and the successive positive charges are fully exposed from the ribosome (Fig. 7). This consideration should be consistent with the two-step recognition of the signal sequences. Some sequences are sufficient for SRP recognition and for ER targeting, but are not sufficient for further progress of membrane translocation (Belin et al., 1996; Jungnickel and Rapoport, 1995). In the SA-I topogenesis, the progression of chain elongation is essential for N-domain translocation after the targeting step.

It has been proposed that the COOH terminus of the H-region of SA-I sequence of the cytochrome P450 is initially located in the lumenal side during SA-I topogenesis. This is based on the observation that a cleavage site which had been introduced to the COOH terminus of the H-region of cytochrome P450 was partially cleaved by the signal peptidase (Monier et al., 1988). However, the minor fraction was processed while the majority fraction remained as an unprocessed form showing SA-I topology in the experiment, thus indicating that the N_{cyt}/C_{exo} transmembrane form could not be an intermediate of SA-I topogenesis. Considering the length between the P site of the ribosome and the processing site (Whitley et al., 1996), Syt-127 is too short to locate the COOH terminus of the H-region to the active site of the signal peptidase (Fig. 7). Thus, it is highly likely that the translocation of the N-domain occurs just when the positive charges following the H-region interact with a component of the translocon.

The nascent chain of Syt-105 was not targeted to ER and glycosylation was inefficient even after release from the ribosome. This suggests that the nascent polypeptide should be targeted to ER as a ribosome complex for efficient translocation of the N-domain. Upon further elongation, the nascent chain-ribosome complex is targeted to the translocon, but its N-domain was still on the cytoplasmic side of the membrane. In this stage, Syt-125 is cross-linked with Sec61α. This cross-linking is stage-specific, since the chain elongation or puromycin treatment caused a loss of reactivity. Thus, this stage is a bona fide productive intermediate of SA-I topogenesis. Interestingly, the free Syt-125 molecule as well as the cross-linked one was precipitated with anti-Sec61α antibody, indicating that the intermediate is in a stable complex of the translocon.

The clustered positive charges beyond the H-region are required for translocation of the N-domain (Fig. 3). These positive charges should emerge from the ribosome and should be recognized by components of the translocon. So far, a systematic analysis of the SA-I sequences demonstrated that the longer H-region and the less positive charges in the N-domain prefer the SA-I topology (Sakaguchi et al., 1992b; Wahlberg and Spiess, 1997). The folding property of the N-domain is also a critical factor in the topogenesis (Denzer et al., 1995). Furthermore, positive charges at the COOH terminus of the stop-transfer sequence have been shown to possess an inhibitory effect on the polypeptide chain translocation (Kuroiwa et al., 1990, 1991). In contrast to these findings, there is no clear observation showing the requirement of positive charges at the COOH terminus of SA-I sequences. Although such a cluster of positively charged residues has been frequently noted following SA-I sequences of microsomal cytochrome P450, we have shown that these positive charges are not essential for the SA-I topogenesis (Sakaguchi et al., 1987). The positive charges beyond the H-region were also demonstrated to show no critical contribution in the topological conversion between SA-II and SA-I sequences (Parks and Lamb, 1991). In this case of Syt II, the N-domain might contain some sequence(s) with a higher energy barrier to be translocated through the translocon, so that even the long H-region would not be sufficient to pull the N-domain and the positive charges are required for fixation of the COOH terminus of the H-region at the entrance of the translocon. It is still an open question as to which components of the translocon interact with these residues and how these residues contribute to the movement of the N-domain through the translocon.

The SA-I function of the hydrophobic segment was not affected by the deletion of at most six residues from the

hydrophobic segment (Fig. 4). Some extra residues are thus required for the other functions, but not for membrane integration. One possibility is that the length of the TM is closely related to the intracellular location of membrane proteins. The length and structure of the transmembrane segment has been demonstrated to be one of the major determinants of intracellular location of the COOH-terminal–anchored membrane proteins (Pedrazzini et al., 1996; Rayner and Pelham, 1997; Yang et al., 1997), as well as SA-II proteins (Munro, 1995). The transmembrane segment of Syt II is much longer than those of the microsomal cytochrome P450s; the H-regions of SA-I of the microsomal P450 so far sequenced comprise <20 amino acid residues. The transmembrane segments of some P450 molecules have been actually demonstrated to be sufficient for ER localization (Ahn et al., 1993; Murakami et al., 1994; Szczesna-Skorupa et al., 1995). The effects of the length of the H-region on the intracellular localization of Syt II are under investigation.

At least one residue of the proline between the N-domain and the H-region is critical for translocation of the N-domain (Fig. 5). The proline residues are often observed within this region in many Syt isoforms (Fukuda et al., 1999; Li et al., 1995) as well as within those in various other species (not shown). The proline residue is not involved in membrane targeting. As shown in Fig. 7, the turn structure should be formed during translocation of the N-domain. This notion is supported by the observation that the proline requirement was not noted with the deletion mutant that had lost its proximal region. Furthermore, the proline can be replaced with Asp and Lys that are of low helical propensity, despite the charges, but not with Leu and Ala with high helical propensity (Liu and Deber, 1998; Monne et al., 1999). These observations suggest that breaking helix is crucial for N-domain translocation.

In summary, we can dissect the membrane topogenesis of SA-I protein as shown in the working model (Fig. 7). Syt-105 is not targeted to the membrane, so that the N-domain is inefficiently translocated even after it is released with puromycin (Fig. 7, stage a). Syt-120 and Syt-125 are targeted to the membrane but are not sufficient in length for integration, so that their N-domains are still on the cytoplasmic side (stage b). These are efficiently chased with puromycin, allowing them to be glycosylated (stage c). The Syt II molecule of more than 127 residues was correctly integrated (stage d). For the completion of the N-domain translocation, further chain elongation is essential so that the positively charged residues beyond the H-region appear from the ribosome. The proline residues between the N-domain and the H-region demonstrate a critical function between stage b and stage d.

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