Function of Positive Charges Following Signal-Anchor Sequences during Translocation of the N-terminal Domain*

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In topogenesis of membrane proteins on the endoplasmic reticulum, the orientation of the hydrophobic transmembrane (TM) segment is influenced by the charge of the flanking amino acid residues. We assessed the function of the positive charges downstream of the hydrophobic segment using synaptotagmin II. The positive charges were systematically replaced with non-charged residues. Although the original TM segment translocated the N terminus, the topology was inverted, depending on the mutations. Orientation was affected in mutants in which 6 Lys were shifted downstream, even when the 6 Lys were 25 residues from the hydrophobic segment. The Lys was functionally replaced by Arg, but not by Asp or Glu. The timing of action during polypeptide elongation indicated that the Lys functions at the ribosome exit sites. We suggest that the commitment of the TM segment to a particular orientation is influenced by far downstream parts of the polypeptide chain and that the positive charges are decoded after exiting the ribosome.

Many membrane proteins are synthesized by ribosomes bound to the endoplasmic reticulum (ER) membrane. The ribosomes are targeted to the ER membrane by the signal recognition particle, and the nascent polypeptide chains are integrated into the membrane, mediated by the translocon (1). The signal sequences, which are primarily defined by a hydrophobic segment (H-segment) in the nascent polypeptide chain, are inserted into the translocon to form the two possible alternative orientations. When the following portion is translocated through the translocon, it results in an Ncyt/Crum orientation in which the N terminus is on the cytoplasmic side and the C terminus is on the lumenal side of the membrane. Cleavable signal peptides and one of the non-cleavable signal-anchor sequences (SA-II sequence) have this orientation. When the preceding portion is translocated, it results in the Nlum/NCyt orientation (2). This type of signal-anchor sequence occurs in various membrane proteins (e.g. microsomal cytochrome P450 isoforms, synaptotagmin isoforms, and some viral membrane proteins) and are termed type I signal-anchor sequences (SA-I sequences) because they have the so-called type I orientation and remain as membrane anchor segments (3).

The H-segments of the signal-anchor sequences are composed of 17–27 residues, and those of signal peptides are 7–14 residues (4). The longer H-segment and fewer positive charges in the N-terminal domain (N-domain) often cause translocation of the N-terminal side and result in an SA-I topology (5). The charged amino acid residues in the flanking regions greatly affect the orientation (6). The effect of positive charge has been also established in statistical analysis as the “positive inside rule”, in which positively charged amino acids tend to be more prevalent on the cytoplasmic than on the extra-cytoplasmic side of TM proteins (5). More specifically, it has been proposed that charge differences in the 15 residues flanking both sides of the H-segment determine the orientation; the more positive side is retained on the cytoplasmic side (7).

In this study, we examined the effect of the number and position of positive charges immediately following the H-segment of SytII on topogenic function and estimated the timing of the action during polypeptide chain elongation. The positive charges had additive effects, irrespective of their position within the flanking region. The 6-Lys cluster was effective on the topology even when the cluster was 25 residues away from the H-segment. When the Lys cluster was shifted downstream, the timing of the N-domain translocation was delayed. A similar position effect was observed with another model protein, neuregulin-1β (NRG-1β). We suggest that the orientation of the H-segment can be suspended until the following polypeptide of more than 25 residues enters the translocon.

EXPERIMENTAL PROCEDURES

Materials—Preparations of rough microsomal membranes (RM) (12) and rabbit reticulocyte lysate (13) were performed as described previously. Cycloheximide was purchased from Sigma. Rabbit antisera for SytII (14) and antisera for dog Sec61α were described previously (10). Antiserum against dog translocating chain-associating membrane protein (TRAM) was raised against a synthetic peptide of the N-terminal 15 residues. The photocleavable amino acid L-4-(3-benzoyl-3H-diazirinyl)-2-phenylethylamine (TMDPhe) was purchased from GL Biochem (Shanghai).

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2 The abbreviations used are: ER, endoplasmic reticulum; C-domain, C-terminal domain; H-segment, hydrophobic segment; N-domain, N-terminal domain; NRG-1β, neuregulin-1β; PrkA, protein kinase A; RM, rough microsomal membrane; SA-I, type I signal anchor; SA-II, type II signal anchor; SytII, synaptotagmin II; TM, transmembrane; (TMD)Phe, L-4-(3-[(trifluoromethyl)-3H-diazirinyl]-2-phenylethylamine; TRAM, translocating chain-associating membrane protein.
flouromethyl]-3H-diazirin-3-yl)phenylalanine ([(Tmd)Phe] was loaded onto amber suppressor tRNA using ([(Tmd)Phe]-pdCpA (Photoprobe) essentially as described previously (15, 16).

Construction of Expression Plasmids—Mouse SytII cDNA was described previously (10). The cDNA was subcloned between HindIII and XbaI sites of pcR-CMV (Invitrogen), which possesses both the T7 RNA polymerase promoter for in vitro transcription and the cytomegalovirus promoter for expression in cultured cells. The sequence from the HindIII site to the initiation methionine was AAGCTTCCAC-

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Substitutions of Lys with neutral polar residues, shifting of the Lys cluster, formations of AIII sites in the open reading frame, and insertion of the amber codon (TAG) at point 111 of 20–6K mutants were performed using the Kunkel method. Insertion of charged amino acid residues (Asp, Arg, and Gla) and the formation of stop codons were performed using the polymerase cycle reaction procedure (18).

The mRNAs coding for the full-length molecule were translated for 1 h at 30 °C in the reticulocyte lysate cell-free system in the presence of 15.5 kBq/μl of EXPRESS protein-labeling mix (PerkinElmer) of [35S]Met and [35S]Cys. The truncated mRNAs were translated at 25 °C for 20–40 min according to length. Translation was terminated by the addition of cycloheximide, and the mixture was further incubated at 25 °C for 30 min to complete the translocation and glycosylation. Proteins were analyzed by SDS-PAGE and subsequent image analysis using BAS180011 (FujiFilm). The intensities of the protein bands were estimated, and the glycosylation efficiency (%) of either site (N32% or N208%) was calculated using the following formula: [glycosylated form] × 100/([glycosylated form] + [unglycosylated form]). Percent glycosylation can be used as an estimation of membrane insertion of either the N- or C-terminal domain (C-domain) (see “Results”). The topology percentage (%) of SA-I was calculated using the following formula: N32% × 100/[N32% + N208%], and the topology percentage (%) of SA-II was calculated using the following formula: N208% × 100/[N32% + N208%].

The truncated mRNAs coding for amber111 mutant of 20–6K protein were translated in the presence of suppressor tRNA with a photoactivatable amino acid, (Tmd)Phe-amber suppressor tRNA. The reaction mixtures were supplemented with 2 mM cycloheximide and transferred to a 96-well plate. They were UV irradiated (365 nm) on ice for 15 min. The irradiated samples were resolved with 1% SDS and diluted with 10 volumes of immunoprecipitation buffer (50 mMTris/Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Trasylol) and then immunoprecipitated using Protein A-Sepharose (Amersham Biosciences) and antiserum against TRAM. The residual supernatants were then immunoprecipitated with anti-Scelα antiserum. The radiolabeled proteins were analyzed by SDS-PAGE and subsequent image analysis.

For proteinase K (ProK) treatment, the translation mixture was incubated with 0.2 mg/ml of ProK for 30 min on ice. The reaction mixture was diluted with 10 volumes of high salt buffer (0.5 mM KOAc, 2.5 mM Mg(OAc)2, 30 mM HEPES/KOH, pH 7.4, 5 mM phenylmethylsulfonyl fluoride) and incubated for 10 min on ice. After centrifugation at 100,000 × g for 10 min at 4 °C, the membrane pellet was solubilized with sample buffer for SDS-PAGE. Aliquots of the membrane precipitates and translation mixtures were subjected to endoglycosidase H treatment according to the manufacturer’s protocol.

Expression in COS7 Cells—COS7 cells were maintained in Dulbec-co’s modified Eagle’s medium (D6429; Sigma) supplemented with 10% fetal bovine serum (JRH Biosciences) under an atmosphere of 10% CO2 at 37 °C. For transfection, COS7 cells were seeded in 3.5-cm wells (2 × 105 cells/well). After culturing for 24 h, the expression plasmids (1 μg) were transfected with FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s protocol. After culturing for 24 h, the cells were pulse labeled with 3.7 MBq/500 μl of medium [35S]Met of EXPRESS protein-labeling mix for 10 min and lysed with 2% SDS. SytII was immunoprecipitated with rabbit anti-SytII antibodies as described previously (22).

RESULTS

Effect of the Number of Positive Charges—To estimate the topology of model proteins derived from SytII, all potential glycosylation sites except one in either the N-domain (Asn15) or C-domain (Asn208) were silenced (Fig. 1A). If the H-segment forms a TM orientation, either site should be glycosylated (Fig. 1B). There are 8 Lys residues among the 12 residues following the H-segment of SytII (Fig. 2A, WT). We first examined the effect of the number of Lys residues on topogenic properties. The 8 Lys residues were systematically replaced with polar, but noncharged, residues (Asn, Ser, or Gln) using the N32 and N208 proteins (Fig. 2A). The plasmid DNAs were linearized with the XhoI site and transcribed in vitro. The synthesized mRNA was translated in a reticulocyte lysate cell-free system in the presence of RM, and radioactive proteins were analyzed by SDS-PAGE and subsequent image analysis (Fig. 2B). The wild-type was glycosylated predominantly in the N-domain (Fig. 2B, lane 1), but not in the C-domain (Fig. 2B, lane 10). In contrast, the mutant in which all 8 Lys were replaced was glycosylated predominantly in the C-domain (Fig. 2B, lane 11) and only slightly in the N-domain (Fig. 2B, lane 2). When 4 of 8 Lys residues were changed (–KKK–), both sites were significantly glycosylated (Fig. 2B, lanes 9 and 18). In all cases, endoglycosidase H treatment shifted the upper bands down (data not shown), confirming that they were glycosylated forms.

To examine the glycosylation efficiency of the glycosylation site translocated into the luminal side, the reaction mixtures were subjected to ProK treatment, which degraded the polypeptide chain on the cytoplasmic side of the membrane. Membrane vesicles were then isolated by ultracentrifugation and analyzed by SDS-PAGE (Fig. 2C). When the wild-type SytII was treated with ProK, a small peptide fragment including the N-domain and the TM segment was observed (Fig. 2C, lanes 2 and 5). Endoglycosidase H treatment shifted the N32 protein down to
the same molecular weight as the N208 protein (Fig. 2C, lanes 3 and 6). The ProK-resistant unglycosylated form was not observed, indicating that the inserted N-domain was completely glycosylated. In contrast, the 0-Lys mutant (--K-----) resulted in a large ProK-resistant fragment including the TM segment and C-domain (Fig. 2C, lanes 8 and 11). Only the fragment of N208 protein was endoglycosidase sensitive. Again, the ProK-resistant fragment was completely glycosylated (Fig. 2C, lanes 11 and 12). The 4-Lys mutant (--KKKK--) gave a pattern that was a mixture
of those of the wild-type and the 0-Lys mutant (Fig. 2C, lanes 14, 15, 17, and 18). In this case, the glycosylation sites in the ProK-resistant fragment were 100% glycosylated. All the ProK protection experiments demonstrated that the glycosylation of the inserted segment was almost 100% in the lumen. It is reasonable to conclude that glycosylation is a direct indication of the membrane insertion. Thus, the glycosylation of wild-type molecules indicated that 80% of the nascent chain was inserted in the SA-I topology, and 20% was not inserted into the membrane (Fig. 2D). In contrast, 10% of the 0-Lys mutant had an SA-I topology and 70% had an SA-II topology. In our cell-free system, an integration efficiency of 80% is standard with SytII. The orientations of other mutants were quantified. The sum of the insertion yields was nearly 80% with all the altered proteins, indicating that they were inserted into the membrane with a similar efficiency of 80% and that the insertion efficiency was not affected by the charge mutations (Fig. 2D). The orientation on the membrane can thus be estimated by the ratio of glycosylation of the N32 and N208 (termed topology percentage) (Fig. 2E). The orientation of the mutants was affected by the number of positive charges, irrespective of the position within this region; i.e. 4-Lys mutations resulted in a similar orientation independent of the positions. The clear additive effects of Lys on the orientation confirmed the topogenic function of the positive charges.

Effect of Positions of the Positive Charges—We next addressed the position effect of the Lys cluster on membrane orientation. Two Lys were kept just after the H-segment to determine the boundary of the H-segment, and the 6-Lys cluster was systematically shifted downstream in 5-residue increments (Fig. 3A). The mutant with only 2 Lys (KK------) had a predominantly Ncyt/Clum (SA-II) orientation (70%), whereas the mutant with the 6-Lys cluster at 5 residues from H-segment had a 100% SA-I orientation (Fig. 3B). The effect gradually decreased as the distance from the H-segment increased. Unexpectedly, the 6-Lys cluster, separated by 25 and 30 residues from the H-segment, still had significant effects on orientation.

To address the effect of the charged residues on topogenesis in living cells, we expressed the altered proteins in cultured COS cells. Newly synthesized proteins were pulse labeled and immunoprecipitated with anti-SytII antibodies. The proteins were analyzed by SDS-PAGE and subsequent image analysis. Glycosylated and unglycosylated forms are indicated by open and closed circles, respectively. The glycosylation (%) of N32 was calculated.
10-min pulse labeling eliminated the possibility of selective degradation of a set of topologic forms. The glycosylation of the inserted domain should be almost complete as shown with the cell-free system. The effect of the 6-Lys cluster position was more apparent in the cultured cells; there were significant effects even when separated by 30 residues from the H-segment (Fig. 4). Although the position effect was somewhat different from that in the cell-free system for unknown reasons, we have concluded that the Nlum orientation was more favorable than the Ncyt orientation in cultured cells and that the Lys cluster was effective even when it was separated by more than 25 residues from the H-segment. Taken together, the 6-Lys cluster affected the topology from more distant positions than previously assumed.

Effect of Other Charged Residues—The effects of other charged amino acid residues were examined at positions 5 or 25 from the H-segment (Fig. 5). Arg and Lys had similar effects on N-domain translocation. When they were separated by 25 residues from the H-segment, Lys had a slightly more prominent effect than Arg. Hermansson et al. (23) reported that when positioned after the H-segment to examine the ability to induce a helical hairpin, the topogenic function of the Asp residue was comparable with Lys. In the context used in the present study, however, both of the negatively charged residues Asp and Glu had significant but very weak effects on topogenesis. The effects of Asp and Glu clusters positioned 5 residues from the H-segment were weaker than those of the Lys cluster positioned 25 residues from the H-segment. The contribution of the negatively charged residues is not clear compared with those of positively charged residues.

Timing of Positive Charge Function—Liao et al. (24) demonstrated that the H-segment of the nascent polypeptide chain can be sensed within the ribosome tunnel. Although the positive charges immediately following the H-segment seem to function at the exit site of the ribosome (10), the Lys residues further downstream might also be sensed within the ribosome. To determine the timing of this action, we examined the effect of the length of the nascent polypeptide chain on N-domain translocation using the 5–6K and 20–6K proteins (Fig. 6). To generate intermediates of polypeptide chain elongation, mRNAs truncated at various positions were obtained by transcription of template DNAs linearized at the AflII site, which was generated at various locations within the open reading frame. The mRNA possessed no termination codon so that the nascent polypeptide chain remained bound to the ribosome as peptidyl-tRNA to form a ribosome nascent chain complex. When translated in the presence of RM, nascent chains with the expected molecular weights were synthesized as major bands (Fig. 6A). A clear difference of glycosylation between the 5–6K and 20–6K proteins was observed at the length of 136 and 141 residues. In the case of the 5–6K, the N-domain was translocated depending simply on the length of the nascent chain. The 136-residue nascent chain was efficiently glycosylated, as reported previously using wild-type SytII molecule (10). In contrast, the N-domain translocation lagged in 20–6K. At
a length of 126 residues, the 20–6K protein was glycosylated by 20%, similar to the 5–6K protein. The integration was highly likely to be independent of the 6-Lys cluster (Fig. 6A). Despite the lag, the 146-residue 20–6K protein was as efficiently glycosylated as the 5–6K protein. In contrast, both proteins translocated the N-domain, when a termination codon was created instead of making the truncated mRNA (Fig. 6A, open symbols). Even when the termination codon was created just after the positive charges, the N-domain was efficiently translocated. The 6-Lys cluster and H-segment in the shorter nascent chain bound to the ribosomes could not function because they were masked by ribosomes. When the nascent chain was released from the ribosomes, the downstream Lys cluster functioned as efficiently as that positioned just after the H-segment. These data indicated that the N-domain translocation up to a nascent chain length of 126 is due to two lysine residues (Lys⁸⁸–Lys⁹⁰). In the 5–6K protein, the 6-Lys cluster closely follows the H-segment, and the resulting curve was monophasic. In the 20–6K protein, however, the 6-Lys cluster emerges 15 residues downstream and is functional only in the longer nascent chains.

To probe the proximity of the Lys cluster to the translocon subunit Sec61α, site-specific photo-cross-linking experiments were performed using various nascent chain mutants. An amber termination codon was created at the fourth codon of the 6-Lys cluster (Fig. 6B). The amber codon was suppressed by translating in the presence of amber-suppressor tRNA that had been charged with phenylalanine modified by a photo-reactive group. In this experiment, the photo-reactive group can be incorporated at a specific position in the nascent chain. After translation and UV irradiation, immunoprecipitation revealed that nascent chains of more than 146 residues were firmly cross-linked with Sec61α (Fig. 6B). No cross-linked product was precipitated with anti-TRAM antibodies, indicating the high specificity of the immunoprecipitation. Thus, the Lys cluster was in the proximity of the Sec61α subunit when the nascent chain was 146 residues long. This is consistent with the idea that the positive charges are decoded outside of the ribosome. Taken together, these findings indicated that the positive charges of the 5–6K and 20–6K proteins are decoded at a similar site, likely at the interface between the translocon and ribosome.

Effect of Charged Residues Assessed with Other SA-I Protein—To examine whether similar effects of positive charges occur with other membrane proteins, we used human NRG-1β (25), which consists of a 247-residue luminal N-domain, a 23-residue hydrophobic TM segment, and a following 375-residue cytoplasmic C-domain (Fig. 7A). It also possesses a cluster of positive charges just after the H-segment (Fig. 7B). Of 9 residues within the cluster, 8 were moved downstream as in Fig. 3. A 147-residue N-terminal deletion was deleted. The topology was examined using a ProK protection assay, in which the translocated N-domain and C-domain were detected as small and large ProK-resistant bands, respectively (Fig. 7C). Wild-type gave the small fragment (Fig. 7C, lane 2). In contrast, the 0-positive charge mutant (Δ8+) gave 23% small ProK-resistant fragments and 77% large Pro-K-resistant fragments, indicating that only 23% of the inserted protein had the SA-I topology. When the 8 positive charges were inserted 20 residues downstream from the H-segment, the mutant resulted in 92% SA-I topology (Fig. 7C, lane 6). They were still effective even when separated by 25 residues from the H-segment (Fig. 7C, lane 8). Thus, the results from NRG-1β support our conclusion with SytII molecule that the positive charges affect the topology even when those were separated by more than 25 residues from H-segment.

DISCUSSION

Positive charge is the most decisive determinant of membrane topology of the TM segment. The charges following the H-segment of SytII have clear additive effects on the orientation of the TM segment. The charges can affect orientation, even when they are separated by more than 25 residues from the H-segment. Arg and Lys residues had similar effects, whereas negatively charged residues, Asp and Glu, had very weak effects. The charges acted adjacent to the Sec61α subunit of the translocon even when they were shifted downstream.

Statistical analysis of naturally occurring signal-anchor sequences indicates that topologies of the N-terminal first TM segments of membrane proteins correlate well with the charge difference within the 15-residue N- and C-terminal flanking regions (7). Our data, however, demonstrated that the positive charges can affect H-segment topology even when they are 25 residues from the H-segment. Hermansson et al. (23) reported that charges separated by more than 30 residues from the H-segment affect its helical hairpin formation on RM. Thus, positive charges are effective as topology determinants at a more distant position than previously assumed. Although complex features of the polypeptide sequence other than charge might further affect the orientation (e.g. Ref. 27) and further comprehensive mutagenesis studies are required to exclude conformational influences, the present conclusions are not affected.

Using ribosome nascent chain complex, we determined the effect of nascent chain length on N-domain translocation. When the charges were adjacent to the preceding H-segment, the dependence showed a

FIGURE 7. Effect of positive charges on the topology of NRG-1β. A, NRG-1β was assessed as another example of SA-I protein. The N-terminal 147 residues were deleted (Δ147). B, the positive charges (bold characters) flanking the C-terminal side of the H-segment were moved downstream as in Fig. 3C, the proteins were expressed in the cell-free system in the presence of RM, and aliquots were treated with ProK. The ProK-resistant fragments of N-domains and C-domains are indicated by up and down arrows, respectively. SA-I(%) was calculated by the formula [N-domain fragment] × 100/[N-domain fragment] + [C-domain fragment]).
simple curve as previously reported with the original SytII molecule. These data indicate that the N-domain is translocated through the translocon immediately after the H-segment and the following charges emerge from the ribosome (10). In contrast, when the charges were shifted downstream 15 residues, the curve became biphasic. The first phase likely corresponds to the 6-Lys cluster-independent translocation of the N-domain, and the second phase corresponds to the translocation mediated by the 6-Lys cluster. The charges of the 20–6K protein functioned only when the nascent chain was longer than 146 residues. Cross-linking experiments further demonstrated that the 6-Lys cluster is just adjacent to the Sec61α at lengths greater than 146 residues. As previously demonstrated (10), targeting to the translocon of the SytII nascent chain occurs prior to the integration of the TM segment. This is also highly likely in the 20–6K protein.

Liao et al. (24) suggested that the H-segment on the translocating polypeptide chain can be sensed within the ribosome and that the information is transferred to the translocon to regulate gating of the lumenal side of the translocon. In contrast to the sensing of the H-segment, the positive charges appear to be recognized at the entrance of the translocon, but not in the ribosome. Yeast Sec61p (corresponding to dog Sec61α) is involved in the recognition of the positive charge (11). The counterpart(s) for the recognition of positive charges might be the negative charges of translocon proteins and those in the phospholipids (28). The charge effect appears to depend, gradually, on the number of positive charges, but not in an all-or-none manner. Thus, the translocon might be sufficiently flexible to generate both orientations.

Our data indicate that the commitment of the H-segment function can be suspended until the following 25 residues are presented to the translocon. Given that the translocating polypeptide chain forms an extended conformation, a sequence of 25 residues seems to be sufficient to allow the H-segment to form a TM α-helix. If so, the helix should be reoriented for N-domain translocation. Alternatively, the TM topology of the H-segment might not be finalized until the following 25 residues emerge from the ribosome and the H-segment is in an intermediate state in which both sides of the H-segment can be translocated through the translocon (Fig. 8b). We hypothesize that once folded into the TM helix in the SA-II topology, the H-segment commits to translocating the following polypeptide chain (Fig. 8c). The positive charges are effective as a topology determinant only prior to the commitment, which is suspended until the following 25–30 residues emerge from the ribosome. When a positively charged cluster does not follow the H-segment, the H-segment itself tends to form a TM helix with a C\textsubscript{terminus} orientation, and once such a stable TM helix conformation is formed, the orientation and the following translocation are committed.

Lys residues at positions distant from the H-segment have a stronger effect in cultured cells than in a cell-free system. Because the chain elongation rate in the cultured cells is much faster than in the cell-free system, it is likely that the positive charges in the more distant position contribute to the topogenic process before the TM helix is committed and the topology is fixed.

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