Reinitiation of Protein Translocation across the Endoplasmic Reticulum Membrane for the Topogenesis of Multispanning Membrane Proteins*

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The reinitiation of the translocation of the growing nascent chain across the endoplasmic reticulum membrane is essential for the topogenesis of multispanning membrane proteins. We investigated the requirements for the reinitiation process using model proteins in which systematically designed sequences were inserted after two preceding topogenic sequences, namely the N-terminal signal sequence (S) and stop transfer sequence (St). The model proteins were translated in vitro in the presence of rough microsomes, and the final topology of the proteins in the microsomal membrane was examined by proteolytic digestion. The structural requirements for S and the reinitiation sequence (R) overlapped to some extent, but substantial differences were noticed. When St and R were separated by a short cytoplasmic segment (58 amino acids), the efficiency of the reinitiation was not affected by the concentration of the signal recognition particle (SRP) in the translation system, even though the sequence inserted as R was an SRP-dependent signal sequence. However, when the cytoplasmic segment was longer (100 amino acids), the reinitiation efficiency was reduced, and the SRP improved the overall efficiency as well as impaired the accessibility of the processing site after the R to the signal peptidase.

In eucaryotic cells, membrane proteins on the endocytotic and exocytotic pathway are co-translationally integrated into the endoplasmic reticulum membrane, and their final membrane topologies are defined during their integration. They are targeted to the endoplasmic reticulum membrane by the signal recognition particle (SRP) and SRP-receptor system (1), and then they are inserted into the endoplasmic reticulum membrane depending on the hydrophilic protein translocation channel, which consists of several protein subunits (2, 3).

The topology of membrane proteins is determined by several functionally defined topogenic sequences (4, 5), the signal sequence (S), the stop-transfer sequence (St), and the signal-anchor sequence (SA). S initiates the protein translocation by functioning at two steps; it is recognized and targeted to the membrane by the SRP system and then initiates the translocation of the following portion of growing peptide by looping into the membrane. St interrupts the ongoing translocation of the nascent peptide chain either by dissociating the protein subunits of the translocation channel or by interacting with the hydrophobic core of the lipid bilayer (6, 7). SA initiates the protein translocation in the same manner as S, but it is not cleaved by the signal peptidase, so that it ends up as the membrane-anchoring sequence. SAs are categorized into Type I (Nin/Cout) and Type II (Nout/Cin) according to their orientation in the membrane (8). Both types of SAs are recognized and targeted to the membrane by SRP (9). The functions of these topogenic sequences are mainly determined by the hydrophobic segment and are modulated by adjacent charged, and mainly positive, amino acid residues. The hydrophobic segments of S are usually 8–12 amino acid residues long, while those of the other topogenic sequences are longer than 20 amino acid residues (10). S prefers the leucine residues as the components of the hydrophobic segment in comparison with the other topogenic sequences (10). The orientation of SA in the membrane depends on the balance between its N-terminal charge and the length of the hydrophobic segment (11, 12). The primary determinant of St is the hydrophobicity of the sequence, and the positively charged amino acid residues after the hydrophobic segment promote the interruption of the protein translocation (13).

For the topogenesis of multispanning integral membrane proteins, the membrane translocation must be reinitiated after the stop-translocation by St. The amino acid sequence responsible for this process is designated here as the reinitiation sequence (R). It has been proposed that multispanning membrane proteins are integrated into the membrane by a series of alternating signal and stop-transfer sequences (14–16). Furthermore, it has also been demonstrated that the membrane integration of proteins is a sequential event of the insertion of the alternative hydrophobic sequences starting from the N-terminal transmembrane segment and that the hydrophobic segment, which is not recognizable by the SRP, can mediate the second translocation (17). It is, however, not clear whether the structural requirements for R are similar to those of S and whether some protease factors, such as the SRP and/or others, participate in the process.

In this study, we systematically and quantitatively examined the reinitiation process using various model sequences whose functions as S or SA had been evaluated in previous studies, and demonstrated that the reinitiation is primarily determined by the hydrophobicity of the sequences as is the case of S, while the structural requirements for R are not identical with those for S. We also demonstrated that the

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1 The abbreviations used are: SRP, signal recognition particle; S, signal sequence; R, reinitiation sequence; SA, signal-anchor sequence; St, stop transfer sequence; IL2, interleukin 2; RM, rough microsomal membranes from dog pancreas; PAGE, polyacrylamide gel electrophoresis.
concentration of the SRP could affect, not only the overall reinitiation efficiency but also the accessibility of the authentic cleavage site for the signal peptidase behind the reinitiation sequence.

**EXPERIMENTAL PROCEDURES**

**Materials**

Plasmids, pSL1 to pSL18 coding for the proteins in which the model sequences were fused at the N terminus of mature IL2 were described (12). pL15 (+) coding for the type I membrane protein in which 15 leucine and two lysine residues (L15 (+) sequence) were introduced into the middle portion of IL2 molecule were described (13). The rabbit polyclonal antibodies against the synthetic peptide of the N-terminal sequence of Ysy6p, MAVQTPRQLANAKP, have also been described previously (18).

**Constructions of Plasmids and Model Proteins**

The proteins constructed in this study are summarized (see Figs. 1 and 4A).

pKL1 to pKL18—EcoRI and EcoT22I sites were created at the termination codon of the L15 (+) protein on the pL15 (+) site by site-directed mutagenesis to obtain pGTP. The DNA fragments coding the model sequences and the mature portion of IL2 were excised with EcoRI and PstI from the pSL series plasmids and subcloned between the EcoRI and EcoT22I site of pGTP. The obtained plasmids were designated sequentially as indicated in the table of Fig. 1A.

**Tagged Constructs**—The four synthetic oligonucleotides designed for the tag sequence were phosphorylated, annealed, and ligated with the plasmids pKL7, pKL9, pKL10, and pKL11, which had been digested with BglII and XhoI as described previously (6). The XhoI site had been created in the middle portion of the second IL2 sequence (12). The resulted plasmids were designated as pKL7, pKL9, pKL10, and pKL11, respectively.

**Marker Constructs**—Each set of appropriate templates and primers was used to obtain DNA fragments by polymerase chain reaction, coding for R + L3, L1 + St + L2 + R, L3, L1 + St, and L1 + St + L2 sequences. The obtained DNA fragments were isolated, digested by Ncol and PstI, and then subcloned into the transcription plasmid, pSP2 m, digested by Ncol and PstI.

pKL9–100 and pKL9–200—The DNA fragments coding N-terminal 40 and 140 amino acids of mature IL2 were amplified by polymerase chain reaction. The obtained DNA fragments were digested with EcoRI and ligated with EcoRI-digested pKL9.

**Topology Assay**

The mRNAs transcribed from the plasmids were translated at 26 °C for 40 min in a wheat germ cell-free system in the presence of RM (2 eq/25 μl) and 35S-labeled amino acids. In some cases, salt-washed RM (KRM, 2 eq/25 μl) was used, and various amounts of SRP were included in the translation system. After the translation, 1 μl of proteinase K (2 mg/ml) was added to the 10-μl translation mixture and incubated at 20 °C for 30 min. The protein precipitated with 10% trichloroacetic acid was solubilized with the sample buffer and analyzed with SDS-PAGE followed by quantification with an image analyzer (BAS 2000, Fuji Film).

**Others**

Immunoprecipitation was carried out as described previously (19). RM and KRM (20), SRP (21), and wheat germ extract (22) were prepared as described. In vitro transcription-translation (23), DNA manipulations (24), and site-directed mutagenesis (25) were all performed according to the described methods.

**RESULTS**

**Construction of Model Proteins Showing a Multispanning Membrane Topology**—The first objective of this study was to systematically examine structural requirements of the reinitiation sequence (R) and to compare them with those of the N-terminal signal sequence (S). For this purpose, we utilized the 18 model sequences that had been constructed in a previous study (12). The model sequences, in which number of N-terminal net charges (−1, +1, +3) and lengths of the hydrophobic stretch (5, 7, 10, 12, 13, 15 amino acid residues) had been systematically changed, were designated according to the net charges and the lengths of the hydrophobic stretch (Fig. 1A).

To examine R function of the model sequences, they were placed between two mature interleukin 2 (IL2) sequences in the protein constructs shown in Fig. 1A. S of IL2 was placed at the N terminus of the constructs, and then the middle portion of the first IL2 sequence was replaced by an St consisting of 15 leucine residues followed by 2 lysine residues (shadowed box), and the systematically designed model sequences (black box) are in the dispositions as indicated. Open boxes represent the spacer sequences. L1 and L2 are parts of the 1st mature IL2 sequence, and L3 is the second mature IL2 sequence. The number of amino acid residues of the spacer sequences is indicated in the open boxes. The arrowhead after the model sequence indicates the signal peptidase processing site in the second IL2 portion. The number of arginine residues (m) and the leucine residues (n) are listed in the lower panel. The model sequences are designated according to the number of net charges and the leucine residues as indicated in the panel. B, the expected membrane topologies of the constructed proteins. The arrowheads indicate the cleavage by the signal peptidase. The open circles indicate the inserted tag-sequence.
tify the protected peptide segments, an epitope tag sequence was inserted into the L3 portion of the four constructs (pKL7, pKL9, pKL10, and pKL11), which gave typical protease protection patterns, to obtain pKL7', pKL9', pKL10', and pKL11'. Supposed topologies of the tag sequence on the membrane were indicated by open circles in Fig. 1B. It is reasonable to assume that the tag sequence did not affect final topology of the proteins in the membrane, since it is short and not hydrophobic.

Assay for Translocation Reinitiation on RM—The constructed cDNAs were transcribed in vitro, and the obtained mRNAs were translated with wheat germ cell-free translation system. Typical results with the tagged constructs are shown in Fig. 2A. Each of the translates from pKL7', pKL9', pKL10', and pKL11' gave the major precursor protein bands of 40 kDa (lanes 1, 6, 11, and 16). Upon the translation in the presence of RM, the KL7' protein showed no additional bands, whereas KL9' and KL10' proteins gave two major additional bands of 20 and 21 kDa (lanes 7 and 12), and KL11' protein also gave the similar additional bands of weak intensity (lane 17). By immunoprecipitation with the anti-tag antibodies, the precursor protein and the 20-kDa protein were precipitated but not the 21-kDa protein (lanes 9, 14, and 19), thus indicating that the 20-kDa peptide was the second IL2 segment (L3), which was split at the junction with the model sequence by signal peptidase, and the 21-kDa peptide consisted of the first IL2 segment and the introduced model sequence, L1-St-L2-R (Fig. 1B).

When the translation products with RM were treated with proteinase K, several membrane-protected bands were detected. KL7' protein gave one major protected band of 18 kDa (Fig. 2A, lane 3). Because the 18-kDa peptide was not precipitated with the anti-tag sequence antibodies, the peptide was identified as the L1-St or the S-L1-St portion (see below) (lane 5). KL9', KL10', and KL11' proteins gave two or three protected bands of 18, 20, and 24 kDa (lanes 8, 13, and 18). Both the 20- and 24-kDa peptides were precipitated with anti-tag antibodies (lanes 10, 15, and 20), which thus indicated that the two protected peptides contained the L3-portion. Judging from the apparent molecular weights, the 20-kDa protein was the L3 portion processed by the signal peptidase after the translocation, and 24-kDa peptide was the unprocessed, membrane protected-form with R (R-L3).

To compare the mobility of the protein fragments described above with those of marker proteins, we constructed five marker proteins consisting of each set of the segments of pKL110' protein and translated them in the absence of RM (Fig. 2B). The 24-kDa protected band (lanes 5 and 6) showed a little bit higher mobility than that of R-L3 protein. This is likely because some of the N-terminal charged residues of R sequence of pKL110' protein were accessible to the protease K on RM membrane. The 21 and 20 kDa processed bands of pKL10' protein (lane 2) showed the same mobility of L1-St-L2-R and L3, respectively, confirming the identity. The 21-kDa processed band of pKL9' protein showed slightly higher mobility because of its reduced number of leucine residues. The 18 kDa protected bands showed unexpected lower mobility than that of the L1-St marker protein. This observation coincides with our previous observation that when IL2 constructs, having the 15 leucine residues in its middle portion as a stop-transfer sequence, was translated with RM and then subjected to proteinase K treatment, the same high molecular mass (18 kDa) membrane-protected band was detected (6). This unexpected mobility of the protected band might be because S of these constructs could not be processed by signal peptidase. Anyway, the facts that the 18 kDa band was detected for all of the protein constructs examined here and that the mobility was same as that observed in the constructs lacking R and L3 portions (6) indicate that 18 kDa bands correspond to the first translocated segments.

The 18 kDa band can be used as an estimate of the total protein translated as it is generated independent of reinitiation. This first translocated segment provided an internal control for the quantitation of the efficiency of the translocation reinitiation. The two membrane-protected bands of 20 and 24 kDa, which could be precipitated with the anti-tag antibodies indicated the translocation reinitiation. Both the L1 and L3 portions contain three methionine residues, and the L2 portion contains no methionine. We thus estimated the reinitiation efficiency (Fig. 3A, %) by calculating the ratio of the 24 kDa (R-L3) band plus the 20 kDa (L3) band to the 18 kDa band.

To confirm that the protease K resistant bands of 24, 20, and 18 kDa were integrated depending on the N-terminal signal sequences, we examined the membrane topology of N-terminal 5 deleted constructs derived from pKL9', pKL10, and pKL11 and found that those proteins gave no or, if any, only faint protease K-resistant bands (data not shown). Thus, it is demonstrated that protein integration into RM observed here.

**Fig. 2. Topology assay of the in vitro translated proteins.** A, the tagged constructs, pKL7', pKL9', pKL10, and pKL11' corresponding to the original pKL7, pKL9, pKL10, and pKL11, respectively, were translated, and the mRNAs were translated in the wheat germ cell-free system in the presence (indicated by +) or absence (indicated by −) of RM. After incubation at 26 °C for 30 min, some aliquots (10 μl) were treated with 200 μg/ml proteinase K at 20 °C for 30 min (ProK+). In some cases, immunoprecipitation with anti-tag IgG was carried out (Imppt+). The proteins were analyzed by SDS-PAGE (15% gel) and the subsequent image analysis. The molecular masses and the compositions of the peptide fragments on the panel are indicated on the right side. B, five marker constructs encoding L1-St, L3, L1-St+L2, L1-St+L2+R, and R-L3 portions were transcribed and translated in the absence of RM. Each aliquot (lanes 8–12) or the mixture except L1-St+L2 protein (lane 7) was subjected to SDS-PAGE. The mRNAs from pKL9', pKL10', and pKL11' were translated in the presence of RM and directly analyzed by SDS-PAGE (lanes 1–3) or subjected to proteinase K treatment and subsequent SDS-PAGE (lanes 4–6). Positions of the molecular size markers are indicated on the left side of the figure.
were calculated by the following formula and are shown on the ordinate: 24 kDa band/18 kDa band \times 100 \text{ plus } 20 \text{ kDa band}/18 \text{ kDa band} \times 100. The results shown in the figure are the averages of two experiments. The lengths of the hydrophobic segments in the model sequences are indicated by the residue numbers (n) on the abscissa. The net charges of the model sequences are also indicated by the symbols (−, +, and +++). The hatched boxes indicate the percentages of the proteinase K-resistant 24-kDa peptide unprocessed after the translocation (Fig. 1B case 2, R + L3). The closed boxes indicate the percentage of the proteinase K-resistant, processed 20-kDa peptide (Fig. 1B, case 3, L3). B, S function of the model sequences. Translocation assays using pSL1 to pSL18 were carried out (12). Each band was quantitated with the image analyzer, and the percentages of translocation (%) were calculated by using the following formula and are shown on the ordinate: precursor form/precursor form + mature form \times 100. The lengths of the hydrophobic segments in the model sequences are indicated by the residue numbers (n) on the abscissa. The number of the net charges preceding the hydrophobic segments are also indicated by the symbols (−, +, and +++). The translocation was started with the amino-terminal S sequence.

Effects of the Lengths of Hydrophobic Segments and Positive Charges—All constructed proteins were synthesized in the presence of RM, and their membrane topologies were assessed. The efficiencies of the inserted model sequences as R are summarized in Fig. 3A. In the figure, contributions of the 24 kDa band and the 20 kDa band were indicated separately (hatched and solid bars, respectively). The reinitiation efficiencies were tightly dependent on the numbers of hydrophobic amino acid residues. The accessibility of the processing site in the second IL2 sequence to the signal peptidase varied depending on the inserted model sequences.

To compare the R activities of the model sequences with their S activity, we also estimated their S activity by using the previously constructed proteins in which the same model sequences were placed at the N terminus of the mature IL2 like the SL9 protein in Fig. 4. As shown in Fig. 3B, the amino-terminal positive charges mitigate the effect of long hydrophobic sequences. In these cases, all of the translocated IL2 portions were completely processed by the signal peptidase, indicating that none of the hydrophobic sequences act as type I SA. It should be mentioned that (−1)L13 and (−1)L15 sequences did not act as S but functioned as type I SA whose final orientation in the membrane (Nin/Cout) was reverse to that of S (12) (see "Discussion").

Short hydrophobic segments of (−1)L5, (+(+L5, and (+3)L5 did not function as R. (−1)L7, (−1)L10, and (−3)L15 showed a relatively low R activity, although these sequences efficiently functioned as S. It should be pointed out that the functional S sequences, (−1)L7 and (−1)L10, which were recognized by the SRP, could not function as R. (−1)L12, (−1)L13, (−1)L15, and (−1)L13 showed an efficient R activity. In most cases, the reinitiation efficiencies of KL9, KL9–100, and KL9–200 proteins were carried out as described in the legend to Fig. 2A, except that various amounts of SRP and salt-washed RM (KRM, 2 eq/25 μl) were included in the translation system. The results shown in the figure represent the average of three experiments. Standard deviations (error bars) were calculated for processed and unprocessed proteinase K-resistant bands, individually. The hatched and closed boxes indicate the percentages of the proteinase K-resistant 24 kDa (unprocessed) and 20 kDa (processed by signal peptidase) peptides, respectively.
reinitiation efficiency (R function) of a model sequence was higher than the translocation efficiency (S function). This is likely because the nascent protein is already associated with the membrane when R acts (see "Discussion"). These results indicate that the structural requirements of S and R largely overlap, but some sequences with efficient S function do not function as R and vice versa.

Spiess et al. (26) reported that stop-transfer efficiency depended on translation systems used, which thus suggested the possibility that different results could be obtained by using reticulocyte lysate translation system instead of the wheat germ extract system. We therefore examined the R efficiency of the constructed proteins with the reticulocyte lysate system and found that the efficiencies of R in the reticulocyte lysate system on the whole were higher than those estimated in the wheat germ extract system, but the results obtained with the two cell-free translation systems were essentially the same (data not shown).

Effects of SRP and Distance between St and R on the Reinitiation Efficiency—We next investigated whether SRP-concentration would affect the R function of (-1)L10 sequence of KL9 protein. It was assumed that under the condition of limited amount of SRP, the externally added SRP should affect the function of S and R. The RM was washed twice with 0.5 M potassium acetate to remove SRP (KRM). The translocation activity of KRM was about one-eighth of the original RM. In vitro translation was performed in the presence of KRM and various concentrations of added SRP. The concentration of SRP did not affect the R activity of (-1)L10 sequence in KL9 protein (Fig. 4B). When the (-1)L10 sequence was attached to the N terminus of mature IL2 (Fig. 4A, SL9 protein), the translocation efficiency was clearly dependent on the concentration of SRP (Fig. 4C), which indicated that the (-1)L10 sequence was recognizable by the SRP. The translocation reinitiation of KL9 protein thus seemed to be SRP-independent.

To test the effect of context, the distance between the St and the R of KL9 protein, 58 amino acids, was elongated to 100 amino acids (KL9–100 protein) and 200 amino acids (KL9–200 protein) (Fig. 4A). The elongation of the L2 portion of KL9 protein resulted in a significant reduction of R efficiency depending on the distance between St and R (Fig. 4B). In the case of KL9–100 protein, total R efficiency was increased by the addition of SRP, whereas SRP did not increase the R efficiency of KL–200 protein.

The processing by signal peptidase after the reinitiation was also affected by the length of the L2 portion. Furthermore, the processing percentage of the KL9–100 protein was affected by the concentration of SRP; the proportion of the processed product decreased as the concentration increased. These observations suggested that SRP might affect the reinitiation process of KL9–100 protein, whereas SRP was apparently not required for the reinitiation process of KL9 protein.

DISCUSSION

Systematically constructed model sequences were fused to the N terminus of mature IL2 and examined for their ability as S (S context) in the previous study (12). In this study, the same sequences were positioned in the R context, where the S of IL2, a St, and the model sequences were sequentially arranged from the N terminus, and the cotranslational incorporation of the constructed proteins into RM was examined with an in vitro system. The model proteins showed the expected topology in the membrane and the topogenic activities of the sequences inserted in the R context were dependent on their structures.

The structural requirements of S and R overlapped to some extent, whereas substantial differences could also be demonstrated as follows. First, in the case of the model sequences containing 13- and 15-leucine segments, the function of the sequences in S context was significantly affected by the number of the preceding positive charges (Fig. 3B and Ref. 12), whereas their function as R was not substantially affected by the charges. Most importantly, (-1)L13 functioned as type I SA in the S context in which the N terminus was translocated to the lumen and the C-terminal portion was left on the cytoplasmic side of the membrane (Nin/Cin). In the R context, both (-1)L13 and (+3)L13 functioned as R in which the N-terminal portion was left on the cytoplasmic side (Nout/Cin). It is likely that the N terminus of the long hydrophobic segment in the S context should be fixed by the positive charges at the entrance of the translocation channel for the S function, whereas the cytoplasmic segment between St and R could efficiently retain the N terminus of the R on the cytoplasmic surface of the membrane even when the hydrophobic segment was long (Fig. 5). Second, in the case of the model sequences containing 7- and 10-leucine segments, the positive charges preceding the segments were required for the R function; (-1)L7 and (-1)L10 showed no R activity and low R activity, respectively. These two short sequences, however, functioned as efficient S in the S context irrespective of the charges. These differences suggested that a unique mechanism is operative in the reinitiation of protein translocation. It is likely that the translocation initiation by S and the reinitiation by R differ in the membrane recognition at the targeting step or in the interaction with the translocation machinery during the looping of the peptides into the membrane.

Naturally occurring transmembrane segments of multispansing membrane proteins are usually longer than 18–20 residues. However, we found longer hydrophobic segments of 15 leucines are less efficient reinitiation activity. This finding indicated that too much hydrophobic segment does not mediate translocation reinitiation. It is worthy to speculate that such overhydrophobic segments have been eliminated during the evolution of membrane proteins.

It was reported that the internal transmembrane segments of opsin could initiate translocation in an SRP-dependent manner in the S context (14, 15). The second reinitiation sequence of the multispansing precursor of the structural proteins of Semliki Forest virus mediated the translocation of the following portion depending on SRP when the sequence was exhibited at the N terminus by the deletion of the preceding portion (27). These observations seem to indicate that the hydrophobic
segments of the multispanning membrane proteins possess the potential to initiate peptide translocation in an SRP-dependent manner, which also suggests the possibility that SRP also mediates the reinitiation of the peptide translocation. However, it was also reported that a mutated transmembrane segment, which was no longer recognized by SRP and lost the S function, functioned as R (17). A truncated mutant of the Semliki Forest virus polyprotein, which had only 28 amino acids after the reinitiation sequence, formed the expected multispanning topology. Since the recognition of the signal sequence by the SRP requires more than 40 amino acid residues after the signal sequence to allow the signal to emerge from the ribosome, it was suggested that the reinitiation process can proceed without SRP recognition (28, 29). These facts therefore seem to indicate that the reinitiation step does not require SRP.

We found in this study that a model sequence, (+1)L10, which can be recognized by the SRP, functioned as R, and the reinitiation efficiency was not affected by the SRP concentration in the translation mixture as shown for KL9 protein. Thus, it is suggested that the reinitiation process of KL9 protein is not dependent on the SRP. On the other hand, the reinitiation of the KL9–100 protein was clearly affected by the SRP concentration, and the overall reinitiation efficiency was restored to a level as high as that of KL9 protein at the saturation concentration of SRP. Furthermore the processing efficiency of the retranslocated portion was also affected by the SRP concentration. It is likely that the R after the long cytoplasmic loop (100 amino acids) can be recognized by SRP, and the reinitiation function is influenced by the SRP concentration. There are two possibilities to explain the relationship between the lengths of the cytoplasmic loops and the effect of SRP. In the case of the short cytoplasmic loop (58 amino acid residues), ribosomes may still be in contact with the membrane when R emerges from the ribosomes, and the SRP cannot interact with the R due to steric hindrance. Alternatively, it may be speculated that the R is directly targeted to the nearby translocation channel, which is still open even after the translocation is stopped by St.

The processing of the protein constructs occurred during the translocation of L3 loop possibly at the original processing site of the second I2L2 sequence after the model sequences. The processing at the beginning of L3 loop indicates that the signal peptidase in the protein translocation channel complex is also in the proximity of the translocating L3 portion. The accessibility of the processing site to the signal peptidase was significantly affected by the structure of the preceding hydrophobic segments and the L2 portion before the hydrophobic segment. This observation is consistent with the previous reports; the deletion of the cytoplasmic portions of asialoglycoprotein receptor H1 and HLD-DR histocompatibility antigen, both of which are single spanning transmembrane proteins anchoring to the membrane by type II SA (SAI1), converted their SAI1 into the cleavable S (30, 31); a point mutation introduced into the hydrophobic segment of the SAI1 of the rat sucrose-isoamylase also resulted in the conversion of the SAI1 to the S (32). The accessibility of internal signal sequences to the signal peptidase was dependent on the lengths of their hydrophobic segments (33). Although the cleavage of the model sequence placed in S context was complete irrespective of the sequences, the R consisting of more than 10 leucine residues were inefficiently cleaved. This fact is in contrast with the previous observations that model hydrophobic segments (up to 17 leucine residues) in the R context were efficiently cleaved (33) and that an internal signal sequence was also efficiently cleaved (31). Although the reason of the inefficient cleavage of our model proteins is not clear, it is possibly due to differences in the constructs.

The functions of the topogenic sequences so far characterized, S, St, SA, and R, are mainly dependent on the segment of hydrophobic amino acids in the sequences. Therefore, one of those topogenic sequences can function as another type of the topogenic sequence according to their position in a protein sequence. The SAI1 of asialoglycoprotein receptor H1 could function as St or R, depending on the disposition in the protein molecule and also the preceding amino acid sequences (17). Some of the seven transmembrane segments of bovine opsin could function as a targeting signal to the membrane (14). In our study, (+1)L10 was able to function as either R or S, and also as St (13). In addition to the structural differences among the topogenic sequences, their disposition in the protein molecule is also an important determinant for their topogenic functions.

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REFERENCES

1. Walter, P., and Johnson, A. E. (1994) Annu. Rev. Cell Biol. 10, 87–119
2. Crowley, K. S., Liao, S., Worrell, V. E., Reinhart, G. D., and Johonson, A. E. (1994) Cell 78, 461–471
3. Hartmann, E., Sonner, T., Prehn, S., Görlich, D., Jentsch, S., and Rapoport, T. A. (1994) Nature 367, 654–657
4. Sabatini, D. D., Krebich, G., Morimoto, T., and Adelsnik, M. (1982) Cell 29, 1–22
5. Blobel, G. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1496–1500
6. Kuroiwa, T., Sakaguchi, M., Mihara, K., and Omura, T. (1990) J. Biochem. (Tokyo) 108, 829–834
7. Lingappa, V. R. (1991) Cell 65, 527–530
8. High, S., and Dobberstein, B. (1992) Curr. Opin. Cell Biol. 4, 581–586
9. High, S., Flint, N., and Dobberstein, B. (1991) J. Cell Biol. 113, 25–34
10. von Heijne, G. (1985) Curr. Top. Membr. Transp. 24, 151–179
11. Sato, T., Sakaguchi, M., Mihara, K., and Omura, T. (1990) EMBO J. 9, 2391–2397
12. Sakaguchi, M., Tomiyoshi, R., Kuroiwa, T., Mihara, K., and Omura, T. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 16–19
13. Kuroiwa, T., Sakaguchi, M., Mihara, K., and Omura, T. (1991) J. Biol. Chem. 266, 9251–9255
14. Audigier, Y., Friedlander, M., and Blobel, G. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 5783–5787
15. Friedlander, M., and Blobel, G. (1985) Nature 318, 338–343
16. Lipp, J., Flint, N., Haueplie, M. T., and Dobberstein, B. (1988) J. Cell Biol. 109, 2013–2022
17. Wessels, H. P., and Spiess, M. (1988) Cell 55, 61–70
18. Sakaguchi, M., Ueguchi, C., Ito, K., and Omura, T. (1991) J. Biochem. (Tokyo) 109, 799–802
19. Sakaguchi, M., Mihara, K., and Sato, R. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3361–3364
20. Walter, P., and Blobel, G. (1983) Methods Enzymol. 96, 84–93
21. Walter, P., and Blobel, G. (1983) Methods Enzymol. 96, 682–691
22. Eriksson, A. H., and Blobel, G. (1983) Methods Enzymol. 96, 38–50
23. Sakaguchi, M., Mihara, K., and Sato, R. (1987) EMBO J. 6, 2425–2431
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 488–492
26. Spiess, M., Handschin, C., and Baker, K. P. (1989) J. Biol. Chem. 264, 1917–1924
27. Melancon, P., and Garoff, H. (1986) EMBO J. 5, 1551–1560
28. Cutler, D. F., and Garoff, H. (1986) J. Cell Biol. 102, 889–901
29. Liljestrom, P., and Garoff, H. (1991) J. Virol. 65, 147–154
30. Lipp, J., and Dobberstein, B. (1986) Cell 46, 1103–1112
31. Schmid, S. R., and Spiess, M. (1988) J. Biol. Chem. 263, 16886–16891
32. Hegner, M., von Kieckebusch-Guck, A., Falchetto, R., James, P., Senema, G., and Mantei, N. (1992) J. Biol. Chem. 267, 16928–16933
33. Nilsson, J., Whitley, P., and von Heijne, G. (1994) J. Cell Biol. 126, 1127–1132
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