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The code for directing proteins for translocation across ER membrane: SRP cotranslationally recognizes specific features of a signal sequence

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Abstract

The signal recognition particle (SRP) cotranslationally recognizes signal sequences of secretory proteins and targets ribosome-nascent chain complexes (RNCs) to the SRP receptor in the endoplasmic reticulum membrane, initiating translocation of the nascent chain through the Sec61 translocon. Although signal sequences do not have homology they have similar structural regions: a positively charged N-terminus, a hydrophobic core and a more polar C-terminal region that contains the cleavage site for the signal peptidase. Here, we have used site-specific photocrosslinking to study SRP-signal sequence interactions. A photoreactive probe was incorporated into the middle of wild-type or mutated signal sequences of the secretory protein preprolactin by *in vitro* translation of mRNAs containing an amber stop codon in the signal peptide in the presence of the N^ε-(5-azido-2 nitrobenzoyl)-Lys-tRNA^{amb} amber suppressor. A homogeneous population of SRP-ribosome-nascent chain complexes was obtained by the use of truncated mRNAs in translations performed in the presence of purified canine SRP. Quantitative analysis of the photoadducts revealed that charged residues at the N-terminus of the signal sequence or in the early part of the mature protein have only a mild effect on the SRP-signal

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sequence association. However, deletions of amino acid residues in the hydrophobic portion of the signal sequence severely affect SRP binding. The photocrosslinking data correlate with targeting efficiency and translocation across the membrane. Thus, the hydrophobic core of the signal sequence is primarily responsible for its recognition and binding by SRP, while positive charges fine-tune the SRP-signal sequence affinity and targeting to the translocon.

Keywords

SRP; signal sequence; protein targeting; protein translocation; photocrosslinking

Introduction

Protein trafficking in eukaryotic cells is a complex process, during which proteins are transported from the place of synthesis to their ultimate destinations. The first stage of this process is protein targeting – selection of secretory or membrane proteins by a targeting factor and directing them to endoplasmic reticulum (ER) membrane. The ensuing process of translocation/integration at the ER membrane is conducted by the Sec61 translocon in eukaryotic cells, and the homologous SecYEG translocon in bacteria [1-7]. The eukaryotic translocon forms an aqueous pore or protein-conducting channel in the ER membrane [8]. Several X-ray and cryo-electron microscopy structures of Sec61 and SecY translocon complexes are available [9-12]. However, many details of the mechanism of protein translocation are still unknown.

In the process of targeting to the ER, secretory or membrane proteins are recognized by a cytoplasmic factor – the signal recognition particle (SRP) – and transferred to the Sec61 translocon [13-16]. The prokaryotic counterpart to SRP, the Ffh/4.5S RNA complex, targets proteins to the SecYEG translocon in the bacterial plasma membrane [4, 17, 18]. Secretory proteins are synthesized as precursors with an N-terminal extension, a signal sequence (or signal peptide) [19, 20]. Recognition of signal sequences by a targeting factor is a very well coordinated and balanced process. When this balance is disrupted by overproduction of secretory proteins their precursors are accumulated in the cytoplasm in bacteria [21, 22]. In mammals, inability of a mutated protein to interact with SRP triggers a specific quality control mechanism, RAPP [23]. It has been estimated that about 20% of all mouse or human proteins have signal sequences [24]. Thus, signal-sequence recognition and targeting is one of the most common and frequent events in protein biogenesis. Many human diseases are caused by mutations in signal sequences of secretory proteins [25].

SRP cotranslationally recognizes signal sequences immediately upon their emergence from the ribosome [15, 26-29]. The eukaryotic SRP is a ribonucleoprotein, consisting of a 7S RNA and six protein subunits (SRP9, SRP14, SRP19, SRP54, SRP68, SRP72). The bacterial counterpart is more simple and consists of only two components, a 4.5S RNA and the Ffh protein [3, 4, 13, 16-18]. The SRP54 subunit of mammalian SRP is directly involved in interactions with the signal sequence [26, 27]. Many different SRP structures, substructures, as well as complexes with the SRP receptor (SR) have been determined [30-37].

The SRP targeting pathway consists of a number of distinct steps [14, 16-18]. SRP first binds to the signal sequence when it emerges from the peptide exit site on the ribosome. The formation of a SRP-ribosome-nascent chain complex leads to a transient elongation arrest, and the complex is targeted to the SR on the ER membrane. GTP binding to SRP is a prerequisite for formation of the SRP-SR complex. The ribosome-nascent chain complex is then transferred from the SR to the translocon. The final step, GTP hydrolysis triggers dissociation of the SRP from the SR, releasing SRP back to the cytosol.

SRP binds signal sequences on ribosome-bound nascent chains with very high affinity, in the range of 0.05-0.4 nM [38]. Yet, signal sequences of different secretory proteins do not have extensive sequence homology but rather conform to a more loosely defined architecture with a positively charged N-terminal n-region, a central hydrophobic h-region and a more polar C-terminal c-region [19, 20] (Fig. 1a). The c-region is necessary for processing and cleavage of the signal sequence by the signal peptidase [39, 40]. Studies of mutated secretory proteins in bacteria have suggested that the integrity of the n- and h-regions may be important for protein translocation [41, 42]. As an example, the LamB-prolactin hybrid protein containing *E. coli* LamB signal sequence could be targeted to eukaryotic microsomes but translocated at low efficiency, whereas mutation of three hydrophobic residues in the h-region to leucines resulted in full translocation activity of the mutated protein [43]. This observation led to the conclusion that a leucine-rich signal sequence is necessary for optimal interaction with SRP. Structural studies have also suggested that hydrophobicity of the signal sequence is sufficient for interaction with SRP [32]. Studies in bacteria have shown that reduction in the positive charge of the n-region decreases the efficiency of SRP recognition, and led to the conclusion that basic amino acids can promote interaction with SRP [44]. Studies in yeast have suggested that the h-region is responsible for directing of secretory proteins to SRP-dependent pathway [45, 46]. However, the sequence characteristics that drive the interaction of signal sequences with mammalian SRP are not well understood, as highlighted in a recent review [3].

The goal of the present work was to study what elements of the signal sequence and surrounding regions are responsible for mammalian SRP-nascent chain interactions. Using a site-specific photocrosslinking technique, we have conducted systematic study of the interaction of canine SRP with ribosome-nascent chain complexes containing mutated signal sequences, and have correlated the effects on the SRP-signal sequence interaction with the efficiency of translocation of the mutated proteins across ER membrane.

Results

Experimental design

A site-specific photocrosslinking approach was used to investigate the role of different parts of the signal sequence for interaction with SRP. The technique is based on the tRNA-mediated incorporation of a modified amino acid carrying a crosslinking reagent into ribosome-nascent chain complexes (RNCs) [23, 26, 47]. In the present study, RNCs of both wild-type and mutated preprolactin (pPL) were synthesized using an *in vitro* translation system. All mRNAs used contained a unique amber-stop codon (UAG) in the codon corresponding to the position 18 of the preprolactin signal sequence (Fig. 1b; Table 1).

Premature termination of translation occurs at the position of the amber codon in the absence of an amber-suppressor, however termination is suppressed in the presence of an amber-suppressor tRNA (Fig. 1b). Photoreactive N^ε-(5-azido-2-nitrobenzoyl)-Lys-tRNA^{amb} (εANB-Lys-tRNA^{amb}) amber-suppressor Lys-tRNA was used for site-specific labeling of pPL nascent chains.

Homogeneous populations of [³⁵S] Met-labeled pPL RNCs were prepared *in vitro* by translation of mRNAs truncated at codon 86. In the absence of a natural stop codon at the end of the mRNA, ribosomes do not dissociate and the nascent chains remain bound. RNCs of wild-type and mutated preprolactin were prepared in the presence of purified canine SRP. Photocrosslinking was induced by UV-irradiation, and SRP-photoadducts were visualized by SDS-PAGE. As it is shown in Fig. 1c, a strong photocrosslinking band, corresponding to the expected molecular weight of a nascent pPL and SRP54 photoadduct (NC-SRP54), was observed when SRP was present in the reaction mix, but not in the control reaction without SRP. The result is in good agreement with earlier studies on pPL-SRP interaction [23, 26, 38]. NC-SRP54 photocrosslinking was strictly probe-dependent, as no adduct was found in the control reaction without εANB-Lys-tRNA^{amb} (Fig. 1c).

As noted in the Introduction, signal sequences have three distinct structural regions: a positively charged n-region, a central hydrophobic h-region and a more polar c-region that contains the signal peptidase cleavage site. In order to delineate the parts of the signal sequence involved in interaction with SRP we designed mutated pPL proteins with (i) alterations in the charge content of the n-region (addition/removal of positively charged residues and/or decrease in the length of the n-region; mutations of this kind are denoted with “n-” in their names and a mnemonic for the kind of mutations made), (ii) alterations in the length of the h-region (deletions are denoted by Δ), (iii) alterations in the charge content of the early part of the mature protein immediately after the signal peptidase cleavage site (mutations of this kind are denoted with “-c” in their names), and (iv) combinations of i and iii. All mutants are listed in Table 1.

Positively charged residues in the n-region have only weak effects on the SRP-signal sequence interaction

The n-region of the wild-type pPL signal sequence contains one negatively charged residue (Asp at position 2) and three positively charged residues (Lys at positions 4 and 9, Arg at position 12), Table 1. Replacement of the Lys and Arg residues by Leu led to a mild decrease of SRP photocrosslinking (mutants n-3LR and n-4L, Fig. 2a). Deletion of the first 10 amino acid residues within the n-region (mutant n-10R, leading to decrease of the charge) gave similarly weak effect on the photocrosslinking efficiency. The introduction of two additional positively charged amino acid residues in the n-region did not significantly influence SRP photocrosslinking (mutant n-3KR, Fig. 2a).

Introduction of charged residues into the early part of the mature protein have only weak effects on the SRP-signal sequence interaction

Statistical studies have shown that there is a strong selection against Arg and Lys in the early part of mature portion of bacterial secretory proteins [48, 49]. Although such strong

discrimination against positively charged amino acids was not observed in eukaryotic signal sequences, long strings of positively charged residues placed downstream of the signal sequence can block translocation of eukaryotic secretory proteins [50-52]. What is the nature of this inhibition of translocation? Does it influence interaction with SRP? Using the photocrosslinking approach, we found that introduction of one or two positively charged Lys residues in the early portion of mature pPL did not influence the SRP-nascent chain interaction (Fig. 2b). Further increasing the number of Lys residues to 3-5 led to a small decrease in the quantity of the SRP-photoadduct. The introduction of negatively charged amino acids (five Asp residues, Fig. 2b) had almost the same, weak inhibitory effect on the SRP-nascent chain photocrosslinking as had five Lys residues. The efficiency of SRP crosslinking to mutant signal peptides carrying extra positively charged residues both in the n-region and in the early part of mature protein (mutant n-3KR-4K-c) was equal to the crosslinking efficiency seen for the 4K-c mutant alone (Fig. 2a and 2b).

Deletions in the h-region have a strong effect on the SRP-signal sequence interaction

Recently, we have found that deletions of one or more Leu residues in the h-region of the pPL signal peptide compromise its interaction with SRP and lead to the activation of a novel, preemptive quality control mechanism called RAPP [23]. Here we conducted a quantitative analysis of the SRP interactions with the same mutant pPL signal sequences (Table 1). Deletion of only one Leu decreased SRP photocrosslinking efficiency almost by one third, while deletion of the second Leu had little effect, but deletion of three and four Leu led to an almost complete inhibition of SRP photocrosslinking (Fig. 3). These results show that the hydrophobicity of the h-region is critical for the SRP-nascent chain interaction. We hypothesized that it is more likely overall hydrophobicity of the hydrophobic core than its length, that is consistent with earlier findings [43]. The data on the hybrid protein LamB-prolactin suggested that a Leu-rich signal sequence is optimal for interaction with SRP [43]. Similar studies also suggested that the h-region is of major importance for the recognition by SRP [32, 33, 35]. Our results are in a good agreement with these observations. Thus, the signal sequence h-region is the key element for SRP-nascent chain interactions in mammalian cells.

Targeting of pPL with mutated signal peptide to the Sec61 translocon

In order to compare the effects on SRP binding with effects on the full translocation reaction elicited by the different signal sequence mutations, *in vitro* translocation experiments with dog pancreas rough ER microsomes (RMs) were conducted. Full-length wild-type and mutant pPLs were translated *in vitro* in the presence of purified RMs, and the efficiency of translocation was determined as the fraction of molecules in which the signal sequence was cleaved by the lumenally disposed signal peptidase. We did not see significant inhibition of cleavage for the proteins with mutations in the signal sequence n-region. All of them were efficiently processed and very little precursor with uncleaved signal sequence was detected (Fig. 4a). These data are in a good agreement with previous findings [53]. However, proteins with charged amino acid residues in the early portion of mature protein were not efficiently cleaved, and in general much more precursor was found for all mutated proteins of that group in comparison with the wild-type protein (Fig. 4b). The uncleaved precursor was not transported across the membrane since it could be digested by proteinase K, as shown for

the 4K-c mutant (Fig. 4d). However, a mutant with extra positively charged amino acids both in the n-region and in the mature part, n-3KR-4K-c, was more efficiently translocated than the 4K-c mutant (Fig. 4a and 4b).

A likely explanation for the translocation defect seen for the mutants carrying extra positively charged residues in the early part of the mature chain is that such mutations may affect the orientation of the signal peptide in the ER membrane, effectively imparting an N_{lum} - C_{cyt} orientation on the signal sequence [48, 54, 55]. In this orientation, the signal sequence will not become a substrate for the lumenally disposed signal peptidase, and the protein will remain accessible to proteinase K.

Mutated pPLs containing different deletions in the h-region of the signal sequence were also tested for translocation across the ER membrane (Fig. 4c; see also Karamyshev *et al.*, 2014 [23]). Earlier, we found that some deletions in the hydrophobic core led to inhibition of pPL translocation into RMs [23]. Here, we tested additional deletions in the signal sequence (1L*, 2L*, 3L*; Table 1) and assayed translocation of these proteins into RMs (Fig. 4c). While only the unprocessed form of pPL was observed in the absence of RMs, the majority of the wild-type protein was processed in the presence of RMs. However, deletions of 1, 2 or 3 Leu residues led to a gradual inhibition of signal sequence cleavage, with practically complete absence of mature form for a mutant with a 3 Leu deletion. The uncleaved precursor protein but not the mature form was susceptible to proteinase K digestion, as expected (Fig. 4d).

We conclude that deletions in the h-region strongly inhibit translocation and/or targeting to the membrane. These results generally correlate with the photocrosslinking results (Fig. 3).

Discussion

Prokaryotic and eukaryotic signal sequences have similar structural features with three distinct regions (the n-, h-, and c-regions) [19, 20]. It is well established that signals sequences usually have one or several positively charged amino acid residues in the n-region [19, 20]. However, the precise role of the positively charged amino acids has not been much studied, especially for mammalian signal sequences. For *E. coli*, it has been shown that alterations in the n-region of Lpp [56], OmpF-Lpp [57], PhoA [41, 58], and OmpA [59] compromise translocation across the membrane, while PhoE appears to be less sensitive to similar changes [60]. It was not determined which step (targeting, translocation or processing) these mutations affected. However, because these proteins are most likely transported by an SRP-independent mechanism, the effect of the mutations can be different than for SRP-dependent proteins and probably involve interactions with translocon components rather than SRP. On the other hand, studies of SRP-dependent hybrid proteins containing mutant signal sequences suggested that positive charges in the n-region can promote interaction with SRP in bacteria [44]. It therefore seems that positively charged amino acids in n-region contribute to interaction with SRP as well as with the translocon components or other targeting factor in bacteria. Positively charged residues located in the early part of the mature protein, next to the signal sequence, are known to block translocation in *E. coli* [61], but have not been much studied in the mammalian system. In

contrast, the role of the h-region for SRP binding and membrane translocation is well documented, both in bacteria, yeast and mammals [13, 43, 45, 46, 62, 63]. It was also shown that the hydrophobicity of the nascent chain is important for selection of SRP targeting or sorting pathway in bacteria [64, 65].

It was shown earlier that alterations in the signal sequence hydrophobic core had a greater affect on pPL translocation than on its interaction with SRP [66]. The authors found that the efficiency of SRP crosslinking to a mutant signal sequence with deletion of 3 leucines was 80% of the wild type protein crosslinking, and 35% for a mutant with deletion of 5 leucines. However, translocation of the mutated proteins was greatly inhibited: for a protein with deletions of three leucines the translocation efficiency was only 0.5-2.5% of the wild-type one, and for the protein with deletions of 5 leucines it was at the background level. The authors used TDBA-Lys-tRNA for incorporation of the TDBA photocrosslinking probe (two lysines of the n-terminal part of the signal sequence were exposed outside the ribosome, and thus involved in SRP crosslinking). In our studies, we have used a single ANB photocrosslinking probe placed in the center of the signal sequence and are able to detect even small changes in SRP crosslinking efficiencies. The higher sensitivity of our experiments is probably because of the different crosslinking probe used as well as its location in the signal sequence. Our data nevertheless show a similar trend to the previous study and support the model that the recognition of signal sequences and selection of proteins for translocation are conducted at least at two levels: by SRP at the ribosome at an early step of translation and at the membrane after release of the SRP from the signal sequence [66].

We found only weak effects of charge mutations in the n-region on SRP crosslinking and translocation efficiency. This is also true when comparing the n-3KR-4K-c and 4K-c constructs, that are both impaired in SRP crosslinking and translocation to similar extents, i.e., the addition of two lysines to the n-region makes no difference in this case (Figs. 2 and 4). Notably, positively charged amino acids in the n-region have been found to promote protein export in bacteria [44, 67]. For instance, though positive charge in the n-region was not absolutely required for MBP export, the export was most efficient for proteins containing at least one positively charged residue in this region [67]. They also proposed that n- and h-regions do not have completely different functions. It was further found that an increase in the hydrophobicity of the signal sequence can compensate for the loss of basic residues [44]. It is possible that positively charged n-terminus amino acid residues in pPL do not have strong effects because of the high hydrophobicity of the pPL h-region. There are obviously a number of combinations of mutations in the n-, h-, and c-regions of pPL that would be interesting to study further.

Our results show that while interaction with SRP was not very sensitive to charged amino acid residues close to a cleavage site, the translocation of the mutated proteins was more strongly affected (Figs. 2b and 4b), indicating that translocon components are more sensitive to charged amino acids in this region than is SRP.

In summary, the experiments reported here demonstrate that positively charged amino acids, both in the n-region and immediately downstream of the signal sequence, have only weak

influences on binding to mammalian SRP and on translocation into RMs. Their main effect seems to be on the orientation of the signal sequence, with the charge-balance across the signal sequence affecting its propensity to orient either N_{cyt}-C_{lum} (favoring translocation of the protein into the lumen of the ER) or N_{lum}-C_{cyt} (preventing translocation) [48, 54, 55]. In accordance with earlier results, we find that deletions in the h-region of the pPL signal sequence dramatically affect both SRP binding and translocation. Signal sequences inactivated by such deletions trigger recently discovered quality control pathway, RAPP [23].

Our data demonstrate that the integrity of signal sequence h-region is the major feature for SRP recognition, and its hydrophobicity is important for translocation. However, many signal sequences have very moderately hydrophobicity signal sequences. How are they recognized, targeted and translocated? There is a common assumption that eukaryotic proteins are targeted by SRP-dependent mechanism. However, accumulating data suggest that more proteins than originally thought could be translocated by SRP-independent mechanisms in eukaryotes [68-71]. Perhaps there are several pathways for SRP-independent translocation, and additional targeting factors may remain to be discovered.

Materials and Methods

Plasmids, DNA techniques, mRNA, and tRNA

Plasmids containing bovine pPL constructs were made from the previously described pSPBP4 plasmid, a 950 bp NcoI-PstI fragment encodes preprolactin inserted into pSP64T vector [8]. Plasmids containing pPLs with an amber-stop codon and deletions in the hydrophobic core were constructed earlier [23]. Standard DNA techniques were performed according to the protocols of Sambrook and coworkers [72]. Site-directed mutagenesis was performed by a PCR technique using Pfu turbo DNA polymerase (Stratagene, La Jolla, CA). All mutations were confirmed by DNA sequencing. DNA fragments for *in vitro* transcription were generated by PCR under standard conditions using *Ex Taq* DNA polymerase (TaKaRa, Shiga, Japan). N^ε-(5-azido-2-nitrobenzoyl)-Lys-tRNA^{amb} (εANB-Lys-tRNA^{amb}) and truncated mRNAs were prepared as before [26, 47].

In vitro translation and site-specific photocrosslinking

In vitro translation for photocrosslinking experiments was conducted in wheat germ cell-free translation system as before [47, 73] with some modifications. Standard reaction (25-50 μl) contained 50 mM HEPES (pH 7.5), 100 mM KOAc, 3.5 mM Mg(OAc)₂, 1.0 μCi/μl [³⁵S]methionine, 1-1.4 pmoles/μl εANB-Lys-tRNA^{amb} (or unmodified Lys-tRNA^{amb}), and other components as described [47, 73, 74]. Where indicated, 0.06 μM canine SRP was added to the reaction mix. Translation was conducted in the dark for 30-40 min at 26°C. Samples were photolyzed on ice for 15 min using a 500 W mercury arc lamp as described [73]. After photolysis, samples were sedimented through a 130 μl sucrose cushion (0.5 M sucrose, 50 mM HEPES (pH 7.5), 40 mM KOAc, 5 mM Mg(OAc)₂) in TLA 100 rotor (Beckman Optima TL centrifuge) at 100,000 rpm for 5 min at 4°C. The pellets were resuspended in SDS sample buffer and analyzed by 10-15% gradient SDS-electrophoresis. [¹⁴C] molecular weight protein markers (Gibco or Amersham) were loaded on the gel and

used for calculation of photoadducts molecular weight. Gels were visualized on a Bio-Rad Molecular Imager FX. Quantity One image software (Bio-Rad) was used for analysis. Quantity of each photoadduct was normalized to the amount of the corresponding nascent chain, and then calculated relatively to wild-type pPL crosslink.

Translations of full-length pPL mRNAs were performed at 30°C for 60 min as described [75, 76] in a total volume of 14 µl containing nuclease treated reticulocyte lysate, 40 units of RNase inhibitor, [³⁵S]Met (0.7 mCi/ml), 70 mM of each amino acid except Met, and 4 equivalents of RMs. Samples were analyzed by 12% SDS-PAGE. Gels were visualized on a Fuji FLA-3000 phosphorimager using the Image Reader V1.8J/Image Gauge V 3.45 software.

Proteinase K treatment

Translocation of polypeptides to the luminal side of the microsomes was assayed by resistance to exogenously added proteinase K and by analysis of constructs for protection. Proteinase K treatment was performed as before [77, 78] by adding CaCl₂ (13 mM final concentration) together with proteinase K (67 µg/ml final concentration) to the translation reaction. After incubating for 30 min on ice, the proteinase K was inactivated by adding PMSF (670 µg/ml final concentration) and the samples were further incubated for 5 min on ice before SDS-PAGE analysis.

Canine SRP, ER microsomes

Canine SRP was purified as described previously [79], except that the sucrose density sedimentation was omitted. SRP concentration was determined using $\epsilon_{280\text{ nm}} = 1.0 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$. Column-washed dog rough microsomes were prepared as before [80].

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Abbreviations

SRP	signal recognition particle
RNC	ribosome-nascent chain complex
SR	SRP receptor
ER	endoplasmic reticulum
pPL	preprolactin
RM	rough microsomes

ϵ ANB-Lys-tRNA^{amb}N^E-(5-azido-2 nitrobenzoyl)-Lys-tRNA^{amb}.

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Highlights

- SRP cotranslationally recognizes signal sequences of secretory proteins.
- Signal sequences have three regions: a positively charged N-terminus, a hydrophobic core, and a polar C-terminal cleavage-site region.
- Alterations at the N-terminus of the signal sequence or near the cleavage site have little effect on the association with mammalian SRP.
- The hydrophobic core is primarily responsible for recognition by SRP.

Fig. 1.

Signal sequence is recognized by SRP and this interaction is detected by site-specific photocrosslinking. (a) Scheme of a typical signal sequence (see text for details). a. a., amino acid residues. (b) Scheme of the site-specific incorporation of photocrosslinking probe into a protein. mRNA contains amber-stop mutation (UAG) in position 18 of pPL (shown as an octagon). Premature termination of protein synthesis at this position is occurred when an amber-suppressor is absent in translation (1). However, in the presence of amber-suppressor tRNA the termination is suppressed at the amber-mutation and specific amino acid is incorporated by the amber-suppressor tRNA (Lys-tRNA^{amb}) in response of an amber-codon (2). When translation reaction contains modified amber-suppressor-tRNA carrying amino acid with photoreactive probe (ϵ ANB-Lys-tRNA^{amb}), a modified amino acid residue is incorporated into unique position of the protein that corresponds to a position of amber-mutation in the mRNA (3). Homogeneous population of RNCs is obtained by translation of mRNAs that are truncated in the coding region (for example, codon 86). Normal termination is not occurred at the end of such mRNAs because natural stop codon is missing and nascent chain is not released from the ribosome. As a result, all ribosome bound nascent chains in the reaction have the same length which is determined by the length of the truncated mRNA. (c) Photocrosslinking of nascent preprolactin (86 amino acid long nascent chain) is probe-specific and SRP-dependent. *In vitro* translation contained unmodified Lys-tRNA^{amb} in the control reaction without photo-crosslinking probe (lane 1), ϵ ANB-Lys-tRNA^{amb} (lanes 2 and 3), canine SRP was added where indicated (lanes 1 and 2). [¹⁴C] molecular weight protein markers are shown in lane 4. Proteins were synthesized *in vitro* by wheat germ translation system in the presence of [³⁵S] methionine. See details for reaction conditions and photolysis in *Material and Methods*. Samples were analyzed by 10-15% gradient SDS-

PAGE. The positions of nascent chains (NC), peptidyl-tRNA (Pt), and photoadduct to SRP54 (NC-SRP54) are shown. Apparent molecular masses are shown in kDa.

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Fig. 2.

Affect of charged amino acid residues in the N-terminal region of signal sequence (a) and in the early part of the mature protein (b) of the nascent pPL on photocrosslinking to SRP54. Stable RNCs containing the first 86 residues of either wild-type (WT) or mutant pPLs with different mutations were synthesized *in vitro* translation system in the presence of canine SRP. A photoreactive probe was cotranslationally incorporated into the middle of the signal sequence (position 18) as shown in Fig. 1b. Following photolysis (UV-irradiation), samples were analyzed by electrophoresis in 10-15% gradient SDS-PAGE and photoadducts were detected by phosphorimager. The quantities of mutant nascent chain-SRP54 photoadducts were calculated in per cents relatively to the photoadduct of the wild-type pPL (n=6 for 1K-c, 2K-c, 3K-c, 5D-c; n=10 for 4K-c, n-3KR-4K-c; n=12 for 5K-c; n=2 for n-3LR, n-4L; n=4 for n-3KR, n-Δ10,R; mean $\pm$ S.D.). See Table 1 for mutations' abbreviations and their positions.

Fig. 3. Hydrophobic core of a signal sequence is a primary feature for SRP recognition. Quantitative analysis of SRP photocrosslinking to mutated pPLs with deletions in the hydrophobic core is shown in the graph (n=6, mean $\pm$ SD). Samples were analyzed as in Fig. 2. See Table 1 for mutations.

Fig. 4. Targeting and translocation of mutated pPLs across ER membrane. Mutations in the n-terminal region (a) of pPL signal sequence as well as in the early portion of mature protein (b) and hydrophobic core (c) influence targeting to translocon, translocation and signal sequence cleavage. (a-c) Full-length polypeptides of wild-type preprolactin (WT) or mutated proteins were translated in reticulocyte lysate in the absence (-) or presence (+) of rough microsomes (RM). See Table 1 for abbreviations of mutations. (d) Proteinase K protection of the proteins translocated into rough microsomes. Full-length polypeptides were translated as in figures (a) – (c) and were treated (+) or not (-) with proteinase K (PK) as indicated. Figures represent efficiency of protein translocation in per cent of the mature form (data are from 2-5 experiments; n.d., not determined). Positions of matured pPL and its precursor are marked by one and two black dots, respectively. Apparent molecular masses are shown in kDa.

Table 1

pPL mutants

Mutant	Sequence (N-terminal part of the preprotein)	
	Signal sequence	Mature protein
	←	→
pPL (WT)	MDSKGSSQKGSRL LLLL#VSNLLLCQGVVS	T----PVCNPGPGN
n-4L	<u>M</u> <u>LS</u> <u>L</u> GSSQ <u>L</u> GSRLLLLL#VSNLLLCQGVVS	T----PVCNPGPGN
n-3LR	<u>M</u> <u>LS</u> <u>L</u> GSSQ <u>L</u> GSRLLLLL#VSNLLLCQGVVS	T----PVCNPGPGN
n- 10,R	MRLLLLL#VSNLLLCQGVVS	T----PVCNPGPGN
1L	MDSKGSSQKGSRL LLLL#VSN--LCQGVVS	T----PVCNPGPGN
2L	MDSKGSSQKGSRL LLLL#VSN--LCQGVVS	T----PVCNPGPGN
3L	MDSKGSSQKGSRL LL--L#VSN--LCQGVVS	T----PVCNPGPGN
4L	MDSKGSSQKGSRL LL--L#VSN--LCQGVVS	T----PVCNPGPGN
1K-c	MDSKGSSQKGSRL LLLL#VSNLLLCQGVVS	T <u>K</u> ----PVCNPGPGN
2K-c	MDSKGSSQKGSRL LLLL#VSNLLLCQGVVS	T <u>KK</u> ----PVCNPGPGN
3K-c	MDSKGSSQKGSRL LLLL#VSNLLLCQGVVS	T <u>KKK</u> ----PVCNPGPGN
4K-c	MDSKGSSQKGSRL LLLL#VSNLLLCQGVVS	T <u>KKKK</u> ----PVCNPGPGN
5K-c	MDSKGSSQKGSRL LLLL#VSNLLLCQGVVS	T <u>KKKKK</u> ----PVCNPGPGN
n-3KR	MDSKGSSQ <u>KKKR</u> LLLLL#VSNLLLCQGVVS	T----PVCNPGPGN
n-3KR-4K-c	MDSKGSSQ <u>KKKR</u> LLLLL#VSNLLLCQGVVS	T <u>KKKK</u> ----PVCNPGPGN
5D-c	MDSKGSSQKGSRL LLLL#VSNLLLCQGVVS	T <u>DDDD</u> ----PVCNPGPGN
n-2LKR	<u>M</u> <u>LS</u> <u>L</u> GSSQ <u>K</u> GSRLLLLLVSNLLLCQGVVS	T----PVCNPGPGN
1L*	MDSKGSSQKGSRL LL--VSNLLLCQGVVS	T----PVCNPGPGN
2L*	MDSKGSSQKGSRL LL--VSNLLLCQGVVS	T----PVCNPGPGN
3L*	MDSKGSSQKGSRL LL--VSNLLLCQGVVS	T----PVCNPGPGN

Altered amino acids are underlined, positively charged amino acid residues are marked in red, and negatively charged are in cyan. #, location of εANB-Lys probe in the constructs for photocrosslinking. Mutants 1L – 4L were constructed earlier [23].