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Signal Recognition Particle Arrests Elongation of Nascent Secretory and Membrane Proteins at Multiple Sites in a Transient Manner*

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The signal recognition particle (SRP) has been shown to target nascent secretory and membrane proteins to the endoplasmic reticulum. In the wheat germ cell-free system, SRP arrests the elongation of the nascent chains until the translational complex is docked to the endoplasmic reticulum membrane where the interaction between SRP and docking protein causes a release of the nascent chain arrest. For two secretory proteins, arrested peptides of 70 amino acids have been identified (Walter, P., Ibrahimi, I., and Blobel, G. (1981) *J. Cell Biol.* 91, 545–550; Meyer, D. I., Krause, E., and Dobberstein, B. (1982) *Nature* 297, 647–650). By using an *in vitro* coupled transcription-translation system, we have analyzed SRP arrest and the resulting peptides of the two secretory proteins lysozyme and granulocyte-macrophage colony-stimulating factor and the membrane protein invariant chain. SRP arrested the elongation of all three proteins at multiple sites, giving rise to ladders of arrested peptides. The size of the arrested peptides increased with the time of translation, resulting in mostly full-length pre-peptides after about 40 min. This suggests that SRP arrest is transient rather than stable. Upon addition of microsomes, the SRP arrest was released, and all the blocked peptides could be chased into mature proteins or full-length precursors.

Signal recognition particle and docking protein provide the receptor system that targets secretory and membrane proteins to the endoplasmic reticulum (for review, see Walter *et al.*, 1984; Hortsch and Meyer, 1986). Wheat germ lysate does not contain sufficient endogenous SRP[1] activity to promote translocation of proteins across exogenously added rough microsomes. It therefore provides an ideal *in vitro* system for analyzing the SRP requirement in the targeting and translocation process. Using such an *in vitro* reconstituted translocation system, it was shown that SRP binds to the amino-terminally located signal sequences of secretory proteins as soon as they extrude from the ribosomes, *i.e.* after the polymerization of approximately the first 70 amino acids. The interaction between SRP, nascent chain, and the ribosome causes an arrest of further chain elongation (Walter *et al.*, 1981). This arrest is released when the SRP in the translational complex binds to the docking protein, an integral membrane protein of the rough ER (Meyer *et al.*, 1982; Gilmore *et al.*, 1982). Translocation of the nascent chain across the ER membrane proceeds by an as yet unknown mechanism.

In an attempt to dissect the functional domains of SRP, Siegel and Walter (1985) showed that an SRP particle reconstituted from the isolated 7SL RNA and only four of the six heterologous protein subunits (*i.e.* lacking the 9-kDa and the 14-kDa polypeptides) was still capable of promoting translocation, even though the chain elongation-arrest activity was abolished. Equally intriguing was the observation that SRP arrest of secretory proteins could be observed only in the wheat germ cell-free system, but neither in reticulocyte nor in HeLa cell lysates (Meyer, 1985). This raised the question whether an SRP-mediated arrest, particularly at a very early time of nascent chain elongation, was really required for secretory and membrane proteins to be targeted to and translocated across ER membranes. In only two instances, namely for preprolactin (Walter *et al.*, 1981) and for pre-IgG light chain (Meyer *et al.*, 1982), SRP-arrested peptides of approximately 70 amino acids had directly been observed since their mRNAs could be obtained in a relatively pure form. Even though it was shown for several other secretory proteins that synthesis of the full-length precursor was inhibited by the presence of SRP during prolonged incubation times, arrested peptides were not demonstrated. A few membrane proteins that do not have a cleavable signal sequence and the NH_2 termini of which are positioned in the cytoplasm (type II membrane proteins) were shown not to be SRP-arrested under such experimental conditions (Anderson *et al.*, 1983; Mueckler and Lodish, 1986). Nevertheless, their efficient translocation across ER membranes was SRP-dependent.

Recently, Ainger and Meyer (1986) suggested that SRP could arrest the elongation of pre-IgG light chain even when approximately two-thirds of the nascent chain had already been completed. Consequently, SRP arrest at a very early time of nascent chain elongation as observed for pre-IgG light chain and preprolactin might not be a general rule. We analyzed in a wheat germ cell-free system the SRP-arrested peptides of several proteins. Among these are the secretory proteins mouse IgG light chain, chicken oviduct lysozyme, and mouse GM-CSF, and the integral membrane protein human invariant chain of the class II histocompatibility antigens. Analysis of SRP-arrested peptides originating from a wide variety of proteins is now technically feasible because *in vitro* transcription systems allow synthesis of mRNAs encoding a single protein species. Translation products obtained from such mRNAs can be analyzed directly, avoiding the necessity of immunoprecipitations.

EXPERIMENTAL PROCEDURES

Materials—L-[^{35}S]Methionine was from Amersham, 7-methylguanosine 5′-monophosphate from Sigma, and wheat germ from General Mills.

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[1] The abbreviations used are: SRP, signal recognition particle; ER, endoplasmic reticulum; Iγ, human invariant chain; GM-CSF, granulocyte-macrophage colony-stimulating factor; ^{7}mG, 7-methylguanosine 5′-monophosphate; SDS, sodium dodecyl sulfate.

Plasmids—The basic expression plasmid was pDS5 (Stueber *et al.*, 1984). Lysozyme mRNA was transcribed from pDS5/4 (Stueber *et al.*, 1984) from which the β-lactamase gene had been deleted. The construction of pIγ has been described (Lipp and Dobberstein, 1986b).

p5/GM-CSF, a *StuI-Hind*III fragment from pGM5′Δ7 (Gough *et al.*, 1985) containing the entire coding sequence of GM-CSF, was subcloned into pDS5. pGM5′Δ7 was a kind gift of N. Gough.

In Vitro Coupled Transcription-Translation—Plasmids were transcribed *in vitro* by *Escherichia coli* RNA polymerase, and the resulting mRNA was translated in a wheat germ cell-free system as described by Stueber *et al.* (1984). Since considerable amounts of β-lactamase are expressed from pIγ, the translation of this mRNA was suppressed by the addition of a 20-mer oligodeoxynucleotide complementary to the sequences around the initiating AUG (Haeuptle *et al.*, 1986). Either SRP (about 0.02 A_{280} unit) or the corresponding SRP buffer was added at the start of translation. In order to test for release of the SRP arrest, the SRP-arrested sample was equally divided. In one sample, elongation was stopped by the addition of 10 mM EDTA and cooling to 0 °C; to the other, salt-washed microsomes (Walter and Blobel, 1980) were added and the incubation continued for another 15 min. Translations were synchronized 5 or 8 min after the start of the incubations by the addition of ^{7}mG to a final concentration of 2 or 4 mM.

Other Methods—IgG light chain mRNA (Blobel and Dobberstein, 1975) was translated in the wheat germ system as described (Warren and Dobberstein, 1978). Synchronization and addition of SRP and salt-washed microsomes were done as described under "transcription-translation."

Dog pancreas SRP was prepared according to Walter and Blobel (1980) and used after elution from DEAE-Sepharose. Salt-washed microsomes from dog pancreas were prepared as described (Warren and Dobberstein, 1978).

The preparation of the antisera directed against the amino- and the carboxyl-terminal domains of Iγ (Lipp and Dobberstein, 1986b), as well as the method used for immunoprecipitation of *in vitro* synthesized proteins (Dobberstein *et al.*, 1979), has been described.

SDS-polyacrylamide gels containing 22% acrylamide, 0.088% bisacrylamide, and 6 M urea were prepared and processed for fluorography as described by Haeuptle *et al.* (1986). Protein bands were quantitated by densitometry of the autoradiograms.

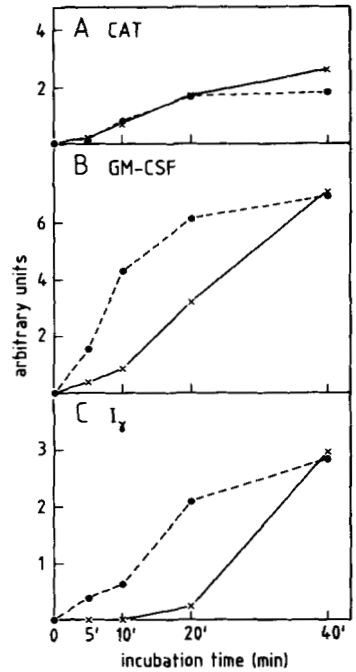

FIG. 1. **Time course of SRP-mediated delay in nascent chain elongation.** mRNAs coding for the cytoplasmic protein chloramphenicol acetyltransferase (*A*), the secretory protein GM-CSF (*B*), or the type II membrane protein Iγ (*C*) were translated either in the absence (●– – –●) or the presence (×——×) of SRP. 5 min after the start of translation, initiation of protein synthesis was blocked by the addition of ^{7}mG to 4 mM. Either immediately (0′) or after 5, 10, 20, or 40 min, aliquots were removed from the incubation mixture, and chain elongation was stopped by the addition of EDTA and cooling on ice. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, fluorography, and densitometry. The relative intensities of the bands representing the full-length pre-proteins were plotted against the time of incubation after synchronization with ^{7}mG.

RESULTS

SRP Retards the Elongation of a Secretory and a Type II Membrane Protein Rather Than Inducing a Complete Arrest—In order to analyze the effect of SRP on the chain elongation of different proteins, mRNAs encoding the secretory protein GM-CSF, the bacterial cytoplasmic protein chloramphenicol acetyltransferase, or the type II membrane protein Iγ were transcribed from plasmid vectors using *E. coli* RNA polymerase. These mRNAs were used to prime protein synthesis in a wheat germ cell-free system. Translation of all three proteins, either in the absence or presence of SRP, was allowed to proceed for 5 min, after which time ^{7}mG was added to the translation mixture in order to prevent further initiation of protein synthesis. Samples were then removed after several time intervals, the newly synthesized proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and the full-length pre-protein bands were quantitated by densitometry. From this experiment (see Fig. 1), it can be concluded that SRP causes a significant retardation of the chain elongation of GM-CSF and Iγ during the first 20 min of incubation, while the cytoplasmic protein chloramphenicol acetyltransferase is unaffected. However, after 40 min of synthesis, no difference could be seen in the amount of full length pre-proteins obtained in the presence or absence of SRP. This result suggests that the SRP arrest is transient and therefore reversible, even in the absence of docking protein which had been shown to cause a release of the SRP-mediated elongation block (Meyer *et al.*, 1982).

The Transient SRP Arrest of the Secretory Proteins Lysozyme and GM-CSF and of the Type II Membrane Protein Iγ Gives Rise to Ladders of Arrested Peptides the Sizes of Which Change with Prolonged Incubation Time—IgG light chain is one of the two secretory proteins for which rather tightly arrested, approximately 70-amino-acid-long peptides had been substantiated (Meyer *et al.*, 1982). If light chain mRNA is translated in wheat germ lysate in the presence of SRP, no full length precursor chain is synthesized (see Fig. 2*A*, *lane b*). Instead, a short peptide of approximately 7 kDa appears which persists for incubation times of 30 min (Fig. 2*A*, *lane c*) and longer. Upon the addition of salt-washed microsomes, the arrested peptide disappears and mostly mature light chain is obtained from which the signal peptide has been removed (Fig. 2*A*, *lane d*). When we performed a similar experiment with mRNA coding for chicken oviduct lysozyme (Fig. 2*B*), translation for 15 min in the presence of SRP alone gave rise to a ladder of short peptides with molecular weights ranging from 7,000–13,000, while little full-length pre-lysozyme was observed. When the incubation was continued for another 15 min, considerable amounts of pre-lysozyme appeared, and the number and average size of arrested peptides increased. When after the first 15-min incubation period microsomes were added to the arrested lysozyme peptides, mature chains from which the signal peptide was cleaved and some pre-chains were obtained. This suggests that the membranes released the SRP-mediated arrest, and that most of the blocked peptides were still translocation-competent.

We have shown that the appearance of full-length GM-CSF and Iγ pre-chains was retarded rather than tightly arrested by SRP (see Fig. 1). The analysis by SDS-polyacrylamide gel electrophoresis of the arrested peptides of GM-CSF

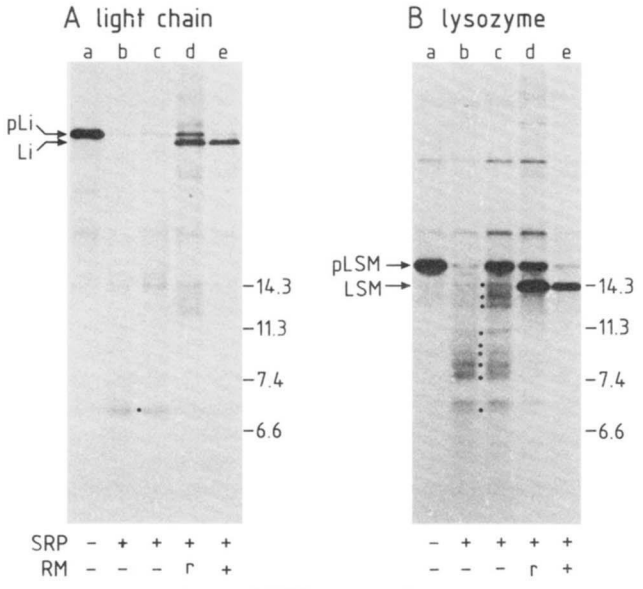

FIG. 2. **Comparison of SRP-arrested peptides of IgG light chain and chicken oviduct lysozyme.** To wheat germ lysate primed with IgG light chain (*A*) or lysozyme (*B*) mRNA, either blank buffers (*lane a*), SRP alone (*lanes b–d*), or SRP and salt-washed microsomes (*RM*, *lane e*) were added. After 8 min of incubation, translation was synchronized with 2 mM ^{7}mG. After another 15 min, the samples containing SRP alone were split into three: to one, EDTA was added in order to stop elongation (*lanes b*); to the second, buffer without membranes was added and incubation continued for 15 min (*lanes c*), to the third, salt-washed microsomes (*RM*) were added and incubation continued for 15 min in order to release (*r*) SRP arrest (*lanes d*). The control samples shown in *lane a* (synthesis of full-length precursors in the absence of SRP and microsomes) and *lane e* (synthesis of mature protein in the presence of membranes and SRP) were incubated for a total of 38 min. M_r values should be multiplied by 10^3. The positions of precursors (*pLi*, pre-light chain; *pLSM*, pre-lysozyme) and mature chains (*Li*, light chain; *LSM*, lysozyme) are indicated by *arrows* and those of SRP-arrested peptides by *dots*.

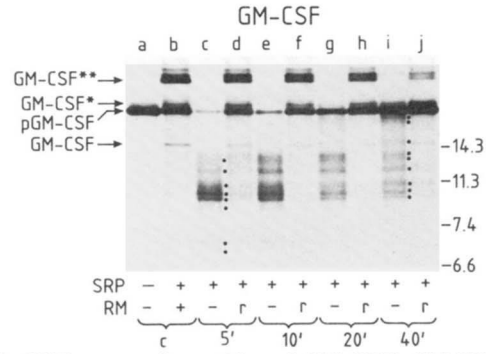

FIG. 3. **SRP-arrested peptides of GM-CSF.** GM-CSF mRNA was translated in a wheat germ cell-free system either in the absence (*lane a*) or in the presence (*lane b*) of SRP and ER membranes or with SRP alone (*lanes c–j*). After 8 min, translation was synchronized by the addition of 2 mM ^{7}mG, and incubation continued for 5, 10, 20, or 40 min. At each time point, 2 aliquots were removed: translation in 1 was stopped by the addition of EDTA and cooling on ice (*lanes c, e, g,* and *i*); to the 2nd aliquot, salt-washed microsomes (*RM*) were added, and incubation continued for another 15 min in order to allow for the SRP arrest to be released (*r, lanes d, f, h,* and *j*). The total incubation time of the control (*c*) samples shown in *lanes a* and *b* was 48 min. M_r values given should be multiplied by 10^3. SRP-arrested peptides are indicated by *dots*, the full-length GM-CSF precursor and its different processed forms by *arrows*. *pGM-CSF*, pre-GM-CSF; *GM-CSF*, mature unglycosylated GM-CSF from which the signal peptide had been cleaved; *GM-CSF**, mature GM-CSF containing one core sugar; *GM-CSF***, mature GM-CSF glycosylated at two sites.

(Fig. 3) and Iγ (Fig. 4) at different times after the start of translation gave results similar to those obtained for lysozyme. Namely, for each protein, a characteristic pattern of blocked peptides was observed within 5 min after synchronization (see Fig. 3, *lane c*, for GM-CSF, not shown for Iγ). Additional larger peptides appeared after longer incubation times, while the shorter peptides became fainter or disappeared altogether (Fig. 3; *lanes e, g,* and *i*). The sizes of the arrested peptides ranged from 10,000–18,000 for GM-CSF and from 11,500–23,000 for Iγ. When, after each time point, membranes were added to the samples, most of the arrested peptides disappeared and mature chains were obtained. For Iγ which has no cleavable signal sequence but two potential *N*-linked glycosylation sites, the M_r of the mature chain increased by approximately 6,000 (Fig. 4, *lane b*). GM-CSF has a cleavable signal sequence of 29 amino acids and two sites for *N*-linked glycosylation. Therefore, three mature forms were observed: one from which the signal sequence had been removed, but no glycosylation occurred, and two where the signal sequence was cleaved and either one or two core sugars were added. Whether the mature chains arose from all the arrested peptides or only from the shortest ones cannot be concluded from the experiments. However, it is noteworthy that, even after 40 min of incubation, when most polypeptides had been "chased" into full-length precursor, significant amounts of mature chain were still obtained upon addition of rough microsomes (Fig. 3, *lanes i* and *j*).

Immunoprecipitation of the SRP-arrested Peptides of Iγ—

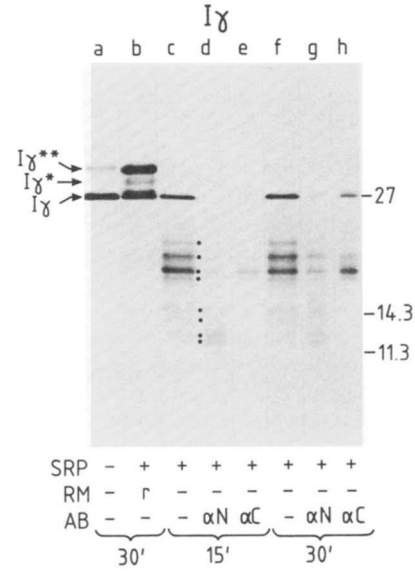

FIG. 4. **SRP-arrested peptides of Iγ and their immunoprecipitation.** A wheat germ cell-free system was primed with Iγ mRNA either in the absence (*lane a*) or the presence (*lanes b–h*) of SRP and synchronized 10 min after the start of translation by the addition of 2 mM ^{7}mG. After 15 or 30 min of further incubation, elongation was stopped by the addition of EDTA and cooling on ice, except for 1 aliquot (*lane b*), to which salt-washed microsomes (*RM*) were added and SRP arrest was allowed to be released (*r*) by incubation for another 15 min. Aliquots of the samples containing SRP-arrested peptides (*lanes c* and *f*) were immunoprecipitated with antibodies directed against either the NH_2-terminus (*αN*, *lanes d* and *g*) or the COOH-terminus (*αC*, *lanes e* and *h*) of Iγ. M_r values given should be multiplied by 10^3. SRP-arrested peptides are indicated by *dots*, precursor and mature chains of Iγ by *arrows*. *Iγ*, unglycosylated Iγ precursor; *Iγ**, mature Iγ containing one core sugar; *Iγ***, mature Iγ glycosylated at two sites.

A major difficulty in showing arrested nascent peptides has arisen from the lack of antisera which recognize the NH_2 terminus of secretory pre-proteins. The signal sequence of Iγ was shown to be located internal to the polypeptide chain, preceded by 30 NH_2-terminal hydrophilic amino acids (Lipp and Dobberstein, 1986a). Therefore, it was possible to raise antibodies against the NH_2-terminal sequence (Lipp and Dobberstein, 1986b). Such an antiserum, as well as one directed against the 144 carboxyl-terminal amino acids of the invariant chain, was used to immunoprecipitate arrested peptides obtained upon translation of Iγ in the presence of SRP. The results in Fig. 4 show that the antiserum directed against the NH_2-terminal 30 amino acids of Iγ recognizes most of the arrested peptides (*lanes d* and *g*), whereas the antiserum directed against the carboxyl terminus of the protein (*lanes e* and *h*) precipitates only the larger arrested peptides and the full-length pre-chain which are present in significant quantities after 30 min of translation. These results show that most or all the peptides detected in the presence of SRP alone, but not after addition of ER membranes (Fig. 4, *lane b*), are elongation-arrested translation products arising from mRNA coding for Iγ.

DISCUSSION

We have shown here, for the two secretory proteins GM-CSF and chicken oviduct lysozyme and the type II membrane protein Iγ, that elongation arrest mediated by dog pancreas SRP in a wheat germ cell-free system was not a tight and static phenomenon, but rather transient. The SRP arrest occurred at multiple sites during chain elongation, giving rise to ladders of arrested peptides the patterns of which changed upon prolonged incubation times. Finally, after about 40 min, only small amounts of arrested peptides remained, and mostly full length pre-chains were observed. Addition of salt-washed microsomes to the SRP-arrested peptides, even 40 min after synchronization of translation, resulted in the disappearance of the arrested peptides and the appearance of processed mature chains. The later the SRP arrest was released by the addition of ER membranes, the less efficiently the nascent chains were translocated.

To our knowledge, a single blocked peptide has been observed only with the two secretory proteins pre-IgG light chain and preprolactin. We therefore suggest that a transient SRP-mediated arrest in elongation at specific sites that gives rise to a ladder of blocked peptides is rather the rule than the exception. The lag period in chain elongation induced by SRP appeared to have a characteristic length for each protein. This indicates that each signal sequence might have a different affinity for SRP, and that this interaction determines the tightness of the SRP arrest.

It is not known how the interaction between SRP and a signal sequence extruding from the translating ribosome results in an elongation arrest. Walter *et al.* (1981) showed that SRP binds with low affinity to the large ribosomal subunit and with high affinity to polysomes synthesizing secretory protein. The simultaneous interaction of SRP with both signal sequence and ribosome appears to cause the elongation arrest.

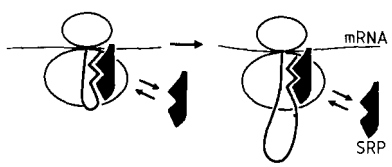

FIG. 5. **Model illustrating a transient SRP arrest.** The interaction between SRP, the signal sequence, and the ribosome is reversible, therefore giving rise to arrested peptides of increasing sizes.

The additional interaction of SRP with docking protein was shown to render the arrest reversible. The potential of SRP for such multifaceted interactions might be explained by an inherent capacity of the particle to undergo conformational changes induced by its binding first with the signal sequence, and later with docking protein. That SRP might exhibit such conformational changes is suggested by recent work on its 7SL RNA backbone (Zwieb and Ullu, 1986). The transient character of the SRP arrest described in this paper suggests a dynamic on-off interaction between SRP, the signal sequence, and the ribosome. Since evidence exists (Lizardi *et al.*, 1979) that polypeptide chain elongation proceeds in a discontinuous rather than a strictly linear fashion, the binding of SRP to the signal sequence and the ribosome might render the halts in the elongation process more pronounced, thus giving rise to the ladder of peptides which we observed.

When, for preprolactin, an SRP-arrested peptide of approximately 70 amino acids was observed, it was speculated that an elongation block ocurring when only the signal sequence extrudes from the translating ribosome (with the remaining 40 amino acids being buried within the ribosome) might be a requirement to assure proper segregation of a secretory protein (Walter and Blobel, 1981). The size of the shortest arrested peptides that we observed for lysozyme (60 amino acids), GM-CSF (90 amino acids), and Iγ (105 amino acids) corresponds to a length such that the signal sequence should just extrude from the ribosome. The lengths of the signal sequences are 18 amino acids for lysozyme, 29 for GM-CSF, and 56 for Iγ (including the NH_2-terminal hydrophilic tail).

When SRP arrest was released by the addition of ER membranes late in translation, *i.e.* when few short arrested peptides were left (see for example Fig. 3, *lanes i* and *j*), a significant amount of mature protein was still obtained. This indicates that the large arrested peptides of the three proteins analyzed in this paper were also translocation-competent, even though it cannot be firmly excluded that only the shortest arrested peptides were translocated and processed. The possibility that large segments of protein can still be translocated was already suggested for IgG light chain (Ainger and Meyer, 1986). A revised model of secretory and membrane protein targeting to ER membranes could then be envisaged as follows (Fig. 5). Nascent chain elongation proceeds in discrete steps which are characteristic for each protein. At each halt in elongation, the SRP has the opportunity to bind with both the signal sequence and the ribosome. By an unknown mechanism, this interaction stabilizes the elongation stop. Because the binding between SRP, signal sequence, and the ribosome is reversible, elongation resumes when SRP dissociates from one or both of its ligands, until the next halt occurs. When ER membranes are added to the arrested translational complexes, SRP binds to docking protein, thereby targeting the arrested peptides to the "translocation site." It then has to be assumed that large segments of protein are translocated, either in an unfolded or in a folded conformation.

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