Direct Probing of the Interaction between the Signal Sequence of Nascent Preprolactin and the Signal Recognition Particle by Specific Cross-linking

Martin Wiedmann, Teymur V. Kurzchalia, Heinz Bielka, and Tom A. Rapoport
Central Institute for Molecular Biology, Academy of Sciences of the German Democratic Republic, 1115 Berlin-Buch, Robert-Rösse-Str. 10, German Democratic Republic

Abstract. We have studied the interaction between the signal sequence of nascent preprolactin and the signal recognition particle (SRP) during the initial events in protein translocation across the endoplasmic reticulum membrane. A new method of affinity labeling was used, whereby lysine residues, carrying the photoreactive group 4-(3-trifluoromethyldiazirino) benzoic acid in their side chains, are incorporated into a protein by means of modified lysyl-tRNA, and cross-linking to the interacting component is induced by irradiation.

SRP interacts through its Mr 54,000 polypeptide component with the signal sequences of nascent preprolactin chains containing about 70 residues, and with decreasing affinity with longer chains as well; it causes inhibition of elongation. Binding of SRP is reversible and requires the nascent chain to be bound to a functional ribosome. SRP cross-linked to the signal sequence still inhibits elongation but does not prevent it completely. We conclude that SRP does not block the exit site of the polypeptide chain on the ribosome. The SRP receptor of the endoplasmic reticulum membrane displaces the signal sequence from SRP and, even if SRP is cross-linked, releases elongation arrest.

The initial events in the synthesis of a secretory protein are believed to proceed as follows (for review see reference 16). The hydrophobic signal sequence, after emerging from the ribosome, is recognized by the signal recognition particle (SRP), which consists of six polypeptide chains and a 7S RNA (20, 22). This recognition causes high affinity binding of SRP to the ribosome (21) and an arrest of polypeptide chain elongation (20). The interaction of SRP with its receptor (docking protein) (12, 6) in the endoplasmic reticulum (ER) membrane then causes SRP displacement and a concomitant release of the elongation arrest (7). The actual process of protein translocation across the membrane appears to proceed without the need of SRP or its receptor (7), but details are unclear as yet.

Several aspects of this scheme of events are still uncertain, mainly because of the lack of methods available to follow the fate of a protein in the process of its synthesis. We have recently developed a new technique of affinity labeling whereby binding partners of a nascent polypeptide can be identified (10). A modified amino acid residue, carrying a photoreactive group in its side chain, is incorporated into a protein, and cross-linking to the interacting component is induced by irradiation. The selectivity of the translational machinery could be overcome by introducing the modification to the amino acid when already bound to the specific tRNA. We have used this method to provide evidence for a direct interaction between the signal sequence of nascent preprolactin and a component of SRP, the 54-kD polypeptide (Mr 54,000) (10).

A disadvantage of the p-azidobenzoic acid (ABA) previously used as a photoreactive group is the fact that the nitrene radical produced by irradiation is rapidly converted into less reactive species that undergo predominantly nucleophilic reactions (2). Therefore, secondary reactions cannot be excluded with components outside the immediate vicinity of the nascent polypeptide.

We now report on the use of 4-(3-trifluoromethyldiazirino) benzoic acid (TDBA), which upon irradiation yields a very reactive carbene radical forming cross-links only with immediate neighbors (5, 13). The improved technique has enabled us to directly probe the interactions of the signal sequence of nascent preprolactin with SRP and thereby to tackle some so far unresolved questions concerning the translocation process. Is the interaction between the signal sequence and SRP reversible? Is the presence of a nascent chain on the ribosome essential for the signal sequence-SRP interaction? Is the recognition restricted to a certain size class of nascent polypeptide chains? Is it dependent on polypeptide chain elongation? What effect has cross-linking to the signal sequence on the functions of SRP? What happens to the signal sequence when docking to the SRP receptor occurs?
Materials and Methods

Materials

SRP was isolated from rough microsomes of dog pancreas (23). It was further purified by hydrophobic chromatography and sucrose gradient centrifugation (24). Its specific activity amounted to ~4.5 x 10^6 U/mg. SRP was isolated from rough microsomes of dog pancreas with 0.5 M potassium acetate.

TDBA was synthesized as described in the literature (13), and was a gift from Dr. A. S. Girshovich and Dr. E. S. Bochkareva, Institute of Protein Research, Pouchchino, USSR. Synthesis of e-TDBA-Lys-tRNA was carried out essentially as described previously for e-ABA-Lys-tRNA (10). Briefly, unmodified tRNA from baker’s yeast was charged with [14C]lysine and purified by phenol extraction and gel filtration. For specific modification of the ε-amino group of lysine, the N-hydroxy-succinimide ester of TDBA was incubated with lysyl-tRNA at pH 10.4 for 3 min. After ethanol precipitation, the modified lysyl-tRNA was purified on a BD-cellulose column by elution with 1 M NaCl in 30% ethanol. The identity of the material was proved by thin layer electrophoresis after hydrolysis (10). Incorporation of TDBA-modified lysine residues into cell-free synthesized preprolactin was demonstrated by enzymatic hydrolysis and subsequent ion exchange chromatography as described (10).

Cell-free Translation and Cross-linking

Translation in a wheat germ system was performed with the following final concentrations: 20% wheat germ extract, 1 mM CaCl_2, 1 mM ATP, 1 mM GTP, 10 mM dithiothreitol (DTT), and 40 mM potassium acetate. The procedure described above was followed. Immunoprecipitation with anti-54-kD polypeptide antibodies was carried out in the same manner with IgG affinity-purified from a rabbit serum (25). Controls were performed with nonimmune serum (2 μl per sample) and subsequently treated in the same way as the test samples.

Samples not subjected to immunoprecipitation were precipitated with TCA and redissolved in SDS gel sample buffer.

Products were separated in SDS gels of various polyacrylamide concentrations (see figure legends) and visualized by autoradiography of the 2,5-di-phenyloxazol-impregnated gels. The following molecular mass standards were used: a cro-β-galactosidase fusion polypeptide coded by the pEX-plasmids (18), 117 kD; T7-RNA-polymerase, 100 kD; bovine serum albumin, 68 kD; catalase, 56 kD; ovalbumin, 45 kD; aldolase, 40 kD.

Results

Reversible SRP Binding Requires Functional Ribosome-bound Nascent Preprolactin

SRP is known to inhibit the translation of preprolactin mRNA when added to a wheat germ cell-free system (21). If the translation is synchronized by permitting initiation for only a short time period, SRP stops the movement of the ribosome when the NH2-terminal portion of preprolactin is about 70 residues long (referred to as arrested fragment [20]). We have previously shown that after translational incorporation of e-ABA-lysine into the arrested fragment and subsequent irradiation, a cross-linked complex is produced with the 54-kD polypeptide component of SRP (10). A similar experiment was performed with the new cross-linking reagent as follows.

A nonsynchronized translation of preprolactin mRNA was carried out in the presence of [35S]methionine (serving as label to facilitate product analysis) and N'-TDBA-Lys-tRNA. The latter is accepted by the translational machinery (not shown). SRP was present from the beginning of translation. Irradiated and nonirradiated samples were separated on an SDS gel and radioactive bands were visualized by fluorography.

As with the older cross-linking reagent, a main band of M, 62,000 was produced by irradiation (Fig. 1 A, lane 2; Fig. 1 B, lane 1), which was absent in controls (Fig. 1 A, lane 1). Up to 10% of the total radioactivity exposed to irradiation was recovered in the 62-kD band and only few other products were seen, indicating that the new reagent was more efficient and more specific than the one previously used. Antibodies directed against the 54-kD polypeptide of SRP or against prolactin precipitated the product of cross-linking (Fig. 2, lanes 3 and 5).

These data confirm that the signal sequence of the preprolactin fragment, which contains the only lysine residues responsible for cross-linking (positions 4 and 9), interacts with the 54-kD polypeptide component of SRP. Since a single cross-linked band was obtained under these conditions, movement of the ribosome must have been inhibited with the first interaction between the signal sequence of the growing chain and SRP, as has been suggested before (20).

A simple dilution experiment demonstrated the reversibility of the interaction between SRP and the nascent polypep-
Figure 1. Interaction of nascent preprolactin with SRP. (A) Translation for the sample shown in lane 2 was carried out for 10 min in the presence of SRP. After addition of 4 mM 7mGp, the sample was irradiated at 0°C. Lane 1 shows a nonirradiated control. Translation for the samples presented in lanes 3–8 was carried out for 20 min in the absence of SRP. Cycloheximide was added and the incubation was continued for 2 min. Finally, SRP was added in different amounts, and, after another 5 min of incubation, the samples were cooled to 0°C and irradiated (irr.) as indicated. All samples were immunoprecipitated by prolactin antibodies before electrophoresis in a 12% acrylamide gel. It should be noted that a five times greater translation volume was used for the samples shown in lanes 3–8 as compared with those in lanes 1 and 2. The arrow indicates the position of the 62-kD band (see text). pPL, preprolactin. (B) Translation of preprolactin mRNA was carried out for 10 min in the presence of SRP. Equal portions received either 0.2 mM cycloheximide or 2 mM puromycin (at 140 mM potassium acetate) and were further incubated for 2 or 10 min, respectively. Half of each sample was incubated at 26°C for 10 min after a 1:10 dilution with a complete translation mixture lacking mRNA, [35S]methionine, and s-TDBA-Lys-tRNA. The other half was cooled down to 0°C and is referred to as undiluted in the figure, despite the fact that it was diluted with an ice-cold translation mixture to the same final volume before further handling. All samples were irradiated in portions of 150 μl, TCA-precipitated, and applied to electrophoresis in a 12% acrylamide SDS gel. The arrow indicates the position of the 62-kD band produced by cross-linking (see text).

tide chain (Fig. 1 B). Dilution of the complex diminished the yield of cross-linked product (cf. lanes 1 and 2).

The nascent chain must be bound to a functional ribosome for SRP interaction, since incubation with puromycin drastically inhibited cross-linking (Fig. 1 B, cf. lanes 3 and 1, or lanes 4 and 2). Release of nascent polypeptides from peptidyl-tRNA under these conditions has been demonstrated previously (8).

SRP Binds to Nascent Polypeptide Chains of Various Length

Is the interaction of SRP with nascent preprolactin restricted to polypeptide chains of ~70 amino acid residues in length? Is it dependent on polypeptide chain elongation? To answer these questions, the following experiment was performed (Fig. 1 A, lanes 3–8). A preprolactin translation was allowed to proceed in the absence of SRP for 20 min, a time at which a steady state is achieved with ribosomes distributed almost equally along the mRNA. The state was frozen by addition of cycloheximide. Then SRP was added to aliquots in different amounts and the mixture was irradiated.

As seen in Fig. 1 A (lane 6 and 7), the main product of cross-linking between SRP and nascent preprolactin was again found at Mr 62,000. However, as the SRP concentration was increased, higher Mr bands were produced up to 80 kD (lane 7). It appears that chains approaching the size of completed preprolactin (Mr, 25,000) can react with SRP but with decreasing efficiency. Completed preprolactin released from the ribosome bound SRP very weakly, if at all (not shown).

These results show that polypeptides, the signal sequence of which has just fully emerged, react best with SRP, but that longer chains can also interact with lower affinity. Our data also indicate that signal peptide recognition does not require ongoing translation.
The SRP Receptor Releases the Translational Inhibition Without Chain Translocation if the Signal Sequence Is Cross-linked to SRP

To test whether cross-linking of SRP to the signal sequence would prevent its interaction with the docking protein in the ER membrane, K-RMs depleted of SRP were added after irradiation of the elongation-arrested translation complex and the incubation was continued. The growth of the cross-linked polypeptide was drastically accelerated (Fig. 4 A, cf. lanes 5–7 with lanes 2–4). The 62-kD band disappeared almost completely and much of the radioactivity was found in the 80-kD band. Of course, processing of preprolactin to prolactin was also observed, since only a percentage of the nascent chains is cross-linked (note the prolactin band in lanes 5–7). With increasing amounts of K-RMs added after irradiation, the 80-kD band became more and more prominent until, at very high concentrations of K-RMs, an inhibition of polypeptide chain elongation was observed (Fig. 4 B).

The release of the translational inhibition was not seen if K-RMs pretreated with NEM were used (Fig. 4 A, lane 8 with lane 9, which shows a mock-treated control; note also the absence of a prolactin band in lane 8). The arrest-releasing activity was also disturbed by pretreatment of K-RMs with low concentrations of trypsin followed by high salt washing (not shown). This provides evidence that the SRP receptor, which contains a sensitive SH group (6) and can be proteolytically severed from the membrane (19), is responsible for the activity of the membranes.

Immunoprecipitation experiments confirmed that the 80-kD band consisted of preprolactin cross-linked to the 54-kD polypeptide of SRP (Fig. 2, lanes 4 and 6). It should be noted that products of higher Mr, notably of 100,000–105,000 (arrowhead), were also immunoprecipitated by both antibodies. The 100–105-kD band might represent a cross-linked product of completed preprolactin with both the 54-kD polypeptide of SRP and either the 19-kD polypeptide of SRP or a ribosomal protein.

Little, if any, of the 80-kD product and of the intermediate products was translocated across or integrated into the membrane since they were accessible to proteases, in contrast to prolactin, which was protected by the vesicle membrane (Fig. 4 C). Furthermore, most of the 80-kD band was recovered in the supernatant after pelleting of the microsomes (not shown).

Polypeptide Chains Cross-linked to SRP Can Still Be Elongated

It was of interest to test whether cross-linking of the signal peptide to SRP would prevent further polypeptide chain elongation.

Translation of preprolactin mRNA was carried out in the presence of SRP for 10 min. After irradiation of the elongation-arrested translation complex, the incubation was continued for different periods of time (Fig. 3 A). The intensity of the 62-kD cross-linking band decreased slowly, and higher Mr bands were produced up to a size of ~80 kD (Fig. 3 A, lanes 4–6). The growth of the cross-linked polypeptide could be prevented by addition of cycloheximide (lane 8). Since preprolactin has an Mr of 25,000, the 80-kD band corresponds to cross-linking of completed polypeptide chains with the 54-kD polypeptide of SRP. Indeed, the 80-kD band does not appear to be associated with tRNA, since it cannot be precipitated by cetyltrimethylammonium bromide, in contrast to the lower Mr bands (not shown).

All bands between Mr 60,000 and 80,000 could be immunoprecipitated with antibodies directed against prolactin and the 54-kD polypeptide of SRP (not shown). It should be noted that a closely spaced band pattern of up to at least Mr 75,000 was seen (corresponding to nascent chains of approximately 200 residues in length; see Figs. 3 and 4).

One may conclude that cross-linking of the signal sequence to SRP does not completely prevent further chain elongation of nascent preprolactin.

The continuation of chain elongation in the presence of SRP could also be demonstrated if the irradiation was carried out after incubation for different time periods (Fig. 3 B). However, compared with the experiment in which the growth of the cross-linked polypeptide was studied, the disappearance of the 62-kD band was much slower, and higher Mr products were seen in only small amounts (cf. lanes 3 and 2, lanes 3 and 4, and lanes 7 and 6). Notably, the 80-kD band was hardly visible, if at all.

The SRP Receptor Releases the Translational Inhibition Without Chain Translocation if the Signal Sequence Is Cross-linked to SRP

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The release of the translational inhibition was not seen if K-RMs pretreated with NEM were used (Fig. 4 A, lane 8 with lane 9, which shows a mock-treated control; note also the absence of a prolactin band in lane 8). The arrest-releasing activity was also disturbed by pretreatment of K-RMs with low concentrations of trypsin followed by high salt washing (not shown). This provides evidence that the SRP receptor, which contains a sensitive SH group (6) and can be proteolytically severed from the membrane (19), is responsible for the activity of the membranes.

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Little, if any, of the 80-kD product and of the intermediate products was translocated across or integrated into the membrane since they were accessible to proteases, in contrast to prolactin, which was protected by the vesicle membrane (Fig. 4 C). Furthermore, most of the 80-kD band was recovered in the supernatant after pelleting of the microsomes (not shown).
Figure 3. Polypeptides cross-linked to SRP can still be elongated. (A) The sample presented in lane 1 was incubated for 60 min without SRP. For all other samples, translation was carried out for 10 min in the presence of SRP. 4 mM 7mGp was added and samples were irradiated (irr.) as indicated. All samples, except those in lanes 2 and 3, were incubated further for the time periods indicated. The sample in lane 8 received cycloheximide before further incubation. (B) Translation was carried out for 10 min in the presence of SRP. The samples shown in lanes 1, 2, 4, and 6 were irradiated before further incubation for the time periods indicated. The samples presented in lanes 3, 5, and 7 were irradiated after the indicated incubation periods. The products were precipitated by TCA and applied to a 12% acrylamide SDS gel. The arrows give the positions of the 62- and 80-kD bands (see text). pPL, preprolactin.

Figure 4. Cross-linking of SRP with the signal sequence allows for release of the elongation arrest by the SRP receptor but not for translocation. (A) Translation was carried out for 10 min in the presence of SRP. After addition of 4 mM 7mGp and subsequent irradiation (irr.), the samples were further incubated in the absence (lanes 2–4) or presence (lanes 5–9) of K-RMs for the indicated time periods, except for lane 1, which was immediately analyzed. K-RMs pretreated with NEM or mock-treated were employed for the samples shown in lanes 8 and 9. (B) Translation was carried out for 10 min in the presence of SRP. After addition of 7mGp and irradiation where indicated, the samples were further incubated for 60 min in the presence of different amounts of K-RMs. (C) A sample was incubated for 60 min in the presence of K-RMs after irradiation. Posttranslational proteolysis was then performed by addition of proteinase K in the final concentrations indicated. After incubation at 25°C for 10 min, phenylmethylsulfonyl fluoride was added to 1 mM before TCA precipitation. All products were separated in a 12% acrylamide SDS gel. The positions of the 62- and 80-kD bands are marked with arrows. pPL, preprolactin; PL, prolactin.
These data indicate that cross-linking of the signal sequence to SRP still allows release of the translational arrest by the SRP receptor, but, of course, does not permit polypeptide chain translocation.

**Interaction of SRP with Its Receptor Leads to Displacement of the Signal Sequence**

Gilmore and Blobel (7) have demonstrated that the solubilized SRP receptor displaces SRP from the ribosome even in the absence of chain elongation. We have tested whether membrane-bound receptors in form of K-RMs displace the signal sequence from SRP. Such an effect would be manifested by the suppression of the appearance of the Mr 62,000 cross-linked product.

The experiment was carried out as follows. Translation of preprolactin mRNA was performed in the presence of SRP for 10 min. Cycloheximide was then added to prevent further chain elongation. K-RMs were added in increasing amounts and the samples were irradiated (Fig. 5 A, lanes 2–6).

It may be seen that addition of K-RMs inhibited the formation of the 62-kD cross-linked product. More than 85% inhibition could be achieved with high concentrations of K-RMs (Fig. 5 B). Membranes pretreated with NEM did not show this effect (Fig. 5, cf. lanes 7 and 8).

One may conclude that binding of the arrested translation complex to the SRP receptor in the membrane has weakened the interaction of the signal sequence with the 54-kD polypeptide.

It should be noted that even after running the samples in gels of different acrylamide concentrations and after careful scrutiny of the fluorograms, we were unable to detect the formation of a new cross-linked product after addition of K-RMs. (Cross-linking to a protein component of ~Mr 17,000 would not have been detectable because of comigration with preprolactin.)

**Discussion**

In the present study we have directly analyzed interactions of the signal sequence with SRP during the synthesis of a secretory protein. A new method, tRNA-mediated protein labeling, whereby chemical groups can be introduced into...
nascen polypeptides by means of aminoacyl-tRNA modified in the side chain of the amino acid residue was employed. The use of TDBA, which upon irradiation gives rise to a very reactive carbene radical, reduces unspecific reactions that apparently occurred to some extent with the ABA reagent previously employed (10). The new reagent also gives better yields of cross-linked product, allows for irradiation at higher wavelengths with less UV damage, and is not sensitive to SH groups.

In agreement with our theoretical prediction on the basis of a mathematical model of the translocation process (Rapoport, T. A., R. Heinrich, P. Walter, and Th. Schulmeister, manuscript in preparation), we have found that SRP can actually interact with a wide spectrum of ribosome-bound polypeptide chains, beginning with nascent chains containing \( \sim 70 \) residues, in a reversible manner. The observation that the binding of SRP becomes weaker with increased chain length may be explained by the assumption that the signal sequence in the nascent chain should not be too far removed from the ribosome. An alternative explanation is the burying of the signal sequence within domains of the polypeptide, which would diminish the accessibility for SRP. Our data show that preprolactin for which a co-translational mode of translocation is obligatory (17) can interact with SRP late in translation and in the absence of chain elongation. The binding of the nascent chain to a functional ribosome appears essential for the SRP interaction to occur. Puromycin-terminated polypeptide fragments and completed preprolactin bind very poorly, if at all.

Inhibition of elongation occurs even with long polypeptides. This is concluded from the fact that SRP cross-linked to the arrested fragment, after further incubation, slowly gives rise to a spectrum of products with \( M_s \) between 62,000 and 75,000, which can all be chased rapidly by K-RMs into the 80-kD product. Preprolactin chains containing as many as 200 residues out of the \( \sim 250 \) present in a completed chain, must therefore be inhibited by SRP in their elongation. On the other hand, the gap in the band pattern generally seen between 75 and 80 kD may indicate that ribosomes very close to the 3'-end of the mRNA cannot be halted anymore by SRP. These results are in agreement with those of Ainger and Meyer (1) who have recently shown that SRP can exert an elongation arrest even late in translation. We have confirmed for preprolactin that addition of SRP to a synchronized translation system inhibits the elongation of nascent polypeptide chains exceeding a length of 70 residues (not shown).

Inhibition of polypeptide chain elongation is shown to occur with the signal sequence covalently linked to SRP. Indeed, elongation arrest was indicated by the fact that translation was drastically accelerated by addition of K-RMs containing the SRP receptor. Consequently, release of the elongation arrest does not directly involve the signal recognition function of SRP. This is in agreement with the observation that a defective SRP, lacking the two smallest polypeptides, does not exert an elongation arrest but is competent for protein translocation (17).

We have found that chain elongation, though inhibited, was not stopped completely by cross-linked SRP. A stronger inhibition was observed with non-cross-linked SRP. Although we cannot exclude the possibility that irradiation has reduced the inhibitory effect of SRP, the result may also be explained by the assumption that binding of SRP to the signal sequence increases the binding strength of SRP to the ribosome, which in turn effects elongation arrest. Equilibrium exchange of SRP in its complex with the ribosome would be reduced by cross-linking, since other SRP molecules would not have access to the blocked signal sequence. Thus, cross-linking would, in essence, decrease the effective concentration of SRP-ribosome complexes. This interpretation implies that the signal sequence–SRP interaction is reversible as found.

Assuming that SRP, to inhibit translation, must be bound to the ribosome, and taking into account that elongation still proceeds after cross-linking, two conclusions may be drawn. First, SRP does not block the exit site of the polypeptide chain on the ribosome, and second, the emerging polypeptide chain assumes a loop shape. It may be surmised that even a non–cross-linked polypeptide chain forms a loop and is inserted in this shape into the ER membrane, as has been suggested before (9).

The displacement of the signal sequence from SRP by the SRP receptor provides further evidence for the transient nature of the signal sequence–SRP interaction and for the formation of the translocation complex in the absence of chain elongation (7). It may also be assumed that a drastic conformational change in the SRP occurs upon docking.

We cannot exclude that a second receptor protein present in the ER membrane accepts the signal sequence from SRP. However, it is possible that the lipid environment interacts with the signal sequence after docking (4), thus explaining the absence of a new cross-linked band. In any case, the data would not exclude the existence of an aqueous pore through which the other parts of the polypeptide chain are transported (3, 15). The new method of affinity labeling employed here may prove valuable for the identification of presumed tunnel proteins.

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