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Photocrosslinking demonstrates proximity of a 34 kDa membrane protein to different portions of preprolactin during translocation through the endoplasmic reticulum

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Photocrosslinking has been used to identify integral proteins of the endoplasmic reticulum membrane that are in proximity to nascent preprolactin during in vitro translocation. A photoreactive lysyl derivative was introduced into truncated preprolactin chains comprising 86 or 115 amino acids. Both with the 86mer, containing the reactive group in the signal sequence, and with the 115mer, containing the probe exclusively in the mature portion of the chain, photocrosslinking occurred to a ~35 kDa transmembrane glycoprotein, the signal sequence receptor (SSR). SSR is identical with a previously isolated abundant and ubiquitous 34 kDa membrane protein that appears to be essential for protein translocation.

Signal sequence; Endoplasmic reticulum; Protein translocation; Photocrosslinking

1. INTRODUCTION

Hydrophobic signal sequences initiate the translocation of secretory and membrane proteins across the ER membrane. They are first recognized in the cytosol by the 54 kDa polypeptide component of the SRP [1–3]. The nascent chain is then targeted to the membrane by interaction of SRP with its receptor (also called docking protein) [4,5]. The signal sequence is released from the SRP [6] and is transferred into close proximity of an integral, glycosylated ~35 kDa membrane protein, the SSR [7].

The environment and the interactions of the nascent chain as it crosses the membrane are as yet unknown. It is not even clear if passage occurs directly through the lipid bilayer or if membrane proteins are involved.

A crosslinking approach has been used previously to investigate interactions of the signal sequence of nascent preprolactin [2,3,6–8]. A photoreactive group was introduced into the nascent polypeptide by means of a modified lysyl-tRNA; subsequent photocrosslinking allowed the identification of proteins located in its proximity. In these experiments, interactions of the signal sequence could be studied exclusively by employing the arrested fragment of preprolactin (~70 amino acids long), produced by translation of mRNA in the presence of SRP; only the lysines in position 4 and 9 of the signal sequence of preprolactin carried photoreactive groups and had emerged from the ribosome. It was demonstrated that the signal sequence was in proximity to the 54 kDa polypeptide of SRP in the absence of microsomal membranes and to the SSR in their presence.

We have now extended the crosslinking approach by employing longer fragments of nascent preprolactin produced by translation of truncated mRNAs. This allowed one to position the photoreactive groups not only into the signal sequence but also into the subsequent portion of the polypeptide chain. In both cases crosslinking to the SSR was observed, indicating that it is a constituent of a protein environment of the nascent chain as it is transferred through the membrane. Furthermore, the SSR is shown to be identical with a recently isolated, abundant and ubiquitous 34 kDa protein that appears to be essential for protein translocation in vitro [9].

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Abbreviations: ER, endoplasmic reticulum; SRP, signal recognition particle; SSR, signal sequence receptor; K-RM, rough microsomes washed with high salt

2. MATERIALS AND METHODS

2.1. *mRNAs*

RNA coding for the first 86 amino acid residues of preprolactin was obtained by in vitro transcription with T7 RNA polymerase of the plasmid pGEMBP1 linearized by the restriction enzyme *Pvu*II [10].

RNA coding for the first 115 residues of preprolactin was produced from poly(A)-RNA of bovine pituitaries by adding to the final translation mixture an oligonucleotide (5'-GGAGCGCAGCAA-CCC-3') complementary to amino acids 115–120 [11] (4 OD/ml). Specific cleavage occurred by an endogenous endonuclease H present in wheat germ extracts [12].

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2.2. *Translation and photocrosslinking*

Translation reactions in the wheat germ system were carried out as described [6] except that the final potassium acetate concentration was 80 mM. Salt-washed microsomal membranes from dog pancreas (K-RM) were present at 6 equivalent/25 μl assay, and canine SRP at 40 nM. Translation was carried out for 5 min at 26°C in the presence of SRP. ^{7}mGp (4 mM) was added to prevent further chain initiation. For experiments with the 70mer arrested fragment, cycloheximide (2 mM) was added instead of ^{7}mGp. After 1 further min of incubation, K-RMs were added for 10 min. Incubations were stopped by placing the tubes in ice water. ε-TDBA-Lys-tRNA (40 nM) was added either at time point zero or right before the membranes (after 5 min). Irradiation was carried out as described [6]. In some experiments translation was continued after irradiation. In this case, before further incubation one-tenth of the volume of the following mixture was added: 0.3 mM lysine, 3.6 mM GTP, 3.6 mM CTP, 20 mM dithiothreitol, 6 mM magnesium acetate.

2.3. *Product analysis*

Alkali extractions and immunoprecipitations with antibodies to prolactin were carried out as described [7].

Immunoprecipitation of the crosslinked products with antibodies directed against the 34 kDa protein was carried out as follows. The translation mixtures (25 μl) programmed with mRNA coding for the 86mer or the 115mer were irradiated. The membranes were solubilized with 1% Nikkol at 0.5 M potassium acetate and the samples were centrifuged through a 60 μl sucrose cushion in a Beckman Airfuge at 30 psi for 30 min. The pellet was treated with 3% SDS at 95°C and was then diluted with 800 μl immunology buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). 5 μl of antiserum directed either against the intact 34 kDa protein [9] or against a C-terminal peptide (LPRKRAQKRSVGSDE; Prehn, S. et al., submitted) were added. Controls were performed with 5 μl nonimmune serum. All subsequent steps were as described [7].

Binding of proteins to concanavalin (Con) A-Sepharose was carried out after solubilization of alkali-extracted membrane pellets in SDS as described [7].

Posttranslational proteolysis of crosslinked products was carried out with proteinase K (250 μg/ml) for 20 min at 4°C.

The products were separated in 10–15% linear acrylamide gels [13], except where indicated, and visualized by fluorography.

3. RESULTS

3.1. *Nascent preprolactin is in proximity to the SSR at different stages of translocation*

Preprolactin chains of either 86 or 115 amino acids long were synthesized in vitro in the presence of SRP and microsomal membranes. Under these conditions, translocating nascent chains remain bound to ribosomes that come to a halt at the end of the truncated mRNAs which lack a termination codon [10]. Photoreactive groups were incorporated into the nascent chains, at the positions where lysine residues occur in preprolactin, by translating the mRNAs in the presence of ε-TDBA-Lys-tRNA. The polypeptides were also labelled with ^{35}S-methionine so that, after irradiation, the crosslinked products could be identified.

With the mRNA coding for the 86mer, irradiation yielded several crosslinked products, including a 60 kDa and a 45 kDa band, which were immunoprecipitable with antiserum to prolactin (fig.1, lane 3). The 60 kDa band corresponds to the crosslinked product of the 70mer arrested fragment of preprolactin (~6–8 kDa) and the 54 kDa polypeptide of SRP [2]. The low amount of 60 kDa product seen in the presence of microsomes (cf. with lane 1) probably originates from inefficient targeting of arrested translation complexes to the ER membrane. Most if not all of the 45 kDa band was found by immunoprecipitation in the redissolved alkali-extracted membrane pellet (fig.1, lane 6), indicating that the nascent preprolactin chain had been crosslinked to an integral membrane protein. In contrast, neither the 60 kDa product nor the other bands could be detected in the membrane pellet. The non-crosslinked 86mer associated with the alkali-extracted membrane pellets (lanes 5–7) only constitutes a small percentage of the total 86mer, as expected for a fragment of a non-membrane protein. The appearance of crosslinked products depended on the presence of modified lysyl-tRNA during incubation (shown for the 45 kDa band in lane 5). The signal sequence of the 86mer must have been involved in the crosslinking since only its lysines (positions 4 and 9 of preprolactin) which carry photoreactive groups could have emerged from the ribosome. Indeed, if the addition of modified lysyl-tRNA was delayed so that the first ~70 residues did not contain photoreactive groups, both the 60 kDa and 45 kDa crosslinked products were greatly diminished (lanes 4 and 7; see also fig.2, lane 6). The 45 kDa crosslinked product corresponds in its size to the sum of the contributions of the 86mer (~10 kDa) and a ~35 kDa integral membrane protein. The latter must be glycosylated since the 45 kDa crosslinked product was bound almost quantitatively to Con A-Sepharose in the absence of α-methyl mannoside (fig.2, cf. lanes 1 and 4), but not in its presence (lane 3). These results are in agreement with the previous characterization of the SSR using the 70mer arrested fragment of preprolactin ([7]; see also fig.1, lane 2).

With the mRNA coding for the 115mer, irradiation yielded mainly two crosslinked products of 60 kDa and 47 kDa which were immunoprecipitable with antiserum to prolactin (fig.1, lane 8). Again, the 60 kDa product originated from crosslinking with SRP, whereas the 47 kDa product contained an integral membrane protein and was recovered in the alkali-extracted membrane pellet (lane 11). The bands were only observed if modified lysyl-tRNA was included in the incubation (cf. lane 10). The size of the 47 kDa product corresponds to the sum of the 115mer (~13 kDa) and a ~34 kDa membrane protein which again was shown to be glycosylated by binding of the crosslinked product to Con A-Sepharose in the absence (fig.2, cf. lanes 7 and 10), but not presence (lane 9) of α-methyl mannoside.

In the case of the 115mer, crosslinking may occur by modified lysines located either in the signal sequence or in the mature part of preprolactin (positions 72, 78 and 99 of preprolactin). The lysines at positions 72 and 78 are expected to have emerged from the ribosome.

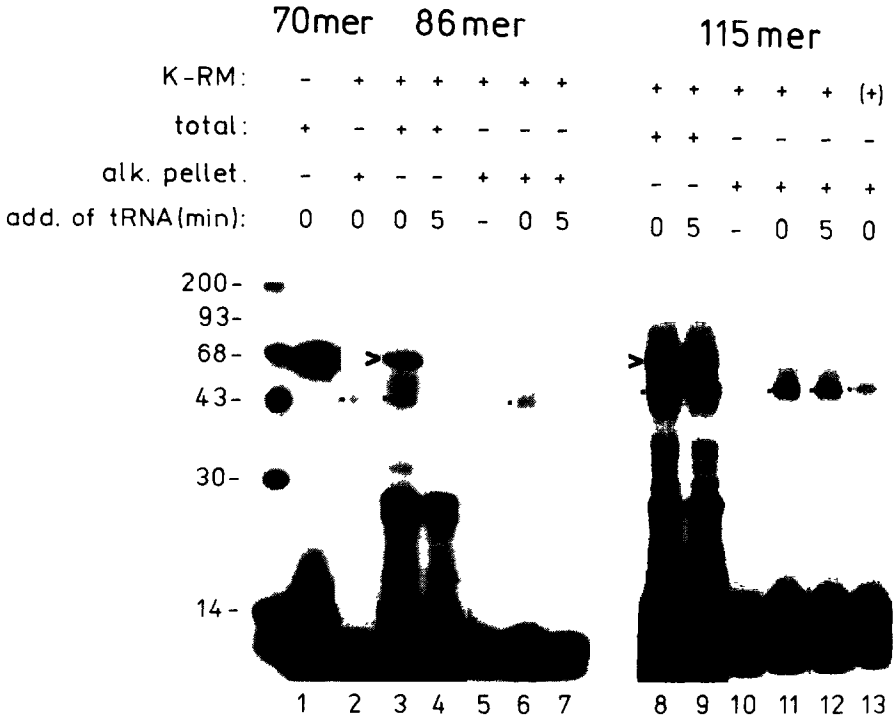

Fig.1. Crosslinking of nascent chains of preprolactin during different stages of translocation. Photocrosslinking was carried out in the presence of K-RM (except lane 1) with the 70mer SRP-arrested fragment (lanes 1,2) or with truncated forms of preprolactin (86mer, lanes 3–7; 115mer, lanes 8–13). The polypeptides contained photoreactive groups either throughout the chain (ε-TDBA-Lys-tRNA added at 0 min) or only beyond amino acid 70 (added at 5 min when the SRP-arrested complex had formed). For sample 13, the 70mer was irradiated at 0°C in the presence of K-RMs, and the polypeptide was further elongated to the 115mer by incubation at 26°C. Either total translation mixtures (tot) or redissolved alkali extracted membrane pellets (alk. pellet) were immunoprecipitated with prolactin antibodies. The arrowheads indicate the position of the 60 kDa crosslinked product. The dots indicate the positions of the crosslinked products with the SSR. Molecular mass standards were (left-most lane): myosin, 200 kDa; phosphorylase A, 93 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14 kDa.

Specific incorporation of ε-TDBA-Lys into these positions was achieved by a staging experiment (Krieg, U.C., Johnson, A.E. and Walter, P., personal communication). Translation was carried out in the absence of modified lysyl-tRNA and in the presence of SRP to produce an arrested fragment of preprolactin that did not carry the photoreactive group in the signal sequence. Then further chain initiation was stopped by ^{7}mGp, and ε-TDBA-Lys-tRNA was added together with microsomal membranes so that the translational arrest exerted by SRP was released and the modified lysines were incorporated into the polypeptide chain during further elongation. As may be seen from fig.1, lanes 9 and 12, and from fig.2, lane 12, the 47 kDa crosslinked product was still observed with almost undiminished intensity, indicating that the mature part of preprolactin was crosslinked to the membrane protein. In contrast, the 60 kDa product was greatly diminished (fig.1, lane 9), as expected for a non-crosslinked interaction of the 54 kDa SRP-polypeptide with the signal sequence.

The assumption that the same ~35 kDa protein (SSR) was crosslinked to different nascent chains was supported by the following experiment. A 70mer arrested fragment was produced with the 115mer mRNA. It was incubated with microsomes at 0°C and irradiated to form crosslinks. Thereafter elongation was continued up to the 115mer. It may be seen that the size of the product is identical with that produced by irradiation of the 115mer (fig.1, cf. lanes 12 and 13).

The SSR must be exposed to the lumenal face of the ER membrane since it contains carbohydrates. It has also a cytoplasmic domain as shown by the data in fig.3. The 45 kDa crosslinked product of the 86mer was degraded by treatment with high concentrations of proteinase K and a ~40 kDa band appeared instead (cf. lanes 1 and 2). Both products were bound to Con A-Sepharose (lanes 3 and 4). The SSR must have been the substrate for the protease since the other component of the crosslinked product, i.e. the 86mer, remained undegraded (see arrow), in agreement with other results [14]. Also, the radioactivity contained in the crosslinked product was not significantly diminished by proteolysis. These results indicate furthermore that the cytosolic domain of the SSR is not involved in crosslinking to the nascent chain. Similar results as with the 86mer were obtained with the 115mer (data not shown).

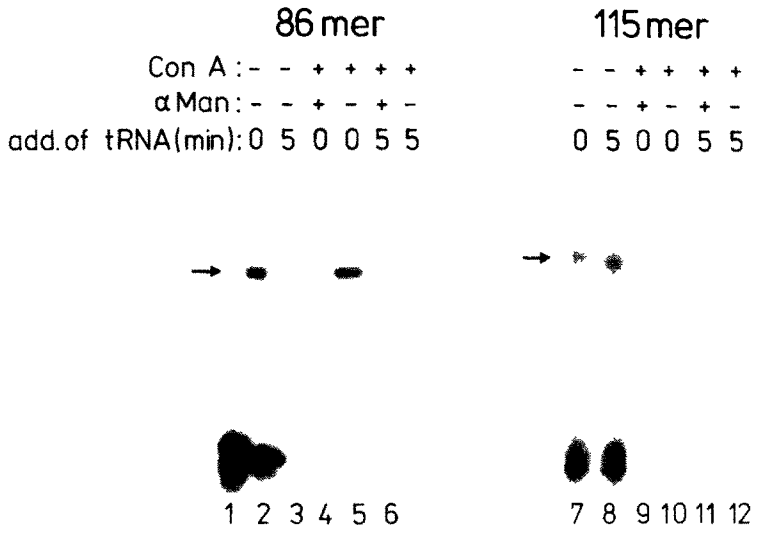

Fig.2. Binding of the crosslinked products to Con A-Sepharose. Translation of truncated preprolactin mRNAs was carried out with the modified lysyl-tRNA added either at time point zero (0) or after 5 min translation in the presence of SRP (5). SDS gel electrophoresis of the crosslinked products was carried out either directly after alkali extraction of the membrane pellet (lanes 1,2,7,8) or after binding to Con A-Sepharose (Con A) (other lanes) in the presence or absence of α-methyl mannoside (αMan) of the dissolved alkali-extracted membrane pellet. Arrows indicate the position of the crosslinked products.

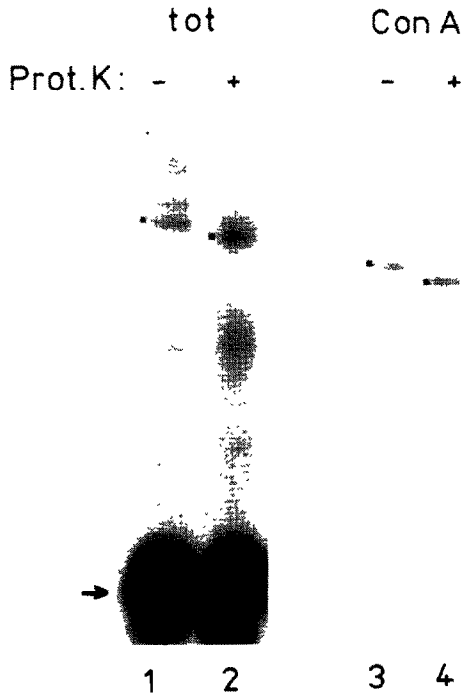

Fig.3. Proteolysis of the crosslinked products. Truncated mRNA coding for the first 86 amino acids of preprolactin was translated in the presence of SRP, K-RMs and ϵ-TDBA-Lys-tRNA. After irradiation, the sample was centrifuged in an Airfuge. The supernatant was discarded and the remainder incubated with or without proteinase K (Prot.K) as indicated. The products were either analyzed directly in a 15–20% polyacrylamide SDS-gel (tot, lanes 1 and 2) or, after binding to Con A-Sepharose, by electrophoresis of the bound material in a 10–15% polyacrylamide SDS-gel (Con A, lanes 3 and 4). The dots show the position of the crosslinked products with the SSR.

3.2. *Identity of the SSR with a previously isolated 34 kDa protein*

The following immunoprecipitation experiment was carried out to test if the SSR identified in the crosslinking experiments is the same as a recently purified 34 kDa protein [9].

The translocation complexes assembled with either the 86mer or the 115mer were irradiated, solubilized with detergent at high salt concentration and sedimented with ribosomes through a sucrose cushion. This procedure separates the crosslinked products from the vast excess of the non-crosslinked 34 kDa protein that would otherwise interfere in the subsequent immunoreaction. The sedimented crosslinked products were dissolved and subjected to immunoprecipitation with antibodies directed against either prolactin (fig.4, lanes 1 and 5), the intact 34 kDa protein (lanes 3 and 7) [9] or against a C-terminal peptide of the 34 kDa protein (lanes 4 and 8) (Prehn, S., Herz, J., Hartmann, E., Kurzchalia, T.V., Frank, R., Roemisch, K., Dobberstein, B. and Rapoport, T.A., submitted). It may be seen that both antisera against the 34 kDa protein recognized selectively the crosslinked products with the SSR. Controls with non-immune serum were negative (lanes 2 and 6). The low efficiency of immunoprecipitation may be due to the still substantial amounts of non-crosslinked 34 kDa protein in the ribosome pellet and/or to changes in the immunoreactivity of the 34 kDa protein after crosslinking. Small amounts of 86mer which were found in the immunoprecipitates with both the antisera and the non-immune serum (lanes 2–4) probably represent unspecific precipitation.

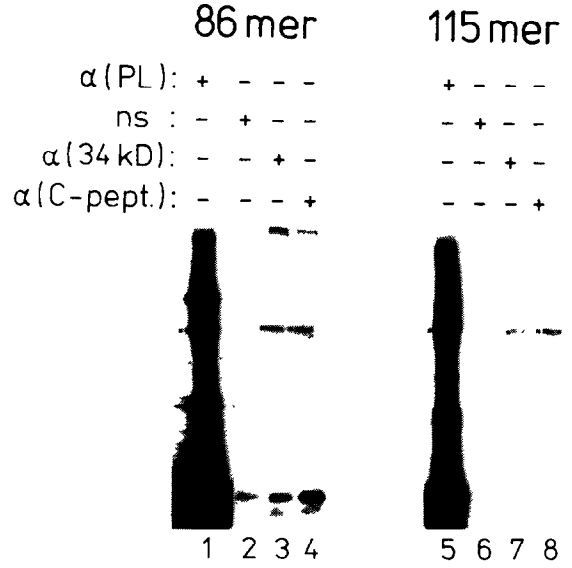

Fig.4. Immunoprecipitation of the crosslinked products by antibodies to the 34 kDa protein. Truncated mRNAs coding for the 86mer or the 115mer were translated in the presence of SRP, K-RMs and ε-TDBA-Lys-tRNA. The samples were irradiated, and processed as described in section 2 for immunoprecipitation with either antiserum against prolactin [α(PL); lanes 1 and 5], non-immune serum (ns; lanes 2 and 6), antiserum against intact 34 kDa protein [α(34 kDa); lanes 3 and 7] or antiserum against a C-terminal peptide of the 34 kDa protein [α(C-pept.); lanes 4 and 8]. The dots indicate the position of the crosslinked product of the nascent chains with the SSR.

4. DISCUSSION

We have used a crosslinking approach to identify membrane proteins located in the proximity of nascent preprolactin. As in previous experiments with the 70mer arrested fragment [7], truncated preprolactin chains of 86 and 115 amino acids long could be crosslinked to a ~35 kDa membrane glycoprotein. Although the crosslinked products are formed in only low yields, they appear to reflect faithfully the behaviour of the translocating polypeptides since they were only observed if the SRP receptor was intact [7]. The high reactivity of the carbene radical employed in crosslinking may have been one reason for the low yields but it ensured specificity.

Both with the 70mer and the 86mer, the signal sequence, which carried exposed photoreactive groups, was crosslinked. It can be concluded that the proximity of the signal sequence to the SSR is not restricted to a certain size of the nascent chain and is maintained for some time during translocation.

The experiments in which the 115mer contained the photoreactive groups beyond amino acid 70 provide decisive evidence that the mature part of preprolactin is close to the SSR at a later stage of translocation. We cannot entirely exclude, however, that the signal sequence in the 115mer was not also still in proximity to the SSR, even though the yield of crosslinked product was not greater if photoreactive groups were present throughout the polypeptide chain (including the signal sequence). In the case of the relations between the signal sequence and the SSR, specific binding interactions may be envisaged. Such an interaction with the SSR appears highly unlikely for the mature portion of the polypeptide chain. Here, crosslinking thus reflects merely physical proximity with the SSR as a component of the protein environment of the translocated chain, perhaps as a constituent of a postulated protein tunnel [15,16].

Krieg et al. have recently identified a 39 kDa membrane protein by a similar crosslinking approach (personal communication). In view of the fact that the size estimates are probably not very accurate, we believe that their protein is in fact identical with the SSR.

The present paper also provides a link between the identification of the SSR from crosslinking experiments ([7] and this paper) and the isolation of a 34 kDa ER protein that appears to be essential for protein translocation in vitro as indicated by the inhibitory effect of antibodies directed against it and of F_{ab}-fragments prepared from the antibodies [9]. Identity of the two proteins is indicated by several observations: (i) both are transmembrane glycoproteins of 34–35 kDa; (ii) two different antibodies directed against the isolated protein precipitate the crosslinked products; (iii) the isolated 34 kDa protein has a cytosolic domain of about 6–7 kDa which corresponds roughly to the size determined from proteolysis of the crosslinked products; (iv) the 34 kDa protein is present in the membrane in at least equivalent amounts to membrane-bound ribosomes [9], consistent with the assumed role of SSR in guiding the nascent chain through the ER membrane; (v) the relative stability of the 34 kDa protein to proteases [9] is also in agreement with results of crosslinking experiments [7]. The identity of the two proteins allows us to conclude that the SSR is an essential component of the translocation apparatus.

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