

Protein Translocation across the Endoplasmic Reticulum Membrane: Identification by Photocross-Linking of a 39-kD Integral Membrane Glycoprotein as Part of a Putative Translocation Tunnel

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Abstract. The molecular environment of secretory proteins during translocation across the ER membrane was examined by photocross-linking. Nascent preprolactin chains of various lengths, synthesized by *in vitro* translation of truncated messenger RNAs in the presence of *N*^ε-(5-azido-2-nitrobenzoyl)-Lys-tRNA, signal recognition particle, and microsomal membranes, were used to position photoreactive probes at various locations within the membrane. Upon photolysis, each nascent chain species was cross-linked to an integral membrane glycoprotein with a deduced mass of 39 kD (mp39) via photoreactive lysines located in either the signal sequence or the mature prolactin sequence. Thus, different portions of the nascent

preprolactin chain are in close proximity to the same membrane protein during the course of translocation, and mp39 therefore appears to be part of the translocon, the specific site of protein translocation across the ER membrane. The similarity of the molecular and cross-linking properties of mp39 and the glycoprotein previously identified as a signal sequence receptor (Wiedmann, M., T. V. Kurzchalia, E. Hartmann, and T. A. Rapoport. 1987. *Nature [Lond.]*, 328: 830–833) suggests that these two proteins may be identical. Our data indicate, however, that mp39 does not (or not only) function as a signal sequence receptor, but rather may be part of a putative translocation tunnel.

THE synthesis of secretory proteins and membrane proteins in eukaryotes is initiated on ribosomes that are free in the cytoplasm and is completed after these ribosomes become bound to the membrane of the ER (Walter and Lingappa, 1986). The selection of ribosomes that bind to the ER membrane is mediated by the signal recognition particle (SRP),¹ which recognizes the signal sequence as it emerges from the ribosome (Walter et al., 1981). This recognition involves the direct binding of the signal sequence to the 54-kD SRP protein (SRP54) (Krieg et al., 1986; Kurzchalia et al., 1986), and can result in elongation arrest (Walter and Blobel, 1981). The SRP-arrested ribosome-nascent chain complex is then targeted to the ER membrane via a specific interaction of SRP with the SRP receptor (also termed docking protein; Gilmore et al., 1982a; Meyer et al., 1982). Both SRP and SRP receptor are then released from the ribosome (Gilmore et al., 1982b), and the signal sequence is presumably handed over to an ER membrane protein that acts as a secondary (i.e., after SRP) signal sequence receptor (SSR; Wiedmann et al., 1987b); protein chain elongation resumes, and translocation is initiated. Although the mechanistic details of this

selection and targeting process are not yet understood, the molecules that accomplish this function have been identified and have been characterized to varying degrees (Walter and Lingappa, 1986).

In contrast, essentially nothing is known about the environment and the interactions of the nascent chain during its passage through the membrane. Even the most basic topographical issue remains controversial, that of whether nascent chain translocation involves direct movement of the nascent polypeptide through the nonpolar core of the bilayer is facilitated, either actively or passively, by a protein(s) in the ER membrane. Experimental evidence to support the postulated existence of a protein tunnel (Blobel and Dobberstein, 1975) or translocon (Walter and Lingappa, 1986), a specific site in the membrane at which translocation proceeds and which is defined by a particular set of proteins, has remained largely elusive. In spite of considerable speculation regarding the direct involvement of membrane proteins in translocation (e.g., Blobel and Dobberstein, 1975; Connolly and Gilmore, 1986; Evans et al., 1986; Singer et al., 1987; Hoffman and Gilmore, 1988; Wilson et al., 1988) and the hydrophilicity of the putative pore, there is only indirect evidence for the latter (Gilmore and Blobel, 1985).

Here we describe a direct approach to examine the molecular environment of a nascent polypeptide chain as it passes through the membrane. Specifically, we have used func-

1. *Abbreviations used in this paper:* AF, SRP-arrested fragment of preprolactin; ConA, concanavalin A; ϵ ANB-Lys, *N*^ε-(5-azido-2-nitrobenzoyl)-Lys; EKRM, salt- and EDTA-washed microsomal membranes; ϵ TDBA-Lys, *N*^ε-4-(3-trifluoromethyl-diazirino) benzoyl-Lys; SRP, signal recognition particle; SSR, signal sequence receptor.

tional, modified aminoacyl-tRNAs (Johnson et al., 1976) that allow us to incorporate photoreactive groups into the nascent chain which, upon irradiation, react covalently with molecules that are located in close proximity to the probes. It has previously been shown that the incorporation of these residues does not impede the ability of the nascent secretory protein to be recognized by SRP and to become properly translocated across microsomal membranes (Krieg et al., 1986; Kurzchalia et al., 1986). The aminoacyl-tRNA analogues have proven valuable in investigations of the interactions of a nascent signal sequence with both SRP (Krieg et al., 1986; Kurzchalia et al., 1986; Wiedmann et al., 1987a) and SSR (Wiedmann et al., 1987b), as well as in studies of aminoacyl-tRNA complexes with elongation factors (Johnson et al., 1978; Johnson and Slobin, 1980; Duffy et al., 1981; Guerrier-Takada et al., 1981) and the ribosome (Johnson and Cantor, 1980). In this work, we have used the approach to investigate the immediate vicinity of several different preprolactin nascent chains, each with photoreactive moieties located within the bilayer and each functionally engaged with the ribosome and the membrane. As detailed below, each nascent chain species reacts covalently with several proteins, including a 39-kD integral membrane glycoprotein that is likely to be a component of the translocon.

Materials and Methods

Translation Components

Photoreactive *N*-(5-azido-2-nitrobenzoyl)-Lys- (ϵ ANB-Lys) tRNA and wheat germ extract were prepared as detailed previously (Krieg et al., 1986). Messenger RNAs coding for truncated preprolactin polypeptides were synthesized by *in vitro* transcription using linearized pSPBP4 DNA (Krieg et al., 1986; Siegel and Walter, 1988). Pvu II (86 amino acids), Mbo II (100 amino acids), Hha I (118 amino acids), Mae I (127 amino acids), and Rsa I (131 amino acids) restriction sites were chosen to obtain run-off transcripts encoding preprolactin fragments of the indicated lengths. Transcription reactions contained: 0.1 ng/ μ l DNA; 0.5 mM, each, ATP, UTP, and CTP; 0.1 mM GTP; 0.5 mM diguanosine triphosphate (G[5']ppp[5']G, Pharmacia Fine Chemicals, Piscataway, NJ); 40 mM Tris (pH 7.5); 6 mM MgCl₂; 2 mM spermidine; 10 mM DTT; 0.5 U/ μ l RNasin (Promega Biotech, Madison, WI); and 0.3 U/ μ l SP6 polymerase (Promega Biotech). The 90-min incubation at 40°C was followed by a chase (15 min, 40°C) during which the GTP concentration was raised to 0.5 mM. Such preparations were used directly to program the translations. Microsomal membranes, salt- and EDTA-extracted membranes (EKRM), trypsinized membranes, purified 52-kD SRP receptor elastase fragment, and SRP were prepared as published elsewhere (Walter and Blobel, 1983a, b; Siegel and Walter, 1985). A sample of *N*⁶-4-(3-trifluoromethyl diazirino) benzoyl-Lys- (ϵ TDBA-Lys) tRNA was a generous gift of Dr. Tom Rapoport.

Translations

The translation incubations were carried out as described (Krieg et al., 1986) in a room illuminated only by a sodium vapor lamp. When included, EKRM were present at 12 equivalents/25 μ l assay, and SRP was used at a concentration of 50 nM. All protein synthesis was performed at 26°C. 2 min after warming the sample to 26°C, 1 μ l of RNA transcription solution was added ($t = 2$ min). If the translation was not pulsed, protein synthesis was allowed to proceed for 15 min in the presence or absence of SRP and/or EKRM. In case of pulsed (synchronized) translations, 7-methylguanosine 5'-monophosphate (Sigma Chemical Co., St. Louis, MO) and edeine (Sigma Chemical Co.) were added to 4 mM and 10 μ M, respectively, at $t = 4$ -7 min. When present in the sample, SRP was added at the beginning of the incubation ($t = 0$ min), while EKRM were added at $t = 11$ min. Reactions were stopped at $t = 21$ min by the addition of cycloheximide to 2 mM.

Photoreactions

Samples were usually split into two aliquots in polypropylene microfuge

tubes. Aliquots serving as negative controls were covered in aluminum foil, and all samples were frozen in liquid nitrogen. Appropriate aliquots were irradiated for 60 min while the tubes were floating in liquid nitrogen \sim 30 cm from a 150 W mercury arc lamp equipped with a 306-409-nm bandpass filter (6% transmission at 312 nm). In several experiments photolysis was carried out at 0°C. In this case, the lids of the microfuge tubes containing the (not previously frozen) samples were opened, and the tubes were kept in an ice-water bath for 20 min while irradiating from above with the mercury arc lamp. The time required to maximize cross-linking at both temperatures under our conditions was determined empirically. The fraction of newly synthesized nascent chains that reacted covalently with nearby proteins in our samples was dependent upon the time allowed for initiation, since that affected the extent of ϵ ANB-lysine incorporation, but typically totaled $>4\%$.

Fractionation

Translation samples (25 or 50 μ l) were diluted to 100 μ l with solution A (140 mM potassium acetate [pH 7.5], 3 mM magnesium acetate, 50 mM triethanolamine [pH 7.5], 1 mM glutathione [pH 7.5]) and layered on a 100- μ l cushion of solution A/0.5 M sucrose. The membranes were pelleted in an airfuge (Beckman Instruments Inc., Palo Alto, CA) at 20 psi for 3 min. For each sample, the top 125 μ l were collected as the supernatant fraction, made 12.5% (wt/vol) in TCA, incubated for 10 min at 0°C, and the acid-insoluble material was pelleted by centrifugation. The remainder of the sucrose cushion was discarded, and the tube was rinsed once with solution A to yield the membrane pellet. The membrane and supernatant pellets were then each analyzed by either immunoprecipitation or lectin binding. When appropriate, fractionation was done in the dark.

Immunoprecipitation

The membrane pellet was solubilized in 40 μ l solution B (3% [wt/vol] SDS, 100 mM Trizma base [Sigma Chemical Co.], 3 mM DTT) at 40°C for 60 min. The acid-insoluble supernatant pellet was solubilized in solution B at 60°C for 30 min. When appropriate, solubilization was done in the dark. After ensuring that the pH of each sample was \sim 7, it was boiled for 5 min. The sample was then diluted (final volume 600 μ l) to a final concentration of 20 mM Tris-HCl (pH 7.5), 0.2% (wt/vol) SDS, 150 mM NaCl, and 1% (vol/vol) Triton X-100. 40 μ l of a 1:1 suspension of Sepharose 4B (Sigma Chemical Co.) in the same solution were added to each sample to adsorb any material that bound nonspecifically to the resin. After 15 min of shaking at room temperature, the Sepharose 4B was removed by centrifugation and then 2 μ l of prolactin antiserum (United States Biochemicals) were added to each sample. After an overnight incubation at 4°C, the antibody-antigen complexes were recovered by adsorption (2 h, room temperature) to protein A-Sepharose (Pharmacia Fine Chemicals). Samples were prepared for electrophoresis by boiling the resin for 5 min in the presence of sample buffer (120 mM Trizma base, 3.6% [wt/vol] SDS, 7.5 mM EDTA, 125 mM DTT, and 15% [vol/vol] glycerol).

Alkaline Extraction

A sodium carbonate solution (133 mM) was adjusted with sodium hydroxide to pH 11.5, and 75 μ l of this solution were added to each 25- μ l translation sample. The samples were then kept on ice for 30 min with occasional mixing before being layered onto a 100- μ l alkali cushion (100 mM sodium carbonate [pH 11.5], 200 mM sucrose) and spun for 10 min in an airfuge at 30 psi. Supernatant and pellet fractions were collected and worked up either (a) as above using immunoprecipitation with antibodies to prolactin, or (b) as detailed in the next section.

Lectin Binding

The pellet fractions of 25- μ l translation incubations were solubilized in 40 μ l of 1% (wt/vol) SDS, 30 mM DTT, 100 mM Trizma base for 90 min at 40°C. Samples were then boiled for 5 min and diluted into 360 μ l solution C (70 mM Tris [pH 7.5], 5 mM CaCl₂, 1 mM MgCl₂, 1.2% [vol/vol] Triton X-100; Wiedmann et al., 1987), while parallel samples were diluted into 360 μ l solution D (solution C containing 0.5 M α -methylmannoside). 40 μ l of a 1:1 suspension of Sepharose 4B (Sigma Chemical Co.) in solution C (or D, as appropriate) were added to each sample to adsorb any material that bound nonspecifically to the resin. After 15 min of shaking at room temperature, the Sepharose 4B was removed by centrifugation and then 25 μ l of a 1:1 suspension of concanavalin (ConA) A-Sepharose (Sigma Chemical Co.) in solution C (or D) were added. The material was adsorbed to the

lectin resin overnight at 4°C with shaking. The beads were washed four times in 1 ml of solution C before the lectin-bound material was recovered by boiling the resin in 25 μ l of sample buffer.

Protease Treatment

Individual membrane pellets from 25 μ l photolyzed translation reactions were each resuspended in 40 μ l of solution A containing 10 mM CaCl₂ using a 10- μ l syringe (Hamilton Industries, Two Rivers, WI) with a flat-tip 22-gauge needle. The suspensions were transferred to microcentrifuge tubes and 0–10 μ l of solution E (20 mM Tris-HCl [pH 7.5], 10 mM CaCl₂) were added to individual tubes. Proteinase K (Bethesda Research Laboratories, Gaithersburg, MD; 1 mg/ml in solution E) was added to achieve a final protease concentration between 0 and 100 μ g/ml in each sample. Proteolysis of the photolyzed membranes was then carried out for 1 h in an ice-water bath. Each reaction was stopped by the addition of an equal volume of 25% (wt/vol) TCA solution, and the samples were processed as described in the lectin binding section. Inactivation of the protease was not necessary, since continued sample degradation was not observed during further work-up. A 10-mg/ml proteinase K stock solution was prepared fresh each time, and a 1:10 dilution of this was incubated at 37°C for 10 min before addition to the membrane samples to inactivate possible lipase contaminants (Hansen et al., 1986).

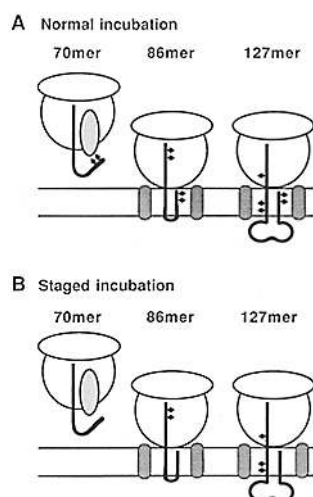


Figure 1. Working model for the disposition of crosslinking probes incorporated into nascent preprolactin chains. Lysine residues (indicated by diamonds) are located in the nascent preprolactin polypeptides at positions -27 and -22 in the signal sequence, and 42, 48, and 69 in mature prolactin (Sasavage et al., 1982). Ribosomes are depicted in the SRP-arrested state (AF = 70 amino acids), or at the end of truncated mRNAs encoding 86 and 127 amino acids, respectively. In the latter two cases, the ribosomes have been engaged in translocation on the ER membrane.

The nascent chains are shown in positions relative to the ribosome and the membrane based on the following assumptions: the COOH-terminal 40 amino acids of the nascent chain are located inside the ribosomal structure (Malkin and Rich, 1967; Blobel and Sabatini, 1970); the ribosome/membrane junction is tight, i.e., does not allow for much "slack" in the nascent chain (Smith et al., 1978; Sabatini and Blobel, 1970); and the extension of the nascent chain through the membrane (amino acids 41 to \sim 63 from the COOH-terminal end) averages 2.5 Å per amino acid. Assuming a different value for the per-residue extension of the nascent chain within the membrane (e.g., 1.5 Å per amino acid for an α -helix) alters the location of the photoreactive lysines in the model depicted in Fig. 1, but does not alter the conclusions of the experiments detailed in the text. The NH₂ terminus of the nascent chain is arbitrarily depicted as being located neither on the cytoplasmic nor on the luminal side of the ER membrane because its location is still uncertain (but see Shaw et al., 1988). Note, that on average only one cross-linking group was incorporated per nascent chain under our experimental conditions. (A) A "normal" translation is shown in which cross-linking reagents were incorporated into all Lys positions. (B) A "staged" translation is shown, where synthesis of the AF was first performed in the absence of ϵ ANB-Lys-tRNA, and synthesis was then continued to the end of the truncated mRNA in its presence. By performing a staged translation, the Lys residues at positions -27 and -22 in the signal sequence are not photoreactive and therefore do not participate in the cross-linking reaction.

Estimation of ϵ ANB-Lys Incorporation

The extent of incorporation of the crosslinking reagent into nascent chains was determined from the amount of ϵ ANB-³H]Lys-tRNA (Krieg et al., 1986) radioactivity recovered in the membrane pellet. The total number of nascent chains synthesized was determined from the amount of [³⁵S]methionine recovered in the same pellet.

Results

Experimental Strategy

We used photochemical cross-linking as a direct approach to determine whether a nascent protein chain was in close proximity to a membrane protein(s) during its translocation into microsomal vesicles. Nascent chains of the secretory protein preprolactin, with photoreactive groups located at specific sites, were synthesized in a wheat germ cell-free translation system using ϵ ANB-Lys-tRNA (Krieg et al., 1986), a Lys-tRNA analogue that provides efficient incorporation of photoreactive ϵ ANB-Lys into protein *in vitro*. To position the photoreactive probes at different locations along the route of the nascent chain, various truncated mRNAs were first prepared by linearizing plasmid DNA containing the preprolactin gene at different points within the preprolactin coding region before transcription with SP6 RNA polymerase. Then polypeptides of different lengths were translated from the different mRNAs. These nascent chains remained ribosome-bound as peptidyl-tRNAs because ribosomes stall at the end of mRNAs that lack a termination codon (Siegel and Walter, 1988). The nascent chain-ribosome complexes were targeted to the membrane by translating in the presence of microsomal membranes and SRP. Using this strategy, we created nascent chains of different, discrete lengths that were functionally engaged with both the protein synthesis and secretion apparatus, and therefore should constitute true intermediates in the translocation process. Photoreactive groups were positioned wherever lysine residues were located in the nascent chain (Fig. 1 A) and, upon activation by UV irradiation, were cross-linked to molecules located adjacent to the nascent chain.

The Preprolactin 127mer Forms Membrane-Dependent Photocross-Links to Proteins

When the translation of preprolactin is stopped at residue 127, the two lysines at positions 42 and 48 in the mature prolactin would, based on the assumptions given in the legend to Fig. 1, be located near the center of the membrane. In addition, depending upon the location of the signal sequence in a 127mer nascent chain, the two lysines in the signal sequence may also be found in the membrane. Thus, the preprolactin 127mer may have as many as four photoreactive lysines positioned in a membrane environment (Fig. 1 A). Since the preprolactin fragment is synthesized using a truncated mRNA in the presence of [³⁵S]methionine, any prolactin-containing radioactive material with a molecular mass larger than the preprolactin 127mer itself (estimated as 14 kD) in an irradiated sample must result from a photocross-linking event between the nascent chain and an adjacent protein.

A number of high molecular mass photoproducts were formed when the preprolactin 127mer was translated and then irradiated in the presence of ϵ ANB-Lys-tRNA, SRP, and microsomal membranes (Fig. 2, lanes 3 and 4). Most

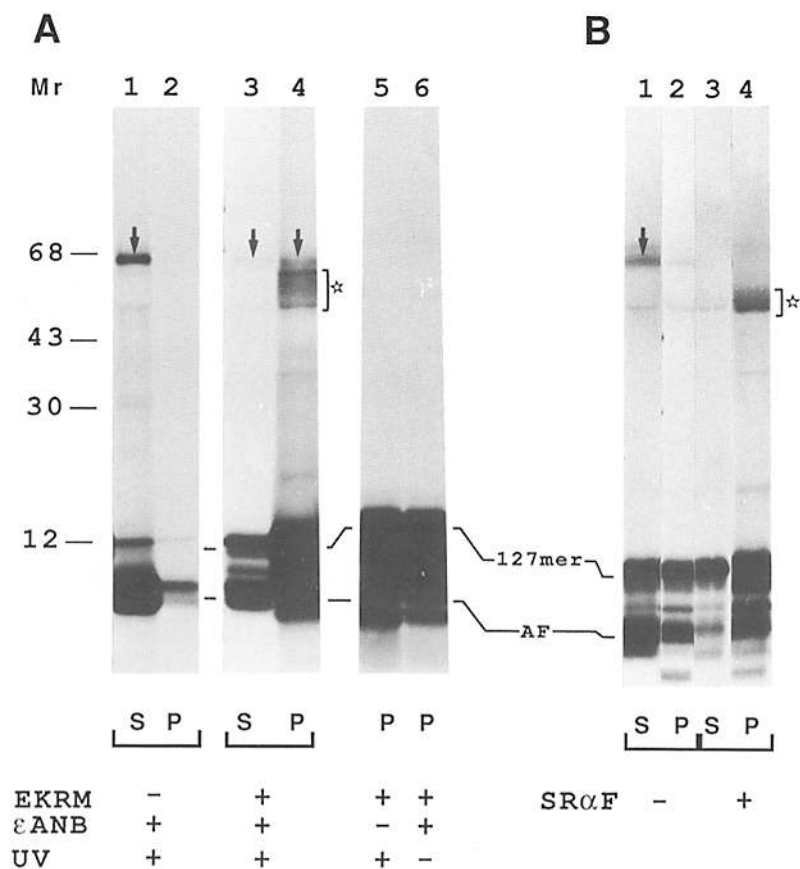


Figure 2. Photocrosslinking of membrane-bound preprolactin 127mer. Unpulsed translations (25 μ l) were carried out as detailed in Materials and Methods and were programmed with run-off transcript obtained by in vitro transcription of Mael-digested pSPBP4 plasmid encoding the first 127 amino acids of preprolactin. The incubations contained EKRM (EKRM; A, lanes 3-6), SRP (A and B, all lanes), and ϵ ANB-Lys-tRNA (ϵ ANB; A and B, all lanes except lane 5, which contained an equivalent amount of unmodified Lys-tRNA). Membranes treated with trypsin (30 μ g/ml) were added to the samples in B, lanes 1 and 2, while the sample in B, lanes 3 and 4, received trypsinized membranes that had been preincubated with purified 52-kD SRP-receptor elastase fragment (SR α F; 100 nM final concentration) (Siegel and Walter, 1985). After 15 min at 26°C, translation was stopped by the addition of cycloheximide. After photolysis at -196°C (the sample in lane 6 was not irradiated), the samples were fractionated into supernatant (S) and pellet (P) fractions (see Materials and Methods). In this experiment, samples were immunoprecipitated with antiserum to prolactin, separated by SDS-PAGE in a 10-20% (wt/vol) gradient gel, and analyzed using fluorography. Exposure was for 10 d. The positions of the arrested preprolactin fragment (AF) and the major 127mer are indicated. The position of the AF-SRP54 cross-link (arrow) and the membrane-dependent 127mer cross-links (star) are indicated. Molecular mass standards: phosphorylase B, 97 kD; BSA, 68 kD; ovalbumin, 43 kD; carbonic anhydrase, 30 kD; cytochrome C, 12 kD.

of the 127mer chains and almost all of the photoproducts were recovered in the membrane pellet fraction after centrifugation, indicating that they were properly targeted to the membrane. After immunoprecipitation with antiserum to prolactin, the primary photocross-linked products traveled as a broad, unresolved protein band with a relative molecular mass from 53 to 62 kD in our gel system (Fig. 2, lane 4, star).

The appearance of these species was dependent both on the presence of the photoreactive Lys-tRNA and on photolysis with UV light, since in the absence of the modified tRNA (Fig. 2, lane 5) or in the absence of irradiation (Fig. 2, lane 6), no photocross-linked products were formed. Also, when the samples were treated with puromycin (Blobel and Sabatini, 1971) before photolysis, no photocross-linked material was observed (data not shown). Hence, the photocross-linking was due only to ribosome- and membrane-bound nascent chains.

When SRP, but not microsomal membranes, was included in the incubation, most of the cross-linked radioactive material was found in the soluble fraction (Fig. 2, lane 1, arrow) as a 62-kD photoproduct. This band could also be immunoprecipitated with antiserum against SRP54, and thus represents the arrested fragment of preprolactin (AF) crosslinked to SRP54 (Krieg et al., 1986; Kurzchalia et al., 1986). The anti-SRP54 serum also precipitated residual 62 kD photocross-link in the pellet and supernatant fractions of the sample shown in lanes 3 and 4 of Fig. 2 (arrow), as well as the 62-kD band in lane 1 and 2 of Fig. 3 B (arrow) (data not

shown). The presence of the 62-kD photoproduct in samples that contained membranes was variable, probably depending on how efficiently the SRP-arrest was released by the membranes before photolysis.

The formation of all photocross-links (except the 62-kD species) was blocked when the translations were supplemented with proteolyzed membranes that lacked a functional SRP receptor (Fig. 2 B, lanes 1 and 2). However, photocross-linking was restored by reconstituting the proteolyzed SRP receptor in the reaction with purified 52-kD elastase fragment of SRP receptor (Fig. 2 B, lanes 3 and 4). Thus, the formation of the photocross-linked species required each of the following: ϵ ANB-Lys-tRNA, light, SRP, SRP receptor, and membranes.

Several Photocross-Linked Products Contain Integral Membrane Proteins

To determine whether translocating nascent chains can be cross-linked to an integral membrane protein(s), we extracted photolyzed membranes with sodium carbonate. Resistance to extraction from membranes at alkaline pH has been widely used to distinguish between integral membrane proteins and proteins that associate only peripherally with membranes (Fujiki et al., 1982).

We found that nascent chains ranging in length from 86 to 131 amino acids each cross-linked to at least one integral membrane protein. Fig. 3 shows the data obtained with the 127mer (A) and 86mer (B) nascent chains. The membrane-

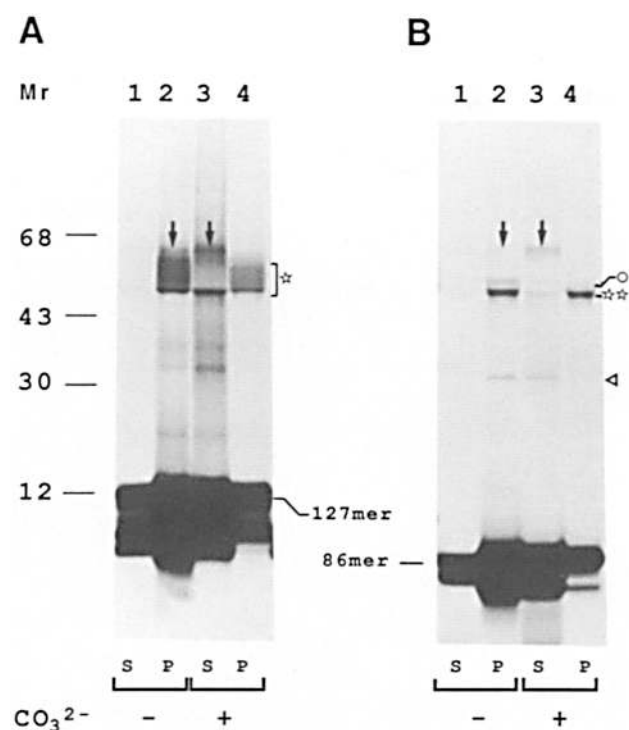


Figure 3. Alkaline extraction of photoproducts from the membrane. Translations (25 μ l), programmed with run-off transcript encoding the preprolactin 127mer (*A*) or the 86mer (*B*), were carried out as described in Materials and Methods in the presence of SRP and membranes, and were not pulsed. After photolysis at -196°C , the samples in lanes 3 and 4 of both *A* and *B* were treated with 100 mM sodium carbonate (CO_3^{2-}) at pH 11.5 as detailed under Materials and Methods. Immunoprecipitations of supernatant fractions (*S*) and membrane pellets (*P*) with antiserum to prolactin were analyzed as in Fig. 2. Exposure was for 10 d. Bands are marked as in Fig. 2, with the addition of the 86mer-mp39 cross-link (*double star*), and the 33-kD (Δ) and 56-kD (\circ) cross-links of the 86mer to putative ribosomal proteins.

bound preprolactin 86mer represents an early stage of polypeptide translocation across the membrane, since only the two lysines in the signal sequence can potentially be both photoreactive and in contact with membrane components (Fig. 1 *A*). The 86mer nascent chain formed distinct photoproducts with masses similar to the high molecular mass photoproducts observed with the 127mer (Fig. 3 *B*, lane 2; a prominent 49-kD [*double star*] and a less abundant 56-kD photoadduct [\circ]), and at least one additional strong band with an apparent mass near 33 kD (Δ). In both sets of incubations, those translating the 127mer and those translating the 86mer, the 62-kD cross-link of SRP54 to the AF was extracted by the sodium carbonate, and was thus recovered in the supernatant fraction (*arrows*, Fig. 3, *A* and *B*, compare lanes 1 and 3). As expected, most of the noncross-linked nascent chains were also extracted from the membranes (Fig. 3, *A* and *B*, lanes 3 vs. lanes 4; in agreement with Gilmore and Blobel, 1985). In contrast, the 127mer-containing photo-cross-linked material in the band at 53–58 kD was nonextractable at high pH (Fig. 3, *A*, lane 4, *star*). In the case of the 86mer, both the 33- and 56-kD photoproducts were extracted (Fig. 3 *B*, lanes 3 and 4), whereas the prominent 49-

kD photoproduct (*double star*) was fully resistant to the high pH treatment (Fig. 3 *B*, lane 4). (As will be shown below, the carbonate-extractable photoadducts are likely to be cross-links to ribosomal proteins.) This experiment shows that the preprolactin 127mer and the 86mer are each located adjacent to at least one integral membrane protein to which they were cross-linked.

The poor resolution of the bands between 53 and 62 kD in lane 4 of both Fig. 2 *A* and Fig. 3 *A* led us to investigate the dependency of these bands on the time allowed for initiation of the translation. We compared cross-linking reactions in translations that had been pulsed between 2 and 10 min, and found that, while the overall pattern of products was unchanged, bands were better resolved at lower pulse times. Henceforth, we limited the time for translation initiation by adding initiation inhibitors to minimize the chain heterogeneity caused by multiple ribosome initiations on the same mRNA.

A Major Photocross-Linked Product Is Glycosylated

Since integral membrane proteins of the ER are often gly-

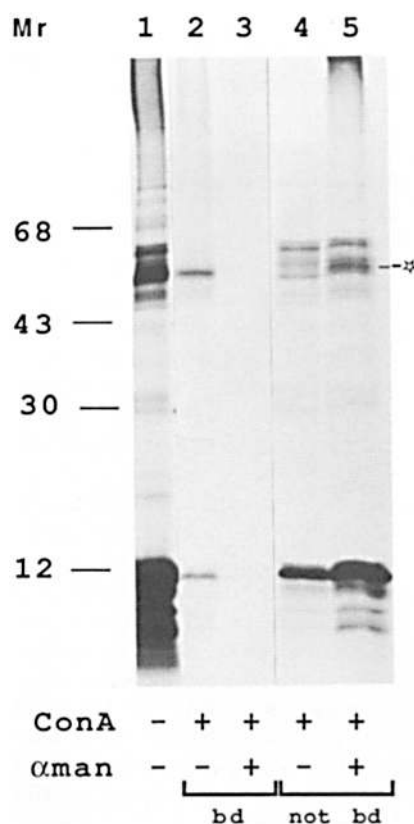


Figure 4. Lectin binding of photoproducts. Translations (25 μ l), programmed with run-off transcript encoding the preprolactin 127mer, were pulsed for 3 min, supplemented with membranes, and photolyzed at 0°C before extraction with sodium carbonate (pH 11.5). The membrane pellets (lane 1) were further fractionated using Con A-Sepharose (*ConA*) in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 0.5 M α -methylmannoside (α *man*). The ConA-bound material (*bd*) (lanes 2 and 3) and unbound fractions (*not bd*; precipitated with 10 vol of acidified acetone [19 parts acetone; 1 part 100 mM HCl]; lanes 4 and 5) were analyzed as in Fig. 2. Exposure was for 3 d. The 127mer-mp39 cross-link is marked with a star.

cosylated, we examined whether any of the 127mer-containing photoproducts bound specifically to ConA-Sepharose (preprolactin itself is not glycosylated). Only a single photoproduct (~53 kD, Fig. 4, lane 2, *star*) from the 53–58-kD region was bound by the affinity resin; all other cross-linked material either was not bound or was adsorbed in only very low amounts. The unbound fraction contained the remainder of the photocross-linked species (Fig. 4, lane 4). When the resin was preincubated with an excess of α -methylmannoside as a competitive inhibitor, none of the photoproducts bound to the ConA-Sepharose (Fig. 4, lane 3), but were recovered in the unbound fraction (Fig. 4, lane 5). After elution from the resin the material that bound to the ConA-Sepharose could be immunoprecipitated with the anti-prolactin serum (data not shown). This demonstrated the presence of both carbohydrate and prolactin in the same photoproduct.

A comparison of the gel in Fig. 4 with those in Figs. 2 and 3 shows that the high molecular mass photoproducts were better resolved when the immunoprecipitation step was omitted (Fig. 4). This resulted from the absence of large amounts of immunoglobulin heavy chains that in the immunoprecipitated samples distorted the electrophoretic mobility of the photocross-linked material, which was present only in radiochemical amounts.

The Glycoprotein Photoproduct Is Exposed to the Cytoplasm

Since the 127mer was cross-linked to an integral membrane protein that is glycosylated, and therefore exposed on the luminal face of the ER membrane, we wished to determine whether the glycoprotein-containing photoproduct also had a cytoplasmic domain(s). This was done by digesting photolyzed membranes extensively with proteinase K. As shown in Fig. 5, proteolysis of the 53-kD cross-linked glycoprotein (*star*) appeared to proceed through a discrete intermediate (lanes 2 and 3) to a final proteolyzed form (lanes 4 and 5). In total, the glycoprotein photoproduct was reduced in size by ~4 kD before becoming insensitive to further proteolysis under these conditions.

Similar results, including the observation of a discrete intermediate form, were obtained when material photocross-linked to the 86mer was proteolyzed as above. At a concentration of 100 μ g/ml of proteinase K, the 86mer-glycoprotein cross-link (49 kD, see below) was digested to a ConA-bound complex with an apparent mass of 45 kD (data not shown).

Ribosomes were not stripped from the membranes in these experiments, and the observed proteolysis did not result from digestion of the nascent chain portion of the photoproduct. Control experiments with unphotolyzed 127mer showed that the nascent chains were not proteolyzed under these conditions (data not shown). Furthermore, the radioactivity associated with the photoproduct was not detectably reduced by the exposure to proteinase K (Fig. 5), which indicates that the nascent chain was not the proteolytic target. Thus, our data show that the membrane glycoprotein to which the nascent chain photocross-links is a transmembrane protein of the ER.

Photoreactive Nascent Preprolactin Chains of Increasing Length Each Cross-Link to a 39-kD ER Membrane Glycoprotein

To map the environment of nascent chains across the ER

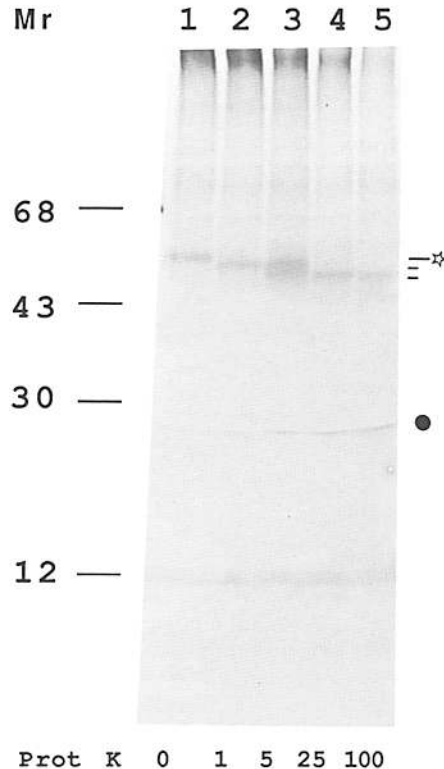


Figure 5. Proteolytic sensitivity of photoproducts. A translation (150 μ l) was programmed with mRNA coding for the preprolactin 127mer and pulsed for 5 min in the presence of SRP. After an additional 4 min incubation in the presence of initiation inhibitors, membranes were added and translation was continued for 10 min before the addition of cycloheximide. After photolysis at 0°C of individual 25 μ l samples of the original incubation, the membranes were pelleted and resuspended (see Materials and Methods). Proteolysis was carried out for 1 h at 0°C in the presence of 0, 1, 5, 25, and 100 μ g/ml proteinase K (*Prot K*). The trichloroacetic acid-insoluble material of each sample was incubated with ConA-Sepharose, and the lectin-bound material was analyzed as in Fig. 2. Exposure was for 3 d. The position of the 127mer-mp39 cross-link is indicated by a star; the digestion products are indicated by lines. ● marks the position in front of the heavily overloaded ConA band (eluted from the resin during sample preparation).

membrane, we have examined the photoproducts that were formed by photoreactive, truncated nascent chains containing 86, 100, 118, 127, and 131 amino acids. By increasing the length of the shortest product studied (the 86mer), the photoreactive lysines were positioned at different sites along the nascent chain pathway (cf. Fig. 1 A). Many of the major and minor photoproducts increased in molecular mass according to the increase in size of the photoreactive nascent chain (data not shown). This molecular mass shift was particularly evident for the glycoprotein cross-link, because it could be purified away from the other photoproducts (Fig. 6). The molecular masses of the major ConA-bound photoproducts ranged from 49 (*double star*) to 53 (*star*) to 54 kD in the samples of Fig. 6 (identical numbers were obtained when the material was analyzed by SDS-PAGE in a 10% gel rather than the 10–20% gradient gel shown here). After subtracting the estimated molecular masses of the cross-linked preprolactin fragment (10–14.5 kD) from the mass of the respective photoproduct, the apparent molecular mass of the target glyco-

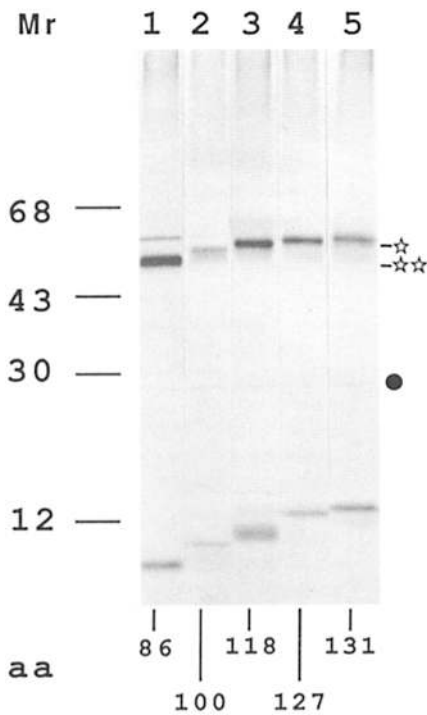


Figure 6. Lectin binding of photoproducts formed by preprolactin fragments of various lengths. Translations (25 μ l) containing SRP were programmed with mRNA coding for preprolactin fragments that were 86, 100, 118, 127, or 131 amino acids in length and were pulsed for 5 min. After an additional 4 min, the incubation received membranes, and translation was continued for 10 min before the addition of cycloheximide. After photolysis at 0°C and carbonate extraction, the ConA-bound material was analyzed as in Fig. 2. Exposure was for 3 d. The positions of the 127mer-mp39 and the 86mer-mp39 cross-links are indicated by a star and double star, respectively. The bands at the bottom of the gel represent a small fraction of the nascent chains that were nonspecifically adsorbed to the resin. Their sizes also indicate that no signal peptide cleavage occurred on nascent chains of this length under our assay conditions. The additional cross-linked protein in lane 1 was bound nonspecifically; almost all of this material was recovered in the non-bound fraction. Glycosylation of 86 mer-mp39 was further confirmed by digestion with endoglycosidase H, which resulted in a small increase in gel mobility of the cross-linked product. A similar digestion of the 127mer-mp39 crosslink did not show a detectable mobility shift, possibly because the resolution of the gel was insufficient or because the cross-linked product was a poor substrate for the enzyme.

protein was found to be 39-kD in each case. This suggests that each nascent chain was cross-linked to the same glycoprotein, which we have termed mp39.

The Staging Experiment

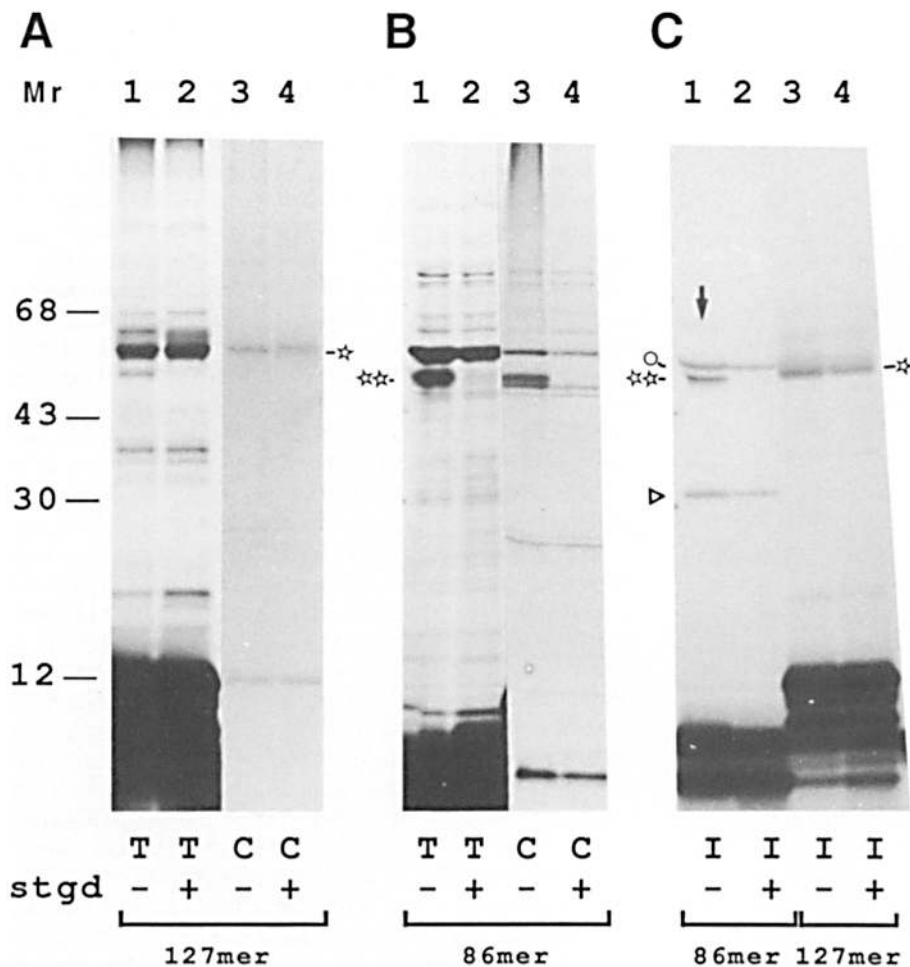
From the data shown in Fig. 6, it appears that lysines in the signal sequence of preprolactin and/or lysines in the mature prolactin sequence are located close to mp39 even when positioned in different locations along the nascent chain pathway. To map the path of the nascent chain through the membrane with precision, it is necessary to know which of the photoreactive lysines in each nascent chain species formed the covalent bonds that yielded the photocross-linked products. As a first step in accomplishing this goal, we set out to distinguish between photocross-links formed by ly-

sines in the signal sequence and by lysines in the mature prolactin sequence. To do this, we used a strategy that we have designated the "staging experiment." In this approach, pulsed translations were first carried out in the presence of SRP but in the absence of ϵ ANB-Lys-tRNA. Under these conditions, protein synthesis proceeded until the SRP-dependent elongation arrest (Walter and Blobel, 1981), and only endogenous unmodified lysines present in the wheat germ translation extract were incorporated into positions -27 and -22 of the signal sequence in the arrested nascent chains. After adding edeine and $p^{7m}G$ to the samples to block further chain initiation, ϵ ANB-Lys-tRNA and membranes were added to the incubations. This released the elongation arrest, and nascent chain synthesis and translocation continued in the presence of ϵ ANB-Lys-tRNA. In these samples the photoreactive lysines were therefore incorporated only at lysine positions beyond the point of SRP-induced elongation arrest in the mature prolactin (Fig. 1 B).

When the products from a staging experiment were analyzed after immunoprecipitation (Fig. 7 C), the 86mer nascent chain was no longer able to form the 49-kD photoproduct with mp39 (*double star*), as indicated by its absence in lane 2. Furthermore, although only a very small amount of AF-SRP54 cross-link is evident in the experiment shown in Fig. 7 C (lane 1, *arrow*), the AF-SRP54 photocross-link was always absent in staged incubations, even upon prolonged exposure of the autoradiogram. Therefore, those two adducts resulted from cross-linking via the preprolactin signal sequence (compare Fig. 1, A with B). This was expected for the AF-SRP54 photocross-link (Krieg et al., 1986; Kurzchalia et al., 1986). This also demonstrates conclusively that under staged conditions, no ϵ ANB-Lys was incorporated into positions -22 and -27 of the nascent chains. Thus, the presence of the 56-kD (\circ) and 33-kD (Δ) bands, which, in contrast to the cross-links described above, are still present in the staged samples (Fig. 7 C, lanes 1 and 2) shows that these photoproducts were formed by lysines 42 and/or 48 in the mature prolactin. Since these lysines are still located within the ribosome (Fig. 1 B), the 56- and 33-kD bands most likely result from photocross-links to ribosomal proteins.

Most of the cross-linked products that are formed by the 127mer nascent chain and that migrate in the 53–62-kD region of the gel are formed whether or not the signal sequence contains ϵ ANB-Lys (compare lane 1 with lane 2 of Fig. 7 A and lane 3 with lane 4 of Fig. 7 C [the bands in Fig. 7 C are distorted due to the presence of IgG heavy chain]). It follows therefore that these products result from cross-linking via the lysines at positions 42 and/or 48 of the mature prolactin. In the 127mer the lysines at positions 42 and 48 of mature prolactin are predicted to be located within the ER membrane (Fig. 1 B) and thus the 53–58-kD photoproducts are membrane-associated targets of the photo-probes, which is in agreement with the carbonate extraction data shown above.

The staging results are more striking when we focus on the behavior of the cross-links with mp39 selected on the ConA resin. Note that the extent of 127mer-mp39 cross-link formation was unaffected by omitting the photoreactive lysines from the signal sequence (lanes 3 and 4 of Fig. 7 A, *star*). In contrast, the formation of the 86mer-mp39 cross-link did not occur when photoreactive lysines were not present in the signal sequence (Fig. 7 B, lanes 3 and 4, *double star*). Thus, assuming that the same 39-kD glycoprotein is cross-linked



Lys-tRNA was added 7 min after the initiation inhibitors. Photolysis was at -196°C . All samples were analyzed as in Fig. 2. The band at 49 kD present in lane 1 and absent in lane 2 in A results from the cross-linking of a shorter (than 127mer) nascent chain to mp39, probably due to multiple ribosome initiations on the same mRNA. T, total pellet; C, ConA-bound; I, immunoprecipitate. Bands are marked as in preceding figures.

Figure 7. The staging experiment: lysines both in the signal sequence and in the mature prolactin cross-link to a 39-kD membrane glycoprotein. (A) Translations (50 μl) of preprolactin 127mer were started in the presence of SRP, but in the absence of membranes; $\epsilon\text{ANB-Lys-tRNA}$ was initially present only in one sample (lanes 1 and 3; *stgd* -). After 3 min, initiation inhibitors were added, and one min later, the staged sample (lanes 2 and 4; *stgd* +) received $\epsilon\text{ANB-Lys-tRNA}$. Membranes were added and the incubation was continued for 10 min. Samples were photolyzed at -196°C and split into two aliquots. From one aliquot total membrane pellets were analyzed by SDS-PAGE (lanes 1 and 2); the other aliquot was first carbonate-extracted and then adsorbed to ConA (lanes 3 and 4). (B) The experiment was done similarly to that in A, except that the mRNA coded for the 86mer and photolysis was done at 0°C . Note that in lanes 3 and 4 a small fraction of some presumably nonglycosylated proteins were nonspecifically adsorbed to the ConA resin. (C) Immunoprecipitations with antiserum to prolactin are shown of the membrane pellets of staged (lanes 2 and 4) and nonstaged (lanes 1 and 3) translation incubations. As above, the incubation was pulsed for 3 min, but in the case of the staged samples, the $\epsilon\text{ANB-Lys-tRNA}$

to both the 86mer and the 127mer preprolactin, it would appear that both the signal sequence and a portion of the mature preprolactin sequence are located adjacent to the same ER membrane glycoprotein during translocation, and, at least to that extent, encounter the same environment during their passage through the membrane.

Photoproduct Formation Depends on Temperature

We discovered during the course of this investigation that the efficiency of formation of the prominent photocross-links depended upon the temperature at which photolysis occurred. Interestingly, however, the temperature dependence showed opposite effects for different photoproducts. The extent of formation of the 62-kD AF-SRP54 cross-link was increased if the samples were kept frozen in liquid nitrogen rather than unfrozen in an ice-water bath during irradiation (compare lanes 1 and 2 in Fig. 8, arrow). In contrast, some 127mer-containing photoproducts behaved in the opposite manner, since the high molecular mass products formed preferentially at 0°C (compare lanes 5 and 6 of Fig. 8). Similarly, photocross-link formation by the preprolactin 86mer nascent chain was more efficient in the membranes that were photolyzed at 0°C (compare lanes 3 and 4 of Fig. 8). The

mechanistic implications of these observations are discussed below.

Discussion

We have used an approach to investigate protein synthesis and secretion which allows us to look at the process from the point of view of the nascent protein chain. The described photocross-linking method permits us to map the path of the nascent chain through the ribosome and then through the ER membrane, identifying, in particular, the immediate molecular environment of newly synthesized polypeptides that are in the process of translocation across the ER membrane. Most importantly, nascent chains generated *in vitro* are efficiently recognized by SRP and engaged with the translocation machinery of the ER. Hence, we can use the functional properties of the *in vitro* system to ensure that the structural conclusions are valid. This is an important consideration because photocross-linking is a low yield procedure and it is therefore necessary to verify that essentially all the nascent chains in the analyzed sample are properly targeted and engaged with the membrane.

Each of the photoreactive nascent chains used in this study

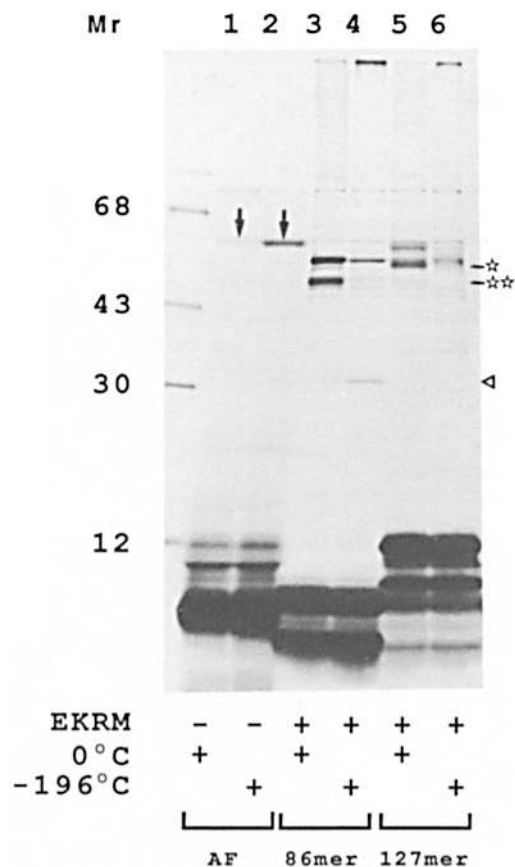


Figure 8. Temperature dependence of photoproduct formation. Pulsed translations (25 μ l) were programmed with run-off transcript coding for the preprolactin 127mer (lanes 1, 2, 5 and 6) or the 86mer (lanes 3 and 4). Initiation was stopped after 2 min. After an additional 4 min, the incubations in lanes 3–6 received membranes, and translation was continued for 10 more min before the addition of cycloheximide. Samples in lanes 1 and 2 received cycloheximide after 10 min of translation. The samples were photolyzed either at 0°C (unfrozen in ice) or at –196°C (frozen in liquid nitrogen). After fractionation, membrane pellets of samples in lanes 3–6 were dissolved directly in sample buffer without immunoprecipitation. Samples 1 and 2 were precipitated with TCA and then solubilized in sample buffer. Note that the time window for initiation was shorter in this experiment than in those shown above. Samples were analyzed as in Fig. 2; exposure was for 3 d. Bands are marked as in preceding figures.

reacted covalently with several proteins (e.g., Fig. 2), some of which are membrane proteins (Fig. 3) and some of which are ribosomal proteins (see below). Most prominent of the photocross-linking targets, both in terms of extent of cross-linking (Figs. 2, 3, and 8) and ease of identification (Figs. 4 and 6), is mp39. This protein appears to be a transmembrane protein of the ER, since it is both glycosylated (Figs. 4 and 6) and therefore exposed to the lumen of the ER, and also sensitive to proteolytic digestion from the cytoplasmic face of the ER membrane (Fig. 5).

The most important finding of this study is that photoreactive lysines positioned at different distances from the carboxy terminus, and therefore, given the functional engagement of these nascent chains with the translocation apparatus, assumed to be at different locations within the membrane, will react

covalently with mp39 (Figs. 6 and 7). This property of mp39 indicates that it is aligned, at least partially, along the pathway of the nascent chain through the ER membrane. Although no function for the protein has yet been identified, its topography and spatial proximity to the nascent chain suggest that mp39 serves as a guide for the translocating nascent protein chain, is part of a scaffold for the translocation machinery, and/or is a component of a protein pore or tunnel through the membrane.

Formally, until a functional role for mp39 is translocation is demonstrated, we cannot rule out the possibility that the photocross-linking of mp39 to the nascent chain results from a random encounter between the two polypeptides. However, if diffusion to the nascent chain were the basis of the observed cross-linking, then we would expect a far more complex pattern of cross-linked species than that shown in Figs. 2 and 3. For this reason, we believe that the small, discrete set of cross-linked proteins identified in our experiments, and mp39 in particular, constitutes a nonrandom arrangement of ER proteins near membrane-engaged ribosomes that collectively promotes nascent protein chain translocation. Our evidence strongly suggests not only the existence of such a site, termed the ER translocon (Walter and Lingappa, 1986), but also identifies mp39 as one of its components.

Wiedmann et al. (1987b) have recently reported the cross-linking of the arrested fragment (70 amino acids long), through photoreactive lysine residues located in the signal sequence, to a 35-kD glycoprotein in the ER that they designated the SSR. We have repeated these experiments and observed a single glycoprotein photoproduct cross-linked to the AF that is smaller than the mp39-86mer photoproduct by about the difference in mass between the two nascent chains (data not shown). In addition, we have examined the protease sensitivity of ER glycoproteins by ConA blotting and found that the major 35-kD glycoprotein was reduced by 29 kD when treated as described in Fig. 5 (Zimmerman, D., and P. Walter, unpublished data), consistent with the proteolytic reduction in size of the mp39 cross-links. In view of these results, and considering the inherent uncertainty in molecular weight determinations, it therefore seems likely that mp39 is the same protein identified as SSR by Wiedmann et al. (1987b). Results recently obtained by M. Wiedmann and T. Rapoport (personal communication) confirm this assignment. Using anti-peptide antibodies to SSR, these workers succeeded to immunoprecipitate nascent chain photoadducts prepared as described here.

Assuming that mp39 and SSR are identical, the data presented here indicate that this glycoprotein does not (or not only) function as a signal sequence receptor as implied by the SSR designation, but rather serves as a tunnel component. This conclusion is based primarily on the ability of nascent chains to cross-link to mp39 even when no photoreactive groups were present in the signal sequence (Fig. 7, A and C). Further support for this notion comes from the apparent insensitivity of the reaction to the position of the cross-linking probe within the membrane (Fig. 6) and from the characteristic temperature dependence of the cross-linking reaction (see below). It is also noteworthy that the yield of the AF-photoadduct, obtained using either ϵ ANB-Lys-tRNA or ϵ TDBA-Lys-tRNA (a carbene-generating photocross-linking reagent used by Wiedmann et al., 1987b), was 10-fold lower than that of the mp39-86mer or mp39-127mer cross-

link (data not shown). Again, this result would not be expected if mp39 bound to the signal sequence specifically. The difference in cross-linking efficiencies could result from a different juxtaposition of AF and 86mer to mp39, or alternatively, the cross-linking in the AF samples may be effected only by a small fraction of nascent chains that have elongated beyond AF due to some leakage though the SRP arrest during the incubation.

The extent of photocross-linking of the ϵ ANB-containing signal sequence to SRP54 was much greater when samples were irradiated in liquid nitrogen than when they were photolyzed in an ice-water bath (Fig. 8, compare lane 1 with lane 2). A similar temperature dependence was observed recently in the photoaffinity labeling of chymotrypsin with an aromatic azide, and the increased photoreaction at -196°C in this model study was ascribed both to temperature-dependent nitrene photochemistry and also the inability of the nitrene to diffuse out of its binding site in the solid matrix (Kanakarajan et al., 1988). In contrast, we observed the opposite temperature dependence for the extent of nascent chain photoreaction with all the protein targets in the membrane and ribosome (Fig. 8, lanes 3–6). This striking observation may reflect the differing arrangements of the nascent chain and its cross-linking targets. Because the nascent chain is not likely to bind tightly to the ribosome or the membrane as it passes through them, we surmise that the higher photocross-linking efficiency at 0°C may result from the ability of the nascent chain to diffuse in the unfrozen sample. Specifically, we propose that in one case (SRP54) the nascent chain is bound tightly and can react covalently with its target in a solid matrix, whereas in the other case (mp39, etc.), the nascent chain is not in direct contact with its targets. Hence cross-linking largely relies upon limited diffusion of the nascent chain during the brief lifetime of the activated aryl azide to make contact with the molecules that line its path. The difference in SRP54 and mp39 cross-linking efficiencies therefore suggests to us that mp39 does not function by binding to either the signal sequence (as would be expected for a bona fide signal sequence receptor) or the mature portion of the nascent chain during the membrane translocation event.

Although this report has focussed on mp39, a close examination of the photoproducts that remain after carbonate extraction indicates that the 127mer was cross-linked to more than one integral membrane protein (Fig. 3 A, lane 4). Several of these carbonate-resistant photoproducts did not bind to ConA (Fig. 4, lane 4) and were formed whether or not the sample was pulsed or staged (Fig. 7 A, lanes 1 and 2). Hence, these photocross-linking targets appear to be specific integral membrane proteins of the ER that are not glycosylated. Also, Robinson et al. (1987) have reported the photocross-linking of a synthetic consensus signal peptide to a 43-kD integral membrane protein of the ER. We have observed photocross-linking to carbonate extraction-resistant proteins of similar size (lane 4 in Figs. 3 A and 4), and it is possible that the same translocon component has reacted covalently both with free peptide and with in vitro-assembled nascent chains. Thus, while there is evidence that the translocon contains more than one ER membrane protein, we will require more specific tools (e.g., antibodies) to resolve, identify, and characterize these proteins individually.

We have also shown, most convincingly in the staging ex-

periment, the photocross-linking of the nascent chain to ribosomal proteins (e.g., Fig. 7 C, lane 2). Two major cross-links, to a 42-kD protein (Fig. 7 C, lanes 1 and 2, \circ) and a 23-kD protein (Fig. 7 C, lanes 1 and 2, Δ), were consistently observed. When nascent chains of different lengths were analyzed in an experiment similar to that shown in Fig. 6, except that the products were not ConA-selected, these cross-linked species shifted in molecular size correspondingly (data not shown). This indicates that, similar to the results described here for mp39, both of these ribosomal proteins are in proximity to multiple regions of the nascent chain. Thus, taken together, our data demonstrate the feasibility of mapping the ribosomal components that form the tunnel through, or the crevice in, the ribosome along which the nascent chain travels, as well as its molecular environment in subsequent stages, including translocation and processing.

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