ER Translocation Intermediates Are Adjacent to a Nonglycosylated 34-kD Integral Membrane Protein

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Abstract. We have used the homobifunctional cross-linking reagent disuccinimidyl suberate (DSS) to identify proteins that are adjacent to nascent polypeptides undergoing translocations across mammalian rough ER. Translocation intermediates were assembled by supplementing cell free translations of truncated mRNAs with the signal recognition particle (SRP) and microsomal membrane vesicles. Two prominent cross-linked products of 45 and 64 kD were detected. The 64-kD product was obtained when the cell free translation contained SRP, while formation of the 45-kD product required both SRP and translocation competent microsomal membrane vesicles. In agreement with previous investigators, we suggest that the 64-kD product arises by cross-linking of the nascent polypeptide to the 54-kD subunit of SRP. The 45-kD product resists alkaline extraction from the membrane, so we conclude that the 11-kD nascent polypeptide has been crosslinked to an integral membrane protein of ~34 kD (imp34). The cross-linked product does not bind to ConA Sepharose, nor is it sensitive to endoglycosidase H digestion; hence imp34 is not identical to the α or β subunits of the signal sequence receptor (SSR). We propose that imp34 functions in concert with SSR to form a translocation site through which nascent polypeptides pass in traversing the membrane bilayer of the rough endoplasmic reticulum.

T HE signal sequence of proteins destined for translocation across or integration into the rough ER (RER)[1] contains the information that initiates sequential interactions with cytoplasmic and membrane bound components of the translocation apparatus. The cytoplasmic ribonucleoprotein signal recognition particle (SRP) cotranslationally selects proteins with RER signal sequences by binding to the nascent polypeptide when the signal sequence emerges from the large ribosomal subunit (Walter and Blobel, 1981*b*; Walter and Blobel, 1982; Walter et al., 1981). The signal sequence recognition activity of SRP has been localized to the 54-kD subunit (SRP54) by photo–cross-linking experiments (Krieg et al., 1986; Kurzchalia et al., 1986). The affinity of the SRP-ribosome complex for the membrane-bound SRP receptor (or docking protein) results in the selective delivery of the ribosome to the membrane surface (Gilmore et al., 1982*b*; Meyer et al., 1982*a*; Walter and Blobel, 1981*a*). SRP receptor displaces SRP from the signal sequence in a GTP-dependent reaction that allows the subsequent membrane insertion of the nascent polypeptide (Connolly and Gilmore, 1989; Gilmore and Blobel, 1983). Although translocation of the polypeptide across the membrane bilayer is a poorly understood process, the preponderance of current experimental evidence suggests that nascent polypeptides undergoing transport are in contact with integral membrane proteins that comprise a RER translocation site. By experimentally blocking complete transport of the nascent protein into the RER lumen, translocation intermediates have been assembled and characterized (Connolly et al., 1989; Gilmore and Blobel, 1985; Görlich et al., 1990; Krieg et al., 1989; Wiedmann et al., 1987*b*). Translocation intermediates are disrupted by aqueous perturbants such as alkaline pH or urea (Gilmore and Blobel, 1985), but are surprisingly stable to solubilization with nonionic detergents (Connolly et al., 1989). Photo–cross-linking experiments have identified a heterodimeric integral membrane glycoprotein named the signal sequence receptor (SSR) (Görlich et al., 1990; Wiedmann et al., 1987*b*) or mp39 (Krieg et al., 1989) that is adjacent to partially translocated nascent polypeptides. Protein sequences deduced from cDNA clones have revealed that the α and β subunits of SSR are polypeptides of 286 and 166 amino acids, respectively (Görlich et al., 1990; Prehn et al., 1990). Each protein subunit contains two consensus sites for asparagine linked glycosylation and a single putative membrane spanning segment (Görlich et al., 1990; Prehn et al., 1990). Another integral membrane protein of ~43 kD has been identified by crosslinking to a synthetic consensus signal sequence containing a photoactivatable probe (Robinson et al., 1987).

Could additional integral membrane proteins be required for nascent polypeptide transport across the RER? Gene fusion experiments have demonstrated that the RER translocation site allows the nonsequence-specific transport of virtu-

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1. *Abbreviations used in this paper*: NEM, *N*-ethylmaleimide; RER, rough endoplasmic reticulum; SRP, signal recognition particle.

ally any polypeptide that is preceded by an RER signal sequence (Lingappa et al., 1984). Conversely, hydrophobic protein segments of sufficient length are recognized as stop–transfer sequences, and are transferred into direct contact with the lipid bilayer (Adams and Rose, 1985). The bifunctional signal–anchor sequence at the amino terminus of type II integral membrane proteins functions both as a signal sequence and is integrated into the membrane with a strictly defined polarity that has been proposed to be controlled by the charge of the flanking protein segments (Hartmann et al., 1989a). These three diverse signal-related functions imply that several integral membrane proteins may be required during the transport and integration stages of the translocation reaction. Indeed, mutations have been isolated in three different yeast genes (sec61, sec62, and sec63 (ptl-1)) that impart a translocation defective phenotype at nonpermissive temperatures (Deshaies and Schekman, 1987; Rothblatt et al., 1989; Toyn et al., 1988). Protein sequence analysis indicates that at least two of these gene products (SEC62 and SEC63) are membrane proteins, based upon the presence of potential membrane spanning segments (Deshaies and Schekman, 1989; Sadler et al., 1989). Biochemical evidence for additional membrane bound translocation components has been provided by treatments that selectively inhibit translocation. N-ethylmaleimide (NEM) alkylation of microsomal membranes can block translocation at two distinct steps (Hortsch et al., 1986; Nicchitta and Blobel, 1989), the first of which corresponds to inactivation of the SRP receptor (Gilmore et al., 1982a; Meyer and Dobberstein, 1980a). The second NEM-sensitive protein functions after membrane insertion of the signal sequence (Nicchitta and Blobel, 1989). Neither the α nor β subunit of SSR contains a cysteine residue; consequently, SSR is not likely to correspond to this second NEM-sensitive site (Görlich et al., 1990; Prehn et al., 1990).

Here, we have attempted to identify additional proteins that are adjacent to partially translocated nascent polypeptides using the homobifunctional amine-reactive cross-linker disuccinimidyl suberate (DSS). Cross-linking of a [35S]methionine labeled nascent polypeptide to a RER protein should yield a new radiolabeled product that can be resolved by gel electrophoresis. Using the cross-linker DSS, we detected a novel 45-kD product that is comprised of the nascent polypeptide cross-linked to an integral membrane protein of ~34 kD. Formation of the 45-kD cross-linked product required the assembly of a functional translocation intermediate. The cross-linked protein could be distinguished from αSSR and βSSR, based upon a lack of asparagine-linked oligosaccharide, and upon a greater resistance to externally added proteinase K.

Materials and Methods

Preparation of Microsomal Membranes, SRP and K-RM

SRP and salt-extracted microsomal membranes (K-RM) were prepared from canine pancreas microsomal membranes as described (Walter et al., 1981). K-RM were subjected to limited protease digestion (5 μg/ml of trypsin for 30 min at 0°C [Gilmore et al., 1982a]) to prepare membranes from which the cytoplasmic domain of the SRP receptor α-subunit (SRα) was removed (T5-K-RM). A protease-derived supernatant fraction containing the 52-kD cytoplasmic domain of SRα was prepared and characterized as described previously (T0.3Sup [Gilmore et al., 1982a]). NEM-K-RM and NEM-T5-K-RM were prepared by incubating K-RM and T5-K-RM respectively with 1 or 3 mM NEM for 30 min at 25°C as described (Gilmore et al., 1982a; Nicchitta and Blobel, 1989). A 150-mM NEM stock solution was prepared by dissolving 18.8 mg of NEM in 50 μl ethanol before adjustment to a final volume of 1 ml with water. Mock alkylated NEM-T5-K-RM were prepared by omitting NEM from the ethanol/water mixture used for the alkylation reaction.

Cell-free Transcription and Translation

The plasmid pG3G containing a cDNA insert for the G protein of vesicular stomatitis virus (VSV) was constructed by insertion of an EcoRI fragment from the plasmid pSVGL (Rose and Bergmann, 1982) into the EcoRI site of pGEM3. The plasmid pG3G was linearized within the protein coding region with AvaII or MboII before transcription with T7 RNA polymerase to obtain transcripts that encode 90 and 200 residues, respectively, of the VSV G protein. The plasmid pGEMBP1 (Connolly and Gilmore, 1986) containing a cDNA insert for bovine preprolactin downstream from the T7 RNA polymerase promoter was linearized within the protein coding region with PvuII, MboII, or RsaI before transcription with T7 RNA polymerase to obtain transcripts which respectively encode 86, 100, and 131 residues of preprolactin. RNA was transcribed with T7 RNA polymerase and the transcripts were purified as described previously (Connolly and Gilmore, 1986).

A 100-μl cell-free translation system contained 30 μl of staphylococcal nuclease digested wheat germ S23 (Erickson and Blobel, 1983), 100 μCi of [35S]methionine (New England Nuclear, Boston, MA), 1 U/μl of placental RNase inhibitor (RNasin; Promega Biotec, Madison, WI) and a mixture of protease inhibitors compatible with in vitro translation (Walter et al., 1981). Cell-free translations were adjusted to 100 mM KOAc, 2.5 mM Mg(OAc)2 and supplemented with 0.002% Nikkol (octaethyleneglycol-mono-N-dodecyl ether; Nikko Chemical Co., Ltd., Tokyo, Japan) to stabilize SRP activity (Walter et al., 1981). Wheat germ translations were supplemented with 48 nM SRP and K-RM (2 eq/25 μl) as noted in individual figure legends. The mRNA transcripts were translated for 45 min at 25°C at a final concentration of 300 ng/25 μl translation reaction. The cell free translation products of the truncated mRNAs are referred to with a nomenclature where pPL-86, pPL-100 and pPL-131 correspond to the amino-terminal 86, 100, and 131 residues of preprolactin, respectively, and pG-90 and pG-200 correspond to the amino-terminal 90 and 200 residues of the VSV G protein.

Cross-linking with Disuccinimidyl Suberate

In vitro-translated nascent polypeptides were cross-linked to adjacent proteins with disuccinimidyl suberate (DSS). An appropriate volume of a freshly prepared stock solution of 50 mM DSS (Pierce Chemical Co., Rockford, IL) in DMSO was added to in vitro translocation assays to obtain a final concentration of 1 mM DSS. The DMSO concentration in the cross-linking reaction did not exceed 2% of the final sample volume. After a 30 min incubation at 25°C, the cross-linking reaction was quenched by a 15-min incubation at 0°C after the addition of one-tenth volume of 1 M glycine in 0.1 M NaHCO3, pH 8.7. Where noted, cross-linking was carried out at 0°C for 1 h after a 10-fold dilution of the translation reaction into a high salt buffer, resulting in a final buffer concentration of 50 mM Tris-Cl pH 7.5, 500 mM KCl, 5 mM MgCl2, at a final concentration of 0.2 mM DSS.

Sucrose Gradient Fractionation of Cross-linked Translation Products

Translation products from cross-linking experiments were separated into membrane-bound pellet (P) fractions and supernatant (S) fractions by centrifugation at 4°C in a Beckman airfuge using an A-100/30 rotor (Beckman Instruments, Inc., Fullerton, CA). The cross-linked translation samples were layered over sucrose cushions before centrifugation. The physiological salt-sucrose cushion was 100 μl of 0.5 M sucrose, 140 mM KOAc, 3 mM Mg(OAc)2, 50 mM triethanolamine-acetate pH 7.5 (TEA), 1 mM DTT. Centrifugation was for 4 min at 20 psi. Samples for EDTA-sucrose cushions were incubated for 10 min at 0°C after adjustment to 10 mM EDTA. The EDTA-sucrose cushion was 50 μl of 0.25 M sucrose, 50 mM TEA, 150 mM KOAc, 25 mM EDTA, 1 mM DTT. Centrifugation was for 5 min at 20 psi. Samples for alkaline-sucrose cushions were incubated at 0°C for 10 min

after the addition of an equal volume of 0.2 M Na₂CO₃, pH 11.5. The alkaline-sucrose cushion was 50 μl of 0.2 M sucrose, 0.1 M Na₂CO₃, 100 mM KOAc, 2.5 mM Mg(OAc)₂. Centrifugation was for 8 min at 30 psi. After centrifugation, supernatant fractions were concentrated by precipitation with TCA. TCA-precipitated supernatant fractions and the membrane pellet fractions were incubated at 50°C for 30 min in 10 μl of 0.5 M Tris base, 6.25% SDS before electrophoresis. The alkaline pH of the buffer hydrolyzes the peptidyl-tRNA linkage. Proteins were resolved on either 12–20% or 10–15% polyacrylamide gradient gels in SDS and visualized by fluorography of the diphenyloxazole-impregnated gel.

Before analysis on linear sucrose gradients, microsomal membranes were recovered from a 150 μl DSS-treated translocation assay by centrifugation through a physiological salt-sucrose cushion. The membrane pellets were resuspended in 150 μl of 50 mM Tris-Cl pH 7.5, 500 mM KCl, 5 mM MgCl₂, 1% Nikkol by incubating for 20 min at 0°C. The cross-linked products were applied to linear 10–30% sucrose density gradient containing 50 mM TEA, 500 mM KOAc, 5 mM Mg(OAc)₂, 0.1% Nikkol, 1 mM DTT. After centrifugation for 4 h at 40,000 rpm in a Beckman SW 40 rotor (Beckman Instruments, Inc.), the gradients were resolved into 15 750-μl fractions using an Isco gradient fractionation system (Lincoln, NE) equipped with a UV-absorbance detector.

Con A Binding and Endoglycosidase H Digestion

Microsomal membranes from cross-linking reactions were recovered by centrifugation through either a physiological salt-sucrose cushion or an alkali cushion before incubation with Con A Sepharose or digestion with endoglycosidase H. Specifically, the membrane pellet was solubilized in 40 μl of 1% SDS, 30 mM DTT, 100 mM Tris base by successive incubation for 30 min at 55°C followed by 2 min at 100°C. A minor modification of the method of Krieg et al. was used for Con A binding experiments (Krieg et al., 1989). The solubilized membrane proteins were diluted ninefold with either 360 μl of Con A binding buffer (150 mM KCl, 70 mM Tris-Cl, 5 mM CaCl₂, 1 mM MgCl₂, 1.2% Triton X-100) or 360 μl Con A binding buffer containing 0.5 M α-methylmannoside. To remove proteins that might nonspecifically bind to Con A Sepharose, the samples were precleared by incubation with 20 μl Sepharose 4B beads. The precleared supernatant was incubated overnight, with agitation, at 4°C after the addition of 20 μl of Con A Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ). The supernatants were removed and precipitated by the addition of one-fifth volume of 100% TCA, and the TCA pellets were washed once with 100 μl acetone/HCl (19:1) to remove Triton X-100. Nonspecifically bound proteins were removed from Con A Sepharose by four washes with 1 ml of Con A binding buffer. The TCA precipitated Con A supernatants and the Con A Sepharose bound glycoproteins were resuspended for PAGE in SDS by incubation for 30 min at 55°C with 20 μl of 0.5 M Tris base, 6.25% SDS.

For endoglycosidase H digestion, resuspended K-RM pellets (40 μl) were diluted with 10 vol of endoglycosidase H digestion buffer (50 mM NaOAc, pH 5.8, 57 mM PMSF). The samples were incubated overnight in the presence or absence of 30 mU endoglycosidase H (kindly provided by Dr. Robert Trimble, New York State Department of Health, Albany, NY) at 37°C. The proteins were then precipitated by the addition of one-fifth volume of 100% TCA and prepared for PAGE in SDS.

Protease Digestion of Translation Products

Protease inhibitors were deleted from translations that were used for protease digestion experiments. Prior to digestion with proteinase K, a 100-μl aliquot from a cross-linking reaction containing membranes was applied to a 1.0 ml Sepharose CL-2B column equilibrated in protease digestion buffer (50 mM TEA, 150 mM KOAc, 2.5 mM Mg(OAc)₂). Microsomal membranes eluted in the void volume fraction. Freshly prepared stock solutions of proteinase K in protease digestion buffer were added to aliquots of the membrane fraction to obtain final protease concentrations ranging between 0.005 and 0.5 mg/ml. After 1 h of digestion on ice, the proteinase K was inactivated by adjustment of the sample to 2 mM PMSF and the protease digestion products were prepared for PAGE in SDS as described (Connolly and Gilmore, 1986). For trypsin digestion, cross-linked translation products were adjusted to a total volume of 50 μl and a final buffer concentration of 50 mM TEA, 150 mM KOAc, 2.5 mM Mg(OAc)₂ by the addition of an appropriate stock solution. Freshly prepared stock solutions of trypsin in protease digestion buffer were added to obtain final protease concentrations ranging between 0.01 and 0.5 mg/ml. After digestion for 1 h on ice the samples were adjusted to 2 mM PMSF.
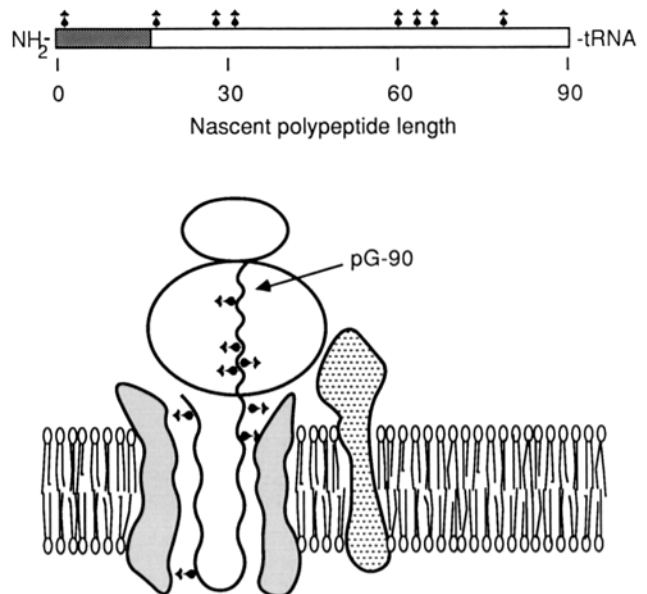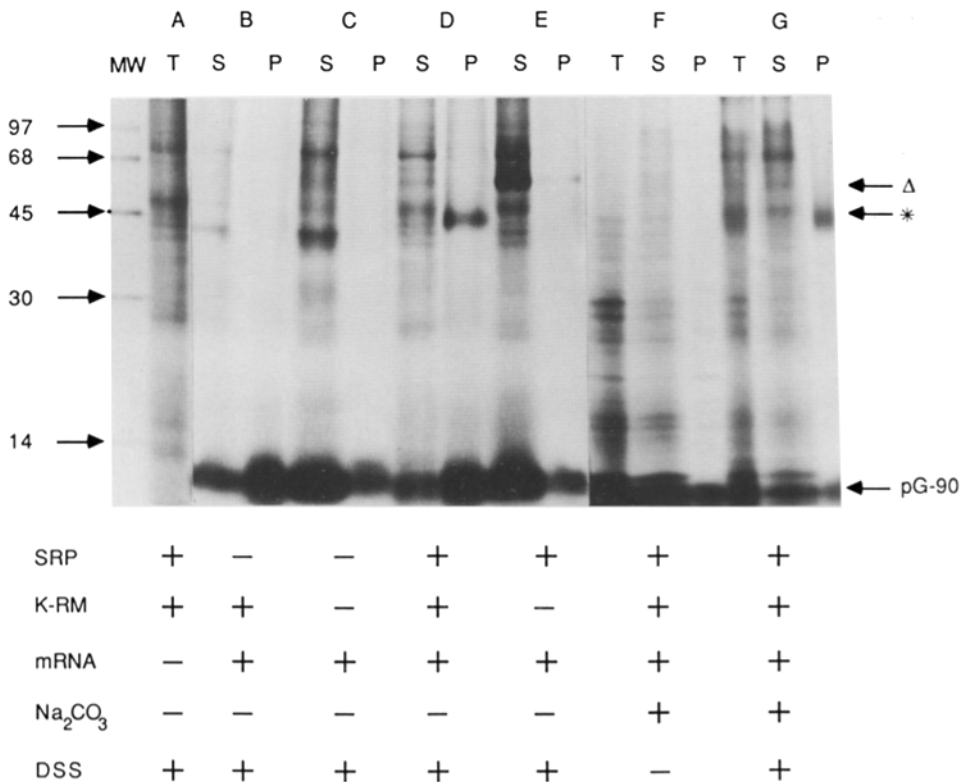

Figure 1. The location of lysyl residues in the pG-90 precursor. Lysines (residues 2, 17, 27, 31, 60, 63, 66, and 79) in the pG-90 nascent polypeptide are indicated (→) in a linear diagram and in a conceptualized diagram of a translocation intermediate. Proteins depicted in the membrane represent potential targets for cross-linking to pG-90. The 16- residue amino-terminal signal sequence of pG-90 is designated by the stippled region in the linear diagram. Protection from proteolysis of at least 30 residues of a nascent polypeptide by the ribosome is in good agreement with previous estimates (Bernabeu and Lake, 1982; Blobel and Sabatini, 1970). Lysyl residues 2, 17, 27, and 31 have emerged from the ribosome and could potentially be crosslinked to adjacent membrane proteins with the homobifunctional cross-linker DSS. Lysyl residue 31 is depicted near the cytoplasmic face of the membrane based upon the partial accessibility of the pG-90 translocation intermediate to digestion with trypsin.

Results

Chemical Cross-linking of Nascent Polypeptides to Translocation Components

We used the homobifunctional chemical cross-linking reagent disuccinimidyl suberate (DSS) to identify proteins that are in close proximity to nascent polypeptides undergoing translocation across the ER. A truncated mRNA encoding the first 90 amino acids of the G protein of vesicular stomatitis virus (VSV) was translated in the wheat germ cell-free system to generate a [³⁵S]methionine labeled nascent polypeptide that is bound to the ribosome as a peptidyl-tRNA (Fig. 1). Previous studies have demonstrated that the amino terminal signal sequence of the pG-90 nascent polypeptide is recognized by SRP (Connolly and Gilmore, 1989), and that translation of the truncated mRNA in the presence of SRP and K-RM leads to the assembly of a membrane-bound translocation intermediate (Connolly et al., 1989). Proteolytic digestion of ribosomes bearing pG-90 indicate that lysines 60, 63, 66, and 79 are inaccessible to trypsin due to ribosomal shielding (data not shown). Therefore, of the eight lysyl residues in pG-90, four could potentially be cross-linked to adjacent endoplasmic reticulum proteins with DSS

Figure 2. Cross-linking of the pG-90 translocation intermediate to a membrane protein. A wheat germ translation system was programmed with either no mRNA (*A*) or the truncated mRNA encoding pG-90 (*B–G*). Individual 25-μl translations contained: SRP and K-RM (*A, D, F,* and *G*), K-RM (*B*), no additions (*C*), or SRP (*E*). After 45 min of translation, all samples except *F* were adjusted to 1 mM disuccinimidyl suberate (*DSS*) and incubation was continued for 30 min at 25°C. Residual DSS was quenched by reaction with glycine (see Materials and Methods). Several samples (*B–G*) were separated into supernatant (*S*) or pellet (*P*) fractions by centrifugation in the airfuge (see Materials and Methods) after treatment with either 10 mM EDTA (lanes *B–E*) or 0.1 M Na$_2$-CO$_3$ (*F* and *G*). Total products (*T*), supernatants (*S*) and pellets (*P*) were prepared for PAGE in SDS. The pG-90 nascent chain and radiolabeled cross-linked products were resolved on a 10–15% gradient polyacrylamide gel. A 64-kD cross-linked product ($\triangle$) was recovered in supernatant fractions and a 45-kD cross-linked product (*) was recovered in membrane pellet fractions. Molecular weight markers (*MW*) are: phosphorylase B (97 kD), BSA (68 kD), ovalbumin (45 kD), carbonic anhydrase (30 kD), and cytochrome *c* (14 kD).

(Fig. 1). These translocation intermediates were treated with disuccinimidyl suberate, and the cross-linking reactions were separated into a membrane pellet (P) and a supernatant (S) fraction by centrifugation in an airfuge after EDTA treatment (Fig. 2). Cross-linking of [^{35}S]methionine-labeled pG-90 to an unlabeled protein yields an additional radiolabeled product that can be resolved by gel electrophoresis. A 45-kD cross-linked product, designated by an asterisk, sedimented with the membrane vesicles when both K-RM and SRP were present during in vitro translation (Fig. 2 *D*). The soluble fraction contained several radiolabeled polypeptides (Fig. 2 *D*) that were also present in total products (T) from a DSS-treated translation that lacked mRNA but contained SRP and K-RM (Fig. 2 *A*) and in the supernatant fraction from a control translation that contained mRNA but did not receive DSS (Fig. 2 *F*). Several of these polypeptides are endogenous wheat germ translation products while others may arise by cross-linking of [^{35}S]methionine to proteins present in the wheat germ extract. The total products of the mRNA deficient translation did not contain the 45-kD cross-linked product (Fig. 2 *A*). By selectively omitting either SRP or K-RM from the in vitro translocation assay, we should be able to identify any cross-linked products that are unique to a pG-90 translocation intermediate. The 45-kD product was not present in either supernatant or pellet fractions when SRP (Fig. 2 *B*) or K-RM (Fig. 2 *E*) were omitted from the translation. Instead, a 64-kD product ($\triangle$) was recovered in the soluble fraction from cross-linking reactions lacking K-RM, but containing SRP (compare Fig. 2, *C* with *E*). Previous investigators have shown that nascent preprolactin poly-

peptides containing a photoactivatable amino acid analogue can be cross-linked to the 54-kD subunit of SRP (Krieg et al., 1986; Kurzchalia et al., 1986; Wiedmann et al., 1987*a*). Based upon these previous studies, and upon the conditions required to generate the 64-kD product, we can identify this product as SRP54 cross-linked to pG-90.

To further characterize the 45-kD product, a cross-linked sample containing the translocation intermediate was extracted with 0.1 M Na$_2$CO$_3$ at pH 11.5 to separate integral from peripheral and lumenal proteins of the RER membrane (Fujiki et al., 1982). After this treatment, the 45-kD cross-linked product sedimented with the membrane (Fig. 2 *G*), indicating that the protein to which pG-90 is cross-linked is an integral membrane protein. The 45-kD product was not observed in total products (T) or in the membrane pellet fraction (P) when a translocation intermediate was treated with DMSO (Fig. 2 *F*) in place of DSS dissolved in DMSO (Fig. 2, *D* or *G*). The cross-linked product typically migrated as a broad band centered around 45 kD. The mobility of the cross-linked product relative to the ovalbumin molecular weight marker was influenced by the acrylamide concentration in the electrophoresis gel, with the cross-linked product migrating more rapidly than ovalbumin in a 10–15% polyacrylamide gel and less rapidly than ovalbumin on a 12–20% polyacrylamide gel. Subtraction of the 11 kD contributed by pG-90 from an average molecular mass of 45 kD for the cross-linked product suggests that the integral membrane protein (imp) has an actual molecular weight of $\sim$34 kD. Until a more accurate molecular weight determination has been made we shall refer to this protein as imp34. Because
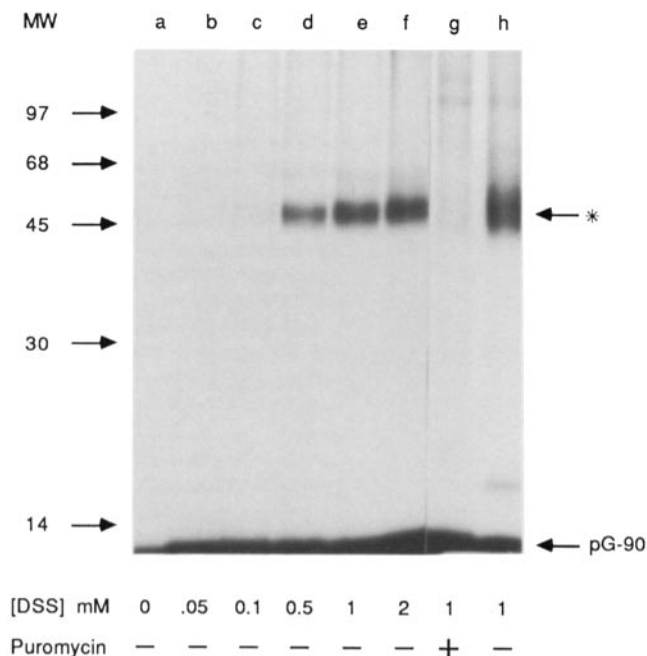
Figure 3. Concentration dependence of cross-linked product formation. The truncated mRNA encoding pG-90 was translated in a wheat germ system supplemented with K-RM and SRP. After 20 min of translation at 25°C, 25-μl aliquots were adjusted to (*a*) 0 mM, (*b*) 0.05 mM, (*c*) 0.1 mM, (*d*) 0.5 mM, (*e*) 1 mM, or (*f*) 2 mM DSS and incubated for 5 min at 25°C before quenching with glycine. For lanes *g* and *h*, two 50-μl translations were adjusted to 2 mM cycloheximide after 30 min of translation at 25°C. The two 50-μl translations were then incubated for 7 min at 25°C in the presence (*g*) or absence (*h*) of 2 mM puromycin followed by a 7-min incubation at 25°C after adjustment to 0.5 M KOAc. The samples in lanes *g* and *h* were cross-linked by incubation in the presence of 1 mM DSS for 20 min at 25°C. Cross-linking of all samples was terminated by the addition of one-tenth volume of 1 M glycine, and membrane pellets were collected by centrifugation in the airfuge through a physiological salt-sucrose cushion. The membrane pellets were prepared for SDS-PAGE and resolved on a 12–20% gradient gel.

the 45-kD product migrates as a broad band, we believe it is possible that more than one protein with a molecular mass of ~34 kD has been cross-linked to pG-90 by DSS.

Cross-linking of pG-90 to imp34 was surprisingly efficient. Conversion of 5–10% of the membrane bound pG-90 polypeptide to the 45-kD product was typical. Greater conversion efficiencies are presumably prevented by the formation of cross-linked products that contain additional protein molecules. Maximal cross-linking of pG-90 to imp34 occurred during 5 min of reaction at 37°C, or during 30 min of reaction at 0°C (data not shown). The DSS concentration used for cross-linking of a 10-fold diluted translation was 0.2 mM, whereas 1–2 mM DSS gave optimal cross-linking for a non-diluted translation (data not shown). The amount of DSS that gave maximal cross-linked product was found to be directly proportional to the quantity of membrane vesicles present in the sample rather than to the aqueous volume of the solution. This observation suggests that DSS partitions into the membranes before cross-linking, rather than reacting as a soluble reagent. Additional radiolabeled products migrating between pG-90 and the 45-kD product were not observed when both
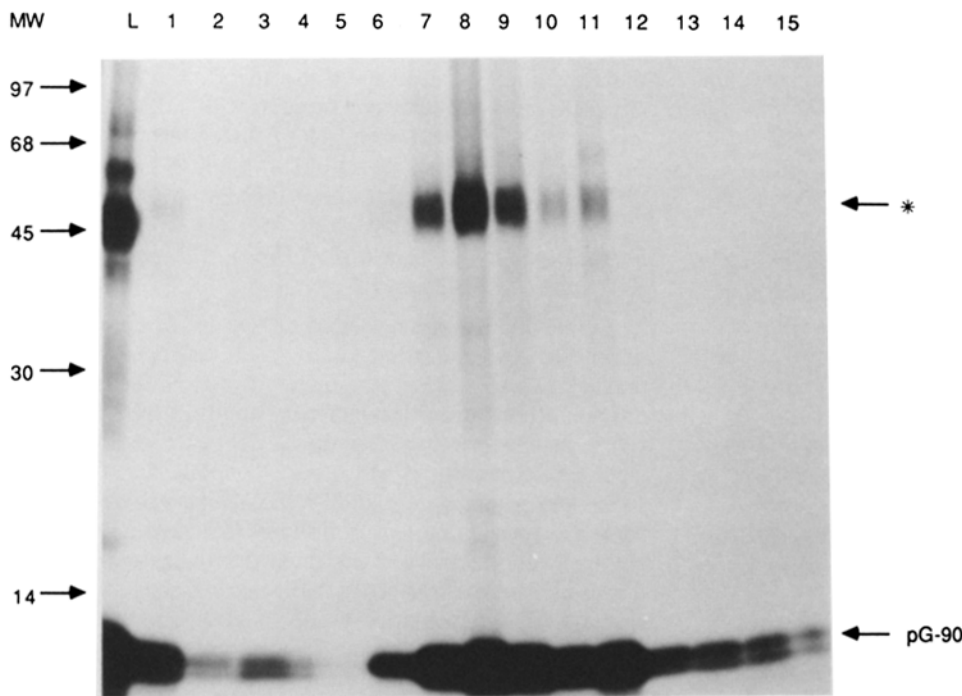
the reaction time and the DSS concentration were reduced (Fig. 3, lanes *a–f*). Cross-linked products of intermediate mobility would be expected if the 45-kD product consisted of pG-90 cross-linked to two proteins with an aggregate molecular mass of 34 kD. The lack of detectable cross-linking intermediates suggests that 45-kD product is a bimolecular rather than a trimolecular cross-linked complex.

Formation of the Cross-linked Product Requires a Ribosome-bound Peptidyl-tRNA

The cross-linking strategy used here is based upon the assembly of a translocation intermediate that persists due to the lack of a translation termination codon on the truncated mRNA. Premature release of the peptidyl-tRNA from the ribosome allows transport of the nascent polypeptide across the RER (Connolly et al., 1989; Connolly and Gilmore, 1986) and potential contact of radiolabeled pG-90 with luminally exposed domains of proteins that may have no role in translocation. Hence, it was important to demonstrate that the 45-kD cross-linked product arises from ribosome-bound peptidyl-tRNA. Formation of the 45-kD cross-linked product was abolished when the nascent polypeptide was released from the ribosome by treatment of an assembled translocation intermediate with puromycin and high salt before the addition of DSS (Fig. 3, lanes *g* and *h*). Membranes from a cross-linking reaction that was not treated with puromycin were recovered by centrifugation in the airfuge and the cross-linked proteins were solubilized by the addition of a nonionic detergent-high salt solution (1% Nikkol in 50 mM Tris-Cl pH 7.5, 500 mM KCl, 5 mM MgCl$_2$). The sedimentation position of the 45-kD cross-linked product relative to ribosome-bound pG-90 was determined by sucrose density gradient centrifugation. Resolution of the labeled proteins by PAGE in SDS demonstrated that the 45-kD product cosedimented with the ribosome bound pG-90 (Fig. 4, lane *8*). A relatively low amount of prematurely released pG-90 was recovered in fraction 1.

IMP34 Is Distinct from the Signal Sequence Receptor

Partially translocated nascent preprolactin polypeptides containing photoactivatable amino acid analogues have been cross-linked to an integral membrane glycoprotein that has been named the signal sequence receptor (SSR [Wiedmann et al., 1987b]) or mp39 (Krieg et al., 1989). Due to the uncertainty in the molecular weight of imp34, it was conceivable that the 45-kD product consisted of pG-90 cross-linked to αSSR or mp39. Further characterization of the 45-kD product was required to address this possibility. Membranes containing the cross-linked pG-90 translocation intermediates were recovered by centrifugation through a sucrose cushion and transferred into a SDS-Triton X-100 mixed micelle detergent solution. The detergent-solubilized proteins were then incubated with Con A Sepharose to determine whether any of the cross-linked products contained the high-mannose oligosaccharide present on RER glycoproteins. Surprisingly, only a small amount of the 45-kD product bound to Con A Sepharose (Fig. 5, lane *b*), while the bulk of the cross-linked protein was recovered in the unbound fraction (Fig. 5, lane *a*). The marginal binding observed here is probably not indicative of a second cross-linked glycoprotein, because a similar level of nonspecific

Figure 4. Cosedimentation of the cross-linked product with the 80S ribosome on sucrose gradients. The truncated pG-90 mRNA was translated in the presence of SRP and K-RM and cross-linked with DSS as in Fig. 2. After quenching with glycine, the membranes were recovered by centrifugation through a physiological salt-sucrose cushion. The membrane pellet was resuspended in 150 μl of 50 mM Tris-Cl pH 7.5, 500 mM KCl, 5 mM MgCl$_2$, 1% Nikkol, and applied to a 12-ml 10–30% sucrose density gradient containing 50 mM TEA, 500 mM KOAc, 5 mM Mg-(OAc)$_2$, 0.1% Nikkol, 1 mM DTT. After centrifugation for 4 h at 40,000 rpm in a Beckman SW 40 rotor (Beckman Instruments, Inc.), the gradients were resolved into fifteen fractions of 750 μl each. Scintillation counting of an aliquot from each of 15 gradient fractions revealed a peak of radioactivity in fraction 8 that co-sedimented with the 80S ribosome peak as detected by absorbance at 254 nm. Gradient fractions were concentrated by precipitation with TCA, washed with acetone: HCl, and pG-90 and the 45-kD cross-linked product (*) were resolved by PAGE in SDS on a 12-20% gradient gel. One-sixth of the load fraction (L) and fractions 1-15 from the sucrose gradient are shown.

binding of pG-90 to Con A Sepharose was observed (Fig. 5, lane *b*). A relatively minor 72-kD polypeptide (♦) bound to Con A in the absence (Fig. 5, lane *b*) but not in the presence (Fig. 5, lane *d*) of α-methylmannoside. The identification of this minor product has not been pursued. To obtain a more satisfactory control, mRNA encoding the first 200 amino acids of the VSV G protein was translated in the presence of K-RM and SRP and subjected to DSS treatment. Asparagine 179 of the pG-200 precursor was glycosylated on the subpopulation of pG-200 polypeptides that were prematurely released from the ribosome, to yield the glycosylated form designated as g-G-200 (Connolly et al., 1989). The glycosylated g-G-200 selectively bound to Con A Sepharose (Fig. 5, lanes *e* and *f*) unless α-methylmannoside was included in the binding buffer (Fig. 5, lanes *g* and *h*). Based upon this control, we conclude that the reagents used for cross-linking did not interfere with the subsequent quantitative and specific binding of glycoproteins to Con A Sepharose.

To confirm the results of the Con A experiment using a different procedure, the 45-kD cross-linked product derived from pG-90 and imp34 was subjected to digestion with endoglycosidase H. As a control, ^{14}C-labeled molecular weight standards were digested in a parallel reaction. The glycoprotein ovalbumin showed an increase in mobility after incubation with endoglycosidase H consistent with removal of an asparagine-linked oligosaccharide (Fig. 5, lanes *k* and *l*), whereas the 45-kD cross-linked product showed no detectable increase in mobility (Fig. 5, lanes *i* and *j*). Endoglycosidase H digestion of DSS-treated g-G-200 demonstrated that the cross-linking reagent does not interfere with a subsequent endoglycosidase digestion (data not shown). Resident RER

glycoproteins contain high mannose oligosaccharide, hence, they bind to Con A and are susceptible to digestion with endoglycosidase H. By these two criteria, the 45-kD cross-linked product appears not to contain high mannose oligosaccharide, a feature that distinguishes imp34 from both αSSR (mp39) (Krieg et al., 1989; Wiedmann et al., 1987b) and βSSR (Görlich et al., 1990).

Having shown that nascent pG-90 can be cross-linked to an integral membrane protein, we asked whether an analogous cross-linked product would be obtained using nascent polypeptides derived from the secretory protein preprolactin. A series of truncated mRNAs encoding the first 86, 100, and 131 amino acids of preprolactin (pPL-86, pPL-100, and pPL-131, respectively) were prepared by in vitro transcription of a preprolactin cDNA that had been linearized within the protein coding region. In vitro assembled translocation intermediates were cross-linked with DSS, and the cross-linked products that correspond to integral membrane proteins were resolved by PAGE in SDS (Fig. 6, lanes *a-c*). A membrane-bound cross-linked product of 46–50 kD (*) was produced for each of the nascent polypeptides (pPL-86, pPL-100, and pPL-131). The incremental increase in apparent molecular weight of the cross-linked product correlated well with the increasing size of the nascent polypeptides (Fig. 6, lanes *a-c*). Radiolabeled products from a DSS treated pPL-86 translation were collected by sedimentation through a physiological salt-sucrose cushion (Fig. 6, lane *d*) and immunoprecipitated with antibody to prolactin (Fig. 6, lane *e*). The unlabeled 50-kD IgG heavy chain in the immunoprecipitate lane is responsible for the distorted migration of the 46-kD cross-linked product. Other radiolabeled polypeptides
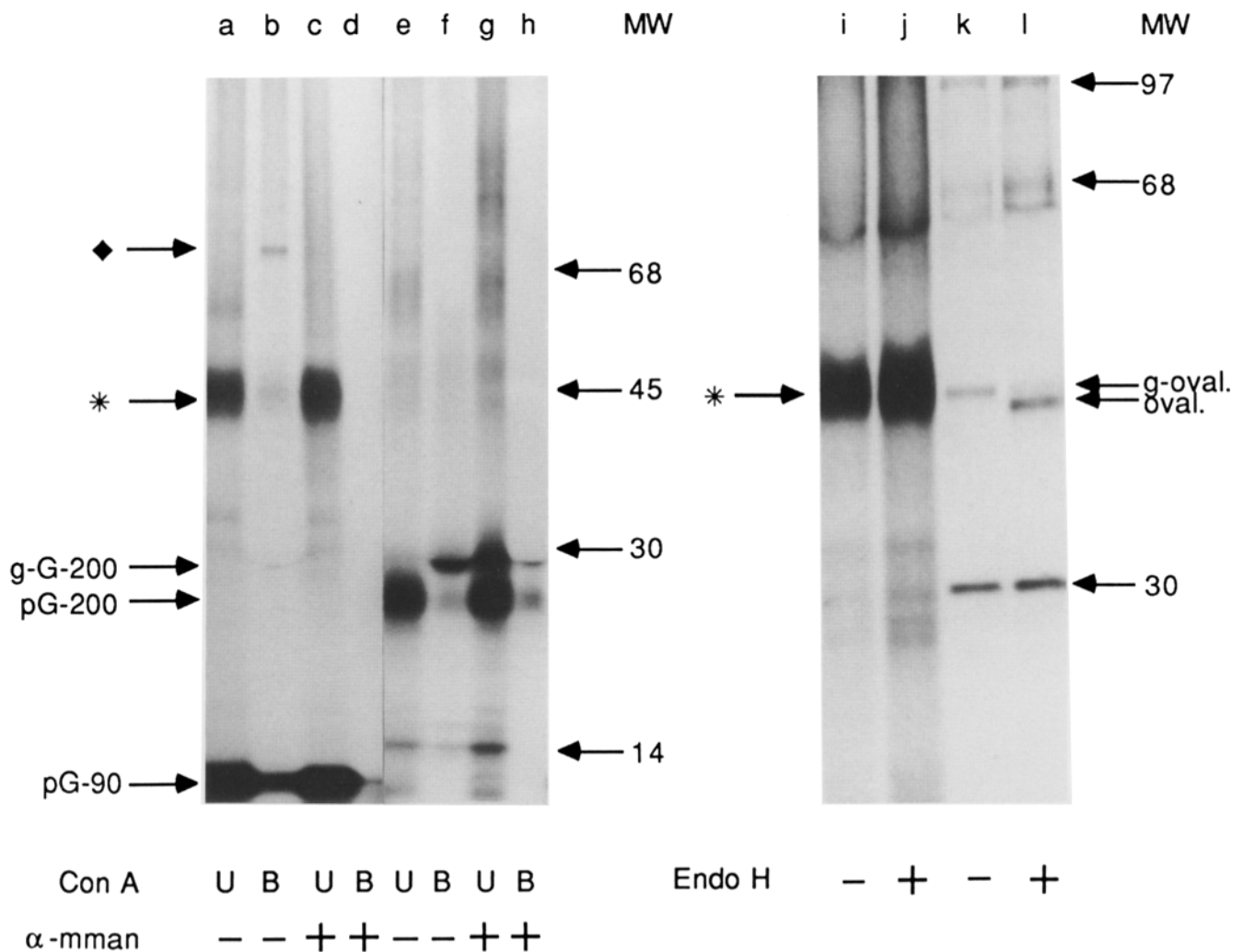
Figure 5. The 45-kD product involves a cross-link to a nonglycosylated membrane protein. Truncated mRNAs encoding pG-90 (lanes *a–d*, *i* and *j*) or pG-200 (*e–h*) were translated in a wheat germ system supplemented with SRP and K-RM. Cross-linking reactions were done as in Fig. 2 for lanes *a–h*. The translation used for lanes *i* and *j* was diluted 10-fold with high salt buffer (see Materials and Methods) before cross-linking for 1 h at 0°C with 0.2 mM DSS. The membranes were recovered after cross-linking by centrifugation through a low salt cushion (lanes *a–j*). The detergent solubilized membrane proteins were incubated with Con A Sepharose either in the absence or presence of 0.5 M α-methylmannoside (*α-mman*). Unbound (*U*) and bound (*B*) proteins were prepared for PAGE in SDS (lanes *a–h*). For endogylcosidase H digestion, the resuspended membrane pellet was diluted into endo H digestion buffer and incubated overnight at 37°C in the presence (lane *j*) or absence (lane *i*) or endoglycosidase H as described in Materials and Methods. A control endoglycosidase H digestion (lane *l*) of ^{14}C-labeled molecular weight standards (lane *k*) is shown for comparison. The migration position of pG-90, pG-200, and the glycosylated g-G-200 are designated by arrows. The 45-kD cross-linked product and a 72-kD glycosylated product are designated by an (*) and a (♦), respectively. The glycosylated (*g-oval.*) and de-glycosylated (*oval.*) forms of ovalbumin are indicated (lanes *k* and *l*).

that were immunoprecipitated with the antibody include pPL-86, prolactin (26 kD), and a 64-kD polypeptide that presumably corresponds to pPL-86 cross-linked to SRP54 (Fig. 6, lane *e*). An incomplete restriction digestion of the preprolactin cDNA with PvuII before transcription is responsible for the small quantity of prolactin obtained in this translation. Formation of the 45-kD cross-linked product containing pPL-86 was prevented by treatment of the translocation intermediate with puromycin before addition of DSS (data not shown). Cross-linked products from a DSS treated pPL-86 translation were alkali stripped, collected by centrifugation through an alkaline–sucrose cushion, and incubated with Con A Sepharose (Fig. 6, lanes *f–i*). The majority of the 45-kD product did not bind to Con A

Sepharose in the absence of α-methyl mannoside (Fig. 6, lane *g*). The rather limited binding of the cross-linked product to the lectin is probably not indicative of a second cross-linked glycoprotein, because a similar proportion of the un-cross-linked nonglycosylated pPL-86 also bound to Con A Sepharose (Fig. 6, lane *g*). The results described above are analogous to those obtained with pG-90; thus, it is likely that pPL-86 and pG-90 have been cross-linked to the same non-glycosylated protein.

Access of Proteases to the 45-kD Cross-linked Product

Proteases have proven to be useful probes for identifying RER proteins with cytoplasmically exposed domains. The
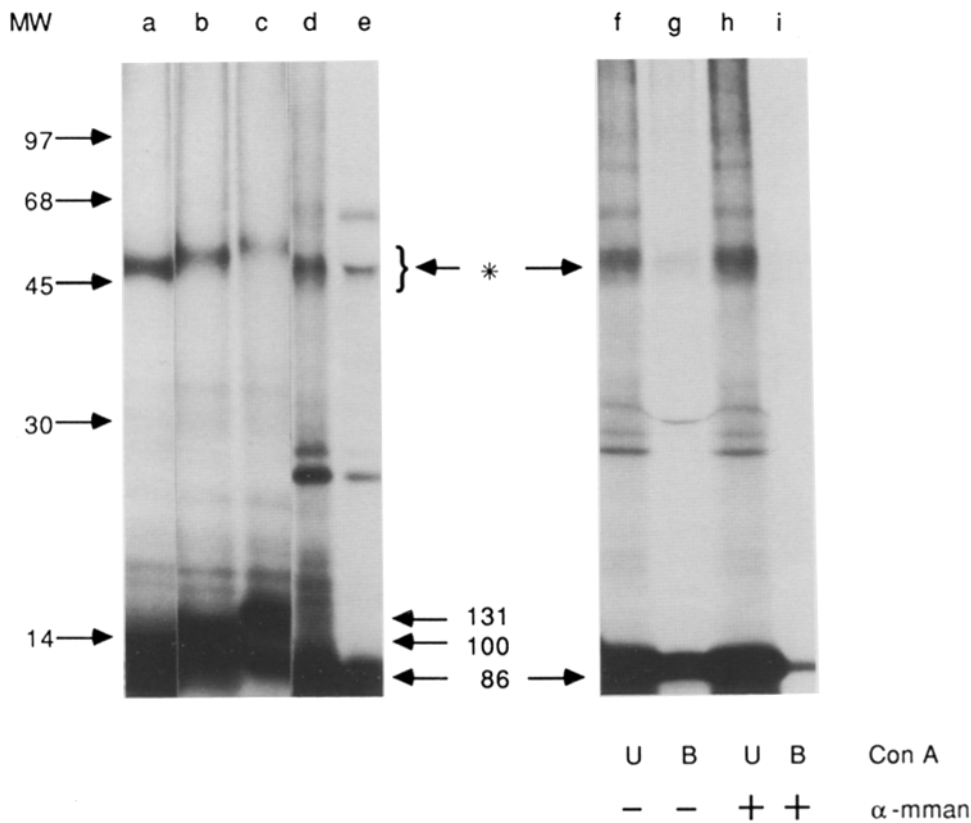
Figure 6. Cross-linking of preprolactin translocation intermediates to a nonglycosylated membrane protein. Truncated mRNAs encoding pPL-86 (lanes *a, d, e, f-i*),pPL-100 (lane *b*) or pPL-131 (lane *c*) were translated in the wheat germ system supplemented with SRP and K-RM. Cross-linking was done as in Fig. 2. After quenching residual DSS, samples *a-c* and *f-i* were adjusted to 0.1 M Na$_2$CO$_3$ and the membrane fraction was collected by centrifugation through an alkaline-sucrose cushion as in Fig. 2 *G*. Alkali pellets were prepared for electrophoresis and separated on a 12-20% gradient gel (lanes *a-c*) or incubated with Con A Sepharose either in the absence or presence of 0.5 M α-methyl mannoside (*α-mman*). Unbound (*U*) and bound (*B*) proteins were prepared for PAGE in SDS (lanes *f-i*). The cross-linking reaction from which samples *d* and *e* are derived was centrifuged through a physiological salt-sucrose cushion. Cross-linked products in the membrane pellet fraction were either analyzed by PAGE in SDS without further purification (lane *d*) or collected by immunoprecipitation with antisera against prolactin (lane *e*) as described previously (Connolly and Gilmore, 1986). The cross-linked product between imp34 and the different nascent preprolactin polypeptides is designated by the asterisk.

molecular weight of the cross-linked product between the α subunit of SSR (mp39) and pPL-86 or pPL-127 is reduced by ~4-7 kD by proteinase K digestion, suggesting that αSSR(mp39) has a cytoplasmically exposed domain of this size (Hartmann et al., 1989*b*; Krieg et al., 1989). The recent sequence determination of αSSR (mp39) indicates a cytoplasmic domain of ~7 kD (Prehn et al., 1990), which agrees well with the proteolysis results. Proteinase K digestion of the 45-kD cross-linked product should allow further comparison with αSSR (mp39), and indicate whether imp34 has a protein domain that is accessible to this protease. Microsomal membranes from a DSS-treated pG-90 translocation reaction were separated from the soluble wheat germ proteins by gel filtration chromatography using a Sepharose CL-2B column. No detectable alteration in the molecular mass of the 45-kD cross-linked product was observed when the proteinase K concentration was varied between 5 and 500 μg/ml (Fig. 7, lanes *b-e*). The 72-kD glycoprotein (♦) and several other radiolabeled polypeptides were more sensitive to proteolysis than the 45-kD product (Fig. 7, lanes *a-e*). All ^{35}S-labeled products were susceptible to proteinase K digestion at 37°C when the sample was dissolved in 1% SDS (Fig. 7, lane *f*). High concentrations of protease reduced the intensity of the 45-kD cross-linked product (Fig. 7, lane *e*) without producing a radiolabeled digestion product that migrated between the 45-kD cross-linked product and pG-90. The pG-90 nascent polypeptide contains methionine residues at positions 1, 61, and 74. Of the three methionine residues, at least one and probably two have not emerged from the protease

inaccessible cleft in the large ribosomal subunit (Bernabeu and Lake, 1982; Blobel and Sabatini, 1970). Proteolytic cleavage near the extreme amino terminus of pG-90 would have little effect on the migration of the cross-linked product, yet the intensity of the cross-linked band would decrease by one third due to loss of the NH$_2$-terminal methionine. Thus, proteolytic removal of the amino-terminal methionine may be responsible for the decrease in the intensity of the cross-linked product (Fig. 6, lane *e*). Previously, we reported that partially translocated pG-90 nascent polypeptides are extremely resistant to digestion by externally added proteinase K (Connolly et al., 1989). This observation was confirmed here, and has now been extended to include imp34: a protein that appears to be adjacent to a pG-90 translocation intermediate. Most importantly, resistance of the 45-kD product to proteinase K digestion provides further evidence that αSSR is not the protein to which pG-90 is cross-linked.

Digestion of a cross-linking reaction with a high concentration of trypsin eliminated the 45-kD product and produced several significantly less intense proteolytic fragments (Fig. 6, lane *i-l*; ● and ■). For this experiment, trypsin was added to the total cross-linked translation mixture, and the proteolytic digestion products that remained integrated in the membrane were subsequently isolated by centrifugation through an alkaline-sucrose cushion (Fig. 7, lanes *g-l*). Lysine residues are located at positions 2, 17, 27, 31, 60, 63, 66, and 79 of pG-90; arginine is located at residue 87. Digestion with a high concentration of trypsin (250 μg/ml) results
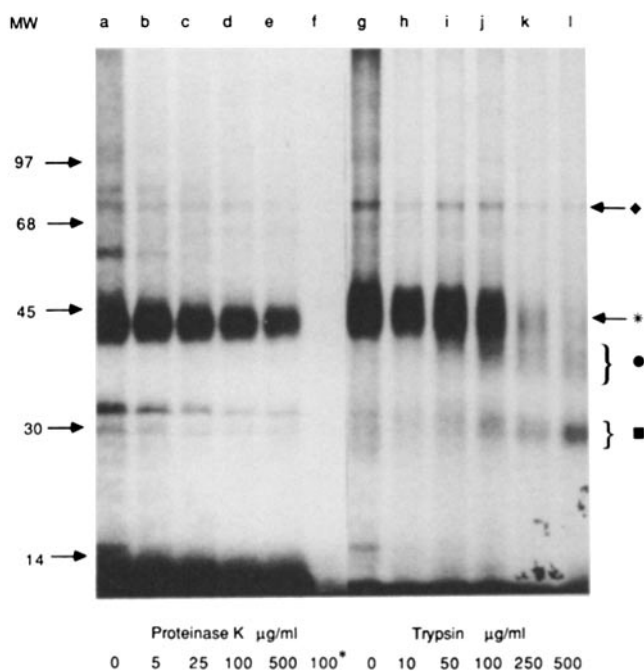
Figure 7. Protease digestion of the 45-kD cross-linked product. The truncated mRNA encoding pG-90 was translated in a wheat germ system supplemented with K-RM and SRP. Cross-linking was done with 1 mM DSS as in Fig. 2. Before proteinase K digestion, the DSS-treated membranes were separated from the translation extract by gel filtration chromatography (see Materials and Methods). Aliquots of the cross-linked membrane fraction were adjusted to final proteinase K concentrations ranging between 0 and 500 μg/ml, as indicated in the chart below the autoradiogram. The sample in lane *f* contained 1% SDS in addition to 100 μg/ml of proteinase K (*100**). One hour digestions on ice (lanes *a–e*) or 10 min at 37°C (lane *f*) were terminated by the addition of PMSF. Proteinase K-digested samples (lanes *a–f*) were resolved by PAGE in SDS using a 10–15% polyacrylamide gradient gel. Trypsin was added to 50-μl aliquots of a DSS treated translation sample to obtain final protease concentrations ranging between 0 and 500 μg/ml (lanes *g–l*). 1-h digestions on ice were terminated by the addition of PMSF. Integral membrane proteins were recovered in the pellet fraction from an alkaline/sucrose gradient and the radiolabeled polypeptides were resolved by PAGE in SDS (lanes *g–l*). The 45-kD cross-linked protein (*), several prominent digestion intermediates (● and ■), and a 72-kD integral membrane protein (♦) were visualized by fluorography of the PPO impregnated gel.
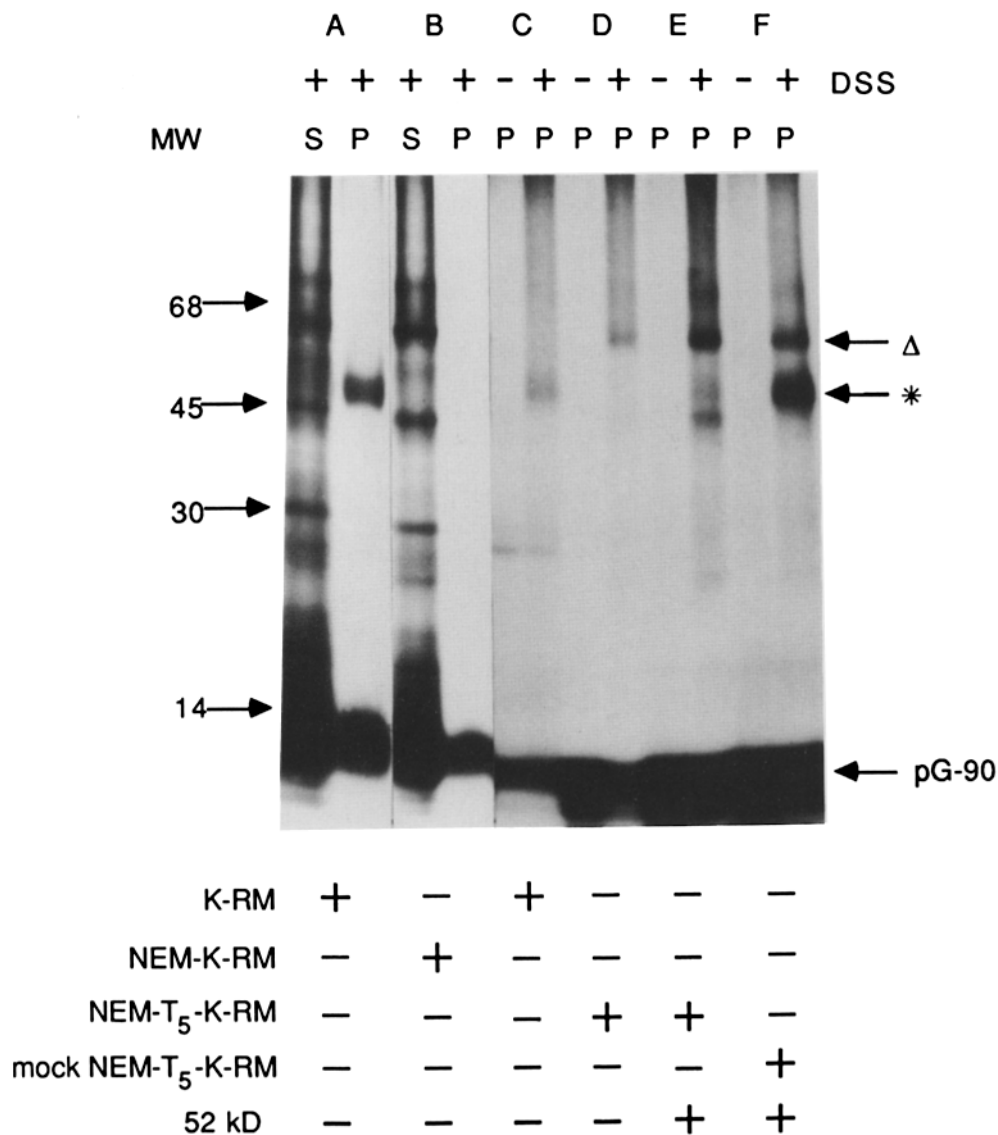
in cleavage of ∼50% of the pG-90 translocation intermediate at either lysine residue 27 or 31 (data not shown). Control trypsin digestions of the pG-90 polypeptide indicate that lysines 60, 63, and 66 are inaccessible due to ribosomal shielding (data not shown). Although cleavage at lysine 27 or 31 may be partially responsible for the reduction in label intensity, it cannot account for the entire 15-kD decrease in size of the radiolabeled product after trypsin digestion. Therefore, we conclude that imp34 probably has at least one trypsin sensitive site located on the cytoplasmic side of the membrane. The actual amount of the molecular weight decrease that can be respectively attributed to cleavage of pG-90 and imp34 cannot be determined from the available data.

Inhibition of Cross-link Formation by NEM

To further elucidate the point in the translocation process

when pG-90 is adjacent to imp34, we selectively blocked transport at two previously defined steps using the reagent NEM and determined whether a 45-kD cross-linked product could still be formed. In the first experiment, the truncated mRNA encoding pG-90 was translated in the presence of SRP and either K-RM (Fig. 8 *A*) or K-RM that had been alkylated with 1 mM NEM (NEM-K-RM, Fig. 8 *B*) to inactivate the SRP receptor (Gilmore et al., 1982*a*). After cross-linking, the samples were adjusted to pH 11.5 and fractionated on alkaline sucrose gradients. The 45-kD cross-linked product was recovered in the pellet fraction from the control translation (Fig. 8 *A*) but was absent from both the supernatant and pellet fraction from a translation containing NEM-K-RM (Fig. 8 *B*, pellet). The yield of the SRP54 cross-link (Δ) was enhanced when NEM-K-RM were present (Fig. 8 *B*, fraction S). The NEM-sensitive site in the SRP receptor has been localized to the SRP receptor α subunit (SRα) based upon the following evidence. Mild proteolysis of K-RM with trypsin or elastase can be used to sever the SRP receptor into two essential fragments: (*a*) a soluble 52-kD domain derived from SRα (Gilmore et al., 1982*b*; Meyer and Dobberstein, 1980*a*; Meyer et al., 1982*b*) and (*b*) a membrane-bound domain consisting of SRβ and the NH₂-terminal 16-kD fragment of SRα (Lauffer et al., 1985; Tajima et al., 1986). The 52-kD domain of SRα can restore translocation activity to trypsin digested K-RM (T-K-RM) that are devoid of intact SRα (Gilmore et al., 1982*a*; Meyer and Dobberstein, 1980*a*; Meyer and Dobberstein, 1980*b*). Although T-K-RM alkylated with 1 mM NEM can be functionally reconstituted with the 52-kD fragment, NEM alkylated 52-kD fragment cannot reconstitute T-K-RM (Meyer and Dobberstein, 1980*a*). Thus, the 52-kD fragment of SRα contains a NEM sensitive site.

A second NEM-sensitive translocation factor was detected when the 52-kD fragment of SRα was used to reconstitute trypsinized membranes which had been treated with higher concentrations of NEM (Hortsch et al., 1986; Nicchitta and Blobel, 1989). Recombining NEM-T₅-K-RM (treated with 3 mM NEM) with the SRα 52-kD fragment yielded a reconstituted membrane which was defective in translocation, yet contained a functional SRP receptor (Nicchitta and Blobel, 1989). The translocation defect in the recombined membrane occurred after the GTP-dependent insertion of the nascent polypeptide (Nicchitta and Blobel, 1989). We prepared T₅-K-RM that had been alkylated with 3 mM NEM (NEM-RT₅-K-RM), and confirmed the translocation defect reported by previous investigators (data not shown). We then asked whether pG-90 nascent polypeptides that were blocked at this point in the translocation process could be cross-linked to imp34. The truncated mRNA encoding pG-90 was translated in the presence of SRP and either intact K-RM (Fig. 8 *C*) or recombined membranes comprised of the SRα 52-kD fragment plus NEM-T₅-K-RM or mock-alkylated NEM-T₅-K-RM (Fig. 8, *D–F*). The translation reactions were divided into two aliquots, one of which was treated with DSS. The samples were separated into a membrane pellet fraction (Fig. 8, *C–F*) and a supernatant fraction (not shown) by centrifugation through a low salt sucrose cushion. The 45-kD cross-linked product was recovered in the membrane pellet fraction when K-RM were included (Fig. 8 *C*) or when mock-alkylated NEM-T₅-K-RM were recombined with the SRα 52-kD fragment (Fig. 8 *F*). The

A   B   C   D   E   F

\+   \+   \+   \+   \-   \+   \-   \+   \-   \+   \-   \+   DSS

MW   S   P   S   P   P   P   P   P   P   P   P   P

68 →

45 →   ← Δ
                                                   ← *

30 →

14 →

                                                   ← pG-90

K-RM   +   −   +   −   −   −

NEM-K-RM   −   +   −   −   −   −

NEM-T₅-K-RM   −   −   −   +   +   −

mock NEM-T₅-K-RM −   −   −   −   −   +

52 kD   −   −   −   −   +   +

Figure 8. NEM treatment of K-RM interferes with crosslink formation. The truncated pG-90 mRNA was translated in a wheat germ system containing SRP. Individual translations also contained: (*A* and *C*), K-RM; (*B*), NEM-K-RM; (*D*), NEM-T₅-K-RM; (*E*), NEM-T₅-K-RM plus T₀.₃-Sup; (*F*) mock alkylated NEM-T₅-K-RM plus T₀.₃-Sup. The T₀.₃-Sup contains the 52-kD cytoplasmic domain of SRα. All of samples *A* and *B*, and one of two aliquots from samples *C–F* were cross-linked with DSS as in Fig. 2. Two samples (*A* and *B*) were extracted at pH 11.5 with Na₂CO₃ and fractionated using alkaline–sucrose cushions into supernatants (*S*) and pellets (*P*). Samples *C–F* were centrifuged on low salt sucrose cushions to yield supernatant and pellet fractions. Pellets (*P*) are shown (lanes *C–F*). The 45-kD cross-linked product (*), pG-90, and pG-90 cross-linked to SRP 54 (Δ) were resolved by PAGE in SDS on a 12–20% polyacrylamide gradient gel. The translation with K-RM (lane *C*) received approximately one-fourth the amount of mRNA used in the translations shown in lanes *D–F.*

decreased signal observed for the K-RM control (Fig. 8 *C*) was due to a fourfold reduction in the quantity of mRNA transcript added to the translation. Little or no 45-kD cross-linked product was obtained when the translation contained NEM-T₅-K-RM alone (Fig. 8 *D*) or when NEM-T₅-K-RM were reconstituted with the SRα fragment (Fig. 8 *E*). The majority of the SRP54 cross-linked product (Δ) and a radiolabeled band that migrated slightly faster than the 45-kD cross-linked product were recovered in supernatant fractions (data not shown).

We sought to determine if the NEM alkylation of T₅-K-RM, rather than preventing assembly of a translocation complex, was chemically modifying the lysyl residue needed to form the cross-linked product (Smyth et al., 1960; Vallee and Riordan, 1969). To address this question, the pG-90 translocation intermediate was assembled by translation of the mRNA in the presence of SRP and K-RM. After translation, the membranes were separated from reducing agents and soluble wheat germ proteins by gel filtration chromatography. The K-RM containing the pG-90 translocation intermediate were then incubated with 3 mM NEM for 30 min

at 25°C, after which the residual NEM was quenched by the addition of 50 mM DTT. Subsequent cross-linking with DSS showed no decrease in the amount of the 45-kD product formed using either the posttranslationally alkylated sample or a mock-alkylated sample (data not shown). Therefore, NEM treatment is not interfering with the cross-linking reaction, per se, but rather with assembly of a transport intermediate that consists of imp34 adjacent to the nascent polypeptide.

Discussion

We have used a chemical cross-linking approach to detect an integral membrane protein, imp34, that is adjacent to nascent polypeptides undergoing translocation across the endoplasmic reticulum. The pG-90 nascent polypeptide used for the majority of these experiments has a total of eight lysine residues that could react with the disuccinimidyl suberate. At least four of the lysine residues have emerged from the large ribosomal subunit and could make contact with nonribosomal proteins. Sequential intermediates in the pro-

tein transport reaction were assembled either by omission of selective modification of a specific protein translocation component. By defining the components required to produce a given cross-linked product, we were, at least in two cases, able to determine the identity or site of function of the protein to which the nascent polypeptide was cross-linked. The validity of the cross-linking method we have used here was demonstrated by the efficient cross-linking of the pG-90 polypeptide to the 54-kD subunit of SRP. The identification of SRP54 as the signal sequence binding site of SRP confirms the previous cross-linking results that were obtained by incorporating photoreactive lysine analogues into nascent secretory polypeptides (Krieg et al., 1986; Kurzchalia et al., 1986; Wiedmann et al., 1987a).

Formation of the 45-kD cross-linked product required the assembly and maintenance of a membrane-bound translocation intermediate. Unlike the cross-linked product involving SRP54, the 45-kD product arose by cross-linking of pG-90 to an integral membrane protein that we have designated imp34. Based upon the 11.5-Å length of the cross-linking reagent, imp34 appears to be in close proximity to nascent polypeptides undergoing transport across the membrane. Several different criteria could distinguish imp34 from the previously identified α(mp39) and β subunits of SSR. The 45-kD cross-linked product containing either pG-90 or pPL-86 did not bind to Concanavalin A Sepharose, unlike αSSR or mp39 (Krieg et al., 1989; Wiedmann et al., 1987b). The 45-kD product was also resistant to Endo H digestion. As βSSR is a glycoprotein of ∼24 kD (Görlich et al., 1990), the protein we have identified here cannot be βSSR. The cross-linked product between imp34 and pG-90 is highly resistant to proteinase K digestion in intact microsomal membranes. In contrast, proteinase K digestion reduces the apparent molecular weight of the αSSR (mp39)-nascent polypeptide cross-linked product by ∼4 kD (Krieg et al., 1989; Wiedmann et al., 1987b). While the proteinase K resistance of the 45-kD product may indicate that imp34 has a small cytoplasmic domain, the ribosome or the cytoplasmic domain of a neighboring protein may shield the cross-linked product from digestion. Unlike proteinase K, trypsin is able to cleave both the 45-kD cross-linked product and the pG-90 nascent polypeptide in intact membranes. Inherent differences in the molecular weight and cleavage specificity of these two proteases presumably account for the different protease digestion results.

NEM alkylation of microsomal membranes and puromycin treatment of the assembled translocation intermediates provided evidence that imp34 functions during the authentic transport pathway for nascent polypeptides. Inactivation of the SRP receptor by proteolytic digestion or by alkylation with NEM prevented the formation of a cross-link between imp34 and pG-90, as did release of the nascent polypeptide from the ribosome by puromycin/high salt treatment. SSR cross-link formation is also blocked by inactivation of the SRP receptor (Krieg et al., 1989; Wiedmann et al., 1987b). The 52-kD fragment of SRα restores translocation activity to a proteolyzed membrane fraction, but not to a NEM-alkylated proteolyzed membrane fraction (Nicchitta and Blobel, 1989). Formation of the 45-kD cross-linked product correlated well with the translocation activity of these recombined membrane samples. Therefore, NEM modification of a protein other than the SRP receptor prevents the as-

sembly of a translocation intermediate in which imp34 is adjacent to pG-90. It is unlikely that SSR is the protein inactivated by NEM treatment, as neither α nor βSSR contains a single cysteine (Görlich et al., 1990; Prehn et al., 1990). The results presented here suggest that imp34 is either the NEM-sensitive translocation component or that the NEM-sensitive component performs an essential function before the association between imp34 and the nascent polypeptide.

Why was a different integral membrane cross-linked to the nascent polypeptide with disuccinimidyl suberate? The photoreactive probes used by Wiedmann et al. (4-(3-trifluoromethyldiazirino)benzoyl lysine [Wiedmann et al., 1987b]) and Krieg et al. (Nᵋ-(5-azido-2-nitrobenzoyl lysine [Krieg et al., 1989]) are carbene and nitrene precursors respectively. These compounds are designed to react in a nonselective manner with amino acids located in the immediate vicinity of the photoactivatable probe via carbene or nitrene insertion into carbon–hydrogen or oxygen–hydrogen bonds. However, carbon–hydrogen insertions appear to occur only in the absence of competing reactions (Brunner et al., 1980). Electron-rich amino acids, such as tryptophan, glutamic acid, or cysteine are preferentially labeled relative to other amino acids (Brunner and Richards, 1980; Hoppe et al., 1983; Ross et al., 1982). Furthermore, the lifetime of both the nitrene group, and the linear diazo isomer formed in high yields from the carbene precursor, allows for the modification of amino acids that are not in the immediate vicinity of the photoactivatable probe at the time of photolysis (Bayley and Knowles, 1978; Brunner et al., 1980). Therefore, a protein containing a reactive nucleophile in the vicinity of the photoprobe will be selectively modified, at the expense of other adjacent proteins. It is likely that SSR contains such a reactive group, resulting in preferential cross-linking of the nascent polypeptide to SSR. Less abundant cross-linked products have been obtained upon photolysis of the nascent polypeptides (Krieg et al., 1989), and one of these nonglycosylated integral membrane proteins may correspond to imp34.

The primary amine specific reagent DSS places a stronger constraint on the possible targets of a cross-linking reaction. Any protein that lacks a properly oriented lysine residue within 11.5 Å of a lysine within pG-90 will not be detected using DSS as a cross-linking reagent. The chemical cross-linker is membrane permeable, and should preferentially react with lysines that are located near the membrane surface. Lysyl residues are located at positions 68, 91, 102, 110, 178, 233, 235, 240, 266, 275, and 279 in αSSR (Prehn et al., 1990). The predicted transmembrane span of SSR corresponds to amino acids 207–229, with the amino terminus located in the ER lumen (Prehn et al., 1990). Thus, the transmembrane and lumenal domains of αSSR appear to be poor targets for cross-linking with DSS, whereas the cytoplasmic domain contains a cluster of lysines near the membrane surface. We would predict that imp34 has at least one and perhaps several lysyl residues located near the membrane surface within the lumen, and this accounts for the efficient chemical cross-linking between imp34 and pG-90. The finding that the photoreactive cross-linking reagents can display selectivity that is quite distinct from the known selectivity of DSS presumably accounts for the differential cross-linking of membrane proteins obtained with these two different classes of reagents. Recently, αSSR has been cross-linked

to nascent polypeptides derived from either preprolactin or β-lactamase using several different chemical cross-linking reagents (Görlich et al., 1990). Further research will need to be conducted to determine whether pG-90 can be cross-linked to αSSR or imp34 using any of these other bifunctional cross-linking reagents.

We propose that imp34 acts in concert with SSR and perhaps other integral membrane proteins to catalyze nascent polypeptide transport. These protein components may be assembled into an aqueous channel in response to the delivery of a ribosome to the membrane surface (Blobel and Dobberstein, 1975). Alternatively, a completely assembled translocation pore may open and close in a regulated manner (Simon et al., 1989). The protein components of the translocation pore could interact with the nascent polypeptide in a sequential manner during the mechanistically distinct phases of polypeptide insertion and transbilayer transport. Although a sequential function of these protein components is an intriguing concept, current data indicates that αSSR (mp39) is in contact with the nascent polypeptide at both early and late phases of a protein transport reaction (Krieg et al., 1989). In the yeast *Saccharomyces cerevisiae*, the proteins encoded by the SEC61, SEC62, and SEC63 genes perform essential functions during protein translocation (Deshaies and Schekman, 1987; Rothblatt et al., 1989; Toyn et al., 1988). The products of these three genes each have two or more putative membrane spanning domains (Deshaies and Schekman, 1989; Sadler et al., 1989). Moreover, the proteins encoded by SEC61, SEC62, and SEC63, in concert with two additional proteins, have been shown to form a multi-subunit membrane-associated complex in *Saccharomyces cerevisiae* (Deshaies et al., 1991). It is quite likely that mammalian microsomal membranes contain proteins that are both structurally and functionally homologous to the SEC61, SEC62 and SEC63 gene products. However, sequence analysis of the α and β subunits of SSR has not revealed sequence homologies to any other proteins sequenced to date (Görlich et al., 1990; Prehn et al., 1990). Imp34 and the SEC62 protein have similar molecular weights; furthermore, the SEC62 protein contains numerous lysines (10 mol%) (Deshaies and Schekman, 1989) that would make it a favorable target for cross-linking reactions with an amine reactive reagent. It should be interesting to determine whether this similarity is significant or merely fortuitous.

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