Stage- and Ribosome-Specific Alterations in Nascent Chain–Sec61p Interactions Accompany Translocation across the ER Membrane

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Abstract. Near-neighbor interactions between translocating nascent chains and Sec61p were investigated by chemical cross-linking. At stages of translocation before signal sequence cleavage, nascent chains could be cross-linked to Sec61p at high (60–80%) efficiencies. Cross-linking occurred through the signal sequence and the mature portion of wild-type and signal cleavage mutant nascent chains. At later stages of translocation, as represented through truncated translocation intermediates, cross-linking to Sec61p was markedly reduced. Dissociation of the ribosome into its large and small subunits after assembly of the precursor into the translocon, but before cross-linking, resulted in a dramatic reduction in subsequent cross-linking yield, indicating that at early stages of translocation, nascent chain–Sec61p interactions are in part mediated through interactions of the ribosome with components of the ER membrane, such as Sec61p. Dissociation of the ribosome was, however, without effect on subsequent translocation. These results are discussed with respect to a model in which Sec61p performs a function essential for the initiation of protein translocation.

In eukaryotic cells, the trafficking of secretory proteins to the extracellular environment is initiated upon translocation across the ER membrane. In addition to providing the entry point into the secretory pathway, the process of protein translocation also functions in the topological assembly of integral membrane proteins (Blobel, 1980; reviewed in Nunnari and Walter, 1992; Sanders and Schekman, 1992; Rapoport, 1992). Recent experimental efforts to elucidate the mechanism of translocation, through genetic, biochemical, biophysical, and electrophysiological approaches, have yielded significant insights into this seemingly complex process.

Genetic selection strategies have allowed identification of three gene products, Sec61p, Sec62p, and Sec63p, which are essential for protein translocation in yeast ER (Deshaies and Schekman, 1987, 1989; Rothblatt et al., 1989; Stirling et al., 1992). The *SEC61* gene encodes a 53-kD polytopic ER membrane protein that is necessary for both secretory and membrane protein translocation (Rothblatt et al., 1989; Stirling et al., 1992). Studies with chemical cross-linking reagents have indicated that Sec61p resides in close physical proximity to translocating nascent chains (Müsch et al., 1992; Sanders et al., 1992). Sec62p and Sec63p are also integral ER membrane proteins (Deshaies and Schekman, 1987, 1989). Screens for synthetic lethality as well as coimmunoprecipitation experiments indicate that Sec62p and Sec63p physically interact with one another, as well as with Sec61p (Rothblatt et al., 1989; Deshaies et al., 1991). Two additional yeast membrane proteins, Sec66p and Sec72p, have been implicated in protein translocation (Feldheim et al., 1993; Kurihara and Silver, 1993; Feldheim and Schekman, 1994). Neither Sec66p nor Sec72p is essential for growth, although the null mutants display disruptions in translocation (Feldheim et al., 1993; Kurihara and Silver, 1993; Feldheim and Schekman, 1994).

Biochemical studies of protein translocation have progressed on two, complementary fronts, chemical cross-linking and biochemical reconstitution. Photocross-linking studies have yielded the identification of two glycoprotein components of mammalian rough microsomes (RM)[1] that reside in physical proximity to translocating nascent chains. One component, SSRα (now referred to as TRAPα; Hartmann et al., 1993), was identified in cross-links to both secretory and membrane protein precursors (Wiedmann et al., 1987; Krieg et al., 1989; Thrift et al., 1991). The func-

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1. *Abbreviations used in this paper*: MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; PDI, protein disulphide isomerase; pPl, preprolactin; RM, rough microsome; SCM, signal sequence cleavage mutant; VSV-G, vesicular stomatitis virus G-protein; WT, wild type.

tion, with respect to protein translocation, of TRAPα is not known, as vesicles reconstituted from detergent extracts lacking the TRAPα complex display unimpaired translocation activity (Migliaccio et al., 1992). An integral membrane glycoprotein similar in molecular weight to TRAPα, termed TRAM, was also identified through photocross-linking (Görlich et al., 1992*a*). TRAM has been demonstrated to promote translocation into vesicles reconstituted from a glycoprotein-depleted detergent extract (Görlich et al., 1992*a*). Cross-linking studies have also been instrumental in the identification of a nonglycoprotein component of mammalian RM as the predominant cross-linking partner of translocating nascent chains (Kellaris et al., 1991; Görlich et al., 1992*b*; Görlich and Rapoport, 1993; High et al., 1993*a*,*b*). This protein, recently identified as the mammalian homologue of yeast Sec61p, is found in association with membrane-bound ribosomes and has been demonstrated to perform a central function in translocation (Görlich et al., 1992*b*; Görlich and Rapoport, 1993). Proteoliposomes containing the signal recognition particle receptor and Sec61p as the only protein components have been shown to translocate precursor proteins, thus indicating that Sec61p likely functions as the ER protein translocation channel (Görlich and Rapoport, 1993).

A prominent focus of current research in the field of protein translocation has been the determination of the molecular environment of the translocating nascent chain. Although originally proposed to occur through a proteinaceous, aqueous channel (Blobel and Dobberstein, 1975), experimental evidence in support of this hypothesis has until recently been lacking. The identification of protein components residing in physical proximity to the nascent chain during translocation has added substantial, although indirect, experimental support for this proposal. Johnson and colleagues have recently analyzed the physical environment of translocating nascent chains through use of environment-sensitive fluorescent probes, covalently incorporated into nascent chains (Crowley et al., 1993, 1994). By analysis of the fluorescence quenching parameters of such probes, Crowley et al. (1993, 1994) were able to determine that the ribosome is tightly associated with the ER membrane and that translocating nascent chains reside in an aqueous environment during translocation. The conclusion that translocation proceeds through an aqueous environment has also received substantial experimental support from electrophysiological studies (Simon and Blobel, 1991, 1992). In studies of the conductance properties of ER vesicles fused into planar bilayer membranes, Simon and Blobel (1991) demonstrated that the release of translocating nascent chains from the ribosome, upon addition of puromycin, was accompanied by a dramatic increase in membrane conductance. The puromycin-sensitive increase in membrane conductance presumably reflects the movement of ions through the unoccupied, protein-conducting channel (Simon and Blobel, 1991).

The remarkable corroboration in the results generated by these differing experimental approaches has supported definitive proposals on the mechanism of protein translocation in the ER. Sec61p is thought to mediate directly the transfer of the nascent chain across the membrane and in this regard function as the protein-conducting channel (Sanders et al., 1992; Görlich and Rapoport, 1993; Mothes et al., 1994). In mammalian systems, translocation appears to proceed from an aqueous tunnel in the ribosome to the channel defined by Sec61p. The direct coupling of the two channel domains is a consequence of a high affinity interaction between the ribosome and Sec61p, which acts to seal the junction from the cytoplasm (Görlich et al., 1992; Crowley et al., 1993, 1994; Kalies et al., 1994). Protein translocation across the ER membrane may thus require a single oligomeric complex, the Sec61p complex (Görlich and Rapoport, 1993; Mothes et al., 1994). TRAM is postulated to promote the assembly of the signal sequence into a loop configuration into the membrane and to disengage from the translocation apparatus during translocation (Mothes et al., 1994). Other ER proteins, such as Sec62p, Sec63p, BiP (Kar2p), and lumenal proteins, have also been proposed to function in protein translocation (Deshaies and Schekman, 1989; Vogel et al., 1990; Sanders et al., 1992; Brodsky et al., 1993; Nicchitta and Blobel, 1993). The precise function of these components has not yet been unequivocally established. It is likely, however, that these and perhaps other components are necessary to sustain protein translocation at in vivo rates and efficiencies.

In this report, we present data demonstrating that the near-neighbor interactions between Sec61p and translocating nascent chains vary as a function of the stage of translocation as well as the state of association of the nascent chain with the ribosome. Short chain translocation intermediates (≤90 amino acids) were found to cross-link to Sec61p at unusually high (60–80%) efficiency. Mapping of the cross-linking sites indicated that cross-links to short intermediates occur through both the signal sequence and the mature region of the protein. In evaluating the cross-linking patterns of translocation intermediates of increasing length, as well as mutants unable to undergo signal peptide cleavage, it was observed that nascent chains reside in close physical proximity to Sec61p at early stages of translocation. This near-neighbor interaction is greatly potentiated by the intact ribosome. When the large and small ribosomal subunits of nascent chain–ribosome–membrane complexes are dissociated by EDTA treatment before cross-linking, the efficiency of nascent chain–Sec61p cross-linking is dramatically reduced. Under these conditions, however, the capacity of the nascent chain to undergo translocation is unaltered.

Materials and Methods

Reagents

Hemin, creatine phosphate, creatine phosphokinase, and CHAPS were obtained from Calbiochem Corp. (San Diego, CA). Staphylococcal nuclease, calf liver tRNA, puromycin, and proteinase K were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Phenylhydrazine hydrate was from Sigma Chemical Co. (St. Louis, MO). *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was obtained from Pierce (Rockford, IL). Restriction enzymes, DNA modification enzymes, and PCR components were obtained from either New England Biolabs Inc. (Beverly, MA) or Promega Corp. (Madison, WI). [^{35}S]Pro-Mix ([^{35}S]methionine plus cysteine) was obtained from Amersham Corp. (Arlington Heights, IL). Nucleotides were obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ).

Generation of Signal Cleavage Mutants

A signal cleavage mutant (SCM) of bovine preprolactin (pPl) containing a Ser to Tyr substitution at −1 and a Val to Phe substitution at +3 was pre-

pared. These mutations were designed to abolish both the wild-type (WT) cleavage site and an additional potential cleavage site located 2 amino acid residues downstream of the WT site. PCR amplification was performed using the WT bovine pPl-containing plasmid pGEMBP1 (Connolly and Gilmore, 1986) as template, an oligonucleotide hybridizing to the T7 promoter as the 5′ sense strand primer, and a mutagenic oligonucleotide (5′ GG CCC ATT GGG ACA GAA GGG GGT GTA GAC CAC ACC CTG GCA C 3′) as the 3′ antisense strand primer. The resulting PCR product (PCR-1) was gel purified, and a second round of PCR was performed using pGEMBP1 as template, PCR-1 as the 5′ sense strand "megaprimer" (Sarkar and Sommer, 1990) and an oligonucleotide hybridizing to the SP6 promoter as the 3′ antisense-strand primer. The resulting PCR product was digested with HindIII and EcoRI, gel purified, and ligated to HindIII- and EcoRI-digested pGEM-4Z, to yield pGEMBP1-1. This plasmid was sequenced and found to encode the desired amino acid substitutions. Upon transcription of pGEMBP1-1 and translation in the presence of RM, it was established that the mutant precursor was processed by signal peptidase at an apparent upstream site that we speculated, based on criteria of von Heijne (1986), to exist between Gly (−4) and Val (−3) (data not shown). An additional round of PCR mutagenesis was therefore performed to convert the Gly (−4) to Asn while retaining the −1 mutation at the WT site. For this construct, PCR was performed using pGEMBP1-1 as template, an oligonucleotide hybridizing to the T7 promoter as the 5′ sense strand primer, and a mutagenic oligonucleotide, (5′ GGG GGT GTA GAC CAC ATT CTG GCA CAA GAG TAG 3′) as the 3′ antisense strand primer. The PCR product (PCR-A) was gel purified and used in a subsequent round of PCR amplification using the WT pGEMBP1 plasmid as template, PCR-A as the 5′ sense strand primer, and an oligonucleotide hybridizing to the SP6 promoter as the 3′ antisense strand primer. The final PCR product was digested with EcoRI and HindIII, gel purified, and ligated to the EcoRI and HindIII sites of pGEM-4Z to yield pGEMBP1-2. The identity of this mutant form of pPl was examined by dideoxy sequencing and was observed to contain, in addition to the two expected mutagenized residues, a PCR error resulting in conversion of Val (+3) to Ile. The final pGEMBP1-2 amino acid sequence therefore contains the following mutations: Gly (−4) to Asn, Ser (−1) to Tyr, and Val (+3) to Ile. A signal cleavage form of the vesicular stomatitus virus G-protein (VSV-G) was prepared as follows. With pT7VSV-G as template, two rounds of PCR, giving rise to overlapping PCR products, were performed. For PCR-I, an oligonucleotide hybridizing to the T7 promoter was used as the 5′ sense strand primer and a mutagenic oligonucleotide (5′ GCA ATT CTC CCC AAT GAA 3′) was used as the 3′ antisense strand primer. For PCR-II, a mutagenic oligonucleotide (5′ AT TGG GGA GAA TTG CAA G 3′) was used as the 5′ sense strand primer, and an oligonucleotide hybridizing to the SP6 promoter was used as the 3′ antisense strand primer. Both PCR products (PCR-I and PCR-II) were gel purified and used as templates in PCR using the T7 and SP6 promoter oligonucleotides as primers. The final PCR product was gel purified and digested with HindIII, and the ends were filled in with Klenow fragment. The product was subsequently digested with XbaI and ligated to pGEMBP1 that had been prepared as follows: pGEMBP1 was digested with EcoRI, and a fill-in reaction was performed with Klenow fragment. The plasmid was subsequently digested with XbaI. The vector was separated from the pPl insert by gel electrophoresis, purified, and ligated to the final PCR product. The identity of the mutant form of VSV-G was established by dideoxy sequencing analysis.

Cell-free Transcription and Translation

The plasmids pGEMBP1 (Connolly and Gilmore, 1986) and pGEMBP1-2, containing cDNA inserts for bovine pPl, were linearized within the coding region with PvuII or RsaI to prepare run-off transcripts encoding polypeptides of 86 and 131 amino acids, respectively. For transcripts encoding the pPl 169-mer, pGEMBP1 and pGEMBP1-2 were digested with EcoRI and MscI, isolated after agarose gel electrophoresis, and religated with T4 ligase. Subsequent digestion with EcoRI and run-off transcription yielded mRNA encoding the pPl 169-mer. The plasmids pGEMVSV-G and pGEMVSV-SCM were digested with AvaII and NdeI and transcribed to yield mRNAs encoding truncated transcripts encoding polypeptides of 90 and 173 amino acids, respectively. Transcription reactions were performed according to the procedure of Weitzmann et al. (1990). Reactions were performed in a buffer containing 40 mM Tris-HCl, pH 8.0, 8 mM magnesium acetate, 25 mM NaCl, 2 mM spermidine, 10 mM DTT, 2.5 mM ATP, CTP, UTP, and GTP, 5 U/ml yeast inorganic pyrophosphatase, and 1 U/μl T7 RNA polymerase. Cell-free translations were performed in a rabbit reticulocyte lysate system as described (Nicchitta and Blobel, 1989). Translations (20 μl) contained 8 μl of nuclease-treated rabbit reticulocyte lysate, 25 μCl of [^{35}S]Pro-Mix (methionine plus cysteine), 0.05 U/μl RNasin, 1 mM DTT, 80 μM-methionine amino acid mix, and where indicated, 1 equivalent of RM, as defined by Walter and Blobel (1983). Reactions were adjusted to 110 mM potassium acetate, 2.5 mM magnesium acetate. Rabbit reticulocyte lysate was prepared by the method of Jackson and Hunt (1983), and canine pancreas RM were prepared by the method of Walter and Blobel (1983). Translations were performed for 30 min at 25°C. Where indicated, puromycin was present at 0.5 mM.

Membrane Association of Translation Products

For analyses of the vesicle localization of translation products, translation reactions were chilled on ice and diluted 10-fold in a buffer composed of 50 mM Hepes, 50 mM CAPS, adjusted with KOH to pH 7.5, 9.5, 10.5, or 11.5. After incubation on ice for 30 min, samples were overlayed onto a cushion of 0.5 M sucrose, 25 mM K-Hepes, pH 7.4 (supernatant/cushion ratio of 3:1), and centrifuged for 10 min at 60,000 rpm in the TLA100 rotor (Beckman Instr., Fullerton, CA). The supernatant and cushion were harvested and fractionated by ammonium sulfate precipitation at 66% saturation. After a 20-min incubation on ice, samples were centrifuged for 10 min at 15,000 *g*, the supernatants were discarded, and pellet fractions were washed in 10% TCA. Samples were processed for SDS-PAGE as indicated in Sample Analysis. In these experiments, paired incubations were performed in the absence of translation to compare the release of the lumenal proteins GRP94 and protein disulphide isomerase (PDI). For these samples, supernatant/cushion fractions were concentrated by precipitation with 10% TCA and prepared for SDS-PAGE, and the protein composition of the supernatant and pellet fractions was determined by densitometric analysis of the Coomassie blue-stained gels using a ScanJet Plus (Hewlett-Packard Co., Palo Alto, CA) and NIH Image software.

Chemical Cross-Linking

Chemical cross-linking of completed translation reactions was performed as follows. Reactions were chilled on ice and diluted 10-fold with a physiological salts buffer consisting of 110 mM potassium acetate, 25 mM K-Hepes, pH 7.4, and 2.5 mM magnesium acetate. Diluted reactions were overlayed onto a 1/3 volume cushion of 0.5 M sucrose, 25 mM K-Hepes, pH 7.4, and centrifuged for 10 min at 60,000 rpm in the TL100 rotor. Supernatant and cushion fractions were discarded, and the membrane pellet was resuspended to 2 equivalents per μl in 0.25 M sucrose, 50 mM potassium acetate, 2.5 mM magnesium acetate. Cross-linking reactions (50 μl) were performed for 30 min at 25°C, or 60 min at 4°C, as indicated, by addition of MBS to a final concentration of 1 mM from a 50 mM stock in dimethylformamide. Reactions were quenched by addition of 1 vol of PBS containing 50 mM DTT and 50 mM lysine and incubation on ice for 10 min. Cross-linking reactions were precipitated by addition of TCA to 10% and processed for SDS-PAGE. In experiments involving ribosome dissociation, isolated ribosome-nascent chain-membrane complexes were incubated on ice for 30 min in the presence of 10 mM EDTA, and chemical cross-linking was performed on ice as described.

Protease Protection Protocol

Completed translation reactions were chilled on ice and diluted to 50 μl in a physiological salts buffer containing 110 mM potassium acetate, 25 mM K-Hepes, and 2.5 mM magnesium acetate. Proteinase K, from a stock concentration of 5 mg/ml, was added to a final concentration of 100 μg/ml, and digestions were performed for 30 min on ice. Digestions were quenched by addition of PMSF to a final concentration of 4 mM from a 150 mM stock in isopropanol. Reactions were fractionated by addition of ammonium sulfate to a final concentration of 66% and processed as previously indicated.

Preparation of Antisera and Immunoprecipitation

Anti-peptide antibodies directed against canine Sec61p and canine TRAM (Görlich et al., 1992*a*,*b*) were prepared according to protocols described by Harlow and Lane (1988). Peptides representing amino acids 2–12 of the amino terminus of Sec61p (Görlich et al., 1992*b*) and the 12 carboxy-terminal amino acids of TRAM (Görlich et al., 1992*a*) were synthesized with carboxy-terminal cysteine residues (Multiple Peptide Systems, San Diego, CA). Peptides were conjugated to keyhole limpet hemocyanin with MBS, as described by Harlow and Lane (1988). Conjugates were gel filtered

in sterile PBS and prepared for injection. Primary injections (1 mg) were performed in Freund's complete adjuvant, and secondary injections (0.25 mg) were in Freund's incomplete adjuvant. Injections were performed at days 0, 14, and 28 and at subsequent 28-d intervals. For affinity purification of IgG, 5 mg of each peptide was coupled to Sulfo-Link resin (Pierce) according to the manufacturer's instructions. 50 ml of crude serum was cycled over the peptide affinity columns overnight at 4°C. Columns were extensively washed with PBS, PBS plus 0.5 M NaCl, and water. Bound IgG was eluted by addition of 0.2 M glycine, pH 2.3. Eluted IgG was immediately neutralized by addition of Tris base, pH 10.0, supplemented with sodium azide to 0.02%, and stored at 4°C. Immunoprecipitation of cross-linked complexes was performed according to the following protocol. Completed, quenched cross-linking reactions were overlayed onto a 0.5 M sucrose, 25 mM K-Hepes, pH 7.4, cushion (3:1, supernatant/cushion) and centrifuged for 10 min at 60,000 rpm in the TL100 rotor. The supernatant and cushion fractions were removed, and the pellet was resuspended in PBS, 1% SDS for 15 min at 37°C. Samples were diluted fourfold with PBS, 1% Triton X-100, 2 mM PMSF, 2.5 μg/ml leupeptin and supplemented with either crude sera (1:200 dilution) or affinity-purified IgG (10 μg/ml). Antibody binding was conducted overnight at 4°C. For indirect immunoprecipitations, 40 μl of a 50% slurry of protein A–Sepharose was added, and samples were incubated for 60 min at room temperature. Protein A–Sepharose beads were collected by centrifugation and washed twice in PBS, 0.1% Triton X-100 and once in PBS. Bound antigen was eluted by addition of 50 μl of 0.5 M Tris, 5% SDS and heating at 37°C for 20 min. Yields for Sec61p immunoprecipitations were highly variable (20–80%); preimmune and nonimmune controls, as well as peptide competition experiments, indicated that binding was specific. The source of the variability has not been identified.

Sample Analysis and Quantitation

To resolve the short chain translocation intermediates, translation products were separated on the Tris-Tricine gel system of Schägger and von Jagow (1987). Precipitated proteins were solubilized in 0.5 M Tris, 5% SDS, 50 mM DTT at 55°C for 20 min. In experiments in which cross-linking was not performed, samples were alkylated by addition of iodoacetamide to 125 mM final concentration and incubation at 37°C for 30 min. After SDS-PAGE using the Tris-Tricine buffer system, gels were fixed in 35% methanol, 10% acetic acid and subsequently equilibrated in 5% glycerol. Quantitation of translation products in the dried gels was performed on a MacBAS1000 phosphorimaging system (Fuji Medical Systems, Inc., Stamford, CT) with version 1.0 software. All depicted translation product gels, with the exception of that shown in Fig. 8, are Fuji MacBAS1000 digital images. Image dimensions were adjusted in Photoshop, version 2.5 (Adobe Systems, Inc., Mountain View, CA). The gel shown in Fig. 8 is a digital image of an autoradiogram obtained by digital scanning of the exposed film on a Hewlett-Packard ScanJet Plus.

Results

Translocation Substrates

The process of protein translocation across the ER membrane can be biochemically dissected into at least three discrete stages; (*i*) targeting of the nascent chain to the ER membrane, (*ii*) assembly of the nascent chain into the translocation apparatus, and (*iii*) transport across the ER membrane. We and others have previously observed that the assembly stage can be biochemically distinguished from the transport stage by chemical alkylation of the ER membrane by the sulphydryl directed alkylating agent *N*-ethylmaleimide (Nicchitta and Blobel, 1989; Zimmermann et al., 1990; Kellaris et al., 1991).

In an effort to identify the membrane component(s) interacting with translocating nascent chains at the assembly/transport transition, chemical cross-linking studies were performed with the heterobifunctional cross-linking agent MBS, which reacts with amine and sulphydryl groups. In these studies, well-characterized translocation intermediates of pPl and VSV-G were used. Fig. 1 depicts the relative location of the susceptible lysyl and cysteinyl residues of the different intermediates, as well as those predicted to reside within the ribosome. For both precursors, artificially truncated nascent chains were synthesized after digestion of cDNAs with appropriate restriction enzymes and subsequent transcription. Nascent chains arising through translation of these transcripts lack termination codons and thus remain in association with the ribosome as peptidyl-tRNA adducts (Perara et al., 1986; Gilmore et al., 1991). The ribosome is known to shield the 30–40 carboxy-terminal amino acids of the nascent chain (Malkin and Rich, 1967; Blobel and Sabatini, 1970), and thus for the pPl 86-mer, the lysyl residues at −27 and −22 and the cysteinyl residues at −6, +4, and +11 are potential substrates for MBS-mediated cross-linking. For the pPl 131- and 169-mer, potential cross-linking residues would also include the cysteinyl residue at +58 and the lysyl residue at + 69 and lysyl residues +106 and +124, respectively. As is apparent from Fig. 1, abundant potential cross-linking sites exist in the two VSV-G constructs studied, the VSV-G 90- and 172-mer.

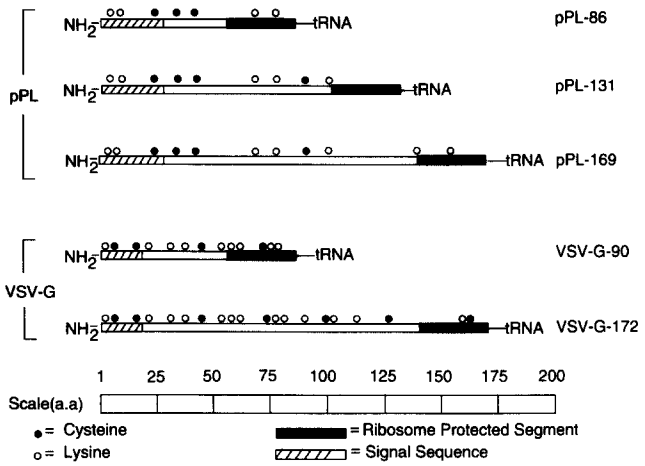

Figure 1. Schematic illustration of translocation intermediates and potential cross-linking sites. Truncated pPl nascent chains of 86, 131, and 169 amino acids (pPl-86, pPl-131, and pPl-169) and VSV-G nascent chains of 90 and 172 amino acids (VSV-G-90 and VSV-G-172) are schematically depicted. The location of the lysyl and cysteinyl residues representing potential cross-linking sites with the heterobifunctional cross-linking agent MBS are illustrated as open and closed circles, respectively. Cross-hatched domains at the amino termini represent signal sequences; the stippled domains at the carboxyl termini/aminoacyl tRNA junctions represent those areas of the nascent chains that lie within the ribosome. The scale (in amino acids) is shown along the bottom of the illustration.

Translocation Behavior of WT and SCM Precursors

At early stages of translocation, the nascent chain assumes a hairpin loop structure in which the amino terminus of the signal sequence is exposed to the cytoplasmic side of the ER membrane (Shaw et al., 1988; Mothes et al., 1994). To aid in the study of these early translocation stages, signal cleavage mutants of pPl and VSV-G were prepared. It was expected that such precursors would prove useful for studying the ER membrane components residing in physical proximity to the signal sequence.

The translocation behavior of the WT and SCM forms of the pPl 86-mer and the VSV-G 90-mer are shown in Fig.

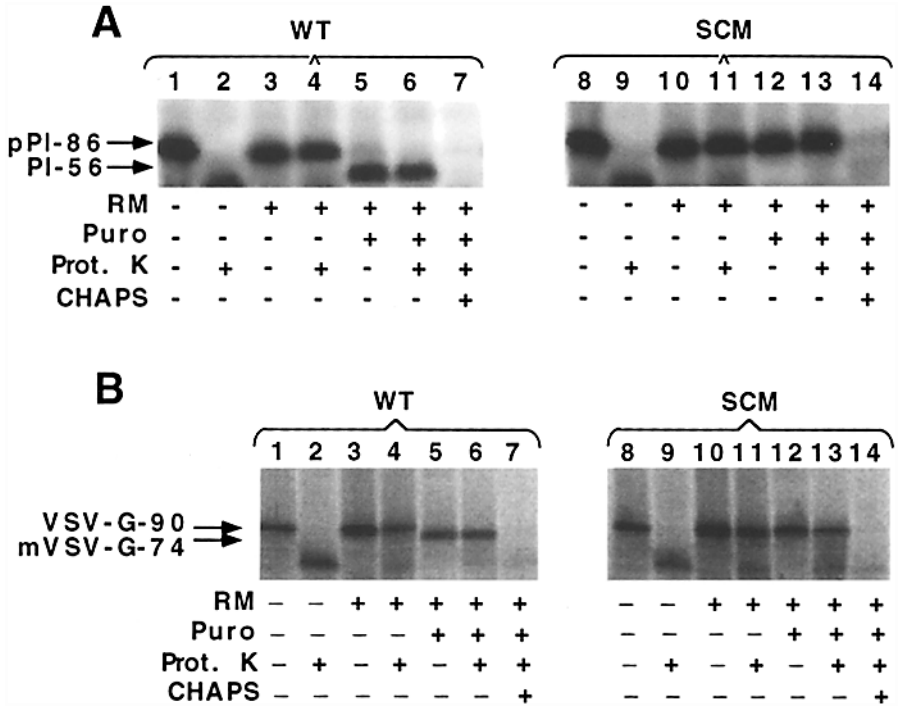

Figure 2. Translocation behavior of WT and SCM forms of short chain translation products. WT and SCM forms of the pPl 86-mer (*A*) and VSV-G 90-mer (*B*) were synthesized in a [^{35}S]methionine-supplemented reticulocyte translation system in the presence or absence of RM (*RM*) for 30 min at 25°C. Processing of nascent chains to mature forms was assessed after addition of puromycin (*Puro*), to a concentration of 0.5 mM, for 10 min at 25°C. Assays were chilled on ice, and translocation was determined by assays of the sensitivity of nascent chains to digestion by proteinase K (*Prot. K*) in the presence (lanes *7* and *14*) or absence (lanes *2*, *4*, *6*, and *9*, *11*, *13*) of detergent (*CHAPS*). Protease protection assays were performed for 30 min on ice at a proteinase K concentration of 100 μg/ml. Samples were processed for SDS-PAGE as described in Materials and Methods. Translation products were separated on 12.5% Tris-Tricine gels, and translation products were quantitated by phosphorimager analysis using a Fuji MacBAS1000 phosphorimager. Digital images of the dried gels, from the phosphorimager analysis, are depicted in *A* and *B*. The faster migrating translation products shown in lanes *2* and *9* represent the ribosome protected limit digestion products observed in the absence of membranes. The relative mobilities of the precursor and mature forms of the translation products are indicated by arrows.

2, *A* and *B*. In the absence of RM, both the pPl 86-mer and the VSV-G 90-mer migrated as single bands (Fig. 2, *A* and *B*, lanes *1* and *8*). Digestion of the polypeptide chains synthesized in the absence of RM with proteinase K yielded limit digestion products, representing those domains of the carboxyl terminus protected from proteolytic attack by the ribosome (Fig. 2, *A* and *B*, lanes *2* and *9*). When translated in the presence of RM, neither translation product was observed to undergo signal peptide cleavage. However, each was recovered in a protease-protected, membrane-bound form (Fig. 2, *A* and *B*, lanes *3* and *4*; *10* and *11*). At this stage, the precursors are of sufficient length to engage the translocation machinery, but the signal peptidase cleavage site is not accessible to the lumenally oriented signal peptidase complex. Release of the nascent chains from the ribosome, by addition of puromycin, resulted in nearly quantitative signal cleavage of the WT but not the SCM translocation intermediates. Both the WT and SCM truncated nascent chains were protected from digestion by exogenous protease in the absence, but not the presence, of detergent, indicating that the translation products had undergone translocation (Fig. 2, *A* and *B*, lanes *5–7* and *12–14*). Based on these criteria, both the WT and SCM forms of the short translocation intermediates are translocated after chain termination.

Membrane Association of WT and SCM Forms

To determine whether the WT and SCM forms of the pPl 86-mer remained in association with ER membrane components throughout translocation, precursor proteins were assembled into RM and treated with puromycin, and the membrane localization of the precursor was assessed after extraction in buffers of increasing pH (Fig. 3). It is well established that exposure of membrane vesicles to alkaline buffers results in the extraction of soluble and peripheral protein components (Fujiki et al., 1982; Lambert and Freedman, 1983; Nicchitta and Blobel, 1993). For comparative purposes, the behavior of the soluble ER lumenal proteins GRP94 and PDI is illustrated in Fig. 3 *A*. After dilution of RM into buffers of increasing pH and subsequent fractionation of the membrane suspension into pellet and supernatant fractions by centrifugation, the relative distribution of the GRP94 and PDI was determined by densitometric analysis of Coomassie blue–stained SDS–polyacrylamide gels. As shown in Fig. 3 *A*, half-maximal release of PDI and GRP94 was observed at pH ~9.5. Nearly complete extraction was observed in buffers of pH 10.5. The pH dependence of the extraction of the WT and SCM forms of the pPl 86-mer is shown in Fig. 3 *B*. For the WT 86-mer, half-maximal extraction was observed between pH 9.5 and 10.5, with ~85% of the processed form recovered in the supernatant at pH 10.5 (Fig. 3 *B*, lanes *2–5*). In contrast to the WT pPl 86-mer, the SCM form was relatively resistant to alkali extraction. In the experiment shown in Fig. 3, ~50% of the SCM form of the pPl 86-mer was recovered in the pellet fraction at pH 11.5 (Fig. 3 *B*, lanes *7–10*), indicating that the SCM forms of the precursor retain a more persistent association with ER membrane components than that exhibited by the WT form. These results are consistent with previous studies demonstrating a stable association of signal cleavage mutants with the ER membrane (Racchi et al., 1993). Under all extraction conditions, ER integral membrane proteins, such as ribophorin I and TRAPα, were recovered in the pellet fraction (data not shown).

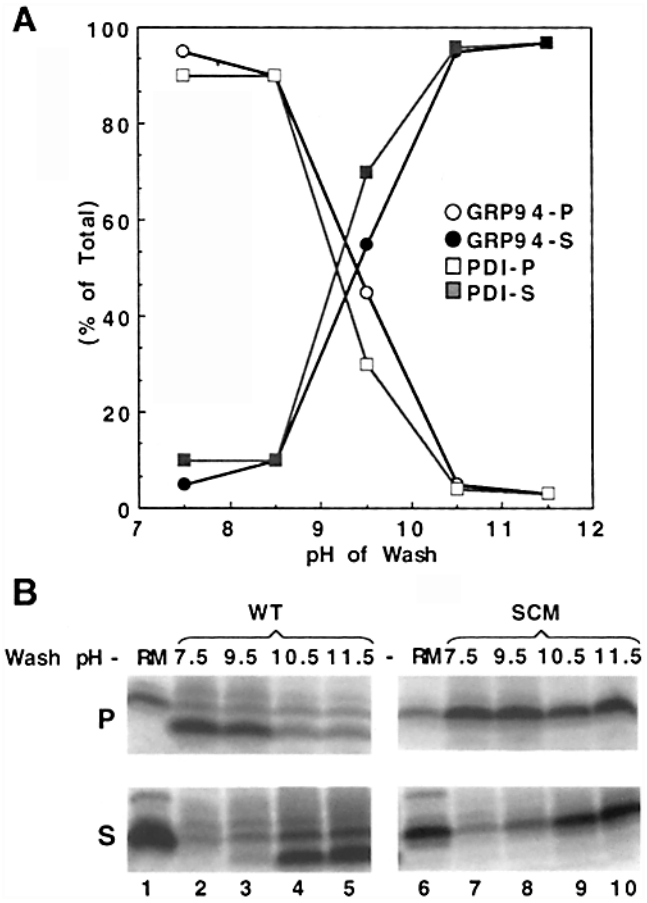

Figure 3. Membrane association of WT and SCM mutant forms of translocated pPl 56-mer and VSV-G 74-mer: comparison with ER lumenal proteins. (*A*) RM were diluted 10-fold in a 50 mM Hepes, 50 mM CAPS buffers adjusted to the indicated pH values with KOH and incubated on ice for 30 min. Membrane-associated (pellet) and released (supernatant) fractions were prepared by centrifugation of the diluted membrane suspension through a 0.5 M sucrose cushion as described in Materials and Methods. Pellet fractions were directly solubilized in SDS-PAGE sample buffer. Supernatant fractions were concentrated by TCA precipitation. Fractions were separated on 10% SDS-PAGE gels, and the relative quantities of the lumenal proteins GRP94 and PDI were determined by densitometric analysis of the Coomassie blue-stained gel. (*B*) WT and SCM forms of the pPL 86-mer mRNAs were translated in a reticulocyte lysate system for 30 min at 25°C. Puromycin was subsequently added to a final concentration of 0.5 mM, and incubations were continued for 10 min at 25°C. Reactions were chilled on ice and diluted 10-fold in a 50 mM Hepes, 50 mM CAPS buffer adjusted to the indicated pH values with KOH. Diluted reactions were incubated on ice for 30 min and overlaid onto a 0.5 M sucrose cushion. The pellet (*P*) and supernatant (*S*) fractions were separated as described in Materials and Methods. Supernatant fractions, comprising the supernatant and cushion, were collected, and the translation products were concentrated by ammonium sulfate fractionation. Pellet fractions were resuspended directly in SDS-PAGE sample buffer. Samples were analyzed as described in the legend to Fig. 2.

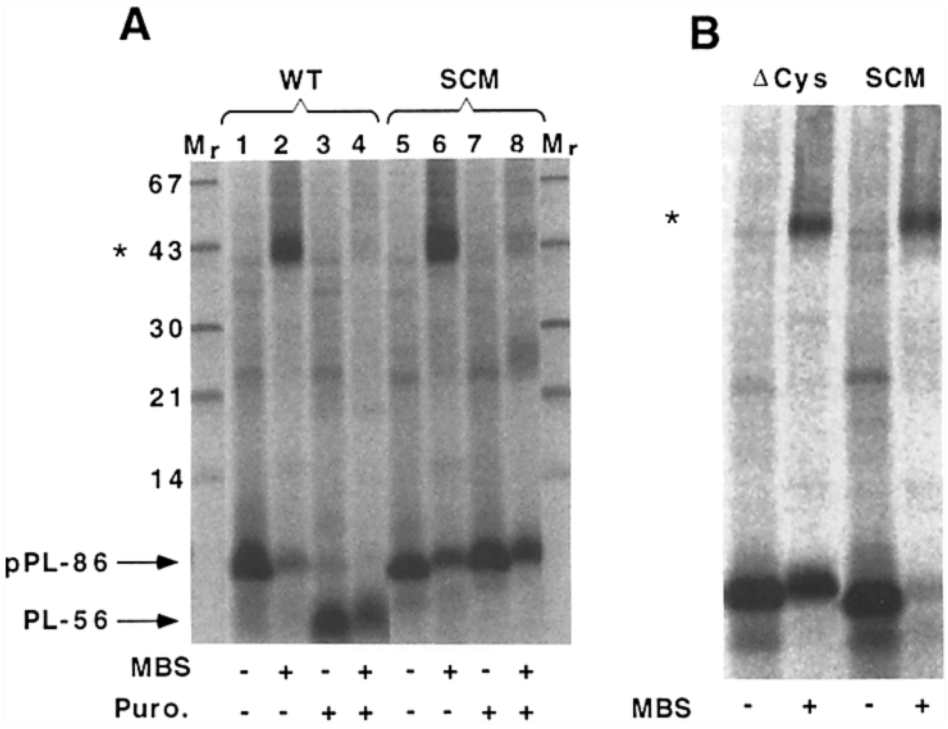

Figure 4. Chemical cross-linking of translocation intermediates of WT and SCM forms of the pPl 86-mer: cross-link localization. (*A*) WT and SCM pPl 86-mer mRNAs were translated in a reticulocyte lysate translation system in the presence of RM, as described in the legend to Fig. 1. After translation and, where indicated, puromycin treatment, reactions were diluted 10-fold in a physiological salt buffer (110 mM potassium acetate, 25 mM K-Hepes, pH 7.4, 2.5 mM magnesium acetate) and collected by centrifugation through a 0.5 M sucrose cushion. Pelleted membrane fractions were resuspended in membrane buffer (0.25 M sucrose, 25 mM K-Hepes, 50 mM potassium acetate, 2.5 mM magnesium acetate) and MBS added to 1 mM. Cross-linking was performed for 30 min at 25°C, and reactions were quenched by addition of 1 vol of PBS, 50 mM DTT, 50 mM lysine and incubation on ice. Samples were TCA precipitated, and translation products were separated by SDS-PAGE using a Tris-Tricine buffer system. A MacBAS1000 digital image is depicted. The migration of the pPl 86-mer and the processed, translocated 56-mer is indicated by arrows. (*B*) Localization of MBS-mediated cross-linking was performed by comparison of the efficiencies of cross-linking between the SCM form of the pPl 86-mer and a pPl mutant lacking the cysteinyl residues at positions +4 and +11 (ΔCys). Cross-linking and sample analysis were performed as described in *A*. For both panels, the asterisk marks the location of the primary cross-linked complex.

Chemical Cross-Linking of Nascent Chains

To assess the molecular environment of the membrane-assembled WT and SCM mutant forms of the pPl 86-mer, chemical cross-linking studies were performed with the heterobifunctional cross-linker MBS. In these studies, membrane-assembled forms of the WT and SCM forms of the pPl 86-mer were prepared by translation in a reticulocyte lysate translation system in the presence of RM. After translation, assays were chilled on ice, and membranes were separated from the translation reaction by centrifugation through a sucrose cushion. Pelleted membranes were resuspended and treated with MBS. As shown in Fig. 4 *A*, treatment of WT pPl 86-mer–RM complexes with MBS resulted in the formation of cross-linked complexes of 43 kD (Fig. 4 *A*, lanes *1* and *2*). The 43-kD cross-linked products were recovered in the pellet fraction after extraction of the cross-linking reaction with 0.1 M Na_2CO_3, pH 11.5, and are thus considered to represent cross-links to an integral membrane protein(s) (data not shown). Cross-linking of the pPl 86-mer with both chemical and photosensitive cross-linking reagents has previously been reported to yield covalent complexes of similar apparent molecular mass (Wiedmann et al., 1987; Krieg et al., 1989; Görlich et al., 1990, 1992). Under the described reaction conditions, formation of the 43-kD cross-linked product was remarkably efficient. Approximately 60–80% of the nascent chains were recovered as the 43-kD cross-linked product. The MBS-dependent covalent cross-links were observed only when the nascent chain was present as a translocation intermediate. Exposure of the WT pPl 86-mer membrane complexes to puromycin before treatment with MBS resulted in a dramatic reduction in cross-linking efficiency (Fig. 4 *A*, lanes *3* and *4*; note the presence of signal cleaved pPl 56-mer). The SCM form of the pPl 86-mer behaved in a manner analogous to the WT form (Fig. 4 *A*, lanes *5–8*). This result was somewhat unexpected in view of the association of the SCM chains with the membrane after puromycin termination.

Topology of MBS Cross-Links

With respect to the pPl 86-mer, cross-linking of the translocation intermediates is expected to involve any or all of the three susceptible residues in the signal sequence, and/or the cysteine residues present at the +4 and +11 positions in the mature region (Fig. 1). To delineate further the cross-linking site(s), reactions were performed with an additional pPl mutant in which Cys (+4) and Cys (+11) were mutated to Ser and Glu residues, respectively. As shown in Fig. 4 *B*, MBS-mediated cross-linking to the ΔCys (+4, +11) pPl 86-mer was observed to occur at high efficiency and to yield a covalent complex of the same M_r as the WT intermediate (Fig. 4 *B*). Phosphorimager analysis of the cross-linked products in Fig. 4 *B* indicated that cross-linking efficiency of the ΔCys (+4, +11) mutant was ~60% of that observed for the WT, indicating that a substantial amount of cross-linking occurs through the signal sequence. These data suggest that the signal sequence of the pPl 86-mer translocation intermediate resides in close physical proximity to a 34–36-kD integral membrane protein. On the basis of the known molecular dimensions of MBS, it can be concluded that the signal sequence of the pPl 86-mer translocation intermediate encounters the 34–36-kD integral membrane protein at a molecular distance of <9.9 Å.

Identity of Cross-Linked Product

Recent cross-linking studies have provided compelling evidence that Sec61p is the predominant integral membrane protein residing in physical proximity to translocating nascent chains (Görlich et al., 1992*b*; Sanders et al., 1992; Görlich and Rapoport, 1993; High et al., 1993*a*,*b*; Mothes et al., 1994). In addition to Sec61p, cross-links to the membrane protein TRAM have been reported to occur in translocation substrates in which the cross-linking moiety is located near the amino terminus (High et al., 1993*b*; Mothes et al., 1994). To determine the identity of the MBS-dependent cross-linked product, the membrane fraction from completed reactions was recovered by centrifugation, solubilized with detergent, and subjected to immunoprecipitation with anti-peptide antibodies directed against either TRAM or Sec61p (Fig. 5). In the experiment shown in Fig. 5, MBS-mediated cross-linking of pPl 86-mer translocation intermediates resulted in efficient formation of a covalent complex (Fig. 5, lanes *1* and *2*). The cross-linked complex was not recovered by immunoprecipitation with the TRAM preimmune serum (Fig. 5, lane *3*); a small fraction (<5%) was, however, present in reactions containing the TRAM immune serum (lanes *3* and *4*). Cross-linked products observed with the anti-TRAM antisera were also observed when the reactions were performed in the presence of excess TRAM peptide and are thus not considered specific (Fig. 5, lanes *4* and *5*). The cross-linked product was efficiently recovered in immunoprecipitation reactions conducted with both unfractionated and affinity-purified Sec61p antisera, whereas no product was recovered with preimmune serum or when reactions were conducted in the presence of the Sec61p peptide (Fig. 5, lanes *6–10*). It is clear from these data that the predominant MBS-dependent cross-linking product obtained with the pPl 86-mer is Sec61p. The differences between the predicted and the apparent Sec61p molecular mass on SDS-PAGE (54 and 34–36 kD, respectively) have previously been reported and reflect the extraordinary hydrophobicity of the protein (Stirling et al., 1992). These data are in general agreement with previous findings reported in studies using photocross-linking agents, with the exception that we have been unable with the chemical cross-linker MBS to detect cross-links to the TRAM protein (Görlich et al., 1992*a*; Görlich and Rapoport, 1993).

Variations in Cross-Linking Efficiency as a Function of Nascent Chain Length

Photocross-linking techniques have proven instrumental in the identification of candidate components of the ER protein translocation apparatus (Wiedmann et al., 1987; Krieg et al., 1989; Thrift et al., 1991; Görlich et al., 1992b; High et al., 1993*a*,*b*; Görlich and Rapoport, 1993). From a theoretical standpoint, photocross-linking reagents, because of their relatively limited reactivity lifetimes upon activation and the expected reduced frequencies of nonspecific cross-linking, are considered the reagents of choice (Ji, 1979; Brunner et al., 1980). These considerations notwithstanding, a unique aspect of the chemical cross-linking studies described here has been the unusually high yields of cross-linked product,

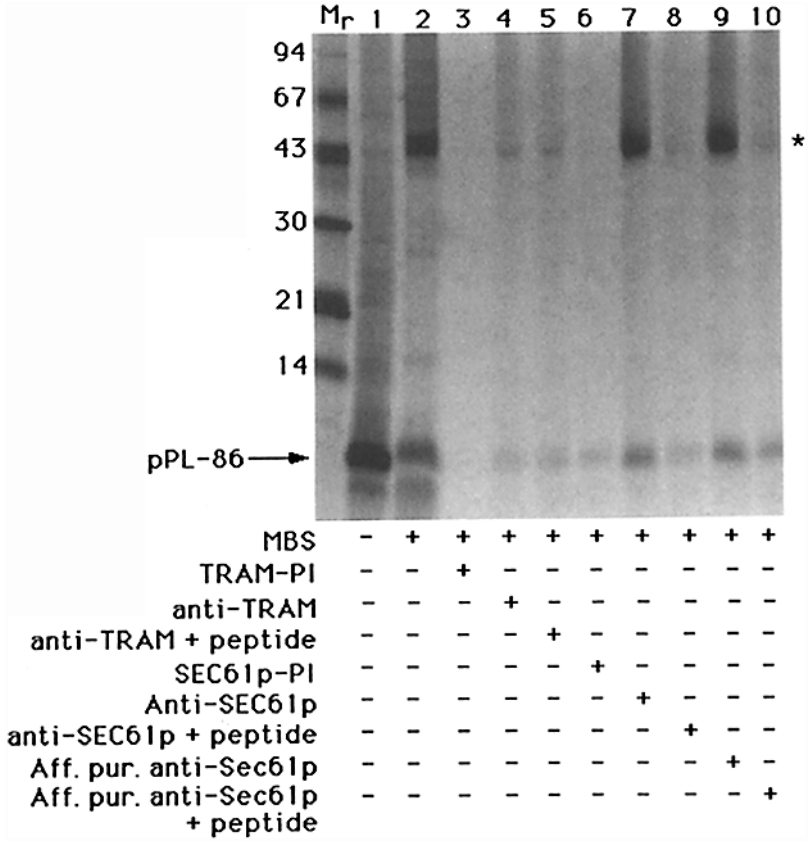

Figure 5. Characterization of cross-linked complexes by immunoprecipitation. pPl 86-mer translocation intermediates were synthesized in a reticulocyte lysate translation system and subjected to MBS-dependent cross-linking as described in the legend to Fig. 4. Quenched samples were centrifuged through a 0.5 M sucrose cushion, and the pellet fraction was processed for indirect immunoprecipitation as described in Materials and Methods. Samples were subject to immunoprecipitation with preimmune (*PI*) sera or anti-peptide immune sera directed against a peptide representing the carboxy-terminal of TRAM or the amino-terminal of Sec61p. Peptide competition experiments were performed by preincubation of serum or IgG fractions with excess peptide.

relative to that obtained by photocross-linking approaches. Indeed, others have reported an increase in cross-linking efficiencies in chemical, versus photochemical, cross-linking studies (Görlich et al., 1990; Kellaris et al., 1991). We have observed that MBS-dependent cross-linking of the pPl 86-mer and the VSV-G 90-mer to Sec61p can be performed under conditions in which extremely high yields (60–80%) of cross-linked products are obtained (Fig. 6). This observation has significant experimental ramifications. For example, in view of such high efficiencies, it is likely that the appearance of the cross-linked product represents a sampling of the physical environment of the majority of translocating nascent chains, rather than that of a small subpopulation. Furthermore, because the molecular environment of the majority of translocating chains is being sampled, additional elements of the translocation process, including analyses of stage- and ribosome-specific effects on nascent chain–Sec61p interactions, can be studied.

Although the SCM form of the pPl 86-mer translocation intermediate can be cross-linked with high efficiency to Sec61p, little or no cross-linking was observed after puromycin treatment, even though the precursor remained in association with components of the ER membrane (Fig. 3). To ascertain whether the puromycin-dependent loss of cross-linking was simply a consequence of chain termination or, alternatively, due to a change in the topology of the precursor in the membrane, translocation intermediates of both the WT and SCM forms of the pPl 86-, 131-, and 169-mer, as well as the WT and SCM forms of the VSV-G 90- and 172-mer, were studied. As shown in Fig. 6, both the WT and SCM forms of relatively short translocation intermediates (pPl 86-mer and VSV-G 90-mer) were cross-linked to Sec61p at the expected efficiencies (Fig. 6, *A* and *B*, lanes *1–4*). When both the WT and SCM forms of the pPl 131-mer were assayed, cross-links to Sec61p were observed at somewhat reduced efficiencies (Fig. 6 *A*, lanes *5–8*). Note that the pPl 131-mer translocation intermediate is of sufficient length to undergo partial signal sequence processing and thus represents a later stage of the translocation process than that assayed with the pPl 86-mer. When the size of the translocation intermediate was extended to either 169 (pPl) or 172 (VSV-G) amino acids, the yields of cross-linked product were dramatically reduced (Fig. 6, *A* and *B*, lanes *9–12* and *5–8*, respectively). Quantitation, by phosphorimager analysis of anti-Sec61p immunoprecipitations, of the data depicted in Fig. 6 indicated that the yields of cross-linked product for the 169- and 172-mer were ~5–10% of those obtained with the 86- and 90-mer, respectively. This result was unexpected, since we had observed that for short nascent chains a substantial portion of the cross-links occurred through the signal sequence. Because the nascent chain is present in the membrane in a loop topology, we had assumed that SCM translocation intermediates would cross-link to Sec61p through the signal sequence, regardless of nascent chain length. It appears, therefore, that the topological relationship between the signal sequence of the translocating nascent chain and Sec61p varies through the course of translocation. In addition, because the cross-linking behavior of the WT and SCM forms of the various translocation intermediates are identical, it can be inferred that the topological relationship between Sec61p and the mature portion of the nascent chain also varies through the course of translocation.

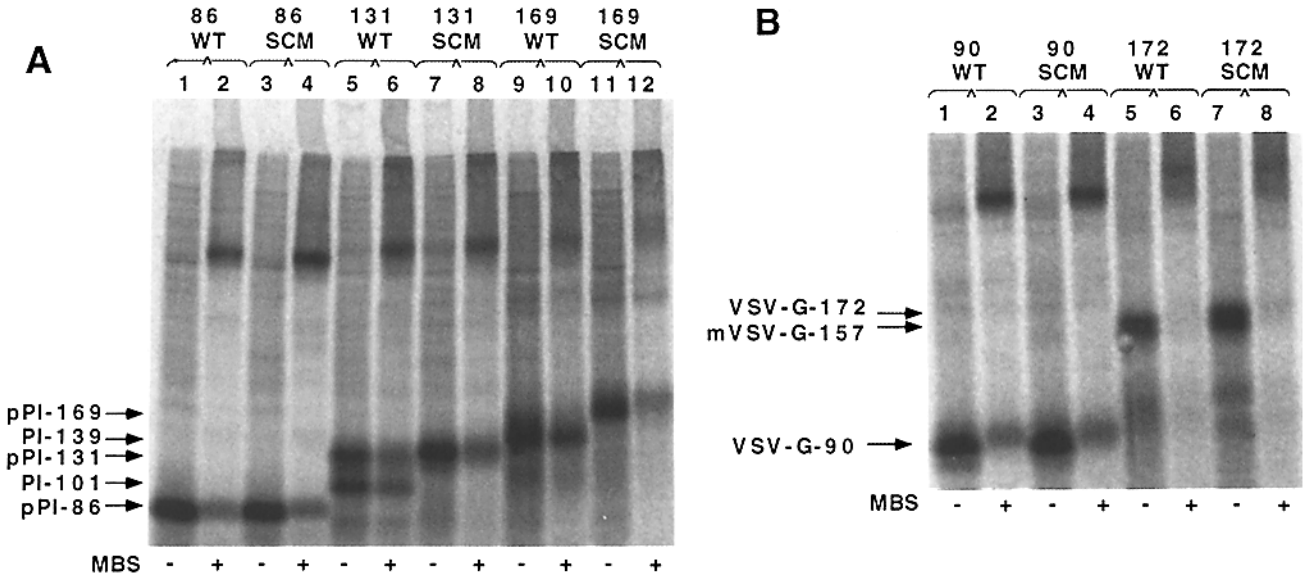

Figure 6. Variations in MBS-mediated nascent chain–Sec61p complex formation as a function of nascent chain length. Translations of WT and SCM forms of the pPl 86-, 131-, and 169-mer (*A*) and the VSV-G 90- and 172-mer (*B*) were performed in a reticulocyte lysate translation system supplemented with RM. After assembly of the translocation intermediates, membrane-associated nascent chains were collected by centrifugation through a 0.5 M sucrose cushion (as described in Materials and Methods) resuspended in membrane buffer, and subjected to cross-linking as described in the legend to Fig. 4. Cross-linking reactions were quenched, and samples were precipitated with 10% TCA. Cross-linked complexes were resuspended in SDS-PAGE buffer and analyzed as described in the legend to Fig. 2. The relative migration of the different translation products is indicated by arrows. Note that the pPl 131-mer is partially processed to the Pl 101-mer and the pPl 169-mer is fully processed to the Pl 139-mer (*A*). For *A*, the stacking gel has been included to ascertain whether the apparent lack of MBS-dependent complex formation was due to the formation of high molecular weight aggregates that were unable to enter the separating gel.

Effects of Ribosome Disassembly on Nascent Chain–Sec61p Interactions

On the basis of the results shown in Fig. 6, it is apparent that near-neighbor interactions between Sec61p and the translocating nascent chain vary over the course of translocation. To gain further insight into this phenomenon, the role of the ribosome in the regulation of nascent chain–Sec61p interactions was examined. Recent experimental evidence indicates that Sec61p, in addition to its role as a potential protein-conducting channel, functions in the binding of ribosomes to the ER membrane (Görlich et al., 1993; Kalies et al., 1994). To determine the contribution of the ribosome to nascent chain–Sec61p near-neighbor interactions, translocation intermediates of the WT and SCM forms of the pPl 86-mer were assembled and chilled on ice, which serves to block any further translocation (Nicchitta and Blobel, 1989). Subsequently, ribosomes were dissociated by treatment with EDTA (Gesteland, 1966). At the EDTA concentrations used in these assays (10 mM), the ribosome dissociates into its component subunits and both the large and the small ribosomal subunits are released from the membrane (Sabatini et al., 1966; Pryme, 1988; data not shown). The topology of the nascent chain was then assayed by protease digestion. The results of these experiments are shown in Fig. 7. For both the WT and SCM forms of the pPl 86-mer, digestion of the ribosome-associated nascent chains, in the absence of membranes, yielded a limit digestion product of 3–4 kD (Fig. 7 *A*, lanes *1* and *2*; *7* and *8*). The limit digestion product represents that part of the nascent chain protected by the ribosome (Fig. 2) (Malkin and Rich, 1967; Blobel and Sabatini, 1970). Translocation intermediates of both the WT and SCM forms of the pPl 86-mer were resistant to proteolytic digestion (Fig. 7 *A*, lanes *3* and *4*; *9* and *10*), an observation consistent with previous reports (Connolly and Gilmore, 1986; Nicchitta and Blobel, 1989). More significantly, however, dissociation of the ribosome under conditions (reduced temperature) in which translocation does not occur resulted in nearly complete accessibility of the precursor to digestion by exogenous protease (Fig. 7 *A*, lanes *5* and *6*; *11* and *12*).

The precise size of the domain of the translocation intermediate exposed after ribosome dissociation has not been determined. This domain is expected to include the 3–4-kD domain that resides within the intact ribosome. Although the Tris-Tricine gel system used in these experiments allows resolution of fragments as small as 1.5–2 kD, we have been unable to identify limit digestion products of the translocation intermediates after ribosome dissociation. It appears, therefore, that the size of the exposed domain may be as large as 6.6 kD.

To investigate the effects of ribosome dissociation on nascent chain–Sec61p near-neighbor interactions, pPl 86-mer translocation intermediates were formed, chilled on ice, treated with EDTA, and subsequently cross-linked with MBS. As shown in Fig. 7 *B*, when pPl 86-mer translocation intermediates were treated with MBS, on ice, efficient formation of the 43-kD cross-linked product, representing a covalent complex of the nascent chain with Sec61p, was observed (lanes *1* and *2*). When the ribosome–nascent chain–membrane complexes were treated with EDTA before cross-linking with MBS, the yield of the cross-linked product was greatly reduced. On average, EDTA pretreatment re-

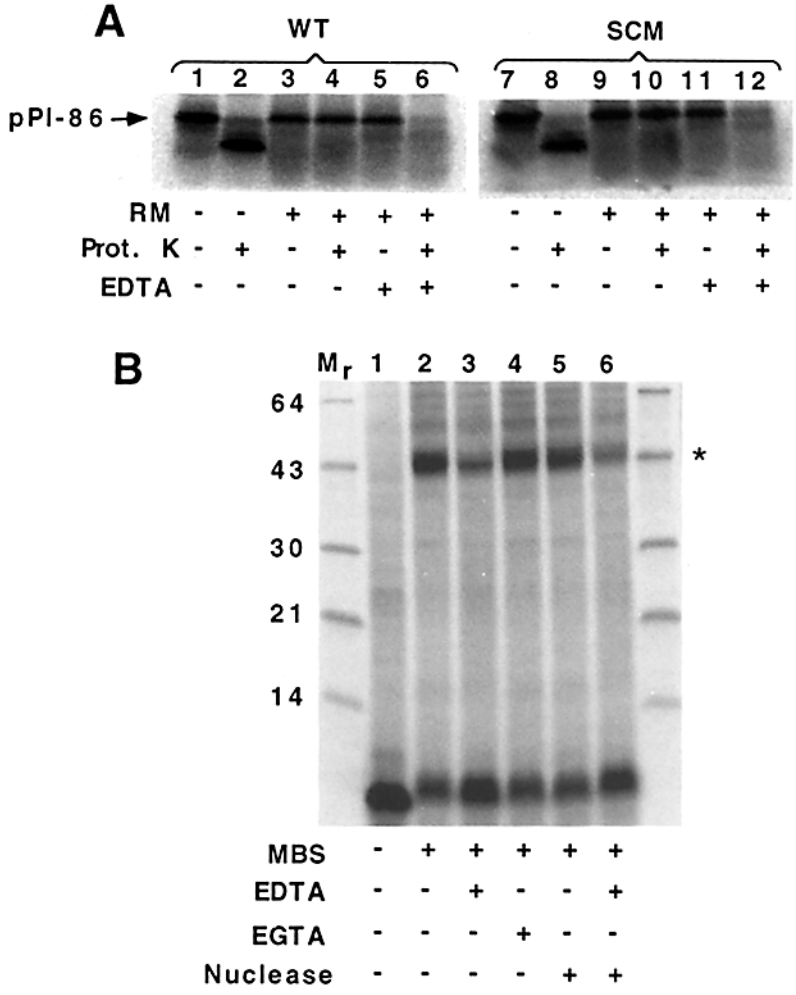

Figure 7. The effects of EDTA-mediated ribosome dissociation on pPl 86-mer processing, translocation, and near-neighbor interaction with Sec61p. (*A*) WT and SCM forms of the pPl 86-mer were translated in the presence or absence of RM in a reticulocyte lysate translation system for 30 min at 25°C. After translation, reactions were chilled on ice and, where indicated, adjusted to 10 mM EDTA. After a 30-min incubation on ice, proteinase K was added, and digestions were performed for 30 min on ice. Protease digestion reactions were quenched and processed for SDS-PAGE as described in Materials and Methods. Samples were analyzed as described in the legend to Fig. 2. The faster migrating form shown in lanes *2* and *8* represents the ribosome protected fragment observed in the absence of RM. (*B*) pPl 86-mer translocation intermediates were assembled as described in *A*. Reactions were chilled on ice and adjusted to either 10 mM EDTA (lane *3*), 10 mM EGTA (lane *4*), 250 U/ml staphylococcal nuclease (lane *5*), or 10 mM EDTA, 250 U/ml staphylococcal nuclease (lane *6*). Incubations were performed for 30 min on ice, and samples were subsequently cross-linked by addition of MBS to 1 mM. Cross-linking reactions were performed for 60 min at 4°C. The primary cross-linked product is indicated by the asterisk.

duced the formation of the nascent chain–Sec61p product by 60–70% (n = 5). This effect was apparently specific for Mg^{2+}, as treatment with equivalent concentrations of EGTA, which exhibits preferential binding to Ca^{2+}, was without effect (Fig. 7 *B*, lane *4*). In addition, treatment with ribonuclease did not alter the yield of the 43-kD cross-linked product (Fig. 7 *B*, lane *2* versus lane *5*). Pretreatment with a combination of EDTA and nuclease resulted in a further, although marginal, decrease in the yield of cross-linked product.

It is apparent that when a ribosome bearing the translocation intermediate is dissociated into its subunits, the efficiency of nascent chain–Sec61p cross-link formation is greatly reduced. These data indicate that efficient near-neighbor interactions between the translocating nascent chain and Sec61p may arise primarily through interactions between the ribosome and Sec61p, a conclusion consistent with the observation that Sec61p is a ribosome-binding protein (Kalies et al., 1994). Because these interactions are to a great extent lost upon dissociation of the ribosome into its subunits, translocation intermediates of the pPl 86-mer were assayed to determine whether disruption of nascent chain–Sec61p interactions would affect translocation of the cytosolic domain of the nascent chain intermediate. The results of a representative experiment are depicted in Fig. 8. In this experiment, translocation intermediates of the pPl 86-mer were first assembled at 25°C, and the efficiency of translocation at 25°C was assessed after either dissociation of the ribosome (plus EDTA) or upon polypeptide termination (plus puromycin). Puromycin addition resulted in both efficient processing and translocation of pPl 86-mer nascent chains (Fig. 8, lanes *2* and *3*). Similarly, dissociation of the ribosome by EDTA addition yielded efficient processing and translocation of the pPl 86-mer (Fig. 8, lanes *5* and *6*). Thus, under the described experimental conditions, disruption of the ribosome by EDTA addition had only marginal effects on the efficiency of translocation.

In experiments in which the effects of ribosome dissociation on near-neighbor, nascent chain–Sec61p interactions were determined (Fig. 7 *B*), the ribosome–nascent chain–membrane complexes were preincubated in the presence of EDTA for 30 min at 4°C before chemical cross-linking. This extended incubation time may have allowed diffusion of the translocating nascent chain from Sec61p and thus the loss of MBS-dependent complex formation. To compare the effects of ribosome dissociation on translocation under conditions comparable to those used for cross-linking, translocation intermediates of the pPl 86-mer were pre-

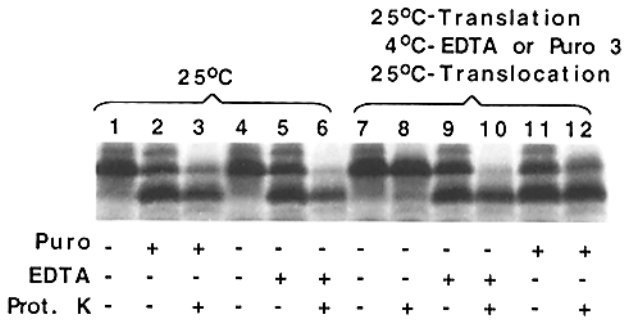

Figure 8. The effects of EDTA-mediated ribosome dissociation on translocation of the pPl 86-mer. Translocation intermediates of the pPl 86-mer were assembled by translation in a reticulocyte lysate system for 30 min at 25°C. After a 30-min translation period, samples were adjusted, as indicated, to 0.5 mM puromycin (lanes *2* and *3*) or 10 mM EDTA (lanes *5* and *6*), and the reaction was continued for 10 min at 25°C. Paired samples were chilled on ice and subsequently adjusted to either 10 mM EDTA or 0.5 mM puromycin (lanes *9* and *10* or *11* and *12*, repectively). After a 30-min incubation on ice, reactions were brought to 25°C for 15 min. To determine the efficiency of translocation, samples were subjected to proteinase K digestion for 30 min at 4°C with 100 μg/ml proteinase K and processed for SDS-PAGE as described in Materials and Methods. In lanes *1–6*, translocation reactions were performed immediately after translation. In lanes *7–12*, reactions were chilled on ice, supplemented as previously described, and subsequently rewarmed to 25°C.

pared, chilled on ice, and treated with either EDTA or puromycin for 30 min. Samples were subsequently warmed to 25°C for 10 min, to allow translocation to continue. As shown in Fig. 8, lanes *9–12*, prolonged dissociation of the ribosome, under conditions in which cross-linking of the nascent chain to Sec61p was markedly reduced was without effect on translocation, as judged by protease protection. For both the EDTA- and puromycin-treated samples, signal peptide processing occurred at 50–70% efficiency, and of the processed chains, 60–80% were protected from proteolytic digestion. These data indicate that disruption of near-neighbor interactions between translocating nascent chains and Sec61p is not accompanied by a loss of translocation activity.

Discussion

Current models of protein translocation across the ER membrane invoke a central role for Sec61p in the translocation of secretory protein precursors across, and the assembly of integral membrane proteins into, the ER membrane (Stirling et al., 1992; Sanders et al., 1992; Görlich et al., 1992b; Görlich and Rapoport, 1993; Mothes et al., 1994). Sec61p, the product of the *SEC61* gene, was initially identified in genetic screens for components of the translocation apparatus necessary for the insertion of secretory and membrane proteins into the yeast ER membrane (Deshaies and Schekman, 1987; Stirling et al., 1992). Subsequently, Sec61p was observed, by photo- and chemical cross-linking, to reside in close physical proximity to translocating nascent chains in both yeast and mammalian ER (Sanders et al., 1992; Müsch et al., 1992; Görlich et al., 1992b; High et al., 1993*a*,*b*). In addition, recent biochemical reconstitution studies have identified Sec61p and the signal recognition particle receptor as the only protein components essential for translocation across the ER membrane, although other membrane proteins, such as TRAM, may further enable the translocation of specific precursors (Görlich and Rapoport, 1993). On the basis of these results, Sec61p has been proposed to function either as a protein-conducting channel (Görlich and Rapoport, 1993; Mothes et al., 1994) or as a component that transports the nascent chain across the ER membrane (Sanders et al., 1992).

To elaborate further a molecular description of the mechanism of protein translocation, we have used chemical cross-linking as a tool to identify ER proteins residing in physical proximity to translocating nascent chains at discrete stages of the translocation process. These studies were performed with WT and SCM forms of pPl and VSV-G. The SCM precursors contained point mutations in the signal cleavage domain that blocked processing of the nascent chain. With relatively short nascent chains (≤90 amino acids), cross-links to Sec61p were observed to occur at remarkably high (60–80%) efficiency. The efficiency of Sec61p translocation intermediate cross-linking decreased, however, as a function of increasing chain length. These data suggest that near-neighbor interactions between translocating nascent chains and Sec61p predominate at early stages of translocation and are consistent with recent models in which precursor proteins are thought to assemble inititially into the translocation apparatus in contact with Sec61p (Sanders et al., 1992). Other investigators have previously identified cross-linked complexes of late stage translocation intermediates and Sec61p, and thus it is clear that such intermediates do encounter, at least for time periods sufficient for cross-linking, the Sec61p complex (Wiedmann et al., 1989; Müsch et al., 1992; Görlich et al., 1992b; Mothes et al., 1994). In these studies, however, cross-linking was performed with photocross-linking reagents, in which the yield of cross-linked product is by nature relatively low. With such low yields, it is difficult to ascertain directly whether the appearance of photocross-linked nascent chain–Sec61p complexes accurately reflects the membrane disposition of the majority population of nascent chains.

To extend further the observation of stage-specific cross-linking, experiments were performed with a series of truncated intermediates containing mutations in the signal sequence cleavage site that blocked processing. The rationale for these experiments was as follows. It is known that the nascent chain assembles into the membrane in a loop conformation (Shaw et al., 1988; Mothes et al., 1994). Furthermore, in studies of a SCM form of VSV-G, it was observed that the loop topology of the nascent chain was maintained throughout translocation and that the amino terminus of the signal sequence remained exposed on the cytoplasmic face of the ER membrane, even after the completion of translation (Shaw et al., 1988). In addition, recent cross-linking studies using a novel suppressor tRNA approach have demonstrated that the signal sequence is immediately adjacent to Sec61p (High et al., 1993*b*). Integrating these observations with current models of the mammalian translocation apparatus, we reasoned that the signal sequence of the SCM forms of translocating nascent chains would remain in association with components of the translocation machinery, such as Sec61p,

throughout translocation. In fact, the SCM forms of the pPl 86-mer and the VSV-G 90-mer cross-link efficiently to Sec61p (Figs. 4 and 6). Site-directed mutagenesis of the potential cross-linking sites in the pPl 86-mer indicated that at least 50% of the cross-links occurred through residues present in the signal sequence, indicating a near-neighbor interaction between the signal sequence and Sec61p, in agreement with High et al. (1993*b*). Although the short chain SCM intermediates could be efficiently cross-linked to Sec61p, late stage SCM intermediate–Sec61p cross-links were observed at very low efficiencies. These data support the conclusion that near-neighbor interactions between the translocating nascent chain and Sec61p predominate at early rather than late stages of the translocation process.

Several interpretations of these data, with respect to models in which Sec61p defines the immediate molecular environment of the nascent chain, are possible. The decrease in cross-linking of the longer SCM intermediates to Sec61p could be reconciled as a simple steric phenomenon, in which an appropriate arrangement of amino acid side chains is lacking in the longer translocation intermediates. Although this explanation may be valid, suitable amino acids are present throughout the pPl intermediates and are especially abundant in the VSV-G intermediates (Fig. 1). Furthermore, a comparison of the relative locations of potential cross-linking sites in the amino termini of the pPl and VSV-G constructs, as well as the carboxy-terminal domains extending from the ribosome protected region towards the amino terminus of the pPl 131-mer and VSV-G 172-mer, shows a nearly identical order of suitable amino acids. On the basis of these similarities, the cross-linking pattern of the pPl 131-mer and the VSV-G 172-mer should be similar. That they are not is likely a reflection of topological alterations in the nascent chain–Sec61p interactions during translocation. Because ~50% of the cross-linking of short chain intermediates was observed to occur through the signal sequence, it is perhaps more likely that at an intermediate stage of translocation, the topological relationship between the signal sequence of the SCM intermediate and Sec61p, is altered. How might this occur? Because the signal sequence of a SCM form of VSV-G has previously been demonstrated to act as a signal anchor domain (Shaw et al., 1988), it is possible that during translocation, the signal sequence of the SCM intermediates diffuses from the protein-conducting channel and integrates into the lipid bilayer. This interpretation places significant constraints on the point at which such topological alterations might occur. For example, pPl 131-mer translocation intermediates undergo partial amino-terminal processing and are therefore exposed to the ER lumen. Cross-linking between Sec61p and the WT and SCM pPl 131-mer is, however, efficient. Should the decrease in cross-linking reflect diffusion of the signal sequence from the channel, these results indicate that diffusion would be delayed until the translocation intermediate exceeds a length of ~131 amino acids.

The high efficiency of MBS-dependent cross-linking of short chain translocation intermediates to Sec61p strongly supports the conclusion that the observed cross-links are reflective of the behavior of the total population of nascent chains, rather than that of a discrete subpopulation. An intriguing aspect of these studies is the strong dependence of high efficiency cross-link formation on the presence of the intact ribosome. In this regard, it is particularly significant that recent studies have demonstrated that Sec61p can function as a ribosome receptor (Görlich et al., 1992b; Kalies et al., 1994). Experiments were therefore performed to characterize the role of the ribosome in the genesis of nascent chain–Sec61p cross-links. Ribosome-associated translocation intermediates of either the WT or SCM forms of the pPl 86-mer were assembled and chilled on ice, and the ribosomes were dissociated, by chelation of Mg^{2+}, into their large and small subunits (Gesteland, 1966). Dissociation of the ribosome results in exposure of the translocation intermediate to the cytoplasmic face of the membrane, as demonstrated by the dramatic increase in the sensitivity of the precursor to digestion by exogenous protease. When the physical interaction of the nascent chain with Sec61p was assayed under similar conditions by chemical cross-linking, the efficiency of cross-linking was reduced by 60–70 %. This decrease in near-neighbor interactions between the nascent chain and Sec61p was apparent even when cross-linking times were extended to 2 h, conditions in which random, collision-dependent cross-links would be expected to occur (data not shown) (Ji, 1979). The relative inability to identify translocation intermediate–Sec61p cross-links after dissociation of the ribosome was not a simple consequence of reduced temperature; cross-linking of ribosome-associated nascent chains to Sec61p was as efficient at 4 as at 25°C. These data, in combination with those previously discussed, best support a model in which near-neighbor interactions between the translocating nascent chain and Sec61p are greatly potentiated by physical interactions between the ribosome and Sec61p. In the absence of such interactions, the translocating nascent chain appears to sample, but not reside in, an environment physically adjacent to Sec61p. This scenario can be easily rationalized if the following criteria are satisfied: (*i*) at early stages of translocation, the nascent chain exit site on the ribosome is in close proximity to Sec61p, thus providing a suitable environment for cross-linking of short nascent chains to Sec61p, and (*ii*) at late stages of translocation, translation and translocation are not efficiently coupled, thereby removing the topological constraint necessary for efficient cross-linking to Sec61p. The predominance of studies demonstrating cross-linking of short chain translocation intermediates to Sec61p clearly supports the first criterion (Wiedmann et al., 1986; Görlich et al., 1992; High et al., 1993*a*,*b*). With regard to the second criterion, translocation intermediates are known to exist in at least two populations, one of which is sensitive to digestion with exogenous proteases (Blobel and Sabatini, 1970). Given the topology of the translocation reaction, the appearance of protease sensitivity must reflect either an uncoupling of translation and translocation, which would allow the precursor to form an exposed loop between the ribosome and the membrane, or dissociation of the ribosome from the membrane during translocation. More recent studies with truncated pPl and VSV-G translocation intermediates have clearly demonstrated that either dissociation of the ribosome from the ER membrane or formation of an exposed domain of the nascent chain occurs when precursors exceed a length of ~100 amino acids (Connolly et al., 1989).

By similar experimental protocols, assays were performed to determine whether disruptions in the near-neighbor nascent chain–Sec61p interaction affected the process of trans-

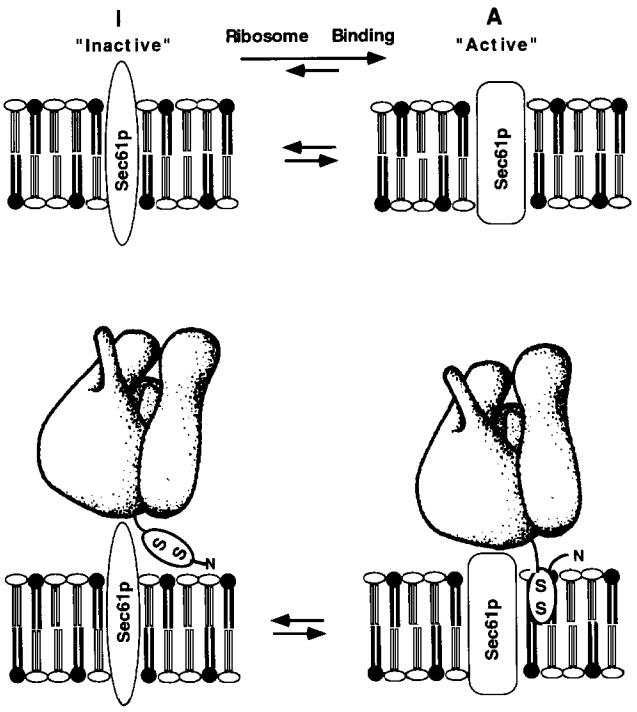

Figure 9. Conformational equilibrium model for Sec61p function. Sec61p is depicted as existing in two conformational states, I and A, representing, respectively, the inactive and active conformations of the protein. In this model, the interaction of the translationally active ribosome with Sec61p promotes conversion of Sec61p from the I to the A state. In the A state, Sec61p assists assembly of the signal sequence with components of the ER membrane at a site distinct from, yet physically near, Sec61p.

location. It was observed that at 25°C, dissociation of the ribosome allowed translocation of the cytosol-disposed region of truncated nascent chains. When translocation intermediates of the pPl 86-mer were chilled on ice, the ribosome dissociated, and when the membrane–nascent chain complexes were subsequently warmed to 25°C, translocation proceeded at identical efficiency. On the basis of these data, it should be considered that translocation across the ER membrane may not require a continuous, direct physical interaction of the nascent chain with Sec61p, but rather Sec61p would serve an essential function in the initiation of translocation, and perhaps other protein components would function in the completion of translocation. Although it is presently unclear what additional factors may assist translocation or provide an environment suitable for transport of the nascent chain, that recent experimental evidence in *Escherichia coli* indicates that translocation across the bacterial inner membrane is driven primarily by the SecA protein, bound to the membrane, in part through interactions with SecY, a bacterial homologue of Sec61p (Lill et al., 1990; Watanabe and Blobel, 1993; Economou and Wickner, 1994; Kim et al., 1994; Stirling et al., 1992; Görlich et al., 1992b). By analogy to *E. coli*, perhaps Sec61p functions in the assembly of additional components to the translocation site, as has been proposed for SecY (Economou and Wickner, 1994; Kim et al., 1994). In this regard, it is interesting to note that in bacterial inverted vesicles, late stage proOmpA translocation intermediates can be chemically cross-linked to SecY (Joly and Wickner, 1993). In this system, the late stage near-neighbor nascent chain–SecY interactions may well be a consequence of a direct physical association of SecA with SecY, rather than interactions between the nascent chain and SecY.

It is clear that at early stages of translocation, nascent chains are in close physical proximity to Sec61p. It appears equally as clear, however, that the apparent near-neighbor relationship between the translocating nascent chain and Sec61p is greatly influenced by the continued association of the nascent chain with the ribosome. Although such a model readily explains the near-neighbor interactions between translocating nascent chains and Sec61p, when such interactions are assayed cotranslationally, experimental observations of posttranslational (i.e., ribosome-independent) interactions between nascent chains and Sec61p are more difficult to rationalize (Sanders et al., 1992; Klappa et al., 1994). To integrate these apparently disparate observations, we propose the following "conformational equilibrium" model, which is schematically illustrated in Fig. 9. In this model, Sec61p is depicted as existing in kinetic equilibrium between two distinct conformational states. In conformation state A, Sec61p defines the site at which translocation is initiated, whereas in conformation state I, Sec61p is inactive and translocation cannot occur. The equilibrium between the two conformational states is depicted as being influenced by the binding of the ribosome, which favors the formation of the A state. Dissociation of the bound ribosome into its component subunits is therefore predicted to result in a shift in Sec61p from the A to the I state and a subsequent alteration in the topological relationship between the nascent chain and Sec61p.

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