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Site-specific Photocross-linking Reveals That Sec61p and TRAM Contact Different Regions of a Membrane-inserted Signal Sequence*

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A chemically charged amber suppressor tRNA was used to introduce the photoactivatable amino acid (Tmd)Phe at a selected position within the signal sequence of the secretory protein preprolactin. This allowed the interactions of the NH_2-terminal, the central, and the COOH-terminal regions of the signal sequence to be investigated during insertion into the membrane of the endoplasmic reticulum (ER). We found that different regions of the nascent chains were photocross-linked to different ER proteins. The TRAM protein (translocating chain-associating membrane protein) contacts the NH_2-terminal region of the signal sequence while the mammalian Sec61p contacts the hydrophobic core of the signal sequence and regions COOH-terminal of this. These results suggest that the ER translocation complex is composed of heterologous protein subunits which contact distinct regions of nascent polypeptides during their membrane insertion.

The translocation of proteins across the membrane of the rough endoplasmic reticulum (ER),[1] or their insertion into it, is mediated by a series of sequential protein-protein interactions. The first step in this process requires the targeting of the protein to the ER membrane. ER-specific signal sequences present within the nascent chain of proteins (von Heijne, 1988) are recognized by a cytosolic ribonucleoprotein complex, the signal recognition particle (SRP), which functions to target the resulting nascent chain-ribosome-SRP complex to the ER. Specific binding of the nascent chain-ribosome-SRP complex to the ER membrane is mediated by a membrane-bound receptor, the docking protein (or SRP receptor) (Rapoport, 1990). Following interaction with the docking protein, the nascent chain is released from SRP, and membrane insertion is initiated (High and Dobberstein, 1992). Recent electrophysiological data suggest that subsequent translocation across the membrane occurs via a protein-conducting channel (Simon and Blobel, 1991).

Photocross-linking has been successfully used to analyze the interactions of nascent chains during their insertion into the membrane of the ER (High *et al.*, 1991; Krieg *et al.*, 1989; Wiedmann *et al.*, 1987). Two groups of ER proteins have been identified as being adjacent to translocation intermediates of both secretory and membrane proteins (for recent review see High, 1992). Glycoproteins of 35–39 kDa were found to be the major cross-linking partners of short nascent chains of the secretory protein preprolactin (Görlich *et al.*, 1992a; Krieg *et al.*, 1989; Wiedmann *et al.*, 1987). The principal glycoprotein which is cross-linked to these nascent chains is the 36-kDa TRAM protein (Görlich *et al.*, 1992a). Glycoproteins of similar molecular weight have also been found in the proximity of some membrane proteins, although often as minor components (High *et al.*, 1991; Thrift *et al.*, 1991). A 37-kDa nonglycosylated protein (P37) has been shown to be the major cross-linking partner of membrane proteins with uncleaved signal-anchor sequences (High *et al.*, 1991, 1993), and a similar protein was observed when two other membrane proteins were used (Thrift *et al.*, 1991). When longer nascent preprolactin chains are analyzed by photocross-linking an interaction with nonglycosylated proteins is also detected (Krieg *et al.*, 1989). The major nonglycosylated protein which is cross-linked to these nascent preprolactin chains has recently been identified as the mammalian homologue of Sec61p (Görlich *et al.*, 1992b). When cross-linking analysis with a homobifunctional reagent was performed a 34-kDa nonglycosylated protein (imp34) was the only detectable neighbor of preprolactin. This interaction was independent of the length of nascent chain which was used (Kellaris *et al.*, 1991).

The biosynthetic photocross-linking approach used to date has depended upon the incorporation of modified lysine residues during cell-free translation (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986). Since several lysine residues are often present in a polypeptide, it is difficult to identify the ER components which contact specific regions of the nascent chain. In addition, acylation of the ϵ-amino group of lysyl residues removes a positive charge, and this may result in altered interactions. Although in the case of preprolactin it has proved possible to distinguish photocross-linking via the signal sequence from

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[1] The abbreviations used are: ER, endoplasmic reticulum; AF, arrested fragment; PPL, preprolactin; SRP, signal recognition particle; (Tmd)Phe, L-4′-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenylalanine; TRAM protein, translocating chain-associating membrane protein; $tRNA^{sup}$, suppressor tRNA; HPLC, high performance liquid chromatography; Boc, *t*-butoxycarbonyl.

photocross-linking via the rest of the nascent chain (Krieg *et al.*, 1989; Wiedmann *et al.*, 1989), it has not been possible to identify components interacting with distinct parts of the signal sequence during membrane insertion.

In this study we make use of a novel method for the incorporation of a photocross-linkable amino acid at a single predetermined position within a nascent chain. A cell-free translation system is supplemented with an amber suppressor tRNA ($tRNA^{sup}$), chemically charged with the photosensitive amino acid L-4′-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenylalanine ((Tmd)Phe). This amino acid is incorporated into the nascent chains in response to a UAG stop codon in the mRNA and, upon photoactivation, forms a covalent cross-link to adjacent components. This technique was applied here to investigate the interactions between three distinct sites within the signal sequence of preprolactin (PPL) and components of the ER membrane translocation complex. During the membrane insertion of the signal sequence, we find clear evidence that different regions are in contact with different ER proteins. The NH_2-terminal region of the signal sequence is in contact with a 36-kDa glycoprotein denoted the "translocating chain-associating membrane" (TRAM) protein (Görlich *et al.*, 1992a). The remainder of the signal sequence, together with a region COOH-terminal of the signal sequence cleavage site, was found to contact the recently identified mammalian Sec61p (Görlich *et al.*, 1992b). We propose that the membrane translocation complex of the ER consists of heterologous protein subunits, and that Sec61p is a core component of this complex.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and yeast $tRNA^{Phe}$ were from Boehringer Mannheim GmbH (Mannheim, Germany). T4 RNA ligase was from Pharmacia LKB GmbH (Freiburg, Germany) and pGEM4 from Promega Corporation (Madison, WI). T7 RNA polymerase was from New England Biolabs GmbH (Schwalbach, Germany) and USB GmbH (Bad Homburg, Germany). RNasin was obtained from USB GmbH (Bad Homburg, Germany). The plasmid pYPhe2 was a gift from Dr. Peter Schultz (University of California, Berkeley, CA). Qiagen columns were from Diagen GmbH (Düsseldorf, Germany). [^{35}S]Methionine and the site-directed mutagenesis kit were from Amersham Buchler GmbH (Braunschweig, Germany). Cycloheximide and 7-methyl-guanosine 5′-monophosphate were supplied by Sigma. HPLC columns were from Applied Biosystems (Foster City, CA) and Bio spin columns from Bio-Rad Laboratories (Glattbrugg, Switzerland). Antibodies against the TRAM protein and mammalian Sec61p were raised against peptides and affinity-purified as described (Görlich *et al.*, 1992a, 1992b).

Preparation of (Tmd)Phe-$tRNA^{sup}$—Oligoribonucleotides, pCpA and CpUpApA, were prepared as described elsewhere.[2] L-Boc-(Tmd)phenylalanine and abbreviated suppressor tRNA missing the 3′-terminal dinucleotide pCpA ($tRNA^{sup}$(−CA)) were prepared as described previously (Baldini *et al.*, 1988; Bruce and Uhlenbeck, 1982; Noren *et al.*, 1989b). The $tRNA^{sup}$(−CA) was also prepared *in vitro* as a run-off transcript from the plasmid pYPhe2 and found to function equally well in suppression of TAG stop codons and subsequent photocross-linking. The scheme for the chemical aminoacylation of $tRNA^{sup}$ with (Tmd)Phe is outlined in Fig. 1*a*. To a solution of 35 mg (94 μmol) of L-Boc(Tmd)Phe in 160 μl of dry tetrahydrofurane were added 16 mg (94 μmol) of 1,1′-carbonyldiimidazole. After activation of the acid (15 min at room temperature), 160 $A_{260\,nm}$ units of pCpA dissolved in a mixture of 175 μl of water and 190 μl of acetonitrile were added, and the mixture was allowed to react for 4 h at room temperature. The solution was extracted with ethyl acetate, and the aqueous phase, containing unreacted pCpA and acyl-pCpAs, was subjected to reverse phase HPLC using an Aquapore RP300 column (10 μm, 4 × 250 mm). Elution of the products was effected using a gradient of 0–100% acetonitrile (solvent B) with 50 mM ammonium acetate, pH 4.5, over 50 min at 1 ml/min (Fig. 1*b*). The material in peaks 3 and 4 was pooled and lyophilized to give a mixture

[2] B. Martoglio and J. Brunner, manuscript in preparation.

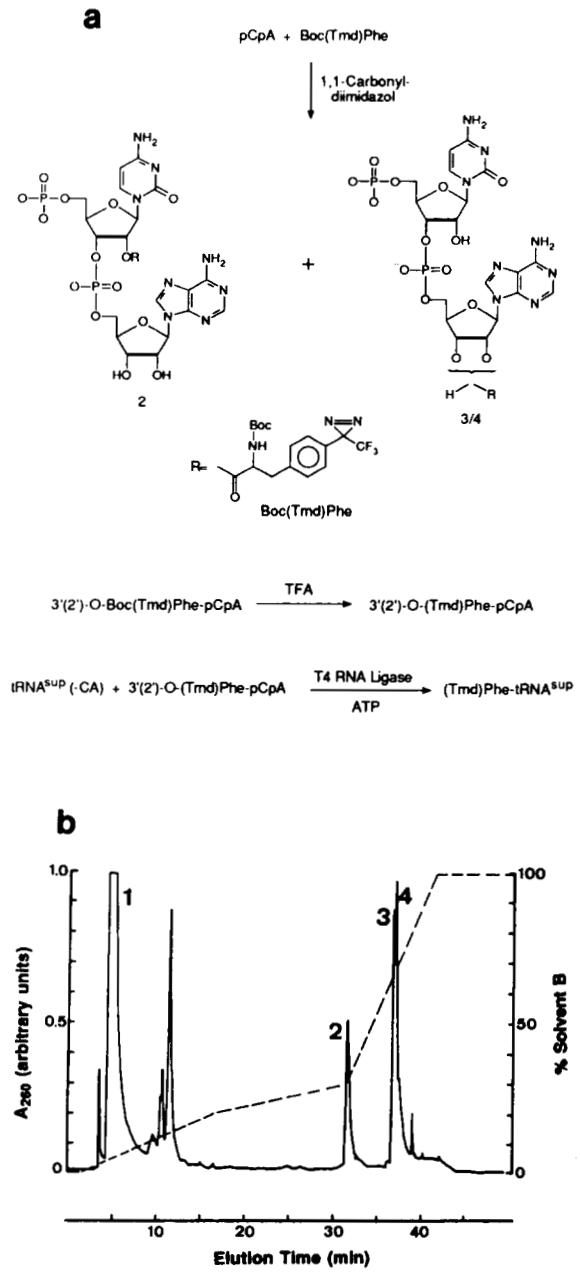

FIG. 1. *a*, outline of chemical aminoacylation of $tRNA^{sup}$ with (Tmd)Phe. The aminoacylation reaction yielded unreacted pCpA (1, not shown) and mixed reaction products (2–4) from which products 3 and 4 were separated by HPLC and used for ligation to $tRNA^{sup}$(−CA) after deprotection as indicated. *b*, HPLC separation of products 1–4 from *a*.

3′- and 2′-*O*-[Boc-(Tmd)phenylalanyl-pCpA in a combined yield of 19 $A_{260\,nm}$ units. To remove the Boc-protecting group, the lyophilized material was dissolved in dry trifluoroacetic acid (10 μl/$A_{260\,nm}$ units), and the solution was kept on ice for 15 min. Trifluoroacetic acid was evaporated by a stream of nitrogen and the deprotected 3′(2′)-*O*-(Tmd)phenylalanyl-pCpA was precipitated by the addition of a small amount of ether and collected by centrifugation. The resulting pellet was dried *in vacuo*, dissolved in 50 mM ammonium acetate, pH 4.5, and lyophilized. For ligation to $tRNA^{sup}$(−CA), the lyophilized 3′(2′)-*O*-(Tmd)phenylalanyl-pCpA and $tRNA^{sup}$(−CA) were dissolved at concentrations of 1 $A_{260\,nm}$ units/100 μl each in buffer containing 55 mM Na-HEPES, pH 7.5, 15 mM $MgCl_2$, 3.3 mM dithiothreitol, 250 μM ATP, 20 μg/ml of bovine serum albumin, and dimethyl sulfoxide (10%, v/v). The mixture was incubated at 37 °C for 10 min with 300 units/ml of T4 RNA ligase and the reaction then quenched by addition of 3 M sodium acetate, pH 4.5, to 10% (v/v). This quenched

solution was immediately extracted with phenol (equilibrated with 0.25 M sodium acetate, pH 4.5), phenol:chloroform (1:1), and then chloroform. The aqueous phase was further purified by gel filtration using a Bio-Spin 6 column, equilibrated with 250 mM sodium acetate, pH 4.5), and the modified tRNA was then recovered by precipitation with ethanol.

Transcription, Translation, and Photocross-linking—To prepare $tRNA^{sup}(-CA)$ as a run-off transcript the plasmid pYPhe2 was first linearized with *Fok*I (Noren *et al.*, 1989b). The transcription reaction contained 0.13 $\mu g\ \mu l^{-1}$ of linearized DNA, 40 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 2 mM each ATP, CTP, GTP, and UTP, 4 units μl^{-1} ribonuclease inhibitor, 4 units μl^{-1} T7 RNA polymerase and was incubated at 37 °C for 2 h. The RNA was purified on a Qiagen 100 column as directed by the manufacturer (Diagen) and then used directly for chemical charging.

The plasmid pGEM4PPL was constructed by subcloning the coding region of pSPBP4 (Siegel and Walter, 1988) into pGEM4 as an *Eco*RI/*Hin*dIII fragment under control of the T7 promoter. Stop codons (TAG) were introduced at codons 11, 18, and 25 of the coding region of PPL to give PPL TAG11, PPL TAG18, and PPL TAG25, respectively. PPL ΔK4 ΔK9 K46 was made by altering codons 4 and 9 of PPL from ones encoding lysine to ones encoding arginine and by replacing the arginine codon at position 46 by a lysine codon. The mutagenesis was performed with an Amersham site-directed mutagenesis kit as directed by the manufacturer and the relevant portions of all constructs were checked by sequencing. Plasmids were linearized with *Pvu*II prior to transcription to give a truncated transcript which encodes the first 86 amino acids of PPL. Transcription was performed as described by the manufacturer (Promega Biotec, Madison, WI). Translation was in wheat germ lysate and when in the presence of N^{ϵ}-4-(3-trifluoromethyldiazarino)benzoyl-Lys-tRNA conditions were as described previously (High *et al.*, 1991). When (Tmd)Phe-$tRNA^{sup}$ was used, 27 pmol of $tRNA^{sup}$/25 μl of cell-free translation reaction mixture were added, otherwise the reaction conditions were as for the N^{ϵ}-4-(3-trifluoromethyldiazarino)benzoyl-Lys-tRNA. After translation the products were irradiated to induce photocross-linking (High *et al.*, 1991).

Analysis of Photocross-linking Products—The extraction of membranes with sodium carbonate solution was as described previously (High *et al.*, 1991). For immunoprecipitation of photocross-linking products with anti-TRAM antibodies and relevant controls, the membrane-associated photocross-linking products were isolated by centrifugation through a cushion (0.25 M sucrose, 0.5 M KCl, 5 mM $MgCl_2$, and 50 mM Hepes-KOH, pH 7.9) 5 min, 100,000 × *g* and 4 °C. The pellet was resuspended in MP buffer (0.25 M sucrose, 80 mM KCl, 3 mM $MgCl_2$ and 10 mM Hepes-KOH, pH 7.5) and then 4 volumes of IP dilution buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, and 1% Triton X-100) and phenylmethylsulfonyl fluoride to a final concentration of 0.2 mg ml^{-1} were added followed by the relevant antibodies or antisera. Samples were incubated at 4 °C overnight, protein A-Sepharose was than added and the incubation continued for 2 h and samples processed as described previously (Lipp and Dobberstein, 1986). Immunoprecipitation with anti-Sec61p antibodies and relevant controls were carried out as described above, except that after suspension in MP buffer SDS was added to 1% (v/v) and samples heated at 95 °C for 5 min prior to the addition of 4 volumes of IP dilution buffer. The anti-Sec61p antibodies had been coupled to Sepharose beads prior to addition (Görlich *et al.*, 1992b). All samples were analyzed on 10–15% SDS-polyacrylamide gels unless otherwise stated. When 22% polyacrylamide gels containing 6 M urea were used, they were prepared as described previously (Haeuptle *et al.*, 1986). All gels were subjected to fluorography with Entensify as directed by the manufacturer (Du Pont NEN).

RESULTS

Suppression of a UAG Stop Codon Using Chemically Aminoacylated Amber Suppressor tRNA—To establish the present methodology, site-directed mutagenesis was used to modify the cDNA encoding PPL so that a TAG stop codon replaced the codons for amino acids flanking the hydrophobic region of the PPL signal sequence (TAG11 and TAG25) or within the center of this hydrophobic region (TAG18) (Fig. 2*a* and Fig. 6). These modified cDNAs were used for transcription and the resulting mRNAs (Fig. 2*a*) were translated in a wheat germ translation system in the presence and absence of (Tmd)Phe-$tRNA^{sup}$. When (Tmd)Phe-$tRNA^{sup}$ was absent, no completed 86-amino acid fragment of PPL (PPL_{86}) was detected (Fig. 2*b*, compare *lanes 2–4* with *lane 1*). In the case of the TAG 25 construct the 24-amino acid truncated product was resolved on the gel system used (Fig. 2*b*, *lane 4*). When synthesis was carried out in the presence of (Tmd)Phe-$tRNA^{sup}$ completed PPL_{86} fragments were observed in all cases (Fig. 2*b*, *lanes 6–8*). In the case of the TAG 25 transcript, a corresponding decrease in the amount of the 24-amino acid product was observed. These results indicated that suppression by the (Tmd)Phe-$tRNA^{sup}$ should be efficient enough to allow visualization of photocross-linking products between the resulting nascent chains and interacting components. The presence of a (Tmd)Phe residue at position 11, 18, or 25 of the PPL nascent chain did not affect the translocation of the full-length protein product across microsomal membranes or influence the accompanying signal sequence cleavage (data not shown). We concluded that the truncated PPL nascent chains to be used for photocross-linking analysis would represent true intermediates of the translocation pathway used by the wild type protein.

During Membrane Insertion Different ER Proteins Are in Contact with Different Regions of the Nascent Preprolactin—Upon the addition of rough microsomes to a nascent chain-ribosome-SRP complex the SRP is released and the nascent chain contacts membrane proteins. The continued presence of the ribosome retains the nascent chain in the translocation site and allows for ER components close to the membrane-inserting nascent chain to be detected (Krieg *et al.*, 1989; Wiedmann *et al.*, 1987). We added rough microsomes to the various PPL nascent chains and photocross-linked them to the ER components with which they were in contact. Since the nascent chains are all of the same length, they should all be trapped at the same stage of the translocation process. Thus, using the site-specifically introduced photocross-linking probes, the nearest neighbors of successive regions of the nascent chain could be determined. As a control we used the modified lysine mediated photocross-linking method for the PPL AF, which had been described previously (Krieg *et al.*, 1989; Wiedmann *et al.*, 1987). The modified lysine residues from which photocross-linking occurs are present exclusively at the NH_2-terminal side of the hydrophobic region of the signal sequence (see Fig. 6).

After photocross-linking of the different PPL nascent chains to ER components, the products were extracted with sodium carbonate solution and centrifuged to separate integral membrane proteins, which are in the resulting pellet, from soluble proteins which remain in the supernatant (Fujiki *et al.*, 1982). The different PPL TAG proteins gave photocross-linking products of different mobilities on SDS-polyacrylamide gels (Fig. 3, *lanes 2–4*), although the mobilities of the nascent chains were all the same (Fig. 2*b*, *lanes 6–8*, and Fig. 3, *lanes 2–4*). The induction of photocross-linking to adjacent components via the incorporated (Tmd)Phe residue was dependent upon UV irradiation as expected (data not shown). When PPL TAG11 was used for analysis, a 43-kDa photocross-linking product was observed (Fig. 3, *lane 2*) which was almost identical in size to the product obtained when modified lysine residues were used for photocross-linking (Fig. 3, *lane 1*). The latter represents the 8-kDa PPL AF covalently attached to a component of about 35 kDa (Krieg *et al.*, 1989; Görlich *et al.*, 1992a). This component consists of at least two glycoproteins, the bulk of the nascent chain being photocross-linked to the translocating chain-associating membrane (TRAM) protein (Görlich *et al.*, 1992a) and a minor portion to the SSRα subunit (Wiedmann *et al.*, 1987). When PPL

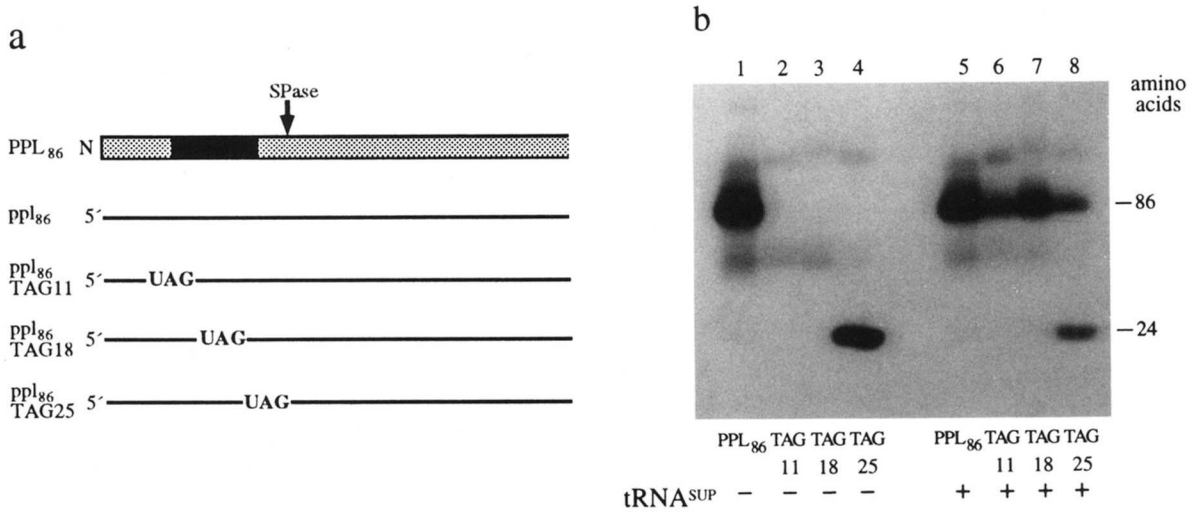

FIG. 2. *a*, outline of PPL_{86} polypeptide (*top*) and ppl_{86} transcripts used to test stop codon suppression by Phe-$tRNA^{sup}$. Phe-$tRNA^{sup}$ was prepared exactly as (Tmd)Phe-$tRNA^{sup}$ except that Phe was used in place of (Tmd)Phe. The *black box* indicates the hydrophobic core of the signal sequence and the site of signal peptidase cleavage is shown (*SPase*). The codon for amino acids 11, 18, and 25 of normal ppl_{86} was replaced in the transcript by a UAG stop codon as indicated (*TAG11, TAG18*, and *TAG25*). *b*, transcripts coding for PPL_{86}, PPL_{86} TAG11, PPL_{86} TAG18, or PPL_{86} TAG25 were translated in the absence (*lanes 1–4*) and presence (*lanes 5–8*) of Phe-$tRNA^{sup}$ (*$tRNA^{sup}$*). The resulting translation products were analyzed on a 22% polyacrylamide gel containing 6 M urea and subjected to fluorography. The calculated number of amino acids in the resulting polypeptides is indicated on the *right*.

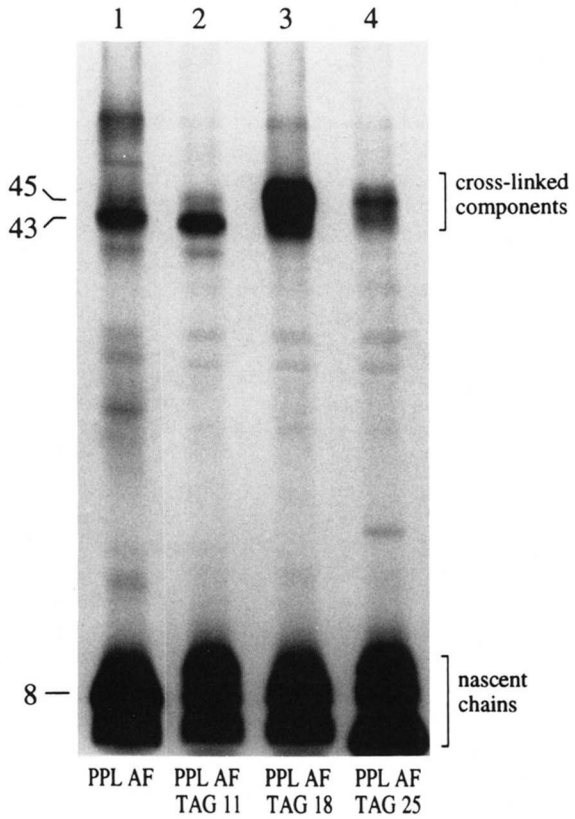

FIG. 3. **Different regions of the signal sequence are in contact with different ER proteins.** The SRP arrested fragment (*AF*) of PPL nascent chains incorporating ε-TDBA-modified lysines at positions 4 and 9 (*lane 1*) or (Tmd)Phe at positions 11 (*lane 2*), 18 (*lane 3*), or 25 (*lane 4*) (see also Figs. 2*a* and 6) were allowed to interact with microsomal membranes and then irradiated with UV. Proteins present in the membrane pellet after extraction with sodium carbonate solution are shown. The relative molecular masses (kilodaltons) of the photocross-linked components are indicated.

TAG18 was used the major photocross-linking product had an estimated molecular mass of 45 kDa (Fig. 3, *lane 3*), suggesting the 8-kDa nascent chain was covalently attached to a component of 37 kDa. The photocross-linking pattern observed with PPL TAG25 showed a major photocross-linked product of 45 kDa together with a weaker 43-kDa product (Fig. 3, *lane 4*). This suggested that the bulk of the nascent chain was cross-linked to a 37-kDa protein, whereas a fraction was cross-linked to a 35-kDa protein.

Identification of the ER Proteins Which Contact Nascent PPL—The 35-kDa ER proteins, which are the major photocross-linking partners of PPL AF, are known to be glycoproteins, since they bind to immobilized lectins (Görlich *et al.*, 1992a; Krieg *et al.*, 1989; Wiedmann *et al.*, 1987). Neither the PPL AF TAG18 nor the PPL AF ΔK4 ΔK9 K46 photocross-linking products were found to bind immobilized lectins, indicating that the photocross-linked components are not glycoproteins (data not shown). To further characterize the protein components which are photocross-linked to the different regions of the PPL nascent chain, the products were immunoprecipitated using antibodies specific for the TRAM protein, the major ER protein found photocross-linked to short truncations of the PPL nascent chain (Görlich *et al.*, 1992a). The photocross-linking products between PPL AF and PPL AF TAG11 and the 35-kDa ER component were efficiently immunoprecipitated by antibodies against the TRAM protein (Fig. 4, *lanes 3* and *6*). The slight difference in mobility of the PPL AF-TRAM protein cross-link and the PPL AF TAG11-TRAM protein cross-link may be due to the nascent chains being photocross-linked to different regions of the TRAM protein. In contrast, the PPL AF TAG18, PPL AF TAG25, and PPL AF ΔK4 ΔK9 K46 photocross-linked components showed no reactivity with the anti-TRAM antibodies (Fig. 4, *lanes 9, 12*, and *15*).

The 37-kDa protein which was photocross-linked to PPL AF TAG18, PPL AF TAG25, and PPL AF ΔK4 ΔK9 K46 was similar in apparent size, and lack of glycosylation, to the *Saccharomyces cerevisiae* Sec61p (Müsch *et al.*, 1992; Sanders *et al.*, 1992) and its recently identified mammalian homologue

(Görlich *et al.*, 1992b). We therefore repeated the analysis of all the photocross-linking products using antibodies specific for mammalian Sec61p (Görlich *et al.*, 1992b). The major 45-kDa photocross-linking products obtained with PPL AF TAG18, PPL AF TAG25, and PPL AF ΔK4 ΔK9 K46 were all efficiently immunoprecipitated with these anti-Sec61p antibodies (Fig. 5, *lanes 9, 12,* and *15*). Thus the 37-kDa nonglycosylated ER protein photocross-linked to these nascent chains was identified as Sec61p. A small amount of the 45-kDa photocross-linking product was also immunoprecipitated when PPL AF and PPL AF TAG11 were used for photocross-linking (Fig. 5, *lanes 3* and *6*). This is consistent with the presence of a faint 45-kDa photocross-linking product which is observed with these nascent chains (Fig. 4, *lanes 1* and *4*; Görlich *et al.*, 1992b) and which is not immunoprecipitated by the anti-TRAM antibodies (Fig. 4, *lanes 3* and *6*).

We conclude that two different ER proteins are in contact with different regions of the translocation intermediate of the nascent secretory protein PPL. One of these is the TRAM protein (Görlich *et al.*, 1992a), whereas the other is mammalian Sec61p (Görlich *et al.*, 1992b) (see Fig. 6).

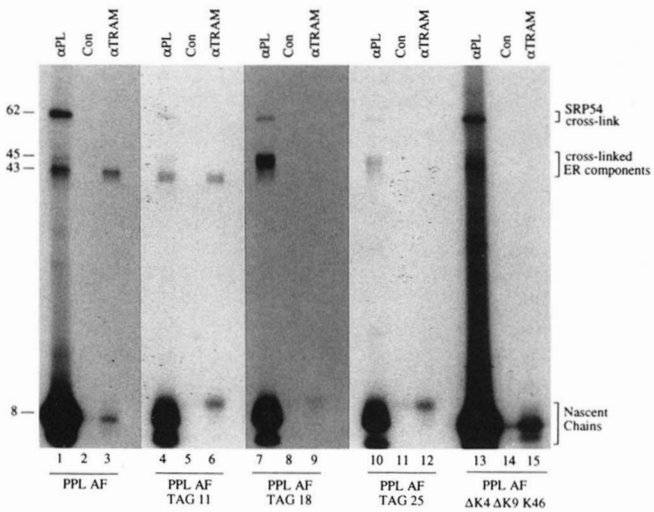

FIG. 4. **Immunoprecipitation with antibodies specific for the TRAM protein.** The SRP arrested fragments of the indicated PPL nascent chains (see Figs. 2*a* and 6) were photocross-linked to ER components. The membrane-bound components were isolated by centrifugation through a high salt cushion, resuspended, and analyzed by immunoprecipitation using an antiserum against prolactin (*αPL*), a control antiserum (*Con*), or antibodies specific for the translocating chain associating membrane protein (*αTRAM*). Within each set of immunoprecipitations (*i.e. αPL, Con,* and *αTRAM*) identical amounts of starting material were used so that the results are directly comparable within these sets. Relative molecular masses (kilodaltons) are indicated.

DISCUSSION

Site-specific Photocross-linking—We have demonstrated here the site-specific incorporation of the photoactivatable amino acid (Tmd)Phe into PPL nascent chains and the successful photocross-linking of these nascent chains to components of the ER translocation complex. The approach used is entirely different from that developed previously (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986) and offers several advantages and the potential for further developments.

A key element of the present study was the preparation of amber suppressor tRNA with (Tmd)Phe. This was accomplished by using the general scheme of Hecht and colleagues (Heckler *et al.*, 1984; Payne *et al.*, 1987; Pezzuto and Hecht, 1980), which involved T4-RNA ligase-mediated coupling of 3′(2′)-*O*-aminoacyl-pCpA to a tRNA lacking the 3′-terminal cytosine and adenosine moieties. The procedure used for the preparation of 3′(2′)-*O*-(Tmd)phenylalanyl-pCpA is essentially that described by Baldini *et al.* (1988) except that the acylation reaction was carried out in a different solvent system (tetrahydrofuran/acetonitril/water). This results in increased yields of the desired 3′(2′)-*O*-(Tmd)phenylalanyl-pCpA. A highly efficient route for the chemical aminoacylation of tRNAs has recently been reported by Robertson *et al.* (1991). Unfortunately the protection/deprotection scheme which

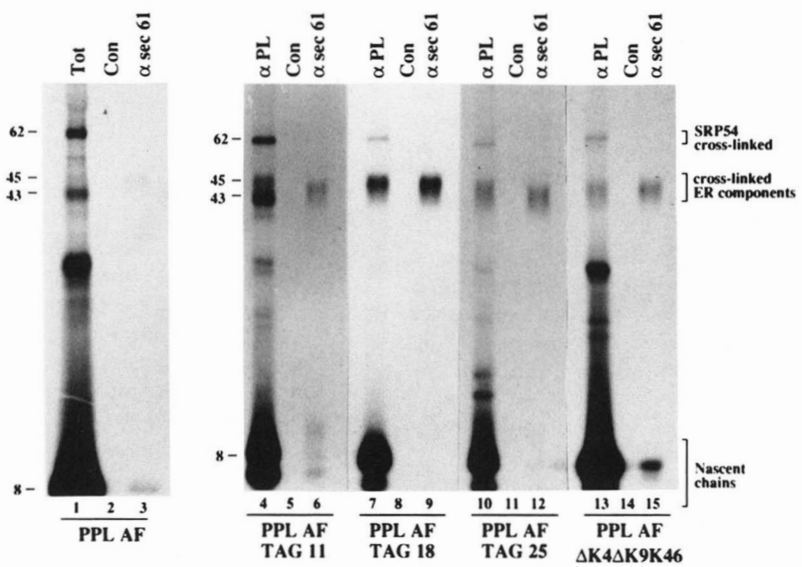

FIG. 5. **Immunoprecipitation with antibodies specific for Sec61p.** The SRP-arrested fragments of the indicated PPL nascent chains (see Figs. 2*a* and 6) were photocross-linked to ER components. The membrane-bound components were isolated by centrifugation through a high salt cushion, resuspended, and the total products analyzed (*Tot*), or the products after immunoprecipitation with an antiserum against prolactin (*αPL*), a control antiserum (*Con*), or antibodies specific for Sec61p (*αSec61*). Within each set of immunoprecipitations (*i.e. αPL, Con,* and *αTRAM*) identical amounts of starting material were used so that the results are directly comparable within these sets. Where total products are shown (*lane 1*) these represent 40% of the amount of material used for immunoprecipitation (*lanes 2* and *3*). The samples shown in *lanes 1–3* were run on a different gel from the remainder of the samples (*lanes 4–15*). Relative molecular masses (kilodaltons) are indicated.

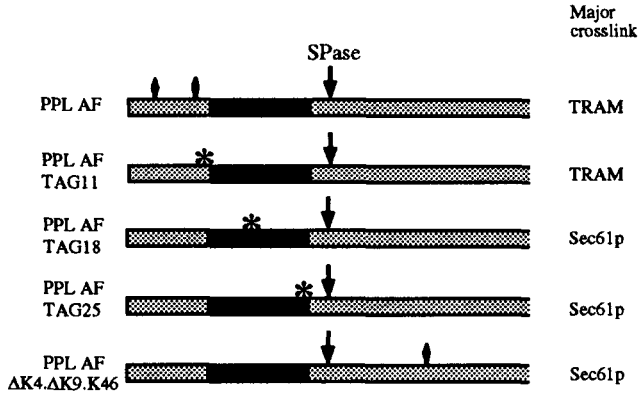

FIG. 6. **Summary of photocross-linking results obtained with PPL and PPL variants.** The *black box* indicates the hydrophobic core of the signal sequence and the site of signal peptidase cleavage is shown (*SPase*). Lysine residues (*black diamonds*) or stop codons which have been suppressed by (Tmd)Phe-tRNA^{sup} (*asterisks*) are indicated. In PPL AF, lysine residues are present at positions 4 and 9 of the nascent chain and in PPL ΔK4 ΔK9 K46, a lysine is present at position 46. For the PPL TAG mutants a (Tmd)Phe was present at residues 11 (*TAG11*), 18 (*TAG18*), and 25 (*TAG25*). The major ER membrane components which was photocross-linked to each nascent chain is shown. TRAM indicates the translocating chain associating membrane protein and Sec61p the mammalian Sec61 protein.

they employed cannot be used for the light-sensitive amino acid (Tmd)Phe.

Suppressor tRNA-mediated site-specific incorporation of non-natural amino acids into proteins has been achieved with *Escherichia coli* extracts and reticulocyte lysate (Bain *et al.*, 1989, 1991a, 1992; Chung *et al.*, 1993; Ellman *et al.*, 1991, 1992a; Ellman *et al.*, 1992b; Mendel *et al.*, 1992; Noren *et al.*, 1989a; Robertson *et al.*, 1991). In this study we have used a wheat germ cell-free translation system, supplemented with chemically charged suppressor tRNA prepared either by the anticodon loop replacement procedure (Bruce and Uhlenbeck, 1982; Noren *et al.*, 1989a) or by run-off transcription (Noren *et al.*, 1989b, Bain *et al.*, 1991b). Our results demonstrate that enough protein could be generated with the present system to easily detect cross-linking products.

(Tmd)Phe is a carbene-yielding structural analogue of phenylalanine, designed for the chemical or biosynthetic incorporation into peptide and protein photoaffinity reagents (Nassal, 1984). Because of its reasonably small size, (Tmd)Phe may be incorporated into proteins without seriously affecting either the structure of the protein or its interaction with adjacent components. Upon photolysis, a very reactive singlet carbene is generated which is capable of inserting even into aliphatic CH bonds (Brunner *et al.*, 1980; Nassal, 1984). The short half-life of the carbene (estimated to be less than 10^{-9} s) ensures that it cross-links only to components in direct contact. Cross-linking as a result of random collisional encounters is very unlikely to occur.

One of the limitations of current biosynthetic photocross-linking approaches is the lack of efficient methods to identify and analyze cross-linked components. This is a particular problem when antibodies recognizing the cross-linked component are not available or when the precise sites of cross-linking need to be determined. To this end we are developing methods which allow the site-specific incorporation of a cleavable, photoactivatable, and radioactively labeled amino acid of high specific radioactivity. The ultimate goal is to combine the present technology with developments in conventional cross-linking (label transfer cross-linking). First promising steps in this direction have been made with the preparation of cysteinyl-tRNA^{Sup}. Alkylation of the thiol group should provide a simple and general method to introduce the required multifunctional residue. While suppressor tRNA-mediated incorporation of photoactivatable amino acids is experimentally more demanding than previous approaches, it has a very much wider potential for studying biological questions relating to protein-protein interactions.

Membrane Components in Contact with Nascent PPL—The immediate environment of a nascent secretory protein as it is translocated across the ER membrane has been investigated in several ways. One approach has been to generate translocation intermediates of different lengths and then analyze their next neighbors by photocross-linking. A detailed study of this kind by Krieg *et al.* (1989) showed that the glycoprotein mp39 was in proximity to nascent chains of PPL ranging from 86 to 131 amino acids in length. As the length of the nascent chain was increased, the number of cross-linked proteins increased, and nonglycosylated proteins were observed in addition to the glycoproteins. The interpretation of the results was complicated by the fact that when longer chain lengths were used, the number of lysine residues from which photocross-linking to ER components could occur was increased.

We have kept the length of the nascent chain in the translocation intermediate of PPL constant (PPL AF = 70 amino acids) and moved the position of the photocross-linking reagent within the nascent chain (Fig. 6). Our results show that the type of ER protein which is photocross-linked to the nascent chain is dependent upon the region of the nascent chain from which the photocross-linking occurs. The simplest interpretation is that the different regions of the nascent chain are in proximity to different ER proteins. However, it is also possible that the three modified amino acids placed into the signal sequence are facing in different lateral directions and that different sides of the nascent chain contact different proteins. If more than one population of membrane-inserted signal sequences exists, then they could each contact one of the two different ER proteins via different regions of the signal sequence and also generate the observed results. Further studies will be necessary to formally disprove any of these possibilities.

At least two different proteins were found to be in contact with different regions of the nascent chain. The NH_2 terminus of PPL (PPL AF and PPL AF TAG11) was photocross-linked to the TRAM protein (Görlich *et al.*, 1992a). A small proportion of nascent chain is also likely to be cross-linked to the previously identified 35-kDa glycoprotein SSRα (Görlich *et al.*, 1992a; Wiedmann *et al.*, 1987). The mp39 glycoprotein which is photocross-linked to the nascent chain of PPL (Krieg *et al.*, 1989) probably represents both of these components.

When the photocross-linking group was placed at the center of the hydrophobic part of the PPL signal sequence (PPL AF TAG18), only the product of cross-linking between the nascent chain and mammalian Sec61p could be detected. Photocross-linking from a position COOH-terminal of the hydrophobic part of the signal sequence (PPL AF TAG25) and from a region after the signal peptidase cleavage site (PPL AF ΔK4 ΔK9 K46) also showed Sec61p as the major cross-linking partner.

In addition to the identification of mammalian Sec61p by Görlich *et al.* (1992b), at least two other studies have found nonglycosylated proteins as a major cross-linking partner of PPL nascent chains during their membrane insertion (Kellaris *et al.*, 1991; Krieg *et al.*, 1989). Although further studies are required, it seems likely that these components also represent cross-linking to the mammalian Sec61p.

Several lines of evidence suggest that the complex which mediates the ER translocation of secretory proteins is either the same as that which is responsible for the insertion of membrane proteins or that it utilizes some common components. We find that Sec61p is in contact with the secreted protein PPL at various stages during its translocation across the membrane (this work; Görlich *et al.*, 1992b) and also with several membrane proteins during their insertion into the ER (High *et al.*, 1991b; High *et al.*, 1993). In *S. cerevisiae* mutations in the SEC61, SEC62, and SEC63 genes cause defects in both the translocation of secreted proteins (Deshaies and Schekman, 1989; Sadler *et al.*, 1989; Rothblatt *et al.*, 1989) and the insertion of membrane proteins (Green *et al.*, 1992; Stirling *et al.*, 1992), consistent with a single ER complex mediating both protein translocation and membrane protein insertion.

Our results suggest that the ER translocation complex consists of heterologous protein subunits and that Sec61p and TRAM are two components of this complex. Indeed the TRAM protein has already been shown to be required for the *in vitro* translocation of some secreted proteins (Görlich *et al.*, 1992a). In *S. cerevisiae* translocation intermediates of a secretory protein can be cross-linked to Sec61p (Müsch *et al.*, 1992; Sanders *et al.*, 1992). Sec61p had been identified previously as a gene product essential for the efficient translocation of secretory proteins into the ER (Deshaies and Schekman, 1987). The frequency with which interactions between nascent secretory or membrane proteins and Sec61p are observed support the view that this protein plays a key role in membrane translocation and membrane insertion. We propose that Sec61p is a core component of the mammalian ER translocation machinery and as such would be absolutely required for the process to occur (High, 1992). This is consistent with the proposal that Sec61p alone, or in combination with other unidentified ER proteins, mediates the translocation of proteins across the ER membrane (see Rapoport, 1992). There is sufficient sequence similarity between mammalian Sec61p and bacterial SecY to suggest that the mechanism of protein translocation may have been conserved (see Görlich *et al.*, 1992a). In *S. cerevisiae*, Sec61p is associated with Sec62p, Sec63p, and two uncharacterized proteins (Deshaies *et al.*, 1991). It is not presently known whether mammalian homologues of these other *S. cerevisiae* proteins exist.

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