# A Nascent Membrane Protein Is Located Adjacent to ER Membrane Proteins Throughout Its Integration and Translation

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*Abstract.* The immediate environment of nascent membrane proteins undergoing integration into the ER membrane was investigated by photocrosslinking. Nascent polypeptides of different lengths, each containing a single IgM transmembrane sequence that functions either as a stop-transfer or a signal-anchor sequence, were synthesized by in vitro translation of truncated mRNAs in the presence of $N^{\epsilon}$-(5-azido-2-nitrobenzoyl)-Lys-tRNA, signal recognition particle, and microsomal membranes. This yielded nascent chains with photoreactive probes at one end of the transmembrane sequence where two lysine residues are located. When irradiated, these nascent chains reacted covalently with several ER proteins. One prominent crosslinking target was a glycoprotein similar in size to a protein termed mp39, shown previously to be situated adjacent to a secretory protein during its translocation across the ER membrane (Krieg, U. C., A. E. Johnson, and P. Walter. 1989. *J. Cell Biol.* 109:2033–2043; Wiedmann, M., D. Goerlich, E. Hartmann, T. V. Kurzchalia, and T. A. Rapoport. 1989. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 257:263–268) and likely to be identical to a protein previously designated the signal sequence receptor (Wiedmann, M., T. V. Kurzchalia, E. Hartmann, and T. A. Rapoport. 1987. *Nature (Lond.).* 328:830–833). Changing the orientation of the transmembrane domain in the bilayer, or making the transmembrane domain the first topogenic sequence in the nascent chain instead of the second, did not significantly alter the identities of the ER proteins that were the primary crosslinking targets. Furthermore, the nascent chains crosslinked to the mp39-like glycoprotein and other microsomal proteins even after the cytoplasmic tail of the nascent chain had been lengthened by nearly 100 amino acids beyond the stop-transfer sequence. Yet when the nascent chain was allowed to terminate normally, the major photocrosslinks were no longer observed, including in particular that to the mp39-like glycoprotein. These results show that the transmembrane segment of a nascent membrane protein is located adjacent to the mp39-like glycoprotein and other ER proteins during the integration process, and that at least a portion of the nascent chain remains in close proximity to these ER proteins until translation has been completed.

In eukaryotic cells, the initial stages of the synthesis of an integral membrane protein appear to be mechanistically identical to those of a secretory protein (for review see Walter and Lingappa, 1986). In each case, protein synthesis is initiated on a cytoplasmic ribosome. After the signal sequence in the nascent chain emerges from the ribosome, a signal recognition particle (SRP)[1] binds to the ribosome (Walter et al., 1981) following a direct interaction between the signal sequence and the 54-kD protein component of the SRP (Krieg et al., 1986; Kurzchalia et al., 1986). The association of SRP with the ribosomal complex either halts or slows further nascent chain elongation (Walter and Blobel, 1981; Wolin and Walter, 1989), presumably to extend the time during which the complex can be productively targeted to the ER membrane. There, an interaction between SRP and a membrane-bound SRP receptor (Gilmore et al., 1982*a*; Meyer et al., 1982) elicits the release of both SRP and SRP receptor from the ribosome, and protein chain elongation resumes (Gilmore et al., 1982*b*). This step is GTP dependent for both secretory and membrane proteins (Connolly and Gilmore, 1986, 1989; Hoffman and Gilmore, 1988; Wilson et al., 1988).

At some point subsequent to the SRP-dependent targeting of the ribosome to the ER membrane, the processing pathways of these two types of nascent proteins diverge. For secretory proteins, translocation continues cotranslationally until the new polypeptide chain has completely traversed the

1. *Abbreviations used in this paper*: εANB-Lys, $N^{\epsilon}$-(5-azido-2-nitrobenzoyl)-Lys; EKRM, salt- and EDTA-washed microsomal membranes; NEM, N-ethylmaleimide; SRP, signal recognition particle; ST, stop-transfer sequence.

bilayer and is released into the lumen of the ER (Walter and Lingappa, 1986). In contrast, the nonpolar transmembrane segments of membrane proteins (also termed "stop-transfer" sequences; Blobel, 1980) do not pass through the bilayer, but are integrated into the membrane. The stop-transfer sequence anchors the nascent polypeptide in the plane of the membrane even though translation continues (Yost et al., 1983; Walter and Lingappa, 1986). Upon completion of protein synthesis, the carboxy-terminal end of the membrane protein may be located on either the cytoplasmic or the lumenal side of the ER membrane, depending upon the orientation of the (final) transmembrane segment in a single-spanning (or polytopic) membrane protein.

The processing pathways for secreted and integral membrane proteins therefore appear to diverge after the nascent polypeptide is localized at the ER membrane. The choice of pathways is dictated by topogenic sequences within the protein being synthesized (e.g., Yost et al., 1983; Rothman et al., 1988; Lipp and Dobberstein, 1988; Szczesna-Skorupa et al., 1988; Haeuptle et al., 1989). Although it is clear that the nascent polypeptide participates in membrane translocation and integration, the nature of its interaction with the ER membrane and its components remains obscure. In particular, there are conflicting views and evidence regarding the extent to which membrane translocation and integration are spontaneous or are mediated by proteins in the ER membrane (e.g., Engelman and Steitz, 1981; Davis and Model, 1985; Mize et al., 1986; Zerial et al., 1987; Müller and Zimmermann, 1987; Singer et al., 1987; Krieg et al., 1989).

To probe the mechanism of integration, we have employed a novel crosslinking approach that has already been used successfully to examine the environment of a nascent secretory protein within the ER membrane (Krieg et al., 1989; Wiedmann et al., 1989). With this approach it was possible to address directly the issue of whether or not a resident ER membrane protein is located adjacent to the nascent chain of a secretory protein as it traverses the bilayer (Krieg et al., 1989). This was accomplished by incorporating photoreactive moieties into nascent preprolactin, positioning them within the bilayer, and then irradiating the sample, because the nascent polypeptide would be crosslinked to protein only if an ER protein was adjacent to the nascent chain during translocation.

Nascent polypeptides were synthesized in vitro using functional Lys-tRNA analogs prepared by a method developed to covalently attach probes to the side chain $\epsilon$-amino group of the Lys in a Lys-tRNA (Johnson et al., 1976). This modification does not significantly impair the interaction of the aminoacyl-tRNA with elongation factors or ribosomes (Johnson et al., 1976, 1978; Johnson and Cantor, 1980; Johnson and Slobin, 1980), and nascent chains containing these abnormally long amino acid side chains have been both successfully translated and translocated (Johnson et al., 1976; Krieg et al., 1986, 1989; Kurzchalia et al., 1986, 1988; Wiedmann et al., 1987*a*,*b*, 1989).

Photoreactive probes were positioned at different locations within the bilayer by synthesizing preprolactin nascent chains of different lengths in the presence of $N^{\epsilon}$-(5-azido-2-nitrobenzoyl)-Lys-tRNA ($\epsilon$ANB-Lys-tRNA) (Krieg et al., 1989). Upon photolysis, the nascent chains reacted covalently, in each case, with ER membrane proteins, and most prominently with a 39-kD integral transmembrane glycoprotein of the ER that was designated mp39 (Krieg et al., 1989). This glycoprotein appears to be the same protein that was designated the signal sequence receptor by Wiedmann et al. (1987*b*) after being crosslinked using a short nascent chain (the arrested fragment, ~70 amino acids long) with photoprobes located only in the signal sequence. Recent experiments have confirmed that nascent chains longer than the arrested fragment also photocrosslink to SSR (Wiedmann et al., 1989). Since preprolactin nascent chains were photocrosslinked to mp39/SSR even when no photoreactive groups were present in the signal sequence (Krieg et al., 1989; Wiedmann et al., 1989), it would appear that the ER membrane glycoprotein does not (or not only) function as a signal sequence receptor, but rather as part of a tunnel or guide for the nascent chain through the ER membrane (Krieg et al., 1989). Whatever the function of this protein, it is clear that nascent chains of secretory proteins are located in close proximity to mp39 and other ER membrane proteins during translocation (Krieg et al., 1989; Wiedmann et al., 1989). These results suggest that translocation occurs at specific sites on the ER membrane that contain these proteins. Such sites have been postulated previously and termed translocons (Walter and Lingappa, 1986).

The observation that secretory and integral membrane proteins share a common targeting pathway (e.g., Yost et al., 1983; Mize et al., 1986; Zerial et al., 1987) suggests that nascent membrane polypeptides may be positioned, at least initially, in the membrane adjacent to the same ER proteins as are nascent secretory proteins. If the mechanism of stop transfer is receptor mediated, i.e., if discrimination between nascent secretory and membrane proteins involves protein recognition of the stop-transfer element, additional protein interactions would be expected. On the other hand, since stop-transfer sequences consist of long nonpolar sequences, it is possible that interactions between ER proteins and the stop-transfer sequences are not required to effect integration of the transmembrane segments of a nascent membrane protein, as has been suggested (e.g., Engelman and Steitz, 1981). As a first step in assessing the possible involvement of ER membrane proteins in integration, we have used the same photocrosslinking approach as in our previous study (Krieg et al., 1989) to determine whether nascent polypeptides being integrated into the ER membrane bilayer are located adjacent to protein components of the ER membrane. To do this, we have used two membrane proteins whose stop-transfer sequences are oriented in opposite directions with respect to the ER membrane, and are located at different positions in the nascent chain. As is documented below, our data show that membrane protein integration occurs adjacent to ER membrane proteins.

## Materials and Methods

### Plasmids

Plasmids coding for the two membrane fusion proteins used in this work were constructed from similar plasmids used in an earlier study (Rothman et al., 1988). The stop-transfer sequence (ST) was derived from the transmembrane region of IgM $\mu$-heavy chain (Early et al., 1980). The DNA sequence encoding this region was fused to that encoding the last 142 amino acids of prolactin (here designated P) as described previously (Rothman et al., 1988). To restrict lysines to only one end of the transmembrane segment, it was necessary to delete the amino terminal lysine from the stop-

transfer element. To accomplish this, the entire coding region was subcloned as a Nco I–Pst I fragment into a vector in which the $AMP^R$ gene was replaced by one for $KAN^R$ because the latter does not contain any Ava II sites. Since there are two Ava II sites in the IgM stop-transfer sequence which flank the sequence encoding the amino terminal lysine, the $KAN^R$ plasmid containing the STP fusion protein sequence was digested with Ava II and religated. The resulting plasmid encoded a modified version of the stop transfer in which the 21 amino acids immediately amino terminal to the hydrophobic core of the element are deleted. This modification removes the single amino terminal lysine from the stop-transfer sequence as well as six other charged residues. For expression in vitro, the complete coding region of the new plasmid was subcloned as a Nco I–Pst I fragment back into the original pSP64T-derived vector described previously (Rothman et al., 1988). The finished plasmid (pST105) encodes a molecule, referred to here as $ST_1P$, that contains the modified stop-transfer sequence of 34 amino acids fused to the last 142 amino acids of prolactin.

A signal sequence was added to the amino terminus of $ST_1P$ to produce a new molecule, $S_LST_1P$, which adopts the opposite topology in the ER membrane. The signal sequence used was that from $\beta$-lactamase of *Escherichia coli* ($S_L$) because this signal of 24 amino acids does not contain lysine. The plasmid used as a source for the lactamase signal sequence was pSPSG1 (Perara and Lingappa, 1985). In this plasmid there is a unique Nco I site separating sequences coding for the lactamase signal sequence and chimpanzee $\alpha$-globin. This Nco I site is in the same reading frame as that in $ST_1P$, and hence, after digesting pSPSG1 with Nco I and Pst I, it was possible to substitute the $ST_1P$ coding sequence for that encoding globin to form a plasmid (pSTP116) that encodes the $S_LST_1P$ fusion protein.

Both plasmids contain upstream SP6 RNA polymerase promoters which permit RNA synthesis in vitro. The full-length preprolactin coding region was also located behind an SP6 RNA polymerase promoter on the pSPBP4 plasmid described previously (Krieg et al., 1986; Siegel and Walter, 1988).

## *In Vitro Transcription*

RNAs encoding the full-length proteins were transcribed from the plasmids described above using SP6 RNA polymerase as before (Krieg et al., 1986, 1989). Plasmids were also digested in the prolactin coding region with various restriction enzymes, and then run-off transcription of the digested plasmids produced truncated mRNAs that coded for nascent polypeptides of defined lengths. For plasmid pSP105, the Rsa I, Taq I, Msc I, Fsp I, Afl II, or Pst I cuts yielded the 78, 90, 111, 152, 162, or 176 (full-length) amino-terminal amino acids, respectively, of the $ST_1P$ protein. The coding sequence for the $ST_1P$ polypeptide contained lysine codons at positions 29, 31, 46, 83, 101, 119, 136, 158, and 164 from the $NH_2$-terminal end. For plasmid pSP116, the Rsa I, Taq I, Fsp I, Esp I, or Pst I cuts produced the 102, 114, 176, 199, or 200 (full-length) amino-terminal amino acids of $S_LST_1P$. Lysines were located in the $S_LST_1P$ polypeptide 53, 55, 70, 107, 125, 143, 160, 182, and 188 amino acids from the $NH_2$-terminal end. Full-length preprolactin polypeptides were synthesized by in vitro transcription using Pst I-linearized pSPBP4 DNA (Krieg et al., 1986, 1989). Pvu II- and Mae I-restriction sites were chosen to obtain run-off transcripts encoding truncated preprolactin fragments that were 86 and 127 amino acids in length, respectively (Krieg et al., 1989).

## *Translation Components*

Photoreactive yeast $\epsilon$ANB-Lys-tRNA, unmodified yeast Lys-tRNA, and wheat germ extract were prepared as detailed previously (Krieg et al., 1986). SRP and microsomal membranes, either rough or salt- and EDTA-extracted (EKRM), were prepared as described elsewhere (Walter and Blobel, 1983*a*,*b*; Siegel and Walter, 1985). For some experiments, EKRM were treated with 3 mM N-ethylmaleimide (NEM) (Gilmore et al., 1982*a*), while sham-reacted EKRM were exposed to 3 mM NEM in the presence of 20 mM DTT.

## *Translations*

The wheat germ–translation incubations were similar to those described previously (Krieg et al., 1986, 1989), and were carried out in a room illuminated only by a sodium vapor lamp. When included, EKRM were present at 9 equivalents/25 $\mu$l assay, and SRP was used at a concentration of 40 nM. Samples were prepared on ice, and translation was initiated by placing them at 26°C. To maximize homogeneity in the lengths of nascent chains, initiation inhibitors were used here with preprolactin mRNA as in our previous study (Krieg et al., 1989) to limit translation to only one nascent chain (ribosome) per mRNA. Specifically, translations of preprolactin were pulsed (synchronized) by the addition of 7-methylguanosine 5′-monophosphate (Sigma Chemical Co., St. Louis, MO) and edeine (Sigma Chemical Co.) to 4 mM and 10 $\mu$M, respectively, after 5 min. Since the initiation of protein synthesis on both the $ST_1P$ and $S_LST_1P$ mRNAs was significantly slower than on preprolactin mRNA, initiation inhibitors were not added to the membrane protein translations in this study. After 30 min, translations were halted by the addition of cycloheximide to 2 mM.

## *Orientation of Membrane Proteins*

Protease protection and sedimentation assays were performed as described previously (Andrews, 1989). Because of the intrinsic protease resistance of $S_LST_1P$, the conditions for the protease reaction were 100–200 $\mu$g/ml Proteinase K for 60 min at 24°C. Under these conditions, ~80% of translocated $ST_1P$ and of the control prolactin are protected by the microsomal membrane from exogenous protease. Additional control experiments confirmed that essentially all of the $ST_1P$ molecules which interacted with membranes (i.e., cosedimented in urea/sucrose gradients) were translocated.

## *Photoreactions*

Samples in open 1.5-ml polypropylene microfuge tubes in an ice-water bath were irradiated from above (~30 cm) with a 150-W mercury arc lamp equipped with a 306–409-nm bandpass filter (6% transmission at 312 nm). Samples were photolyzed for 30 min to obtain maximal crosslinking to membrane proteins. Aliquots serving as "minus light" controls were brought to 100 mM DTT and incubated for 30 min on ice in the dark to inactivate the azide groups chemically.

## *Alkaline Extraction*

Translations (25 or 50 $\mu$l) were diluted with sodium carbonate to achieve a final volume of 100 $\mu$l and a final concentration of 0.1 M $Na_2CO_3$ at pH 11.5. The samples were then kept on ice for 30 min with occasional mixing before being layered onto a 100 $\mu$l cushion (100 mM sodium carbonate, 0.2 M sucrose at pH 11.5) and spun for 10 min at 4°C in an airfuge (Beckman Instruments, Palo Alto, CA) at 30 psi in an A-100 30° rotor. Supernatant and pellet fractions were analyzed by SDS-PAGE or were fractionated, either by immunoprecipitation with antibodies to prolactin or by binding to Con A-Sepharose.

## *Immunoprecipitation*

The carbonate pellet or Con A pellet was solubilized in 40 $\mu$l of 1% (wt/vol) SDS, 100 mM Tris base (Sigma Chemical Co.), 50 mM DTT by boiling for 5 min. The sample was then diluted to a final volume of 400 $\mu$l and a final concentration of 1% (vol/vol) Triton X-100, 20 mM Tris, 0.1% (wt/vol) SDS, 150 mM NaCl, 5 mM DTT, 0.02% (wt/vol) $NaN_3$, and 10 mM EDTA at pH 7.5. Samples were precleared with Sepharose CL-4B (Pharmacia Fine Chemicals), and then 15 $\mu$l of protein A-Sepharose beads (Sigma Chemical Co.), to which prolactin antibodies (United States Biochemical Corp., Cleveland, OH) had been covalently coupled (Harlow and Lane, 1988), were added to each sample. After being rocked overnight at 4°C, the beads were washed twice with 1 ml of 1% (vol/vol) Triton X-100, 20 mM Tris, 150 mM NaCl, 10 mM EDTA (pH 7.5), and twice with 1 ml of 0.1 M Tris-HCl (pH 7.4) before preparation for SDS-PAGE.

## *Lectin Binding*

The carbonate pellet fractions of 25-$\mu$l translation incubations were solubilized in 40 $\mu$l of 1% (wt/vol) SDS, 30 mM DTT, 100 mM Tris base by boiling for 5 min. Each sample was then taken to 400 $\mu$l and a final concentration of 0.35% (wt/vol) SDS, 1 M NaCl, 0.1 M Tris, 3 mM DTT, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1% (vol/vol) Triton X-100, and 0.02% (wt/vol) $NaN_3$ at pH 7.6 (this constitutes solution A). SDS was included in solution A to minimize the nonspecific adsorption of proteins to Con A-Sepharose. Individual samples were precleared with Sepharose CL-4B, and then rocked for 10 h with 15 $\mu$l of Con A-Sepharose (Pharmacia Fine Chemicals) at 4°C in solution A or solution A containing 0.4 M $\alpha$-methylmannoside. The Con A-Sepharose beads were then pelleted in a microfuge, and washed twice with 1 ml of solution A and twice with 0.1 M Tris-HCl (pH 7.4) before the lectin-bound material was eluted from the beads for analysis by SDS-PAGE.

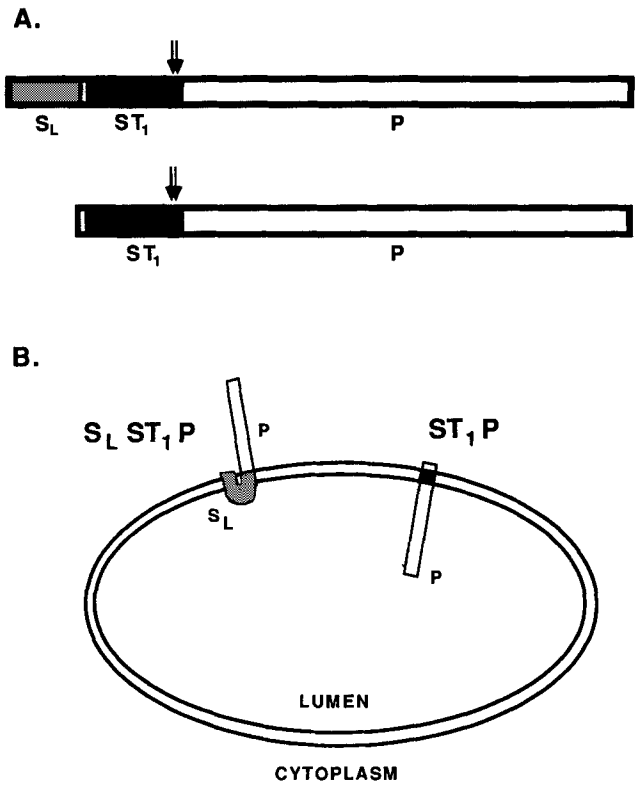


*Figure 1.* Membrane fusion proteins. (*A*) Linear arrangement of the components of the two fusion proteins used in this study: the lactamase signal sequence ($S_L$), an IgM transmembrane sequence ($ST_1$), and the last 142 amino acids of prolactin (*P*). The arrows show the positions of the first two lysine residues in each polypeptide, located at the carboxy-terminal end of the transmembrane sequence. (*B*) The orientation of the two fusion proteins after in vitro translation in the presence of SRP and microsomal membranes. The hydrophobic core of the stop-transfer sequence is indicated by a black box. The lactamase signal sequence (*shaded box*) is shown associated with the membrane, but its actual location is unknown. The polypeptide segment lengths are not to scale.

## Results

### Experimental Strategy

Are nascent membrane proteins situated adjacent to protein components of the ER membrane during integration? We examined this issue directly by asking whether nascent chains containing photoreactive moieties at one end of their transmembrane sequence were able to crosslink to ER membrane proteins when the integration process was halted at an intermediate stage and the sample irradiated. Nascent membrane proteins of defined length were synthesized in vitro by the use of mRNAs that had been truncated within the coding region (e.g., Connolly and Gilmore, 1986; Krieg et al., 1989). The lack of a stop codon prevented normal termination of protein synthesis when the ribosome reached the end of the mRNA, and therefore each nascent chain, its length dictated by the length of the truncated mRNA, remained bound to its ribosomal complex as a peptidyl-tRNA (cf. Krieg et al., 1989). When SRP and microsomal membranes were present in incubations, the ribosomes translating RNAs encoding membrane proteins were bound to the ER membrane, and the nascent membrane polypeptides were translocated into the bilayer while translation continued to the ends of the RNAs. This created a specific membrane-bound intermediate in the integration process.

The two membrane fusion proteins chosen for this study (Fig. 1 *A*) were identical, except that the β-lactamase signal sequence ($S_L$) was present at the $NH_2$-terminal end of one protein ($S_LST_1P$) and not the other ($ST_1P$). Each protein contained a single copy of a modified IgM μ-heavy chain transmembrane region ($ST_1$) that was positioned just ahead of the carboxy-terminal 142 amino acids of prolactin. This prolactin sequence (P) provides a means both to immunoprecipitate the nascent chain (Rothman et al., 1988) and to extend its length a variable amount beyond the stop-transfer sequence. The IgM transmembrane sequence can, in the absence of a signal sequence, initiate nascent chain integration (Mize et al., 1986), and hence it is able to function either as a stop-transfer sequence or as a signal-anchor sequence. The same transmembrane domain therefore permitted us to examine each of the two orientations of the stop-transfer sequence (Fig. 1 *B*), as discussed below.

Because the carboxy-terminal end of the IgM heavy chain stop-transfer sequence contains two lysine codons, photoreactive probes were incorporated into the transmembrane sequences of $ST_1P$ and $S_LST_1P$ by translating in the presence of εANB-Lys-tRNA, a functional Lys-tRNA analogue which has a photoreactive azido group attached to the side chain of the amino acid (Krieg et al., 1986, 1989). The β-lactamase signal sequence was chosen for $S_LST_1P$ be-

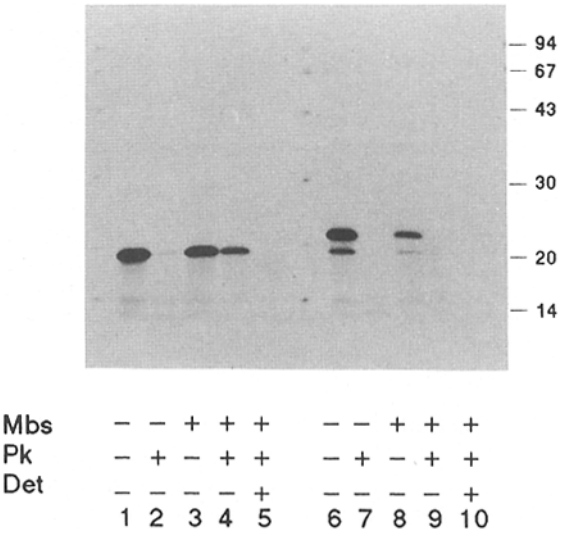


*Figure 2.* Translocation assays for $ST_1P$ and $S_LST_1P$. Full-length fusion protein mRNAs were translated in wheat germ extract for 90 min as described in Materials and Methods; reactions were supplemented with rough microsomes (*Mbs*) as indicated. Translation was terminated by adding cycloheximide to 100 μM and products were subjected to proteolysis with Proteinase K (*Pk*). The detergent Triton X-100 (*Det*) was added to 1% (vol/vol) to some reactions as a control. The less intense band in lanes *6* and *8* migrating at ~20 kD is presumed to result from an internal initiation of translation at the methionine at the junction between the signal and stop-transfer sequences, instead of at the methionine at the amino terminus of the signal sequence. The migration positions of molecular weight markers are indicated in kD at the side. $ST_1P$, lanes *1–5*; $S_LST_1P$, lanes *6–10*.

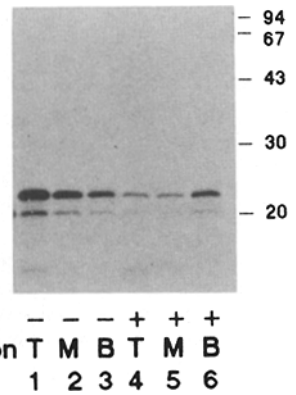


*Figure 3.* Gradient fractionation of translocation reactions. $S_LST_1P$ was synthesized as in Fig. 2 in the absence or presence of rough microsomes (*Mbs*). Samples were adjusted to 2 M urea and fractionated by centrifugation over sucrose cushions. Gradient fractions are indicated as top (*T*), middle (*M*), and bottom (*B*) fractions below the corresponding lanes. The less intense band migrating at ~20 kD is presumed to result from internal initiation.

cause it contains no lysine codons. Thus, no photoreactive azide moieties were incorporated into the nascent chains of either $ST_1P$ or $S_LST_1P$ until translation reached the carboxy-terminal end of the IgM transmembrane sequence (Fig. 1 *A*). Upon continued translation, azido photoprobes could be incorporated at natural lysine codons in the prolactin passenger sequence of $ST_1P$ and $S_LST_1P$ if they were present in a truncated mRNA.

Azido photoreactive groups were therefore positioned at either the cytoplasmic (with $S_LST_1P$) or the lumenal (with $ST_1P$) end of the transmembrane segment when its translocation into the ER membrane was complete (see below). After photolysis, samples were examined for photoadducts that contained both the nascent chain and an ER membrane protein, because any ER component crosslinked to the nascent chain had to be located in close proximity to it after translation and translocation had ceased. Note that by following this strategy, nascent protein chains are created in situ, and hence are functionally engaged with active components of the translation, translocation, and integration machinery.

### *Membrane Protein Topology*

To examine the potential asymmetry of the integration machinery in the ER membrane, we constructed, from the region of the IgM $\mu$-heavy chain previously characterized as a stop-transfer element (Yost et al., 1983; Rothman et al., 1988), transmembrane sequences that were flanked by lysines on only one end. There are three lysines in the wild-type IgM region (Early et al., 1980), one amino terminal and two carboxy terminal to the hydrophobic core sequence. The single lysine near the stop transfer was removed from a plasmid employed earlier (Rothman et al., 1988) by deleting the sequence that encoded a charged region (and contained the lysine) amino terminal to the hydrophobic core of the transmembrane region. Based upon data obtained previously (Rothman et al., 1988), it was expected that the modified stop-transfer sequence, $ST_1$, would adopt opposite orientations with respect to the cytoplasm during the biogenesis of $ST_1P$ and of $S_LST_1P$.

To confirm this, the orientation of the molecules was determined using protease protection (Fig. 2) and sedimentation (Fig. 3) assays (Andrews, 1989). For $ST_1P$, the translocated molecules were protected from exogenous protease when rough microsomal membranes were present in the incubation (compare lanes *1* and *2* with lanes *3* and *4* in Fig. 2). However, when the microsomal membrane was solubilized by including the nonionic detergent Triton X-100, the $ST_1P$ molecules were digested (Fig. 2, lane *5*). Densitometry of lanes *3* and *4* in Fig. 2 indicated that ~30% of the synthesized $ST_1P$ molecules were protected from the protease. Sedimentation experiments similar to those shown below for $S_LST_1P$ confirmed that most of the protease accessible $ST_1P$ molecules had not interacted with the membranes (data not shown). The remainder were lost because of the relatively harsh proteolysis conditions employed, as shown by comparison with protease protection assays for the control prolactin molecules (data not shown). Thus, essentially all of the $ST_1P$ molecules that interacted with the membrane were oriented with the carboxy-terminal prolactin portion in the interior of the microsomal vesicles (Fig. 1 *B*).

Protease data for $S_LST_1P$ were less informative than for $ST_1P$, since they indicate only that the $S_LST_1P$ molecule was not fully translocated when microsomes are added to the reaction (Fig. 2, lanes *6–10*). Moreover, there was no change in apparent molecular weight during membrane insertion of full length $S_LST_1P$ (Fig. 2, compare lanes *6* and *8*), suggesting that the lactamase signal sequence was not cleaved. For this reason, the proteolytic assay did not distinguish between molecules which had interacted with the membrane and those which had not. To demonstrate that $S_LST_1P$ was efficiently integrated into the ER membrane in vitro, we used a sedimentation assay, the results of which are shown in Fig. 3. Although $S_LST_1P$ was cytoplasmically exposed (Fig. 2, lanes *6–10*), the molecule sedimented to the bottom of sucrose step gradients in 2 M urea only when microsomes were added during the translation reaction (Fig. 3, compare lanes *1–3* and lanes *4–6*). This demonstrated that $S_LST_1P$ had assembled on the membrane, with its prolactin portion exposed on the cytoplasmic side of the ER membrane. The deduced orientations for the two polypeptides are shown in Fig. 1 *B*.

### *Photocrosslinking of $ST_1P$ to Microsomal Proteins*

Truncated RNA coding for a nascent chain of $ST_1P$ that was 90 amino acids in length (a 90mer) was translated in the presence of [$^{35}$S]methionine and $\epsilon$ANB-Lys-tRNA. Since a ribosome protects the carboxy-terminal 40 amino acids of a ribosome-bound nascent chain from protease digestion (Malkin and Rich, 1967; Blobel and Sabatini, 1970), about 50 amino acids of each 90mer were exposed outside the ribosome (including the signal-anchor sequence at the N terminus of the nascent chain) after it reached the end of its RNA and translation ceased. When the translations were carried out in the presence of both SRP and EKRM, the photoprobes in the transmembrane sequence of the nascent chain were positioned near the lumenal surface, assuming that the stop-transfer sequence was integrated into the bilayer as shown in Fig. 1 *B*.

Proteins photocrosslinked to nascent chains in irradiated samples were identified by the fact that any prolactin-con-

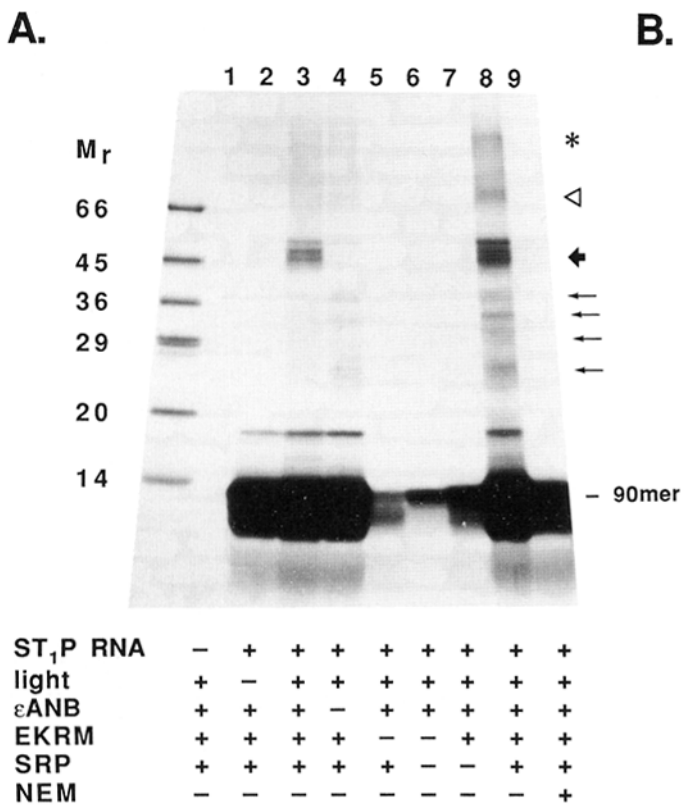


*Figure 4.* Photocrosslinking of $ST_1P$ 90mer. (*A*) Translations (25 μl) were carried out as described in Materials and Methods, photolyzed (except for the sample in lane *2*), extracted with sodium carbonate at pH 11.5, separated by SDS-PAGE in a 10–20% (wt/vol) gradient gel, and analyzed using fluorography. The incubations were programmed with run-off transcript encoding the first 90 amino acids of $ST_1P$ (except for that shown in lane *1*), and contained SRP (except for the samples in lanes *6* and *7*), EKRM (except lanes *5* and *6*), and εANB-Lys-tRNA (except lane *4*, which contained an equivalent amount of unmodified Lys-tRNA). The EKRM added to the sample in lane *9* had been treated with 3 mM NEM before use (Gilmore et al., 1982*a*), while the EKRM used in lane *8* had been sham reacted. The most prominent unresolved band of photocrosslinked material with $M_r$ values between 45 and 53 kD contains at least four different species. The arrowhead, open triangle, and asterisk indicate the positions of photoproducts that contain the nascent chain and membrane glycoproteins with apparent molecular weights of 37–38, 70, and 140 kD, respectively. Four smaller EKRM-dependent photoadduct bands in lane *8* that contain the nascent chain crosslinked to proteins with apparent molecular weights near 15, 20, 24, and 27 kD are indicated with arrows. The extent of photoadduct formation in samples lacking the ANB probe was found to be somewhat variable; in this particular set of experiments, there was more photoadduct formation in the sample of lane *4* than was normally observed. (*B*) Two samples equivalent to those in lane *3* were alkaline extracted, and one was exposed to Con A-Sepharose. The carbonate-insoluble material (lane *10*) and the lectin-bound material (lane *11*) were then each immunoprecipitated with antibodies to prolactin as described in Materials and Methods, followed by analysis as in *A*. The arrowhead and open triangle show the locations of the nascent chain photoadducts with the 37–38- and 70-kD ER glycoproteins, respectively. Molecular mass standards: lysozyme, 14 kD; β-lactoglobulin, 18 kD; trypsin inhibitor, 20 kD; carbonic anhydrase, 29 kD; glyceraldehyde-3-phosphate dehydrogenase, 36 kD; ovalbumin, 45 kD; BSA, 66 kD; phosphorylase B, 97 kD; myosin, 200 kD.

taining radioactive species with a molecular mass greater than that of the 90mer must have arisen via a covalent reaction between a nascent chain and an adjacent protein. Numerous photocrosslinks were observed when we examined the total contents of a sample (data not shown). However, since we were interested primarily in photoadducts between nascent chains and membrane proteins of the ER, we restricted our investigation to proteins that could not be extracted from membranes at alkaline pH, employing a sodium carbonate (pH 11.5) procedure that has been widely used to isolate integral membrane proteins (Fujiki et al., 1982).

Several carbonate-resistant photocrosslinked species were found in the membrane pellet when the $ST_1P$ 90mer was translated in the presence of SRP, εANB-Lys-tRNA, and microsomal membranes, and then irradiated (Fig. 4 *A*, lanes *3* and *8*). The most prominent band of photoadducts contained at least four species (identifiable at low exposure times and partially separable using Con A; see below) with apparent molecular masses between 45 and 53 kD, while less abundant crosslinked species had $M_r$ values near 25, 30, 34, 37, and 80 kD. These photoproducts were immunoprecipitated with polyclonal antibodies against prolactin (Fig. 4 *B*, lane *10*), which confirmed the presence of prolactin epitopes, and therefore nascent chains, in the photocrosslinked species. Low-intensity bands containing higher molecular weight adducts (*asterisks* in Fig. 4 and see Fig. 6) with $M_r$ values near 150 kD were often, but not always, observed.

A set of control experiments was performed in parallel to ensure that the observed photoreactions were specific for fully assembled functional complexes. No photocrosslinks were observed when the incubations were deprived of $ST_1P$ mRNA (Fig. 4 *A*, lane *1*), SRP (lanes *6* and *7*), or ER membranes (lanes *5* and *6*). Furthermore, no photoadducts were found in the membrane pellet if the microsomal membranes were first inactivated by NEM (compare NEM-reacted EKRM in lane *9* with sham-reacted EKRM in lane *8*). Exposure of ER membranes to NEM has been shown previously to prevent them from functioning in targeting, translocation, or integration (Gilmore et al., 1982*a*). Thus, the existence of the above photocrosslinks was dependent upon the biochemical components that are required for the assembly of an intermediate in the integration process. We conclude that the photocrosslinked species represent the crosslinking of $ST_1P$ 90mer to proteins that are located adjacent to the nascent chains during translation and integration.

The nascent chain crosslinking also required light: no

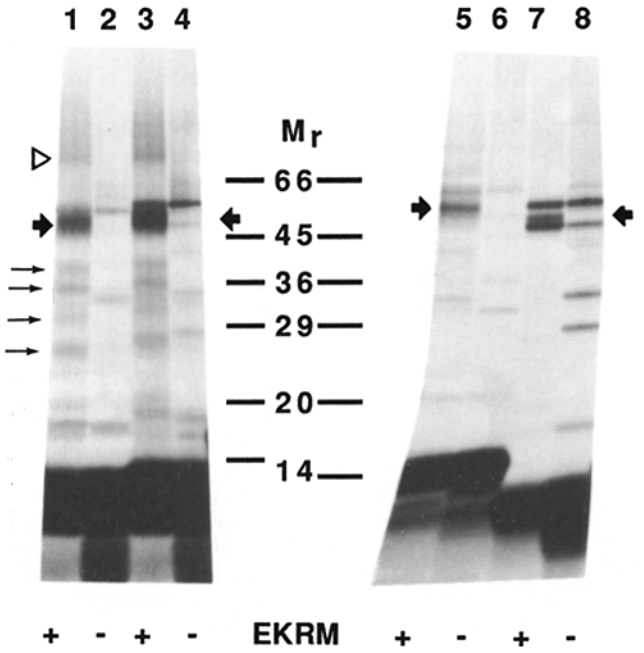


*Figure 5.* Microsome-dependent photoproducts. Translations (25 μl) were programmed with run-off transcripts coding for the $ST_1P$ 78mer (lanes *1* and *2*), $ST_1P$ 90mer (lanes *3* and *4*), preprolactin 127mer (lanes *5* and *6*), or preprolactin 86mer (lanes *7* and *8*), either in the presence (lanes *1*, *3*, *5*, and *7*) or absence (lanes *2*, *4*, *6*, and *8*) of EKRM and SRP. After photolysis, EKRM-containing samples were extracted with carbonate and analyzed as in Fig. 4. Samples lacking EKRM were centrifuged through a 100-μl cushion containing 0.2 M sucrose in an airfuge (A-100 30° rotor; Beckman Instruments) at 30 psi for 30 min at 4°C, and the resulting ribosomal pellets were analyzed directly using SDS-PAGE. The symbols are defined in Fig. 4; the arrowheads indicate the positions of the photoadducts that contain mp39 (Krieg et al., 1989) and a preprolactin nascent chain (lanes *5* and *7*) or mp39-like ER glycoprotein and a $ST_1P$ nascent chain (lanes *1* and *3*).

crosslinking was observed when the samples were not irradiated (Fig. 4 *A*, lane *2*). In contrast to our previous results with preprolactin (Krieg et al., 1989), a small amount of photocrosslinking to some, but not all, of the ER protein targets was observed in samples in which Lys-tRNA replaced ϵANB-Lys-tRNA (lane *4*). Thus, a natural chromophore(s) in either the nascent chain and/or the target proteins can be photoactivated and is favorably positioned for reaction with a nearby polypeptide. This result does not weaken our conclusions, because covalent bond formation between two polypeptides, with or without the azido probe, requires that they be in close proximity.

Irradiation of samples that contained SRP, but lacked functional membranes, resulted in the formation of photocrosslinks between the $ST_1P$ nascent chain and the 54-kD subunit of SRP that were soluble in sodium carbonate at pH 11.5 (data not shown). This stop-transfer sequence therefore mimics, as anticipated (Mize et al., 1986), the interaction of a signal sequence with the SRP in an elongation-arrested complex (Krieg et al., 1986; Kurzchalia et al., 1986).

Since each nascent chain has a transmembrane domain, one might expect that some or all photocrosslinked species, including those between ribosomal proteins and integrated $ST_1P$ polypeptides, would be resistant to carbonate extraction from the membrane. Therefore it was important to identify photoadducts in the carbonate pellets of the experiments shown in Fig. 4 *A* that resulted from nascent chain crosslinking to ribosomal proteins rather than to microsomal proteins. To determine which $ST_1P$ 90mer photoproducts were microsome dependent, we compared the radioactive species found in the carbonate-extracted membrane pellet of a sample containing SRP and EKRM to those found in the ribosomal pellet of a sample that lacked SRP and EKRM. As shown in lane *4* of Fig. 5, a few photoadducts, including two in the 45–53-kD range, were recovered in the ribosomal pellet and hence result from nascent chain crosslinking to ribosomal proteins. However, a comparison of the photoadducts in lanes *3* and *4* (and the comparable photoadducts of the $ST_1P$ 78mer shown in lanes *1* and *2*) of Fig. 5 demonstrates that most of the photocrosslinks observed in the carbonate pellet are in fact microsome dependent. Thus, these species constitute crosslinks between $ST_1P$ nascent chains and protein components of the microsomes. In particular, the broad band of photoadducts with $M_r$ values between 45 and 53 kD (lane *3*) contains at least two species that resulted from covalent reactions between $ST_1P$ 90mers and two microsome components, one a 37–38-kD glycoprotein (see below) and one a nonglycosylated 39-kD protein.

The $ST_1P$ 78mer contains only three lysines: two outside the ribosome at the carboxy-terminal end of the transmembrane domain, and one inside the ribosome, 32 amino acids from the carboxy-terminal end of the nascent chain. Thus, the microsome-dependent and probe-dependent photocrosslinks observed with the 78mer (in particular, those to the 37–38-kD glycoprotein and the 39-kD nonglycosylated protein) were formed by covalent reactions between the photoprobes located at the end of the transmembrane sequence and the target ER proteins (Fig. 5, compare lanes *1* and *2*; and data not shown). Since these two probes are likely to maintain their locations relative to the plane of the surface during continued protein synthesis, and since the transmembrane domain is not likely to be involved in protein folding, it is reasonable to expect that these probes will continue to form crosslinks to the ER proteins adjacent to the integration site, even if the length of the nascent chain increases, until the stop-transfer sequence diffuses laterally away from its crosslinking targets. Consistent with this view, longer nascent chains were also photocrosslinked to the same primary microsomal protein targets (e.g., compare lanes *1* and *3* of Fig. 5; also data not shown), based on the identical apparent molecular weight values (see below) of the target proteins obtained by subtracting the apparent molecular weight of the nascent chain from the apparent molecular weight of the photoadduct.

We conclude, therefore, that when the transmembrane sequence of $ST_1P$ is inserted into the membrane of the ER, it is close enough to protein components of the ER to react covalently with them. Specifically, nascent $ST_1P$ is located adjacent to microsomal proteins with apparent molecular masses of 37–38, 39, and 70 kD, and is able to contact proteins with apparent molecular weight values of ~15, 20, 24, 27, and 140 kD. The first three proteins were found crosslinked to $ST_1P$ nascent chains of all lengths, while crosslinks to the latter five proteins were not always obvious as distinct bands on gels of samples containing long (>152 amino acids) nascent chains, possibly because heterogeneity

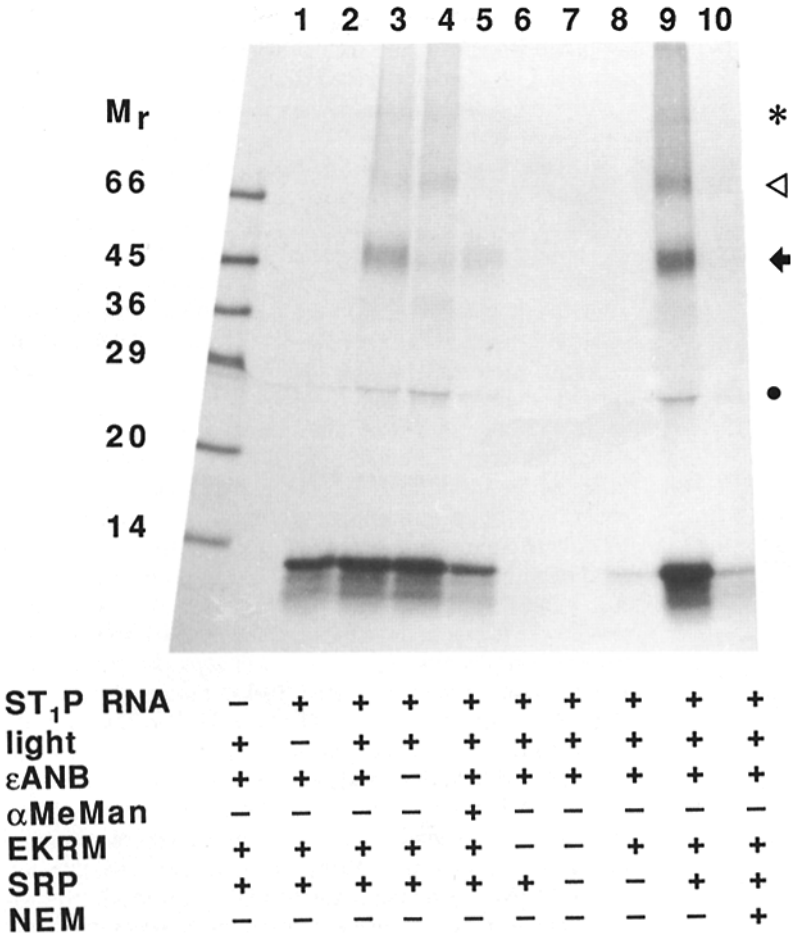


*Figure 6.* Photocrosslinking of $ST_1P$ 90mer to glycoproteins. Portions of the same translations described in Fig. 4 were, after irradiation and extraction with carbonate, incubated with Con A-Sepharose in the absence (lanes *1–4*, *6–10*) or presence (lane *5*) of 0.4 M α-methylmannoside (*αMeMan*) (Materials and Methods). Con A-bound photoproducts were analyzed directly by SDS-PAGE as in Fig. 4. The extent of photoadduct formation in samples lacking the ANB probe was found to be somewhat variable; in this particular set of experiments, there was more photoadduct formation in the sample of lane *4* than was formally observed. Sample contents are as described in the legend to Fig. 4, except that the samples in lanes *6–10* are equivalent to those in lanes *5–9* of Fig. 4, respectively, and the sample in lane *5* was the same as that in lane *3*. The symbols are defined in Fig. 4; (•) marks the front of the heavily-overloaded band of Con A eluted from the resin during sample preparation.

in the lengths of the nascent chains resulted in broadened and less intense bands. Only a limited number of proteins in the microsome were crosslinked to the nascent membrane protein. This rules out the possibility that the photocrosslinks were formed randomly following lateral diffusion of transmembrane sequences in the ER membrane, because one would then have expected to observe crosslinks to virtually every microsomal protein. Instead, the integration of nascent membrane proteins appears to occur at specific sites in the membrane that contain a limited and discrete set of microsomal proteins.

It appears that a nascent membrane protein undergoing integration is in close proximity to more microsomal proteins than a nascent secretory protein undergoing translocation, since more microsome-dependent photocrosslinks were found to $ST_1P$ nascent chains than to preprolactin nascent chains (compare lanes *1–4* with lanes *5–8* in Fig. 5). However, it remains possible that the differences between the photoproducts of $ST_1P$ and of preprolactin are caused by differences in the positions of the photoprobes in these samples. It is also possible that both $ST_1P$ and preprolactin nascent chains photocrosslinked to a peripheral membrane protein(s), but that only the $ST_1P$ adduct(s) was recovered in the carbonate pellet because of its stop-transfer sequence.

### *$ST_1P$ Nascent Chain Photocrosslinking to Microsomal Glycoproteins*

Because many integral membrane proteins of the ER are glycosylated, we identified the photocrosslinked species (Figs. 4 and 5) that contained glycoproteins by their ability to bind to lectin (cf. Krieg et al., 1989). Since none of the nascent chains used in this study were glycosylated, only radioactive nascent chains that were photocrosslinked to ER glycoproteins would be bound to Con A-Sepharose. At least three $ST_1P$ 90mer photocrosslinked species bound to Con A, as shown by the broad bands centered at apparent molecular weight values of 48, 80, and 150 kD in Fig. 6 (lanes *3* and *9*). The binding of these photoproducts to Con A-Sepharose was effectively blocked by an excess of α-methylmannoside, a competitive inhibitor for glycoprotein binding to Con A (lane *5*). The formation of these adducts required light, mRNA, SRP, and EKRM (lanes *1*, *2*, *6–8*), and formation of the 48-kD adduct was strongly dependent upon the presence of the azido group in the nascent chain (lane *4*). To demonstrate that these photocrosslinked species contained both nascent chain and carbohydrate, the material bound to the Con A-Sepharose was eluted from the resin and immunoprecipitated with antibodies to prolactin. All three photoproducts were immunoprecipitated successfully with antibodies to prolactin (Fig. 4, lane *11*; and data not shown). When ribosomal pellets were examined in samples that lacked EKRM as in Fig. 5, no radioactive Con A-binding species were found (data not shown). Thus, as expected, the glycoproteins in the photoadducts shown in Fig. 6 are microsomal in origin.

When the calculated molecular mass of the $ST_1P$ 90mer (10.3 kD) was subtracted from the apparent molecular weight

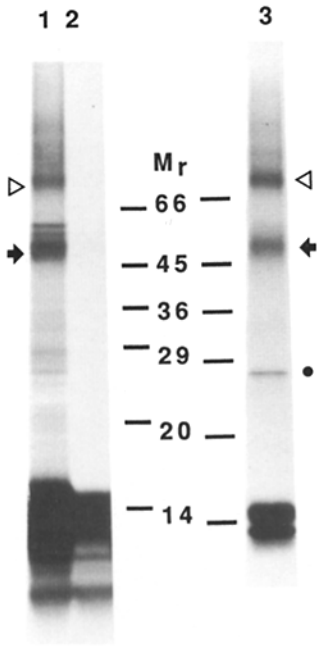


*Figure 7.* Photocrosslinking of $S_LST_1P$ 114mer to microsomal proteins. Translations (25 μl) containing SRP, ϵANB-Lys-tRNA, and EKRM that had either been treated with NEM (lane *2*) or sham reacted (lanes *1* and *3*) were carried out as described in Materials and Methods. After photolysis, carbonate extraction (all samples), and fractionation with Con A-Sepharose (sample in lane *3* only), samples were analyzed using SDS-PAGE as in Fig. 4. The carbonate-insoluble material is shown in lanes *1* and *2*, while the Con A-bound material is shown in lane *3*. The symbols indicate the positions of the photoadducts containing the same photocrosslinking targets identified in Fig. 4; (●) marks the front of the Con A band.

of the glycoprotein-containing crosslinks, the apparent molecular masses of the target glycoproteins were 37–38, 70, and 140 kD. Furthermore, four other $ST_1P$ nascent chains (78, 111, 152, or 162 amino acids in length) each photocrosslinked to two nonribosomal glycosylated species with net values of 37–38 and 70 kD (data not shown). In addition, when the Con A supernatants were examined, microsome-dependent photoadducts were observed that contained a nonglycosylated ER protein with a net value of 39 kD (data not shown). Hence, the broad band in lane *3* of Fig. 4 contains at least four photocrosslinking targets: a 37–38-kD glycoprotein and a 39-kD nonglycosylated protein that are microsomal in origin, as well as two ribosome-derived proteins.

In our previous investigation of the environment of a nascent secretory protein within the bilayer, we found that the major photocrosslinking target of the nascent preprolactin was a 39-kD integral transmembrane glycoprotein of the ER membrane termed mp39 (Krieg et al., 1989). Since the mp39-preprolactin 86mer crosslink has essentially the same apparent molecular weight as a species containing the $ST_1P$ 90mer crosslinked to an ER glycoprotein (Fig. 5, compare lanes *3* and *7*), it is very likely that the $ST_1P$ photoadduct contains mp39. The Con A-bound photoproduct band on the gel is much broader for an $ST_1P$ sample than for a preprolactin sample. This apparent heterogeneity may result either from $ST_1P$ 90mer crosslinking to more than one ER membrane glycoprotein with an $M_r$ ~39 kD and/or from heterogeneity in the lengths of the $ST_1P$ nascent chains involved in the crosslinking. In either case, mp39 is apparently located adjacent both to nascent chains undergoing translocation across the ER membrane and to those undergoing integration into the bilayer. Antibodies specific for mp39 will be required to establish unambiguously that both $ST_1P$ and preprolactin nascent chains crosslink to the same ER protein, but our data strongly suggest that nascent secretory proteins and nascent membrane proteins use either the same or similar translocation machinery in the ER membrane.

### *$S_LST_1P$ Nascent Chain Photocrosslinking to Microsomal Proteins*

Incubations containing [$^{35}$S]methionine and ϵANB-Lys-tRNA were programmed with truncated RNAs coding for a 114mer of $S_LST_1P$, translated in the presence of SRP and EKRM, photolyzed, and then examined for the presence of photocrosslinks to microsomal proteins. In these samples, the ~74 amino acids of nascent chain exposed outside the ribosome contained two topogenic sequences: the 24-residue β-lactamase signal sequence at the amino-terminal end, followed by the IgM stop–transfer sequence. The photoprobes in the transmembrane sequence of this nascent chain were therefore predicted to be located on the cytoplasmic side of the ER membrane where they could react covalently with the cytoplasmic domains of integral membrane proteins and with cytoplasmic peripheral membrane proteins, but not with lumenal proteins (Fig. 1 *B*).

Several photoadducts were found in the carbonate pellet of the $S_LST_1P$ sample containing active microsomes (Fig. 7, lane *1*), but not in that containing inactive microsomes (Fig. 7, lane *2*). Thus, the carboxy-terminal end of the $S_LST_1P$ stop–transfer sequence is located adjacent to several integral or peripheral membrane proteins of the ER. Interestingly, the photocrosslinking profile of the $S_LST_1P$ incubation was similar to that of the $ST_1P$ incubation, with the most abundant carbonate-resistant crosslinks appearing as a broad band between 45 and 56 kD in the SDS-PAGE gels (compare Fig. 7, lane *1* with Fig. 4, lane *8*). However, the photocrosslinking was not identical for the $ST_1P$ and $S_LST_1P$ polypeptides, for their extents of crosslinking to target proteins differed (e.g., $S_LST_1P$ nascent chains reacted more efficiently with the 70-kD ER membrane glycoprotein, and less efficiently with the 27- and 24-kD targets observed in Fig. 5).

The $S_LST_1P$ photocrosslinks were also analyzed for carbohydrate content. Since the photoreactive moieties were predicted to be located on the opposite side of the ER membrane from the glycosylation machinery and any glycosylated protein domains, any photocrosslinked glycoproteins would likely be transmembrane proteins of the ER. Two primary Con A-binding photoproducts were found, one that contained a 70-kD ER glycoprotein and one with a 37–38-kD ER glycoprotein that appeared to be mp39 (Fig. 7, lane *3*; Fig. 8 *B*, lanes *1* and *2*). Since mp39 is a transmembrane protein (Krieg et al., 1989), it is reasonable to expect that mp39 would react covalently with nascent chain probes presumed located either at the cytoplasmic surface with $S_LST_1P$ or at the lumenal surface with $ST_1P$. Furthermore, it is possible that mp39 is identical to SSR, which has been shown to be a transmembrane protein with a lumenal domain of 206 amino acids, a cytoplasmic domain of 56 amino acids, and a single membrane-spanning sequence predicted from the sequence and proteolysis data (Prehn et al., 1990). Our crosslinking of the 37–38-kD glycoprotein with probes predicted to be positioned on either face of the ER membrane is consistent with the target molecule being a transmembrane protein.

In control experiments analogous to those of Figs. 4 and 6, the photocrosslinking of $S_LST_1P$ nascent chains did not occur in the absence of light, $S_LST_1P$ mRNA, SRP, or microsomal membranes (data not shown). As with $ST_1P$, a small amount of photocrosslinking to some of the target pro-

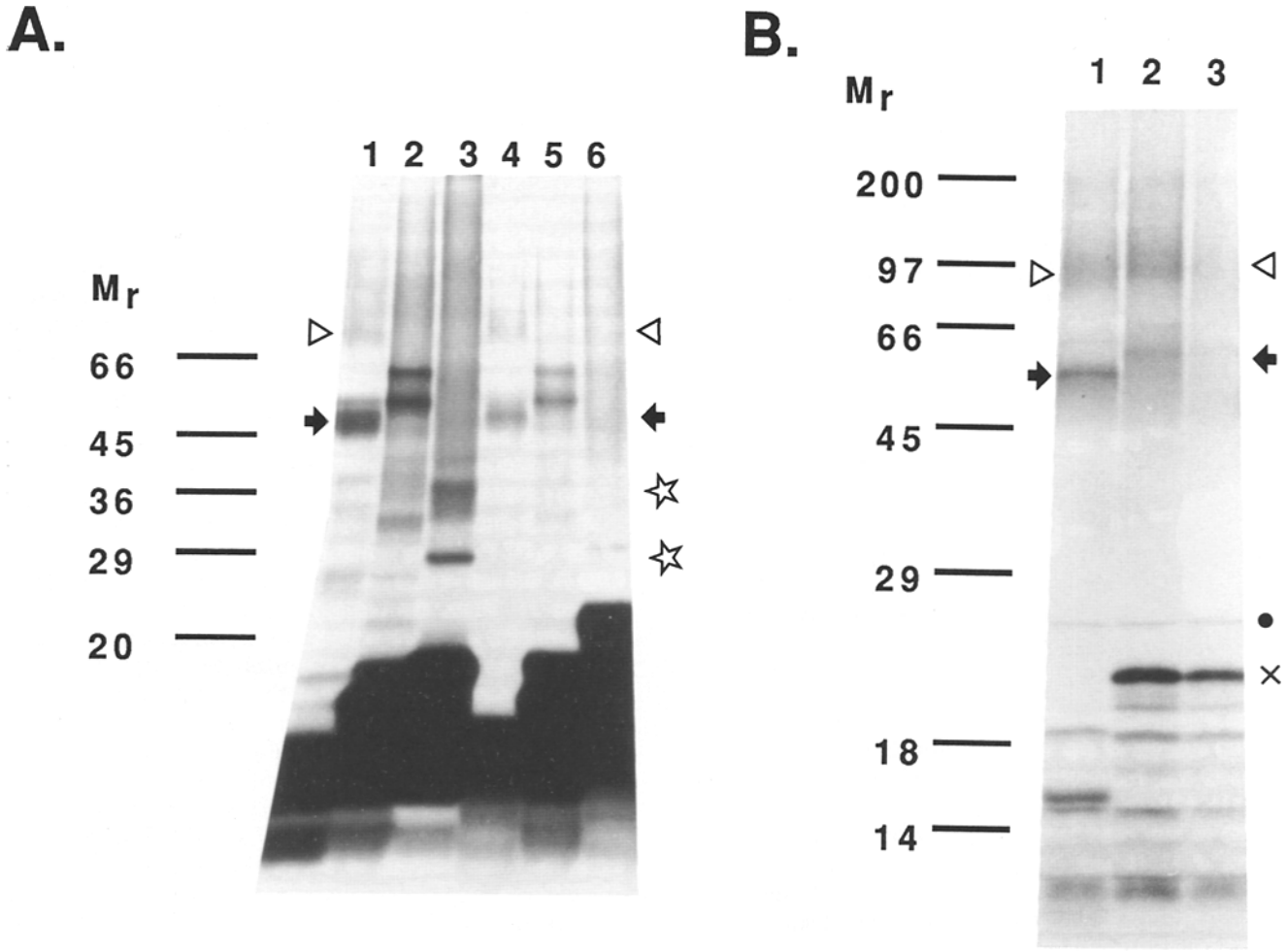


*Figure 8.* Photocrosslinked species formed by full-length $ST_1P$ and $S_LST_1P$ and by their nascent chains of various lengths. Translations (25 μl) containing EKRM, SRP, and ϵANB-Lys-tRNA were incubated, irradiated, extracted with carbonate (*A* and *B*), treated with Con A-Sepharose (*B* only), and analyzed as described in Figs. 4 and 6. Samples were programmed with RNAs encoding the 90mer of $ST_1P$ (*A*; lane *1*), the 152mer of $ST_1P$ (*A*; lane *2*), the full-length 176mer of $ST_1P$ (*A*; lane *3*), the 114mer of $S_LST_1P$ (*A*; lane *4*), the 176mer of $S_LST_1P$ (*A*; lane *5*; *B*, lane *1*), the 199mer of $S_LST_1P$ (*B*, lane *2*), or the full-length 200mer of $S_LST_1P$ (*A*, lane *6*; *B*, lane *3*). *A* (carbonate pellets): the arrowheads indicate the locations of photoadducts containing a 37–38 kD ER glycoprotein in lanes *1* and *4*; the stars mark the migration points in lane 3 of two photoadducts seen frequently with full-length $ST_1P$. *B* (Con A–bound species): the arrowheads indicate the locations of photoadducts containing the 37–38-kD glycoprotein in lanes *1* and *2*; "x" marks the location of residual $S_LST_1P$ 199mers and 200mers adsorbed to the ConA-Sepharose in lanes *2* and *3*, respectively; (●) marks the front of the heavily-overloaded band of Con A eluted from the resin during sample preparation.

teins (e.g., to the 70-kD glycoprotein, but not to the 37–38-kD glycoprotein) was observed when samples were translated and irradiated in the presence of Lys-tRNA instead of ϵANB-Lys-tRNA (data not shown), presumably mediated by chromophores present naturally in the nascent chain and/or target proteins.

Thus, the $S_LST_1P$ nascent chain forms several photocrosslinks that are SRP and membrane dependent. One major target of the nascent chain photoreaction appears to be mp39. The similarity in the photocrosslinking targets of nascent chains of $ST_1P$ and of $S_LST_1P$ indicates that, during integration, the transmembrane sequence is adjacent to some of the same proteins, independent of the final orientation of the newly synthesized stop–transfer element. This similarity also indicates that the same or very similar ER proteins are localized next to a nonpolar transmembrane sequence when it is either the first or the second topogenic sequence in a nascent membrane protein.

### *When Does the Nascent Membrane Protein Leave the Translocon?*

At some point, the transmembrane sequence of a membrane protein must leave its site of insertion and diffuse laterally to its ultimate site of action. We decided to take advantage of the nascent chain photocrosslinking to ER proteins to attempt to determine when the stop–transfer sequence and nascent chain leave the translocon. Specifically, the strategy was to increase stepwise the length of $S_LST_1P$ nascent chain that was synthesized after the transmembrane sequence, on the presumption that the longer the cytoplasmic portion of

the nascent chain, the more likely the stop-transfer sequence was to move away from its site of integration. Then, after photolysis of these different samples, one could determine at what length of nascent chain one could no longer detect crosslinking to mp39 or the mp39-like molecule. Crosslinking to this protein can be used as an indicator of proximity to the site of integration because the 37–38-kD ER membrane glycoprotein is a primary crosslinking target with short nascent chains (Figs. 4–7), the covalent reaction of the nascent chain with this glycoprotein is probe dependent (Figs. 4 and 6), and the 37–38-kD glycoprotein is photocrosslinked via the two lysine positions at the carboxy-terminal end of the stop-transfer segment (Fig. 5, lane *1*; and data not shown).

$S_LST_1P$ nascent chains (still attached covalently to a tRNA and bound noncovalently to a ribosome), ranging in length from 102–199 amino acids, were synthesized from truncated mRNAs as above and then photolyzed. In a parallel incubation, full-length $S_LST_1P$ (200 amino acids) was synthesized, released from the tRNA and ribosome via normal stop codon–mediated termination of protein synthesis, and then irradiated. The nascent membrane proteins, even the one which is only one amino acid short of termination, each crosslinked to the 37–38-kD ER glycoprotein (Fig. 7, lane *1*; Fig. 8 *A*, lanes *4* and *5*; and data not shown). In contrast, the completed full-length membrane protein no longer formed crosslinks with the 37–38-kD glycoprotein (Fig. 8 *A*, lane *6*). The lack of photoreaction between the mp39-like ER membrane protein and completed full-length $S_LST_1P$ was also demonstrated by the absence of any Con A-bound photoproducts in that sample (Fig. 8 *B*, lane *3*), even though the nascent chains reacted covalently with the mp39-like glycoprotein (Fig. 8 *B*, lanes *1* and *2*; and data not shown). This shows that a portion of the nascent chain remains in the vicinity of the 37–38-kD ER membrane glycoprotein, and hence the putative translocon, until translation is complete.

This conclusion was extended to nascent membrane proteins with the opposite orientation by comparing the photocrosslinking targets of completed full-length (176 amino acids) $ST_1P$ with those of $ST_1P$ nascent chains that ranged in length from 78–152 amino acids. Once again, each nascent chain reacted covalently with the 37–38-kD ER glycoprotein (Fig. 5, lanes *1* and *3*; Fig. 8 *A*, lanes *1* and *2*; and data not shown), but a major photoadduct with an apparent molecular weight near 58 kD (the apparent molecular weight of the 37–38-kD glycoprotein plus the apparent molecular weight of the full-length $ST_1P$) was not observed with the completed full-length $ST_1P$ (Fig. 8 A, lane *3*). The absence of full-length $ST_1P$ crosslinking to the 37–38-kD ER glycoprotein was confirmed by the fact that no significant radioactive material with a relative molecular weight value >20 kD was bound to Con A in the sample shown in Fig. 8 *A*, lane *3* (data not shown).

Interestingly, two photocrosslinked species were observed in samples containing full-length $ST_1P$ (Fig. 8 *A*, lane *3*; migration points indicated by the *stars*) that were virtually absent in samples of $S_LST_1P$. Since the photoreactive moieties in full-length $ST_1P$ are predicted to be located on the lumenal side of the ER membrane, this result is consistent with these two photoadducts containing target proteins (predicted $M_r$'s of 15 and 9 kD) that are localized completely (or primarily) within the lumen.

## Discussion

The interactions involved in the integration of a nascent membrane protein into the ER membrane have been difficult to examine because no experimental approach existed that permitted an investigation of this aspect of protein processing. We have applied an approach to the study of membrane proteins that has been used before to examine secretory proteins (Krieg et al., 1989; Wiedmann et al., 1989). Specifically, this approach allows us to "see what the nascent membrane protein sees" by the incorporation of probes, in this case photoreactive moieties, into specific sites of membrane nascent chains that are halted at various points in the integration process. Photolysis of the sample then permits an examination of the immediate environment of the nascent membrane proteins because the formation of photocrosslinks requires that two molecules be closely juxtaposed in order to be linked covalently.

A major advantage of this approach is that the probes in these experiments, the $\epsilon$ANB-Lys-containing nascent chains, are created in situ and can be bound only to functional membranes (Fig. 4, lanes *8* and *9*; Fig. 7, lanes *1* and *2*). This approach therefore ensures that structural data are obtained only from active integration intermediates. This is an important consideration because the efficiency of photocrosslink formation is typically low, and structural conclusions based on photocrosslinking results are justified only if one is confident that the covalent reactions occurred in functional and therefore properly structured complexes.

Using this approach, we have discovered four important properties of nascent chains undergoing integration at the ER membrane. First, the transmembrane sequence of each nascent chain is located in close proximity to several proteins of the ER. Second, similar subsets of the ER proteins are adjacent to the transmembrane domain of the nascent chain in either of its two possible orientations in the membrane. Third, similar subsets of the ER proteins are adjacent to a transmembrane sequence that is the first topogenic sequence in the nascent chain (i.e., has entered the bilayer first and has functioned as both a signal and a stop-transfer sequence) and to a transmembrane sequence that is the second topogenic sequence in the nascent chain (i.e., has entered the bilayer after a signal sequence, and has functioned only as a stop-transfer sequence). Fourth, at least a portion of the nascent chain of a membrane protein remains in close proximity to these ER proteins until the nascent polypeptide has been completely translated.

These observations provide both direct information about the integration process and also form the basis for some initial conclusions about the nature and mechanism of integration. The close proximity of a select group of ER proteins to the transmembrane domain of different nascent membrane proteins suggests that protein components of the ER are involved in and facilitate, either actively or passively, the integration of nascent membrane proteins into the phospholipid bilayer. Furthermore, the nascent membrane protein remains close to these ER proteins, even when the ribosome-bound nascent chain is long enough for the transmembrane segment to diffuse laterally away from its site of insertion. Therefore the transmembrane domain and/or another segment of the nascent polypeptide remains adjacent to the translocon until translation of the polypeptide is complete.

Photoreactive moieties located at the carboxy-terminal end of the transmembrane sequence react covalently with ER proteins, and with the mp39-like protein in particular (Fig. 5, lane *I*). Thus, one possible explanation for the observed photocrosslinking of the 37–38-kD ER membrane glycoprotein by all lengths of nascent chain except the full-length polypeptide (Fig. 8 *B*) is that the transmembrane segment of the nascent protein remains in the translocon until translation is complete. If this is the case, the retention of the stop-transfer sequence of $S_LST_1P$ in the translocon would presumably not be because of limitations in the size of the nascent chain, because the 199-residue nascent chain contains 141 amino acids (494 Å of polypeptide polymer if fully extended) after the stop-transfer sequence on the cytoplasmic side of the membrane and this should be sufficient to allow the transmembrane sequence to diffuse laterally a considerable distance from its site of insertion. Instead, retention would presumably result from at least one of the proteins of the translocon interacting with the transmembrane domain to limit its diffusion before the termination of translation. On the other hand, since photoreactive moieties are also found in the nascent chain after the stop-transfer sequence, it is conceivable that the longer nascent chains crosslink to the 37–38-kD glycoprotein via these lysine positions. If this is the case, then, even if the transmembrane segment leaves the translocon, photocrosslinking to the mp39-like protein may occur until a completed chain diffuses away from the translocon and/or folds in such a way that its probes are no longer accessible to the 37–38-kD glycoprotein.

The above conclusions about the nature of the integration process have been made without reference to any specific ER membrane proteins, and are valid even though the identities of the ER proteins that have been crosslinked to the nascent membrane proteins have yet to be established. If, as appears likely, one of the primary photocrosslinking targets of the nascent membrane proteins is mp39, then the translocation machinery used by secretory proteins and by membrane proteins before integration are the same or are constructed using similar ER protein components.

More microsome-dependent photocrosslinks were observed with the nascent membrane proteins than with the nascent secretory proteins (Fig. 5). One explanation of this observation is that the environments of the two types of nascent chains differ in the ER membrane, either because of the existence of two different types of translocon, or, if all translocons are the same, because the transmembrane domain moves to a position from which a secretory protein is excluded, possibly via receptors which recognize the stop-transfer sequence and mediate integration. Alternatively, the increased number of photocrosslinking targets observed with the membrane proteins may result from differences in the locations of the photoprobes in the nascent chains relative to the membrane and/or from differential recovery of photoadducts (see Results).

In summary, the most important conclusions of this work are that nascent membrane proteins integrate into the ER membrane adjacent to proteins of the ER, and that a segment(s) of a nascent membrane protein remains in close proximity to these ER proteins until translation of the nascent chain is complete. The processing pathways of both nascent secretory and membrane proteins therefore appear to involve the participation of ER membrane proteins (Krieg et al., 1989; and this work). Further experiments using the approach employed in this work may lead to the identification of the mechanism and ER components, if any, that effect the divergence of these pathways.

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*Note Added in Proof*: S. High and colleagues (personal communication) have also observed photocrosslinking between SSR/mp39 and photoreactive nascent membrane proteins in either orientation, and between a Type I nascent membrane protein and a non-glycosylated ER membrane protein that is slightly larger than SSR/mp39 (High et al., In press).

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