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The Protein-Conducting Channel in the Membrane of the Endoplasmic Reticulum Is Open Laterally toward the Lipid Bilayer

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Summary

Lipids and proteins were found to contact a nascent type II membrane protein, as well as a nascent secretory protein, during their insertion into the membrane of the endoplasmic reticulum. This suggests that the protein-conducting channel is open laterally toward the lipid bilayer during an early stage of protein insertion. Contact to lipids was confined to the hydrophobic core region of the respective signal or signal anchor sequence. Thus, the nascent polypeptide is positioned in the translocation complex such that the signal or signal anchor sequence faces the lipid bilayer, whereas the hydrophilic, translocating portion is in proteinaceous environment.

Introduction

The insertion of membrane proteins into, as well as the translocation of secretory proteins across, the membrane of the mammalian endoplasmic reticulum (ER) is initiated in the cytoplasm by binding of the signal sequence of the nascent chain to the signal recognition particle (SRP; Walter and Blobel, 1981a). Specific binding of the ribosome nascent chain–SRP complex to the ER membrane is mediated by the membrane-bound SRP receptor (docking protein) (Gilmore et al., 1982; Meyer et al., 1982), whereupon the nascent polypeptide is transferred into the ER membrane (Connolly and Gilmore, 1989; Rapoport, 1992).

Membrane insertion of secretory proteins and type II membrane proteins is thought to involve the formation of a loop structure, with the N-terminus remaining in the cytoplasm and the growing C-terminal part being continuously translocated across the membrane (Shaw et al., 1988). In case of a secretory protein, cleavage of the signal sequence then releases the mature N-terminus of the polypeptide into the ER lumen. Membrane insertion of a type II membrane protein is thought to occur in a similar manner, except that instead of a signal sequence, a signal anchor (SA) sequence functions in targeting and membrane insertion. The SA sequence is not cleaved by the signal peptidase, and it anchors the mature protein in the lipid bilayer such that the protein spans the membrane with its N-terminus in the cytoplasm and the C-terminus in the ER lumen.

Passage of the nascent polypeptide across the membrane is thought to occur through a proteinaceous, aqueous channel (Blobel and Dobberstein, 1975). This view is strongly supported by experiments showing that nascent polypeptides can be removed from the membrane by agents disturbing protein–protein interactions (Gilmore and Blobel, 1985). Furthermore, electrophysiological studies have demonstrated the presence of large aqueous channels in rough ER membranes (Simon and Blobel, 1991), and fluorescent probes incorporated into the hydrophilic portion of a translocating polypeptide report an aqueous interior (Crowley et al., 1993, 1994). Direct evidence that proteins are involved in the translocation process comes from cross-linking experiments as well as from reconstitution studies. Sec61α has been shown to line the postulated translocation pore (Mothes et al., 1994). In addition to the Sec61p complex (subunits α, β, γ) (Görlich et al., 1992b), the TRAM protein (Görlich et al., 1992a) and the SRP receptor (Meyer et al., 1982) are essential for translocation of polypeptides into reconstituted proteoliposomes (Görlich and Rapoport, 1993).

Whether lipids, as well as proteins, also function in the translocation process, especially in membrane insertion of membrane proteins, is not known. Lipids could be expected in the vicinity of SA sequences, as they anchor the mature protein in the membrane. Signal and SA sequences both have a central core of hydrophobic amino acid residues (h region; von Heijne, 1985a, 1986). Whereas h regions of eucaryotic signal sequences are typically 7–15 residues long, h regions of SA sequences are typically longer than 20 residues (von Heijne, 1985b).

We demonstrate here an experimental approach by which contact between proteins and lipids can be identified and characterized. Using a highly reactive, photoactivatible cross-linking reagent that can attack lipid side chains (Brunner, 1989) as well as proteins, we show that a signal and a SA sequence are in contact with lipid molecules during early stages of the membrane insertion process. This result suggests that the protein-conducting channel is open laterally toward the lipid bilayer.

Results

Experimental Strategy

To characterize the molecular environment of the SA sequence and adjacent hydrophilic segments of the type II membrane protein invariant chain (Ii) and of the signal sequence of the secretory protein preprolactin (PPL), translocation intermediates of defined length were prepared and site specifically cross-linked to neighboring components. In the presence of microsomal membranes, transmembrane-arrested intermediates were obtained by translating mRNAs truncated in their coding sequence (Gilmore et al., 1991). Because no termination can occur,

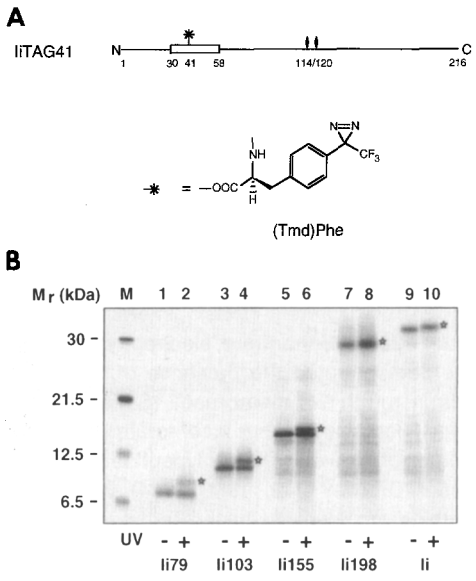

Figure 1. Photocross-Linking with Ii Translocation Intermediates to Membrane Components

(A) Outline of IiTAG41, with photoactivatible amino acid (Tmd)Phe (asterisk) site specifically incorporated at position 41. The open box indicates the hydrophobic region of the SA sequence; potential N-glycosylation sites are indicated by diamonds.

(B) Photocross-linking experiments with Ii translocation intermediates. Radiolabeled IiTAG41 chains of 79 (lanes 1 and 2), 103 (lanes 3 and 4), 155 (lanes 5 and 6), 198 (lanes 7 and 8), and 216 (lanes 9 and 10) amino acids were synthesized in vitro in the presence of SRP and canine pancreas rough microsomes. After UV irradiation, membranes were analyzed for membrane-associated cross-link products (stars).

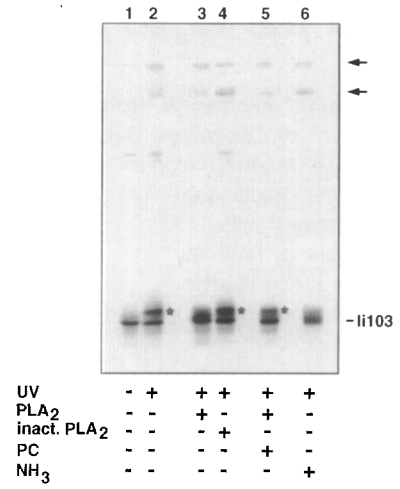

Figure 2. Characterization of Phospholipid as Cross-Link Partner

Membrane-associated IiTAG41/103 cross-link products (lane 2) were treated with phospholipase A_2 (PLA_2; lane 3), with manoalide-inhibited PLA_2 (lane 4), and with PLA_2 in the presence of excess phosphatidylcholine (PC; lane 5). Cross-link products were also treated with ammonia to chemically hydrolyse fatty acyl ester bonds (lane 6). Stars indicate cross-links to lipids; arrows indicate cross-links to proteins.

the translation product remains attached to the ribosome as peptidyl-tRNA. These translocation intermediates presumably span the membrane twice, once with the signal or SA sequence and once with a following part of the polypeptide. The orientation in the membrane is such that both the C-terminus (bound to the ribosome) and the N-terminus face the cytosolic side.

For site-specific photocross-linking (Brunner, 1993a; High et al., 1993a), the photoactivatible amino acid L-4'-(3-[trifluoromethyl]-3H-diazirin-3-yl)phenylalanine ((Tmd)Phe; Figure 1A; Baldini et al., 1988) was incorporated at single, selected positions within the membrane-spanning segments. Ultraviolet (UV)-dependent activation of (Tmd)Phe generates a highly reactive carbene ($t_{1/2}$, ~1 ns) that can attack even aliphatic fatty acyl side chains (Brunner, 1993b). This probe would therefore cross-link to any adjacent component, independent of whether it is a protein, a lipid, or a water molecule. The incorporation of (Tmd)Phe into the nascent polypeptide was mediated by a chemically aminoacylated suppressor tRNA. Hence, each polypeptide contains the photoactivatible amino acid, and therefore, cross-linking efficiencies can be quantified.

The SA Sequence of Ii Is Cross-Linked to a Low Molecular Weight Component

To identify nearest neighbors of the SA sequence of Ii during its membrane insertion, we introduced (Tmd)Phe within the Ii h region at position 41 (IiTAG41 mutant; Figure 1A). Translocation intermediates of increasing length (79, 103, 155, and 198 amino acids), as well as the full length protein, were subjected to UV activation of the cross-linking reagent. Cross-link products were identified on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to their reduced mobility relative to noncross-linked polypeptides. With the full-length protein, we observed a cross-link product with a small reduced mobility consistent with the attachment of a lipid molecule (Figure 1B, lane 10). We also found a major cross-link to a small component (~0.5 kDa) with the translocation intermediates li79, li103, li155, and li198 (Figure 1B, lanes 2, 4, 6, and 8).

The SA Sequence of Ii Interacts with Phospholipids

The small size of the major membrane component that was cross-linked to IiTAG41 translocation intermediates suggested it to be a lipid molecule. The most abundant type of lipids in the rough ER membrane are phospholipids (Alberts et al., 1988). To test whether the low molecular weight cross-link partner is a phospholipid, we treated IiTAG41/103 translocation intermediates after photocross-linking with bee venom phospholipase A_2 (PLA_2). This enzyme specifically cleaves all common types of phospholipids at position C2 into fatty acid and lysophospholipid. If the shift in molecular weight were due to addition of a phospholipid, it should have been reduced by PLA_2 treatment. Indeed, the proportion of low molecular weight cross-link product was significantly reduced upon incubation with PLA_2 (Figure 2, lane 3). A fuzzy intermediate-sized cleavage product accumulated. This probably reflected the fact that one of the PLA_2 cleavage products, either fatty acid or lysophospholipid, remained attached to the translocation intermediate.

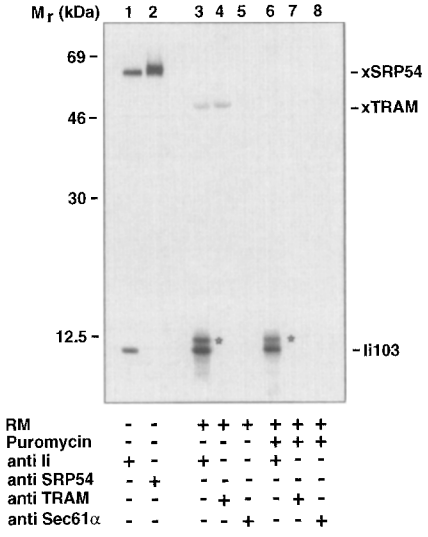

Figure 3. Photocross-Linking with liTAG41/103 to SRP and Membrane Components

Radiolabeled li103 was synthesized in vitro in the presence of SRP (lanes 1 and 2) and in the presence of SRP and canine pancreas rough microsomes (lanes 3–8). Nascent chains were released by puromycin (lanes 6–8). Samples were then UV irradiated, and the cross-link products were immunoprecipitated with anti-li and anti-SRP54 antibodies for translations without microsomes (lanes 1 and 2) and with anti-li, anti-TRAM, and anti-Sec61α antibodies for translations in the presence of microsomes (lanes 3–8). Stars indicate cross-links to lipids.

Table 1. Quantification of Cross-Linking to SRP and to Membrane Components

Mutant	Cross-Linking Efficiencies of li103 or PPL86 to			
	SRP54	Lipid	TRAM	Sec61α
liTAG41 (with SRP only)	~45%	–	–	–
liTAG41	<2%	~45%	~5%	None
liTAG41/Puromycin	None	~45%	None	None
liTAG50	<2%	~35%	<2%	~5%
liTAG59	<2%	~3%	None	~3%
liTAG67	<2%	None	None	~3%
PPLTAG11	~4%	~5%	~8%	<2%
PPLTAG18	~5%	~15%	None	<2%
PPLTAG25	~3%	~17%	None	<2%

Measurements were taken from experiments shown in Figures 3, 5, and 6B. Cross-links to TRAM and to Sec61α with PPL86 translocation intermediates have been characterized previously (High et al., 1993a). The radioactivity in the respective bands was quantified using the phosphoimager and Fuji MacBas V1.0 software. The radioactivity in li103 or PPL86 before cross-linking was taken as 100%.

To demonstrate the specificity of PLA_2 and to exclude the action of contaminating proteases, we inhibited PLA_2 with manoalide (Glaser and Jacobs, 1986). This specific inhibitor covalently modifies the active center of the enzyme. Manoalide-treated PLA_2 did not affect the low molecular weight cross-link product (Figure 2, lane 4). To confirm the lipase activity of PLA_2 further, we supplemented the PLA_2 reaction with excess phospholipid. As expected, the cross-link product was not affected by PLA_2 anymore (Figure 2, lane 5).

Phospholipids as cross-linking partners can also be characterized chemically by alkaline hydrolysis of their labile fatty acyl ester bonds. After treatment with ammonia, the low molecular weight cross-link product was converted into a compound with slightly increased electrophoretic mobility (Figure 2, lane 6). This is consistent with cleavage of the phospholipid and one of the fatty acyl side chains remaining bound to li103. It should be noted that neither the PLA_2 treatment nor alkaline hydrolysis affected cross-link products with proteinaceous components (Figure 2, lanes 2–6).

Besides being cross-linked to lipids, liTAG41/103 was cross-linked to the TRAM protein, indicating localization at the translocation site (Figure 3, lane 4). When the nascent chain was released from the ribosome by puromycin, liTAG41/103 was cross-linked only to lipids, suggesting that it had left the proteinaceous environment of the translocation site (Figure 3, lane 6). Cross-linking to lipids was very efficient (~45%) for ribosome-bound as well as ribosome-released li103 (Table 1). Such high cross-linking efficiencies can be considered to reflect extensive hydrophobic contact between protein and lipid (Brunner, 1989). A cross-linking efficiency of ~45% was also obtained between the li SA sequence and the 54 kDa subunit of SRP during targeting to the ER membrane (Figure 3, lanes 1 and 2; Table 1). Thus, small quenching molecules are largely excluded from the site at which the SA sequence interacts with SRP54 or with membrane components.

Verification of Translocation Intermediates

Translation of truncated mRNA may result in some polypeptides that are released from the ribosome, owing to hydrolysis of the peptidyl-tRNA. To show unequivocally that lipids contact ribosome-attached polypeptides and not only released ones, we inserted SRP-arrested nascent chains into the membrane, induced cross-linking by UV light, and continued translation. For these experiments, we used liTAG41/155, which was translated in the presence of SRP but without microsomes. Under these conditions, SRP arrests translation after about 100 amino acids have been polymerized (Lipp et al., 1987; Walter and Blobel, 1981b). Further translation was blocked with cycloheximide, and microsomes were added to allow insertion of the nascent chains into the membranes. After UV irradiation, the membranes were purified by centrifugation through a sucrose cushion to remove cycloheximide. Fresh wheat germ extract was added, and translation continued. With completed li155, the cross-link to a lipid molecule was clearly detectable (Figure 4, lane 4). Cross-links to lipids with SRP-arrested fragments could not easily be detected because these fragments were not of uniform size (Figure 4, lane 2).

li155 contains two potential N-glycosylation sites at positions 114 and 120. As long as the polypeptide is attached to the ribosome, no glycosylation occurs. Both sites are probably buried within the membrane and therefore not accessible to the oligosaccharyltransferase. However, release of the translocation intermediates by puromycin resulted in an increased molecular weight, consistent with

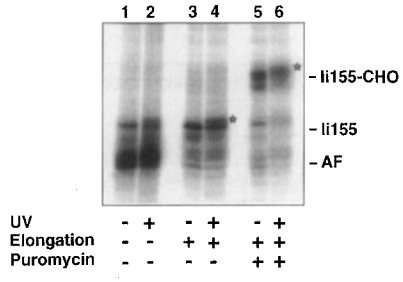

Figure 4. Elongation of SRP-Arrested Nascent Polypeptides

SRP-arrested nascent liTAG41/155 (AF) was inserted into microsomal membranes in the presence of cycloheximide and cross-linked to neighboring components (lanes 1 and 2). After removal of cycloheximide, elongation was continued (lanes 3 and 4). To one aliquot, puromycin was added to release nascent chains from the ribosome and allow glycosylation (lanes 5 and 6). Stars indicate cross-links to lipids; li155-CH indicates glycosylated li155.

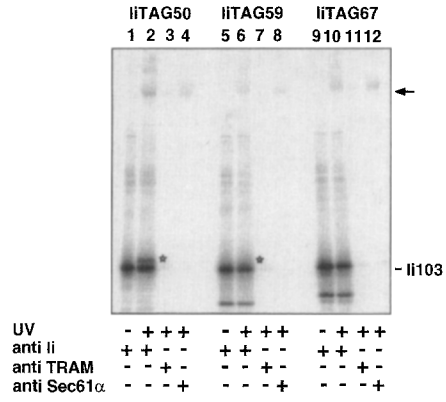

Figure 5. Photocross-Linking with liTAG50/103, liTAG59/103, and liTAG67/103 to Membrane Components

(A) Radiolabeled li103 translocation intermediates with (Tmd)Phe at positions 50 (lanes 1–4), 59 (lanes 5–8), and 67 (lanes 9–12), respectively, were synthesized in vitro in the presence of SRP and canine pancreas rough microsomes. Samples were UV irradiated and immunoprecipitated with anti-li, anti-TRAM, and anti-Sec61α antibodies. Stars indicate cross-links to lipids; the arrow indicates cross-links to TRAM or Sec61α.

the addition of two carbohydrate side chains (Figure 4, lanes 5 and 6). Taken together, these results demonstrate that at the time of UV irradiation, essentially all the nascent chains were attached to the ribosomes and located in the translocation site.

Components Interacting with Other Regions of li103

To see whether lipids also contact other regions of translocation intermediate li103, we tested mutant liTAG50 in which (Tmd)Phe was incorporated into the C-terminal half of the h region. As with liTAG41, we obtained efficient cross-linking to lipid molecules (Figure 5, lane 2; Table 1). In contrast to liTAG41/103, which was also cross-linked to TRAM, liTAG50/103 was cross-linked to Sec61α as well as to lipids (Figure 5, lane 4; Table 1).

To identify components in proximity to the segment C-terminally following the h region, two other TAG mutants were constructed. (Tmd)Phe was incorporated at position 59 (liTAG59), the C-terminal end of the h region, and at position 67 (liTAG67) in the adjacent hydrophilic portion. Translocation intermediates li103 of these two mutants were both found to cross-link to Sec61α (Figure 5, lanes 8 and 12). A small amount of cross-links to lipid molecules was found with liTAG59/103 but none with liTAG67/103 (Figure 5, lanes 6 and 10; Table 1). This is consistent with previous results showing that hydrophilic segments of li translocation intermediates are in contact with Sec61α during membrane insertion (High et al., 1993b).

The Signal Sequence of PPL Is Also in Contact with Lipids

The proteinaceous environment of the signal sequence of PPL has been characterized previously with PPL TAG mutants, in which (Tmd)Phe was site specifically incorporated at positions 11, 18, and 25, respectively (High et al., 1993a). Here, we used these mutants to investigate contact with lipid molecules also. PPL86 translocation intermediates (Figure 6A) of the three TAG mutants were subjected to UV activation of the cross-linking reagent, and membrane-associated cross-link products were identified on SDS-PAGE. PPL86/TAG25 was found to be efficiently cross-linked to a component of low molecular weight (Figure 6B, lane 6). To assess whether this low molecular weight cross-link partner was a phospholipid, we treated the PPL86/TAG25 translocation intermediates after photocross-linking with PLA_2. The low molecular weight cross-link product essentially disappeared upon treatment with PLA_2 (Figure 6B, lane 7). Thus, the signal sequence of the PPL translocation intermediate is also in contact with the lipid bilayer. Cross-linking to lipid molecules was also found with PPL86/TAG18 and, to some degree, with PPL86/TAG11 (Figure 6B, lanes 2 and 4; Table 1). We also found the previously identified cross-link products with TRAM (PPL86/TAG11) and with Sec61α (PPL86/TAG11, PPL86/TAG18, and PPL86/TAG25), which were not further characterized (Figure 6B, lanes 2, 4, and 6).

Discussion

The existence of a protein-conducting channel in the membrane of the endoplasmic reticulum has been demonstrated by electrophysiological studies (Simon and Blobel, 1991). The biochemical nature of such a channel has been elucidated with different cross-linking reagents (High et al., 1991, 1993a; Mothes et al., 1994) and with fluorescent probes (Crowley et al., 1993, 1994). Results from these studies have led to the model of a hydrophilic translocation pore, in which proteinaceous components shield the inserting/translocating polypeptide from the lipid bilayer. Our finding that lipids, as well as proteins, line part of the nascent polypeptide suggests that the protein-conducting channel is open toward the lipid bilayer during the early stage of insertion of nascent secretory proteins, as well as nascent type II membrane proteins. The degree of opening

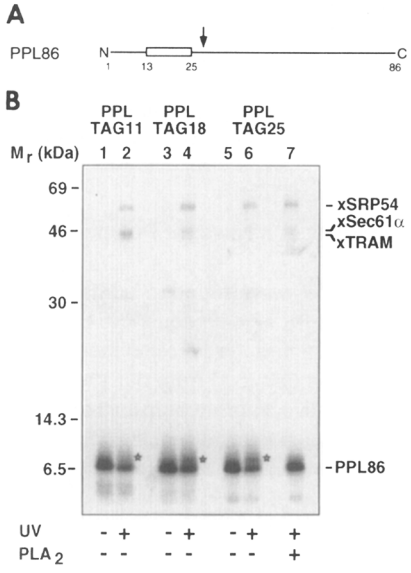

Figure 6. Photocross-Linking with PPL86 Translocation Intermediates to Membrane Components

(A) Outline of PPL86. The hydrophobic core region of the signal sequence is indicated as an open box; the cleavage site is indicated by an arrow.

(B) Radiolabeled translocation intermediates of PPL86 with (Tmd)Phe at positions 11 (lanes 1 and 2), 18 (lanes 3 and 4), and 25 (lanes 5, 6, and 7), respectively, were synthesized in vitro in the presence of SRP and canine pancreas rough microsomes and subjected to UV irradiation. Membrane-associated PPL86/TAG25 cross-link products were treated with PLA_2 (lane 7). Samples were immunoprecipitated with anti-PPL antibodies. Stars indicate cross-links to lipids.

may change depending on the hydrophobic nature of the signal or signal anchor sequence and during different stages of the translocation process.

Detection of Protein and Lipid in the Protein-Conducting Channel

Although proteins and lipids have been suggested to be involved in membrane insertion and translocation of proteins (Blobel and Dobberstein, 1975; Engelmann and Steitz, 1981; Wickner, 1979), assay systems to test for lipids in or near the translocation site have been lacking. We demonstrate here that suppressor tRNA–directed incorporation of a single photocross-linking reagent and carbene-mediated cross-linking (Brunner, 1993a) can be used to characterize precisely the molecular composition of proteins and lipids of the immediate environment of a nascent polypeptide during its membrane insertion. The highly reactive carbene used in this study has been shown to attack neighboring molecules irrespective of their chemical nature. In particular, such reagents are efficiently linked to aliphatic side chains of lipids (Brunner, 1989).

Site-specific photocross-linking allows quantification of cross-linking efficiencies. Translation proceedes only if the suppressor tRNA has successfully delivered the photoactivatible amino acid to the nascent polypeptide (High et al., 1993a). As a consequence, all polypeptide chains

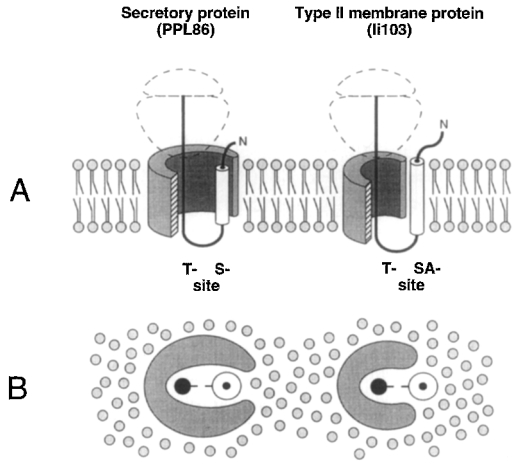

Figure 7. Postulated Arrangement of Proteins and Lipids in the Protein-Conducting Channel

During the early stage of membrane insertion of a secretory and a type II membrane protein, the nascent polypeptide is arranged in a loop-like configuration. Whereas the translocating portion of the nascent chain essentially faces protein (T site), the signal or signal anchor sequence (white barrels indicate their h region) is in contact with protein and lipid (S/SA site). The degree of opening of the channel toward the lipid bilayer is likely to vary according to the hydrophobic property of the signal and signal anchor sequence, respectively. (A) side view; (B) top view.

contain the cross-linking reagent and participate in the cross-linking reaction. Probing the environment of the nascent chain in the translocation complex, we obtained cross-linking efficiencies to membrane components up to 50% (Table 1). This is consistent with results from hydrophobic photolabeling studies with radiolabeled diazirin-containing reagents in other biological membranes. In these experiments, hydrophobic cross-linking reagents were linked to membrane components with an efficiency of 50%–60%, of which >90% was associated with lipid (Brunner, 1989). The remaining portion of the labeling reagent was either quenched by small molecules dissolved in the membrane or was photoisomerized to nonreactive species. Thus, a 50% cross-linking efficiency may be indicative for a complete enclosure of a membrane-spanning segment by lipid or protein.

As lipid molecules are rather small, a nascent chain–lipid adduct is not easily detectable by an increase in molecular weight. However, using small protein fragments (M_r = 7–30 kDa), we were able to detect cross-links to lipids by SDS-PAGE. The apparent increase in molecular weight was in the range of about 0.5 kDa. That this shift was indeed due to a cross-link to a lipid molecule was confirmed by cleavage with PLA_2 and alkaline hydrolysis. Whether a specific lipid or a mixture of lipids are in proximity to the SA sequence of Ii remains to be determined.

Membrane Insertion of a Secretory Protein and a Type II Membrane Protein

Membrane insertion of secretory and type II membrane proteins is thought to involve the formation of a loop across the membrane (Shaw et al., 1988). In this loop, two seg-

ments span the membrane, the signal or SA sequence and the translocating portion of the growing polypeptide chain. Accordingly, we can distinguish two sites in the translocation complex, one in which the signal or SA sequence is retained in the membrane S/SA and one through which the nascent chain traverses the membrane (T site; Figure 7). For a type II membrane protein, we show here that these functionally different sites are also different in their molecular composition. The SA sequence of the model protein Ii is in contact with lipids at any tested stage of its membrane insertion. This indicates that at an early stage of membrane insertion, the protein-conducting channel is already open such that the SA sequence partitions into the lipid bilayer. There is also protein in contact with the SA sequence, as a small percentage of the translocation intermediates was found to be cross-linked to TRAM or to Sec61α. The fact that a single site of the SA sequence can be cross-linked to two components probably reflects a certain degree of rotational, lateral, or perpendicular mobility of the nascent polypeptide in the protein-conducting channel.

The Environment of a Nascent Polypeptide in the T Site

The translocating parts of nascent type II membrane proteins and of nascent secretory proteins are in a hydrophilic environment and contact Sec61. This is concluded from several lines of evidence. We have described in this study that the major cross-link partner of the hydrophilic segment of membrane-inserted Ii is Sec61α. The efficiency of this cross-linking is rather low (~3%) when compared with cross-linking efficiencies obtained when the interaction between the two reaction partners is tight (e.g., nascent chain × SRP; Table 1). We interpret the low cross-linking efficiency to Sec61α to reflect random collision with the wall of the protein-conducting channel formed by Sec61 and quenching of the UV-activated cross-linker by water molecules. Proximity to Sec61α was also described previously when modified, photoactivatible lysine residues were incorporated into several positions C-terminal of the h region of Ii and at one N-terminal position (High et al., 1993b).

The translocating portion of a secretory protein also was found to be in contact with Sec61α. Mothes et al. (1994) systematically probed the hydrophilic portion of translocating nascent PPL chains by a site-specific photocross-linking approach. In this case, modified, photoactivatible lysine residues were incorporated into single, selected positions of nascent polypeptides. Sec61α was found to be the major ER membrane protein in proximity to the translocating polypeptide. Cross-links to lipids were not reported. The environment of a nascent secretory protein during translocation across the ER membrane has also been probed by incorporating fluorescent probes into the hydrophilic portion of nascent chains. It was found that the nascent polypeptide occupies an aqueous pore that spans the entire membrane (Crowley et al., 1993, 1994). For secretory protein translocation across the inner membrane of Escherichia coli, a proteinaceous environment for a translocating polypeptide has also been demonstrated. Joly and Wickner (1993) have shown that SecYp, the bacterial equivalent of Sec61α, is adjacent to the hydrophilic portion of a translocating polypeptide. Lipids were not detected, and therefore, it was concluded that the translocating portion of a polypeptide is shielded from the lipid bilayer.

The Environment of a Nascent Polypeptide in the S/SA Site

Lipids and proteins were found to contact the Ii SA sequence and the PPL signal sequence. How exactly proteins and lipids are arranged around the signal or SA sequence cannot be deduced from our data. However, as we found quantitative differences in lipid cross-linking with signal and SA-sequences, this could suggest that the protein-conducting channel is, to a variable degree, open toward the lipid bilayer. The PPL signal sequence was cross-linked to lipids with an efficiency of up to 17%, whereas the Ii SA sequence was cross-linked to lipids with an efficiency of up to 45% (Table 1). Differences in the environment of signal and SA sequences during their membrane insertion were proposed recently by Nilsson et al. (1994). These authors determined the distance between a signal or SA sequence and the center of the oligosaccharyltransferase and found that the positions of a signal sequence or a SA sequence relative to the oligosaccharyltransferase are different. Furthermore, it was shown that the signal peptidase has access to signal sequences with h regions of less than 15 amino acids but not to extended ones of greater than 17 amino acids typical for SA sequences. Thus, it appears that the arrangement of the components in the translocation complex is determined by the hydrophobic properties of signal and SA sequences of nascent polypeptides. SA sequences with extended h regions might be more exposed toward the lipid bilayer than signal sequences with short h regions (Figure 7).

The architecture of the protein-conducting channel may not only vary depending on the type of the protein inserted but also during different stages of the insertion process: stage I, during docking of the ribosome nascent chain onto the membrane; stage II, during membrane insertion of the nascent polypeptide; stage III, after cleavage of the signal sequence; stage IV, after dissociation of a SA-sequence from the translocation site; and stage V, after chain termination and completed translocation. Additional rearrangements of the translocation complex may occur during the insertion of proteins, with a stop transfer sequence and proteins spanning the membrane several times. In this study, we have investigated the second one of these stages (stage II). The combination of site-specific cross-linking with synchronized membrane insertion and translocation will allow the characterization of these postulated rearrangements of the protein-conducting channel.

Experimental Procedures

Construction of Plasmids

Plasmid pGEM3Ii (High et al., 1993b) was used for site-directed mutagenesis to introduce a single amber (TAG) codon by overlap extension using polymerase chain reaction (Ho et al., 1989). Codon 41, 50, 59, or 67 of the coding region of Ii was replaced by the TAG codon to give

pliTAG41, pliTAG50, pliTAG59, and pliTAG67. Plasmid pGEM4PPL and PPL TAG mutants prepared from them (pPPLTAG11, pPPLTAG18, and pPPLTAG25) were described previously (High et al., 1993a).

Amber Suppressor tRNA

(Tmd)Phe-tRNA^{Sup} was prepared by chemical aminoacylation according to Baldini et al. (1988). Aminoacylated (Tmd)Phe-pCpA (Baldini et al., 1988) and abbreviated suppressor tRNA [tRNA^{Sup}(–pCpA)], which was prepared in vitro by runoff transcription of PstI-linearized plasmid pJDB2 (Bain et al., 1991), were ligated with T4 RNA ligase (High et al., 1993a). Aminoacylated suppressor tRNA was recovered by precipitation with ethanol and 250 mM sodium acetate (pH 4.5) following extraction with phenol/chloroform (1:1) and chloroform.

Transcription

mRNAs were synthesized by in vitro transcription of linearized plasmids using T7 RNA polymerase (High et al., 1993a). For transcripts encoding nascent chains with defined length, plasmids were cleaved in the coding region as described (Gilmore et al., 1991). To obtain nascent Ii chains (Ii79, Ii103, Ii155, Ii198, and Ii216), relevant IiTAG plasmids were linearized with HindIII, NcoI, AflII, PpuMI, and BamHI, respectively. For transcripts encoding nascent PPL (PPL86), PPL TAG plasmids were linearized with PvuII.

Translation and Photocross-Linking

Wheat germ extract was prepared as described by Erickson and Blobel (1983). Microsomal membranes and SRP were prepared from dog pancreas (Siegel and Walter, 1985; Walter and Blobel, 1983). The conditions for translation were described previously (High et al., 1993a), except that ~50 pmol (Tmd)Phe-tRNA^{Sup} were added per 25 µl translation reaction, and the magnesium concentration was raised to 3.5 mM. Translations were performed for 10 min at 25°C in the presence of [^{35}S]methionine, suppressor tRNA, SRP, and rough microsomes. Cycloheximide was then added to a concentration of 4 mM, and the samples were UV irradiated (364 nm) for 2 min on ice (High et al., 1991).

In one experiment (see Figure 4) translation of cross-linked nascent chains was continued. In this case, IiTAG41/155 mRNA was translated as described above in the presence of SRP but without microsomes. Further elongation of the SRP-arrested nascent chains was inhibited by the addition of cycloheximide (4 mM). The mixture was supplemented with rough microsomes, incubated for an additional 5 min at 25°C, and UV irradiated for 2 min on ice. Membranes were extracted with high salt and sedimented through a sucrose cushion (see next paragraph). The pellets were resuspended in new wheat germ extract without [^{35}S]methionine, suppressor tRNA, SRP, and mRNA and incubated for 10 min at 25°C. To one aliquot, 1 mM puromycin was added, and the mixture was further incubated for 10 min at 25°C.

Analysis of Photocross-Link Products

Membranes were extracted with high salt by the addition of KOAc to 0.5 M and incubation for 5 min on ice and recovered by centrifugation through a 50 µl sucrose cushion (0.5 M sucrose, 50 mM HEPES-KOH [pH 7.6], 0.5 M KOAc, 5 mM Mg[OAc]$_2$) by spinning for 3 min at 100,000 × g in a Beckman TLA100 rotor at 4°C.

For immunoprecipitation, proteins were solubilized in IP buffer (10 mM Tris–HCl [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% [v/v] Triton X-100, 0.2 mg/ml phenylmethyl sulfoxide) and incubated with the relevant antibodies (High et al., 1993b). Antigen–antibody complexes were adsorbed to protein A–Sepharose and recovered by centrifugation (High et al., 1993b). Antibodies were raised against peptides corresponding to the 27 N-terminal amino acids of Ii (Claesson et al., 1983), amino acids 136–152 of SRP54 (Römisch et al., 1989), the 14 C-terminal amino acids of TRAM (Görlich et al., 1992a), and amino acids 99–117 of Sec61α (Görlich et al., 1992b). The antibody against PPL was directed against the N-terminus of prolactin and was kindly provided by Sharon Tooze (High et al., 1993a).

Phospholipid Analysis

After high salt extraction, membranes were resuspended in 100 µl 100 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$, 1% (v/v) Triton X-100 per 25 µl initial translation mix. Dissolved membranes were incubated for 5 min at 41°C either with 2 U PLA_2 (Boehringer Mannheim), 2 U manoalide (Calbiochem)-treated PLA_2 (Glaser and Jacobs, 1986), or 2 U PLA_2 in the presence of excess phosphatidylcholine (20 mg/ml). Proteins were subsequently precipitated by the addition of 1 vol 20% (w/v) trichloroacetic acid, 50% (v/v) acetone.

For alkaline hydrolysis, high salt extracted membranes of a 25 µl translation mix were resuspended in 50 µl 12% (w/v) ammonia, 1% (w/v) SDS and incubated at 37°C for 5 hr. Following evaporation of NH_3, proteins were precipitated with trichloroacetic acid as described above.

Electrophoresis

High salt extracted membrane pellets, pellets from immunoprecipitations containing protein A–Sepharose, or trichloroacetic acid–precipitated proteins were solubilized in 30 µl 125 mM Tris-HCl (pH 6.8), 5 mM EDTA, 50 mM dithiothreitol, 5% (w/v) glycerol, 2% (w/v) SDS and incubated for 10 min at 65°C. Samples were analyzed by SDS-PAGE using 12%–18% gradient gels (Laemmli, 1970). Polypeptides containing [^{35}S]methionine were detected by either autoradiography using Kodak XAR-5 film or imaging using a Fujix BAS1000 phospholmager.

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