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Signal Sequence Recognition in Posttranslational Protein Transport across the Yeast ER Membrane

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Summary

We have analyzed how the signal sequence of prepro-α-factor is recognized during the first step of posttranslational protein transport into the yeast endoplasmic reticulum. Cross-linking studies indicate that the signal sequence interacts in a Kar2p- and ATP-independent reaction with Sec61p, the multispanning membrane component of the protein-conducting channel, by intercalation into transmembrane domains 2 and 7. While bound to Sec61p, the signal sequence forms a helix that is contacted on one side by Sec62p and Sec71p. The binding site is located at the interface of the protein channel and the lipid bilayer. Signal sequence recognition in cotranslational translocation in mammals appears to occur similarly. These results suggest a general mechanism by which the signal sequence could open the channel for polypeptide transport.

Introduction

Signal sequences direct polypeptides either co- or posttranslationally across the membrane of the endoplasmic reticulum (ER). In the cotranslational pathway, signal sequences are recognized in two consecutive steps, first by the signal recognition particle (SRP) in the cytosol, and a second time at the membrane (for review, see Rapoport et al., 1996). Experiments with reconstituted proteoliposomes containing purified mammalian components have shown that the second recognition step involves the heterotrimeric Sec61p complex, the major component of the translocation apparatus of the ER membrane, and the translocating chain-associating membrane protein (TRAM) (Jungnickel and Rapoport, 1995; Voigt et al., 1996). Signal sequence recognition in the posttranslational pathway has not yet been studied, but it does not involve SRP or TRAM.

Posttranslational protein transport across the ER membrane has been best analyzed in the yeast *S. cerevisiae*. It requires a seven-component complex, the Sec complex, which consists of the trimeric Sec61p complex (containing Sec61p, Sbh1p, and Sss1p) and the tetrameric Sec62/63p complex (containing Sec62p, Sec63p, Sec71p, and Sec72p), the latter unique to the posttranslational pathway (Deshaies et al., 1991; Panzner et al., 1995). Transport can be reproduced with reconstituted proteoliposomes containing only the purified Sec complex and the luminal ATPase Kar2p (BiP) (Panzner et al., 1995). The Sec61p complex presumably forms the protein-conducting channel, since, in the presence of the Sec62/63p complex, it adopts oligomeric ring structures in the plane of the membrane (Hanein et al., 1996). Homologs of two subunits, Sec61p and Sss1p, exist in all organisms and are essential for protein transport and cell viability (for review, see Rapoport et al., 1996). Sec61p and its bacterial homolog SecY span the membrane ten times (Wilkinson et al., 1996, and references therein). Sss1p and most of its homologs are small, single-spanning membrane proteins; where larger homologs exist (e.g., SecE in *E. coli*), the additional segments are not essential (Murphy and Beckwith, 1994). While the multispanning Sec61p/SecY component is thought to form the channel, the function of the conserved smaller subunit is unclear.

Posttranslational translocation occurs in distinct phases. In an initial binding reaction, a translocation substrate interacts with the cytosolic face of the Sec complex in a signal sequence–dependent but ATP- and Kar2p-independent manner (Lyman and Schekman, 1997; Matlack et al., 1997). Subsequently, Kar2p and ATP are required to move the substrate through the channel. Here, we have used a cross-linking approach to investigate how the signal sequence of the translocation substrate prepro-α-factor (ppαF) is recognized by the Sec complex during the first phase of its posttranslational transport across the yeast ER membrane.

Results

Signal Sequence–Dependent Interactions of ppαF with the Sec Complex

To examine interactions of ppαF posttranslationally bound to the Sec complex, we employed a photo-cross-linking approach. ppαF was synthesized in vitro in a reticulocyte lysate system in the presence of ^{35}S-methionine and modified lysyl-tRNA carrying a carbene-generating probe in the side chain of the amino acid. Photoreactive lysine derivatives are thus incorporated at positions of the polypeptide chain where lysines normally occur (Mothes et al., 1998). After removal of the ribosomes by sedimentation, the full-length polypeptides were incubated with proteoliposomes containing the purified Sec complex but lacking Kar2p. Under these conditions ppαF binds efficiently to Sec complex, but little or no translocation occurs (Matlack et al., 1997). The samples were then irradiated to induce cross-links to neighboring proteins and subsequently solubilized in digitonin, a detergent in which the Sec complex remains

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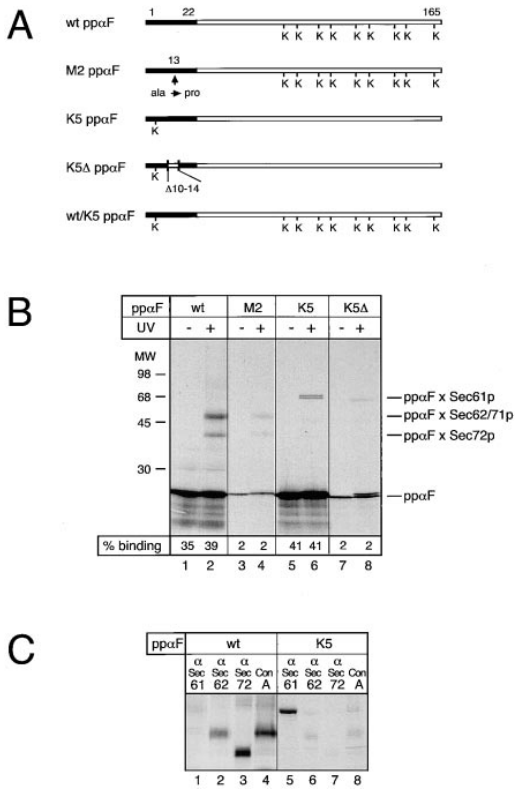

Figure 1. Signal Sequence–Dependent Interactions of ppαF with the Sec Complex

(A) To probe the environment of ppαF bound to the Sec complex by cross-linking, the indicated lysine residues (K) were replaced by photoreactive lysine derivatives. Wild-type (wt) ppαF contains nine lysines in the C-terminal part (approximate positions indicated). K5 ppαF bears a single lysine at position 5 of its signal sequence (filled portion). wt/K5 ppαF is a hybrid of the two. M2 and K5Δ ppαF are signal sequence mutants of wt ppαF and K5 ppαF, respectively.

(B) Different ppαF proteins (see [A]) were synthesized in vitro in the presence of ^{35}S-methionine and lysyl-tRNA with a photoreactive probe in the side chain of the amino acid. They were incubated with proteoliposomes containing the purified Sec complex and irradiated with UV light (UV) as indicated. The vesicles were solubilized in digitonin, and bound and cross-linked ppαF was coimmunoprecipitated with the Sec complex and analyzed by SDS-PAGE and autoradiography. The positions in the gel of non–cross-linked ppαF and of its cross-links to the Sec proteins are indicated. Cross-links to Sec62p and Sec71p comigrate in the gel. The percentage of total ppαF associated with the Sec complex is given below the gel (% binding).

(C) A similar experiment as in (B) was performed with wt and K5 ppαF, except that after irradiation the cross-linked products were denatured in SDS and analyzed by immunoprecipitation with various antibodies (αSec61, αSec62, and αSec72) or by binding to concanavalin A (ConA), which detects cross-links to the glycosylated Sec71p.

intact and maintains its association with ppαF (Matlack et al., 1997). The Sec complex was immunoprecipitated, and associated non–cross-linked and cross-linked ppαF was analyzed by SDS-PAGE. With wild-type ppαF, which contains all its lysines in the C-terminal portion (Figure 1A), cross-links were observed to Sec62p and/or Sec71p (which have approximately the same molecular weight) and to Sec72p, but not to Sec61p (Figure 1B, lane 2 vs. 1). Immunoprecipitation after SDS denaturation demonstrated cross-linking to Sec62p and Sec72p, and binding to concanavalin A Sepharose indicated cross-linking to the glycoprotein Sec71p (Figure 1C, lanes 1–4).

To identify proteins interacting with the signal sequence, we mutated all the lysines in wild-type ppαF to arginines and introduced a single lysine at position 5 of the signal sequence (K5 ppαF; Figure 1A). This mutant gave strong cross-links to Sec61p but almost no cross-links to Sec62/71p or Sec72p (Figure 1B, lanes 5 and 6; Figure 1C, lanes 5–8). Thus, in this initial stage of translocation, position 5 of the signal sequence contacts primarily Sec61p, while the C-terminal part of ppαF contacts Sec62p, Sec71p, and Sec72p. No cross-links between bound ppαF and other components of the Sec complex (Sec63p, Sbh1p, and Sss1p) were produced with either substrate (Figure 1B).

To test the specificity of cross-linking, two ppαF mutants with defective signal sequences were examined (M2 and K5Δ ppαF; Figure 1A). With both mutants, binding and cross-linking to Sec proteins were strongly reduced (Figure 1B, compare lanes 3 and 4 vs. 1 and 2, and lanes 7 and 8 vs. 5 and 6). As an additional control, we demonstrated that bound ppαF is a precursor of translocated material: when ppαF bound to the Sec complex was treated with Kar2p and ATP, the substrate was released and cross-linking to all proteins was much reduced (data not shown).

Next we tested interactions of ppαF with the two separated subcomplexes of the Sec complex. A mutant of ppαF was employed that carries all the lysines of the wild-type protein plus the additional lysine at position 5 (wt/K5ppαF; Figure 1A). Analysis of the cross-linking products after enrichment of membrane proteins by alkali extraction demonstrated that only when both subcomplexes were present did cross-linking to Sec61p and Sec62/71p occur (Figure 2A, lanes 2 and 4 vs. lanes 1 and 3). Since Sec72p is not an integral membrane protein and is thus lost during alkali extraction, we analyzed cross-links to it by immunoprecipitation (Figure 2B); as with the other components, cross-links to Sec72p were only seen when both subcomplexes were present (lanes 2 and 4). Taken together, these results show that binding and cross-linking of the translocation substrate to the Sec complex require both a functional signal sequence and the association of the two subcomplexes. Since only both subcomplexes together form a channel structure detectable in electron micrographs (Hanein et al., 1996), the data also suggest that signal sequence recognition requires an intact channel.

Probing the Environment of ppαF Bound to the Sec Complex with Photoreactive Lysine Derivatives

To examine in detail how the substrate interacts with the Sec complex, we generated a series of ppαF mutants each with a single lysine codon at a different position, allowing us a scan of the molecular environment of the bound substrate by systematic cross-linking. Lysines were introduced throughout the signal sequence and in the mature region. In the signal sequence, lysines were

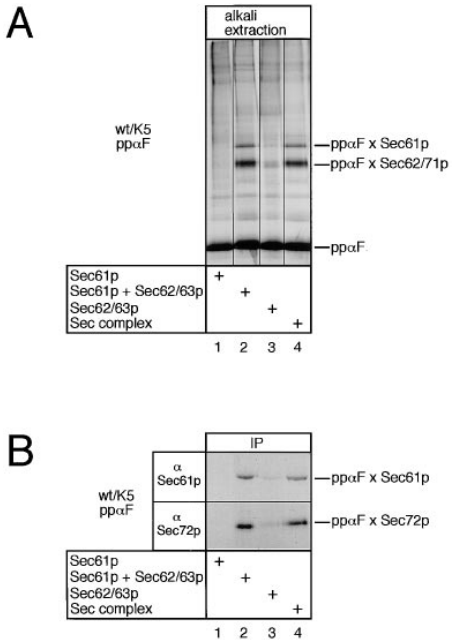

Figure 2. An Intact Sec Complex Is Required for Interactions with ppαF

(A) Proteoliposomes containing either the intact Sec complex, one of the two subcomplexes, Sec61p complex (Sec61p) or Sec62/63p complex (Sec62/63p), or the two subcomplexes together (Sec61p + Sec62/63p) were incubated with wt/K5 ppαF (see Figure 1A) containing photoreactive lysine derivatives. The subcomplexes were used at equivalent concentrations as present when the intact Sec complex was used. After irradiation cross-linked products were analyzed by alkali extraction of the membranes and SDS-PAGE. The low level of cross-linking seen with the Sec62/63p complex (lane 3) is due to some contamination with Sec61p complex.

(B) To analyze cross-linked products of the peripheral membrane protein Sec72p, parallel samples of the experiment in (A) were denatured in SDS and analyzed by immunoprecipitation (IP).

placed from position 8 up to position 22, at which it would be normally cleaved. Each mutant protein was synthesized in vitro in the presence of modified lysyl-tRNA, incubated with proteoliposomes containing the Sec complex, and subjected to irradiation. After solubilization in digitonin, the Sec complex and any ppαF bound or cross-linked to it was immunoprecipitated with antibodies against Sec62p.

Analysis of the protein cross-links showed that most positions gave multiple bands (Figure 3A). Several of them are Sec61p cross-links (indicated by brackets), as demonstrated by immunoprecipitation after SDS denaturation (data not shown). At some positions within the signal sequence, a slow or fast mobility Sec61p cross-linked band predominated (indicated by arrows). In addition, the intensity of the Sec61p cross-links often differed dramatically between neighboring positions (e.g., position 10 vs. 11). Quantitation of the cross-linking yields relative to the amount of ppαF bound to the Sec complex demonstrated that the Sec61p cross-links displayed a striking periodicity within the signal sequence (Figure 3D); positions 10 and 14 gave prominent slow mobility bands, and positions 9, 12, and 15 gave mostly fast mobility bands. The quantitative and qualitative differences among positions were highly reproducible (eight independent experiments, see insets). Differences among positions 17 to 29 were less pronounced, each giving rise to multiple bands. Beyond position 40, cross-linking to Sec61p became insignificant (Figure 3D). If one plots positions 9 to 17 of the signal sequence on a helical wheel (Figure 7A), positions that gave prominent slow and fast mobility Sec61p cross-linked bands were located in distinct patches on opposite sides of the helix. Taken together these results suggest that (1) the signal sequence is not oriented randomly but rather contacts Sec61p in a specific manner, (2) different positions of the signal sequence contact distinct regions of the Sec61p molecule, explaining differences in electrophoretic mobility, (3) a portion of the signal sequence adopts a helical structure, and (4) a region following the signal sequence also contacts Sec61p.

The periodic pattern of signal sequence cross-linking to Sec61p was seen not only with reconstituted proteoliposomes but also with native yeast microsomes (Figure 3C). In this case, ppαF was bound to the membranes in the absence of ATP to prevent translocation, and the cross-links were analyzed after denaturation in SDS and immunoprecipitation with Sec61p antibodies. At several positions, differences to the results with the purified system were noted (e.g., at position 10 the slow mobility band was less prominent than with the purified Sec complex), but in general there was good agreement (compare with Figure 3A).

Cross-links to Sec62p/71p were analyzed with the reconstituted system (Figures 3A and 3D). They were seen with lysines at positions 8 to 26 and with residues at the C terminus, but not with those at positions 27 to 29. Again, a highly reproducible periodic pattern seen in eight experiments was found within the signal sequence: peaks of cross-linking yields occurred with positions 11, 14, 18, and 21. Positions with the highest cross-linking yields were again located on a small surface patch of an assumed helix (Figure 7A). When Sec62p and Sec71p were analyzed separately by denaturing immunoprecipitation and binding to concanavalin A-Sepharose, respectively, they behaved essentially identically at most positions, suggesting that they are associated with each other (Figure 3E). However, with wild-type ppαF containing probes at its C terminus, cross-links to Sec71p were significantly more prominent than those to Sec62p.

Significant cross-linking to Sec72p was only observed with C-terminal residues (position 56 and higher) (Figures 3A and 3D), consistent with the fact that Sec72p is a peripheral protein on the cytosolic face of the ER membrane. As judged from the analysis of the native immunoprecipitations (Figure 3A), cross-linking to either Sec63p, Sbh1p, or Sss1p was insignificant (below 0.1%).

We also analyzed cross-links to lipids, which are seen as small irradiation-induced mobility shifts of ppαF in short exposures of the autoradiograms (Figure 3B). The identity of the lipid cross-links was verified by cleavage with phospholipase A2 (data not shown). Quantitation shows that lipid cross-links occur with some variation throughout the entire signal sequence but not beyond it (Figure 3D).

All cross-linked products (to Sec61p, Sec62/71p, and lipids) appeared with the same kinetics during the incubation of ppαF with the Sec complex (data not shown),

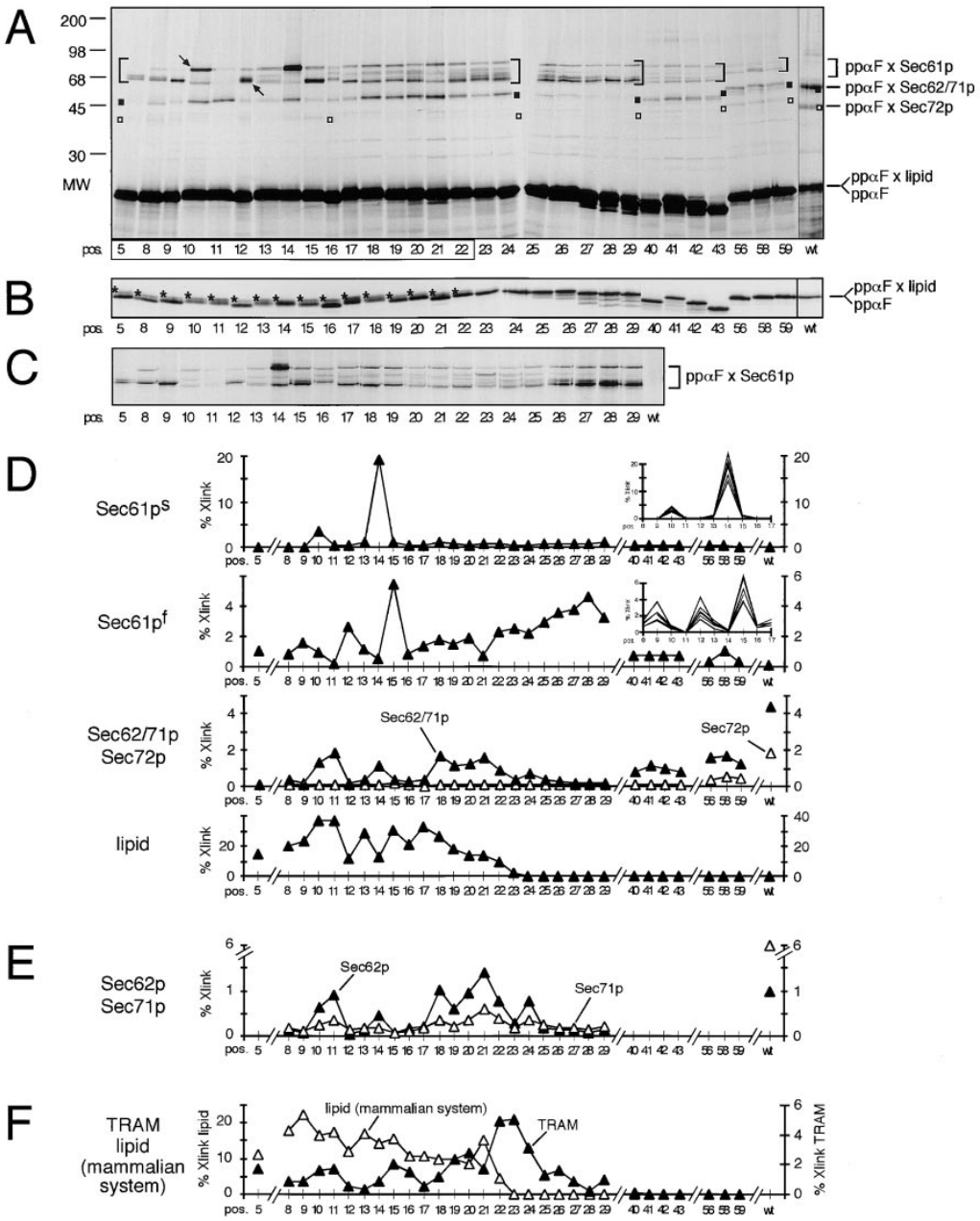

Figure 3. Systematic Probing of the Molecular Environment of ppαF Bound to Sec Complex Using Photoreactive Lysine Derivatives

(A) ppαF mutants were generated, each containing a single lysine codon at the indicated positions (pos.; the signal sequence region is boxed). Mutant proteins or wild-type (wt) ppαF with photoreactive lysine derivatives were incubated with proteoliposomes containing the Sec complex and irradiated. After solubilization in digitonin, bound and cross-linked ppαF was coimmunoprecipitated with the Sec complex and analyzed by SDS-PAGE. Cross-linked bands containing Sec61p are indicated by brackets; the arrows point to examples in which the slow and fast mobility bands predominate. Cross-links to Sec62/71p and Sec72p are indicated by filled and open squares, respectively. Several minor bands visible in the gel were not dependent on irradiation. Note that non–cross-linked ppαF runs somewhat differently depending on the position of the lysine codon.

(B) To visualize lipid cross-links (stars), the experiment was performed as in (A) with a short exposure (1 hr) of the autoradiogram. Only the relevant part of the gel is shown. Bands below ppαF at positions 25–42 were also seen without irradiation.

(C) A cross-linking experiment similar to that in (A) was performed with native yeast microsomes. The different ppαF proteins were incubated with yeast membranes in the absence of ATP to prevent translocation, then irradiated, and cross-links to Sec61p were analyzed after denaturation in SDS by immunoprecipitation. Only the relevant part of the gel is shown.

(D) Quantitation of the various cross-linked products in (A) and (B) was performed with a phosphoimager. Yields of cross-links to Sec61p, Sec62/71p, Sec72p, and lipids were expressed relative to the amount of ppαF coimmunoprecipitated with the Sec complex. Cross-links to Sec61p with a slow or fast mobility in SDS gels (Sec61p^s and Sec61p^f, respectively; see arrows in [A]) were analyzed separately. Reproducibility of the periodic cross-linking pattern is demonstrated in the insets (quantitation of eight experiments).

(E) To distinguish between cross-links to Sec62p and Sec71p, which comigrate in SDS gels, a similar experiment as in (A) was performed

indicating that the signal sequence does not contact one component prior to another.

Probing the Environment with Photoreactive Phenylalanine Derivatives

To exclude the possibility that our results were exclusive to the specific cross-linking probe employed, we used another site-specific cross-linking approach (Martoglio et al., 1995). Stop codons were introduced at various positions of the signal sequence-coding region and suppressed in vitro by translation in the presence of a modified phenylalanyl-suppressor tRNA. This results in the selective incorporation of carbene-generating photoreactive probes at the stop codons. These have a significantly shorter (7 Å vs. 13 Å) and less flexible side chain than the lysine derivatives used before. With the phenylalanine probes the periodic pattern of the Sec61p cross-links from positions within the signal sequence was even more pronounced (Figure 4A; quantitation shown in Figure 4C), again suggesting a helical structure of the bound signal sequence (Figure 7B). Interestingly, compared to the results with the lysine probe, the peaks of the slow and fast mobility Sec61p cross-linked bands were shifted by one position: highest yields of the slowly migrating band were found at positions 11 and 15, rather than 10 and 14, and the strongest intensities of the faster migrating band were seen at positions 13 and 16, rather than 12 and 15 (compare Figures 4C and 3D). Thus, again the signal sequence contacts Sec61p in a specific, nonrandom manner, but its precise location in the binding pocket appears to be dependent on the nature of the side chains of the amino acids involved in the interaction.

With the phenylalanine probes, lipid cross-links were again seen with each tested position of the signal sequence (Figures 4B and 4C), but, in contrast to the lysine derivative, almost no interactions with Sec62/71p were detected, except with residues at the C terminus of the signal sequence (Figures 4A and 4C). Thus, the results with the probes in short phenylalanine side chains indicate that most of the interactions of the signal sequence occur with Sec61p.

Mapping the Cross-Linking Sites in Sec61p

Next we wished to identify the approximate regions of the Sec61p molecule that contact the signal sequence. To this end, we made use of a set of Sec61p mutants, each of which contains a single cleavage site for the protease factor Xa in one of the cytosolic or luminal loops between the ten transmembrane (TM) domains (Wilkinson et al., 1996). The cross-linked products of ppαF and the Sec61p-Xa mutants can be specifically cleaved with the protease, allowing us to determine to which of the two fragments of Sec61p cross-linking has occurred. Combining the results from different Sec61p-Xa mutants we can thus map the cross-linking sites in Sec61p. Preliminary experiments indicated that the major cross-links occurred to either the N- or C-terminal regions, and we therefore concentrated on four Sec61p-Xa mutants with cleavage sites in the loops between TM domains 1 and 2 (N1/2C), 2 and 3 (N2/3C), 6 and 7 (N6/7C), and 7 and 8 (N7/8C). The Sec complex was purified from the four mutants, reconstituted into proteoliposomes, and incubated with the various radioactively labeled ppαF constructs that each contain a photoreactive phenylalanine derivative at a single position. After irradiation, cross-links were immunoprecipitated under denaturing conditions with antibodies directed against the C terminus of Sec61p and analyzed with or without treatment with factor Xa (Figure 5, lanes 2, 4, and 6 vs. lanes 1, 3, and 5 of each panel). In the case of the mutant with a cleavage site in the loop between TM domains 6 and 7, identification of the cross-linking site was complicated by the fact that the two fragments generated had almost the same size; these samples were therefore first treated with factor Xa and then subjected to immunoprecipitation with antibodies against the C or the N terminus of Sec61p (Figure 5, lanes 8 and 9 of each panel). A portion of the sample remained untreated (lane 7 of each panel).

To illustrate the mapping procedure, we will consider ppαF with a photoreactive probe at position 9. When this construct was cross-linked to the N1/2C mutant, subsequent treatment with factor Xa resulted in the label being mostly in the large C-terminal fragment (Figure 5, marked 2C). Thus, the major cross-linking site must be in a region between TM domain 2 and the C terminus. With mutant N2/3C most of the label was in the small N-terminal fragment (Figure 5, marked N2). Together, these data indicate that cross-linking must have occurred to TM domain 2 or, more precisely, to the region between the factor Xa site in the first luminal loop and the factor Xa site in the following cytosolic loop. The results with the other Sec61p-Xa mutants are consistent with this conclusion. This analysis is summarized in the table below the autoradiogram.

Position 10 gave different results. With the N1/2C mutant, the cross-links were contained in the large fragment (2C). With N2/3C, most of the label was in the large fragment (3C), but some cross-linking to TM domain 2 was indicated by the labeling of the small fragment. With N7/8C, essentially all label was in the large N-terminal fragment (N7). Thus, most of the cross-linking must have occurred to the region between TM domains 3 and 7. With the mutant N6/7C, most of the label was found in the C-terminal fragment (7C). Together these results show that the major cross-linking site is in TM domain 7 or the neighboring cytosolic or luminal regions.

A similar analysis was performed with positions 11 to

with selected ppαF mutants, and the cross-linked products were analyzed after denaturation in SDS by immunoprecipitation with antibodies to Sec62p or by binding to concanavalin A (for Sec71p). The cross-linking yields were expressed relative to the total ppαF synthesized.
(F) Cross-linking experiments were performed with ribosome-associated ppαF chains containing the first 86 amino acids, each with a single photoreactive lysine probe at the indicated position. The ribosome/nascent chain complexes were synthesized in vitro in the presence of SRP and canine pancreatic microsomes. After irradiation, cross-links to lipid were analyzed directly by SDS-PAGE and those to TRAM after immunoprecipitation. Cross-linking yields are given relative to the total radioactivity in the chains of 86 amino acids.

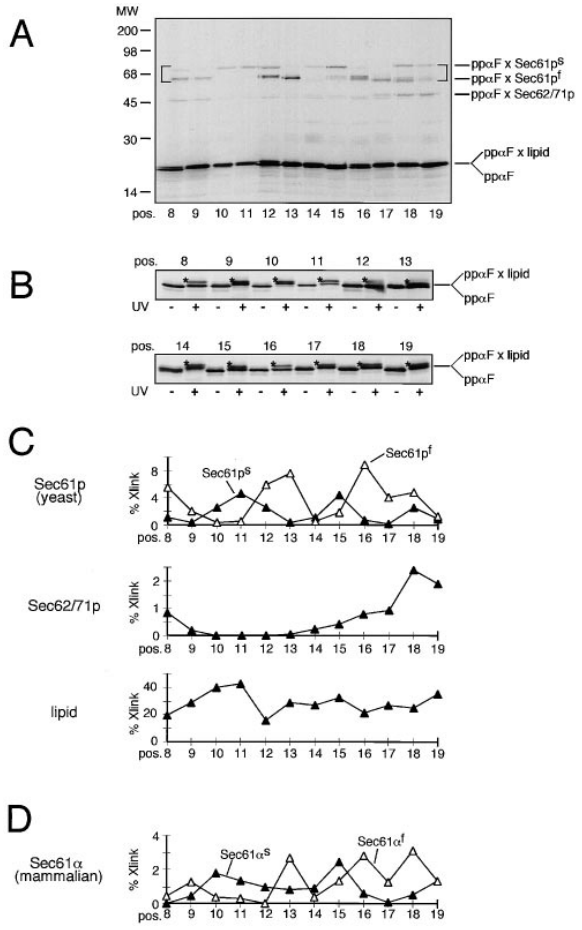

Figure 4. Systematic Probing of the Molecular Environment of ppαF Bound to Sec Complex Using Photoreactive Phenylalanine Derivatives

(A) Single stop codons were introduced at the indicated positions (pos.) of ppαF. mRNAs were translated in vitro in the presence of a modified suppressor Phe-tRNA containing a photoreactive group in the side chain of the amino acid. ppαF proteins were incubated with proteoliposomes containing the Sec complex and irradiated. The vesicles were solubilized in digitonin, and bound and cross-linked ppαF was coimmunoprecipitated with the Sec complex and analyzed by SDS-PAGE and autoradiography. Cross-linked bands containing Sec61p are indicated by brackets. Sec61p^s and Sec61p^f indicate the positions of slow and fast mobility cross-linked products, respectively. Bands not labeled, including a band running at almost the same position as Sec62/71p, were also seen without irradiation (data not shown).

(B) To visualize lipid cross-links (stars), the experiment was performed as in (A). The samples were subjected to irradiation with UV light (UV) as indicated. Only the relevant part of the gel is shown.

(C) Quantitation of the various cross-linked products in (A) and (B) was performed with a phosphoimager as described in Figure 3D.

(D) Cross-linking experiments were performed with ribosome-associated ppαF chains containing the first 86 amino acids. Nascent chains were synthesized in vitro in the presence of modified phenylalanine suppressor-tRNA, SRP, and canine pancreatic microsomes. After irradiation, cross-links to Sec61α, the mammalian homolog of Sec61p, were analyzed after immunoprecipitation by SDS-PAGE. Three Sec61α cross-linked bands of different mobility were visible, and the quantitation of the slowest (Sec61α^s) and fastest (Sec61α^f) is shown. The band of intermediate mobility largely correlated with the fast mobility band. Cross-linking yields are given relative to the total radioactivity in the chains of 86 amino acids.

19 of the signal sequence (Figure 5). In most cases, the analysis was straightforward, but with the position 15, mutant N1/2C indicated significant cross-linking to TM domain 1, whereas mutant N2/3C suggested only very low cross-linking yields with the region containing TM domains 1 and 2. An explanation is provided by the observation that the mutants had a different cross-linking pattern before factor Xa cleavage: the faster migrating band was more prominent than the slower one for mutant N1/2C, whereas the opposite was true for the other Sec61p-Xa mutants. It thus appears that position 15 of the signal sequence shifts its location depending on the insertion of factor Xa sites into Sec61p, being closer to TM domain 1 with mutant N1/2C than with the other mutants. On the other hand, the data with the mutants N2/3C, N7/8C, and N6/7C all indicate that a major cross-linking site is contained in TM domain 7.

Together, these data indicate that all major cross-links from positions 9 to 19 occur to the two TM domains 2 and 7 or their neighboring loop regions. In addition, significant cross-linking of the signal sequence was seen to TM domain 1. In most cases weak cross-linking to other domains could not be excluded. Within the region of positions 9 to 19, there was a good correlation between cross-linking to TM domain 2 and the occurrence of a prominent fast mobility Sec61p cross-linked band in SDS gels. Similarly, cross-linking to TM domain 7 correlated with the appearence of a slow mobility band. The weaker cross-linking to TM domain 1 corresponded to a fast mobility band that sometimes could be separated in SDS gels from that generated by TM domain 2. When slow and fast mobility bands occurred simultaneously, the mapping experiments indicated cross-linking to TM domain 7 as well as TM domains 2 and/or 1. The ratio of the slow and fast mobility bands corresponded even quantitatively to the relative intensities of cross-linking to TM domains 7 and 2/1, respectively (data not shown). In agreement with the previous analysis based on gel mobility (Figure 4C), the strongest cross-links to TM domains 2 and 7 were found on opposite sides of an assumed helix of the signal sequence (Figure 7B).

We also mapped the cross-linking sites in Sec61p with lysine probes in ppαF, using either proteoliposomes containing the Sec complex or native yeast microsomes. The clearest results were obtained for positions 9 and 14 because each gave mostly one cross-linked band of fast or slow mobility (Figures 3A and 3C). Using ppαF with the probe in position 9 and native microsomes from the mutant N1/2C, the label was found in the large fragment of Sec61p (2C) and with mutant N2/3C in the small fragment (N2, Figure 6A). Thus, the cross-linking site was contained in TM domain 2 or its neighboring loop domains, in agreement with the results obtained with the phenylalanine probe (Figure 5).

The results with a lysine probe at position 14 were different from those with a phenylalanine probe at the same position, as expected from the previous analysis of gel mobilities demonstrating that the peak of the slow mobility band was shifted by one position (positions 14 and 15 with the lysine and phenylalanine probes, respectively; see Figures 3D and 4C). The mapping analysis with the lysine probe demonstrated that most cross-links were contained in the region comprising TM

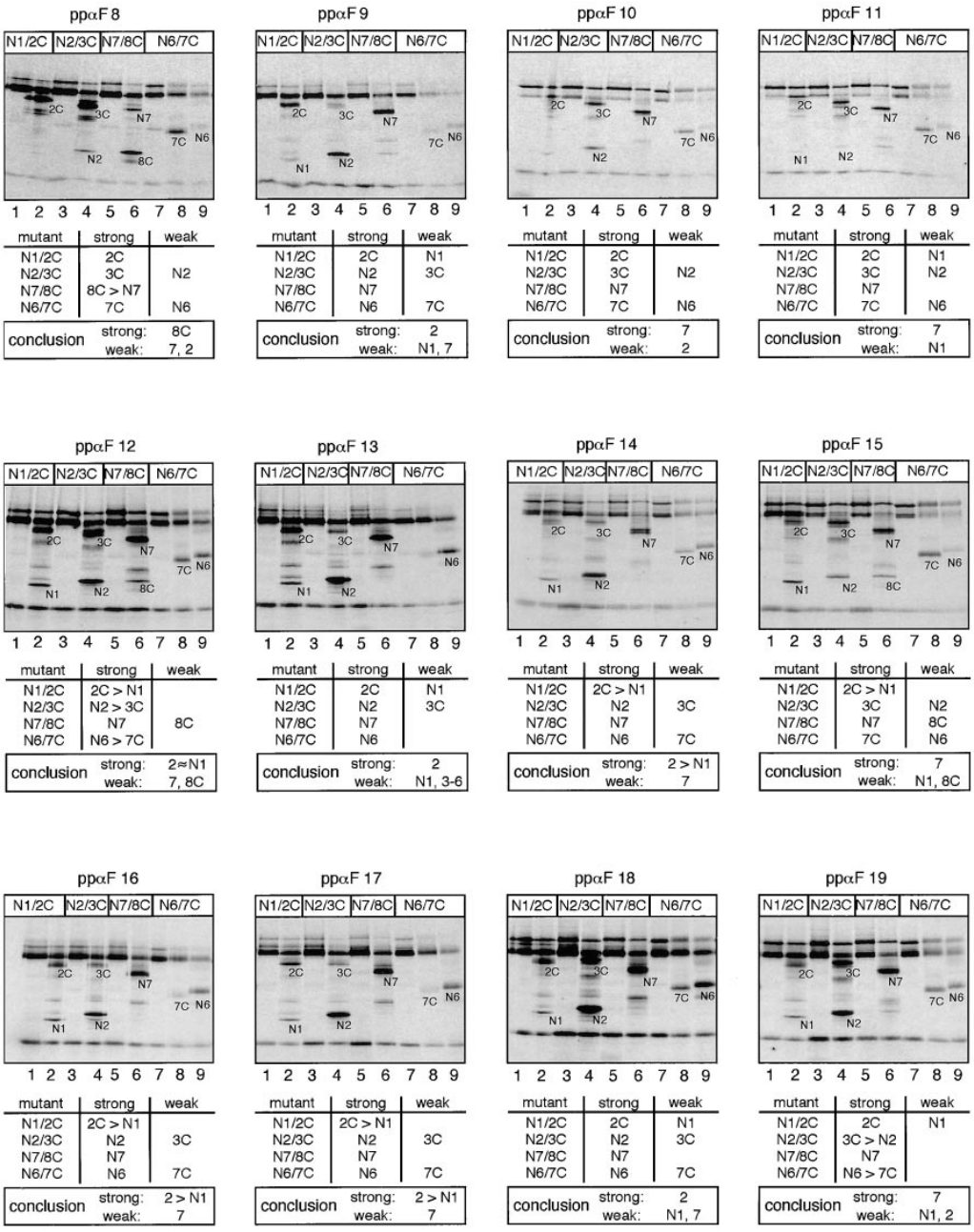

Figure 5. Cross-Linking of ppαF Containing Photoreactive Phenylalanine Derivatives to Specific Regions of Sec61p

The Sec complex was purified from yeast mutants that bear a single factor Xa cleavage site in Sec61p in loops between the indicated TM domains (e.g., N1/2C contains the cleavage site between TM domains 1 and 2). Proteoliposomes containing the various Sec complexes were incubated with different ppαF proteins, each containing a single photoreactive phenylalanine derivative (positions of the probes given above the panels), and irradiated. In each panel, the samples shown in lanes 1–6 were denaturated in SDS, immunoprecipitated with antibodies to the C terminus of Sec61p, and then analyzed with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) treatment with factor Xa. For analysis of mutant N6/7C (lanes 7–9), samples were split into three equal portions. One was analyzed by immunoprecipitation with antibodies to the C terminus of Sec61p (lane 7), and the other two were incubated with factor Xa, denatured in SDS, and immunoprecipitated with antibodies against either the C terminus (lane 8) or the N terminus (lane 9) of Sec61p. Labels in the autoradiograms indicate the fragments of Sec61p that contain the cross-linking sites (e.g., 2C indicates cross-linking to a Sec61p fragment comprising TM segment 2 to the C terminus). The tables below the lanes summarize the mapping analysis for each position. The analysis is based on a quantitation performed with a phosphoimager and included a correction for the different efficiencies of immunoprecipitation with antibodies against the N and C termini of Sec61p. Cross-links are indicated as strong if they contained more than 25% of the total radioactivity in the two fragments generated by factor Xa cleavage. If two strong cross-links occurred, the more intense one is listed first. Weak cross-links are given if they contained between 5% and 25% of the total radioactivity in both fragments. Minor cross-linking to TM domains 3–6 cannot be excluded. The final conclusions are given in boxes under the tables.

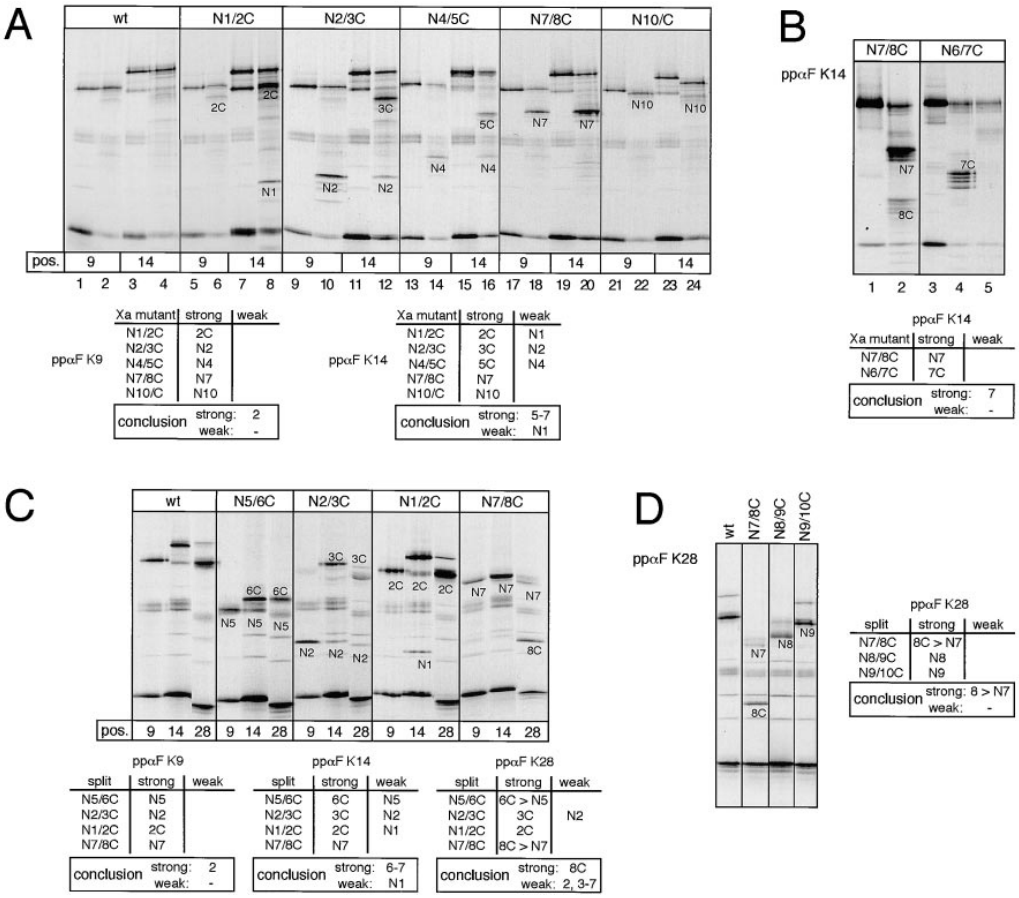

Figure 6. Mapping of Cross-Linking Sites of ppαF Containing Photoreactive Lysine Derivatives

(A) ppαF with photoreactive lysine derivatives at positions (pos.) 9 or 14 (K9 ppαF or K14 ppαF) was incubated in the absence of ATP with native microsomes isolated from either wild-type yeast (wt) or mutants that each contain a single factor Xa cleavage site in Sec61p in loops between the indicated TM domains. After irradiation and denaturation with SDS, the cross-linked products were immunoprecipitated with antibodies against the C terminus of Sec61p. They were analyzed with or without factor Xa cleavage (even and odd lane numbers, respectively). Labels in the autoradiograms indicate the fragments of Sec61p containing cross-linked ppαF. The tables below the autoradiogram summarize the conclusions of the mapping analysis for each position.

(B) To further define the cross-linking site with K14 ppαF, the protein was incubated with proteoliposomes containing the purified Sec complex from either the N7/8C or the N6/7C Sec61p-Xa mutants and irradiated. The samples shown in lanes 1 and 2 were immunoprecipitated with antibodies to the C terminus of Sec61p and analyzed with (lane 2) or without (lane 1) treatment with factor Xa. Samples shown in lanes 3–5 were either directly immunoprecipitated with antibodies to the C terminus of Sec61p (lane 3) or first incubated with factor Xa before immunoprecipitation with antibodies against either the C terminus (lane 4) or the N terminus (lane 5) of Sec61p.

(C) ppαF proteins, each with a single photoreactive lysine derivative at the indicated position (pos.), were incubated in the absence of ATP with native microsomes isolated from either wild-type yeast (wt) or from split mutants that express Sec61p as a pair of complementary N- and C-terminal fragments (e.g., N5/6C contains the breakpoint between TM domains 5 and 6). Cross-links to the N- or C-terminal fragments of Sec61p were analyzed after denaturation in SDS by immunoprecipitation with antibodies against the C terminus of Sec61p (both fragments contain the 12 most C-terminal residues of Sec61p).

(D) To further define the cross-linking site with K28 ppαF, a similar experiment as in (C) was performed with microsomes from various split mutants of Sec61p.

domains 5–7 (Figure 6A). Weaker cross-links occurred to the N-terminal region (N1). Cross-linking to the N-terminal region is probably exaggerated because the insertion of factor Xa sites in the mutants N1/2C and N2/3C increases the yield of the fast mobility band (compare with wild-type or the other Sec61p-Xa mutants). Since the cross-linked products with microsomes from the mutant N6/7C were very weak (data not shown), we used proteoliposomes containing the mutant Sec complex to further define the site of the major cross-links (Figure 6B). These data showed that with the lysine probe at position 14 the major cross-links were to TM domain 7. This is the same domain to which position 15 could be cross-linked with the phenylalanine probe, supporting our conclusion that signal sequences containing different probes bind to the same sites of Sec61p in a slightly different orientation. With the other lysine mutants, the mapping analysis also indicated mainly cross-linking to TM domains 2 and 7, although the analysis was often more complicated because of the occurrence of multiple bands.

To further confirm the cross-linking sites, we used "split" *sec61* mutants in which a functional Sec61p molecule is assembled in vivo from separately expressed

N- and C-terminal fragments (Wilkinson et al., 1997). The mutants each contain a breakpoint in one of the cytosolic or luminal loops of Sec61p at the same position where factor Xa sites were located before. ppαF proteins with lysine probes at different positions were incubated with microsomes from these mutants, and the cross-links to Sec61p were directly analyzed by denaturing immunoprecipitation (Figure 6C). With the probe at position 9, the mutant with a breakpoint between TM domains 1 and 2 (N1/2C) gave labeling in the large C-terminal fragment (2C), and the mutant N2/3C gave labeling in the small N-terminal fragment (N2, Figure 6C). Thus, we conclude again that cross-linking from position 9 occurs mainly to TM domain 2. With position 14, a similar analysis demonstrated major cross-links to TM domains 6–7, consistent with the results obtained with the lysine probe and the Sec61p-Xa mutants. As before, weak cross-linking was seen to TM domain 1. Using other ppαF lysine mutants with the split mutant N1/2C, we found similar weak cross-links to TM domain 1 with positions 10 and 15–19, but not with positions thereafter (data not shown). These data confirm that TM domain 1 is only in proximity to the signal sequence. Taken together, our results with different cross-linking and mapping techniques and either native microsomes or reconstituted proteoliposomes show that positions 9 to 19 of the signal sequence can be cross-linked mainly to TM domains 2 and 7, and more weakly to TM domain 1.

We also mapped the cross-linking sites with mutants containing the probes in the mature region following the signal sequence. With a lysine probe in position 28 and native microsomes from the split *sec61* mutants, the major site was found in a region comprising TM domain 8 to the C terminus, although other cross-links occurred to the N terminus (Figure 6C). Using additional split mutants, the major cross-linking site was identified as TM domain 8 (Figure 6D). Cross-linking to this TM domain started with position 25 and was seen with positions up to 29 (data not shown).

A lysine derivative in position 8 of ppαF could also be cross-linked to TM domain 8 (data not shown), consistent with the mapping analysis using photoreactive phenylalanines at this position (Figure 5). Taken together, these results indicate that TM domain 8 does not interact with the central portion of the hydrophobic core of the signal sequence (positions 9–17) but contacts both a residue preceding it (position 8) as well as several residues following it (25–29).

Interactions of the Signal Sequence during Cotranslational Translocation in Mammals

We tested whether signal sequence recognition is similar in co- and posttranslational translocation pathways. Ribosome-associated nascent polypeptides of ppαF mutants containing the first 86 amino acids of the protein were synthesized in vitro and incubated with SRP and canine pancreatic microsomes. Under these conditions the ribosomes bind to the mammalian Sec61p complex, and the nascent chains are inserted into the channel (Mothes et al., 1998). With phenylalanine derivatives at different positions within the signal sequence, a periodic pattern of cross-links to the mammalian homolog of Sec61p, the α-subunit of the Sec61p complex (Sec61α), was observed (Figure 4D). The highest yields of the fast and slow mobility bands were obtained at approximately the same positions as in the yeast posttranslational system. When cross-links to the TRAM protein were analyzed with lysine probes, a periodic pattern was also seen (Figure 3F). As in yeast, lipid cross-links were seen with all positions of the signal sequence but not beyond it (Figure 3F). Taken together, these results show that the signal sequence of ppαF interacts in the cotranslational system with the mammalian Sec61p complex in a similar manner as in the posttranslational system with the yeast Sec complex.

Discussion

We have analyzed how the signal sequence of ppαF is recognized during the first step of its posttranslational transport across the yeast ER membrane. Interaction requires a functional signal sequence and an intact Sec complex but neither Kar2p nor ATP. Systematic photocross-linking demonstrated that, upon binding to the Sec complex, the signal sequence contacts primarily Sec61p, the multispanning membrane protein likely to be the major constituent of the translocation channel. In fact, with the shorter of the two cross-linking probes (phenylalanine derivative), cross-links of the core of the signal sequence were restricted to Sec61p. When bound to Sec61p, the region between positions 9 and 17 of the signal sequence seems to adopt a helical structure that is contacted on different sides mainly by TM domains 2 and 7 of Sec61p (Figure 7) and somewhat more weakly by TM domain 1. Thus, the signal sequence appears to be recognized by intercalation between TM segments. When the longer of the two cross-linking probes (lysine derivative) was used, one side of the putative helix formed by the signal sequence was cross-linked to Sec62p and Sec71p (Figure 7A). None of the other components of the Sec complex gave significant cross-links to ppαF and are thus not likely to participate in signal sequence interactions. Each residue of the signal sequence could be cross-linked to lipid, indicating that the signal sequence–binding site must be located at the interface between the channel and the surrounding lipid phase. Our data also show that the signal sequence of ppαF is recognized in a similar manner by the mammalian Sec61p complex in the cotranslational translocation system.

Our results suggest that the signal sequence is recognized ultimately by protein–protein interactions, since it is precisely positioned with respect to Sec61p as well as Sec62/71p or TRAM at this initial stage of translocation. In particular, the putative helical region of the signal sequence from position 9 to 17 must have a preferred orientation with respect to the TM domains of Sec61p. The helix contains the hydrophobic core of the signal sequence and comprises about two to three turns (Figure 7). Other signal sequences could interact with the site in a similar manner, and the minimum length of the hydrophobic core might be determined by the requirement of at least two turns of a helix (6–7 residues). However, because of the wide variation in composition

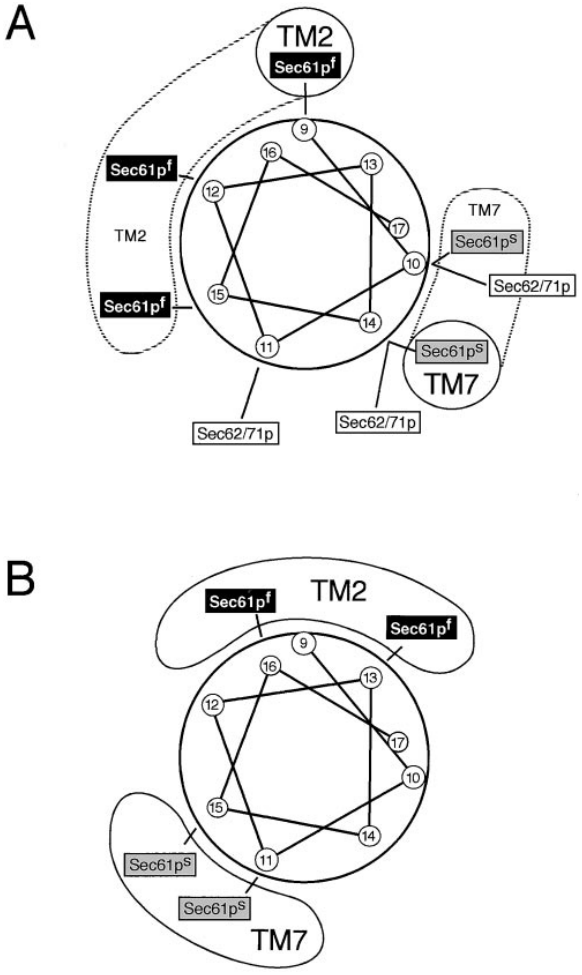

Figure 7. Schematic Illustration of Signal Sequence Interactions

(A) Residues 9–17 of the signal sequence of ppαF are plotted as an α helix (top view shown), although different helical structures are conceivable. Cross-linking partners found with photoreactive lysine derivatives in the yeast system (Sec61p, Sec62p, and Sec71p) are indicated (see Figure 3D). Sec61p^s and Sec61p^f designate positions at which slow and fast mobility Sec61p cross-linked bands, respectively, were prominent. Solid circles indicate interactions with TM domains of Sec61p that have been mapped with *sec61* mutants (Figure 6). The areas surrounded by dotted lines are presumed to map to the same domains on the basis of similar gel mobilities of cross-linked bands.

(B) As in (A), except that the cross-linking partners in the yeast system of ppαF with photoreactive phenylalanine derivatives are shown (Figure 4C). As with the lysine probes, TM domain 2 and 7 are on opposite sides of the helix but are slightly shifted (Figure 5).

and length of signal sequences, each might be oriented slightly differently. It may not require much energy to change the position of a signal sequence, explaining why different signal sequences may be recognized by the same site. This idea is supported by our observation that the incorporation of different probes into the signal sequence of ppαF shifted its orientation slightly relative to the Sec61p molecule, although the same TM domains were still involved in the interaction. Similar small differences were observed between the Sec complex from wild-type and some Sec61p-Xa mutants, as well as between proteoliposomes containing the purified Sec complex and native microsomes. Thus, it seems that relatively small differences may determine the precise orientation of the signal sequence within the binding site provided by TM domains 2 and 7.

Our results suggest that recognition of the signal sequence and its insertion into the channel are the same process. The bound signal sequence is likely to be oriented perpendicular to the plane of the membrane because it contacts two TM domains of Sec61p over a considerable distance through the membrane. Insertion into the channel is also consistent with the observation that at least seven residues following the signal sequence (positions 23–29) contact Sec61p, and that each position of the signal sequence can be cross-linked to lipids. These data can be explained with a loop model of polypeptide chain insertion; the N terminus of the chain would stay in the cytosol, the C terminus of the signal sequence would be located close to the luminal end of the channel, and the next segment of the polypeptide would therefore be dragged into the channel. The latter, in contrast to the signal sequence, is not specifically bound to Sec61p, giving almost uniform cross-linking patterns at each position, and is not in contact with lipids. In addition, TM domain 8 of Sec61p contacts mainly this region, but not the signal sequence. These data suggest that the interior of the channel through which the hydrophilic portions of the polypeptide chain presumably pass is shielded from the lipid phase and formed by TM domains different from those involved in the binding of signal sequences.

Full insertion of the signal sequence into the channel at an early stage of translocation is also suggested by the similarity of the cross-linking pattern in yeast with that in the cotranslational, mammalian system. The ppαF chains of 86 amino acids employed in the cotranslational system are protected against proteolysis, indicating that they are located within the channel (Mothes et al., 1998). Other experiments have shown that this state can only be reached with a functional signal sequence (Jungnickel and Rapoport, 1995) and that the channel is open toward the lumen (Crowley et al., 1994). We therefore assume that in both translocation pathways an interaction of the signal sequence with Sec61p opens the channel. Opening of the channel for ions by an interaction with a signal peptide has also been reported for the related bacterial system (Simon and Blobel, 1992).

Interaction of the core of the signal sequence with Sec61p via TM domains 2 and 7, as well as TM domain 1, is intriguing because mutations in the equivalent domains of the bacterial homolog SecY allow the secretion of proteins with defective or deleted signal sequences. Most of these mutations (called *prlA* mutations) map to the loop between TM domains 1 and 2, TM domain 7, or to TM domain 10 (Osborne and Silhavy, 1993). TM domain 7 of SecY may be of particular importance, since it has been shown to be crucial in the recognition of the signal sequence of staphylokinase (Sako, 1991) and to have most of its *prl* mutations on one side of a presumed helix (Osborne and Silhavy, 1993). TM domains 2 and 7 also contain two of the three sequences in Sec61p/SecY best conserved throughout evolution. Together with our observation that the signal sequence is similarly positioned in the yeast and mammalian systems, it seems

that the mechanism of signal sequence recognition is highly conserved.

Our data suggest that the bound signal sequence is located at the interface between the channel and the surrounding lipid. Lipid cross-links were quantitatively coimmunoprecipitated with the Sec complex, indicating that they were not produced from ppαF molecules totally released into the lipid phase. All positions of the signal sequence may simultaneously contact both proteins and lipids, or there may be an equilibrium between different populations, with each position of the bound signal sequence contacting either only protein or only lipid. Our data indicate that the channel's walls are not uniform, because Sec62/71p in yeast and TRAM in mammals contact the helix formed by the signal sequence on only one side. The location of Sec62/71p/TRAM may be where the channel opens laterally toward the lipid bilayer.

Previous cross-linking experiments suggested that the initial contact of the signal sequence occurs with Sec62p, Sec71p, and Sec72p, and that contact with Sec61p requires a subsequent Kar2p- and ATP-dependent step (Müsch et al., 1992; Sanders et al., 1992; Lyman and Schekman, 1997). However, these experiments employed wild-type ppαF, in which all the cross-linkable lysines are in the C-terminal portion, and did not therefore allow detection of interactions of the signal sequence. Our results now show that the function of Kar2p is not required for signal sequence recognition or insertion of the polypeptide chain into the channel. The previous data can be explained by the movement of the C-terminal domain of ppαF into the Sec61p channel during the actual translocation reaction mediated by Kar2p and ATP.

A Model for Initiation of Translocation

Synthetic lethality between certain *prl* mutations in *secY* and *secE* (Flower et al., 1995), as well as experiments with a dominant negative *secY* mutant (for review, see Ito, 1995), suggests that the regions of SecY that contain *prl* mutations, and are thus presumably involved in signal sequence recognition, are the same as those required for the interaction between SecY and SecE, the homolog of Sss1p. In yeast, Sss1p can be cross-linked with a bifunctional reagent to TM domains 6–8 of Sec61p (Wilkinson et al., 1997), and overexpression of Sss1p rescues a *sec61* mutant with a breakpoint between TM domains 7 and 8, which would otherwise be lethal (Wilkinson et al., 1997). We therefore postulate that the signal sequence and Sss1p/SecE bind to the same or overlapping regions in Sec61p/SecY. We propose that Sss1p/SecE acts as a surrogate signal sequence when the Sec61p/SecY channel is in its closed form in the absence of translocating protein. When the signal sequence of a substrate arrives, it would displace Sss1p/SecE from its binding site in Sec61p/SecY and thus open the channel for polypeptide transport. *prl* mutations in SecY would have a weakened interaction with SecE, allowing the channel to be opened more easily. Opening of the channel may, however, require additional input signals, such as an interaction with SecA in *E. coli*. We assume that TM domains 2 and 7 of SecY/Sec61p are primarily involved in interactions with the signal sequence and SecE/Sss1p, but they may be of lesser importance for the formation of the passageway for the hydrophilic portions of the polypeptide chain.

In support of our hypothesis, Sss1p/SecE looks similar to a signal sequence; its essential region consists of no more than a TM domain, whose precise amino acid sequence is not important, and a few surrounding residues (Murphy and Beckwith, 1994). Upon arrival of the signal sequence, Sss1p/SecE may not be completely released from Sec61p/SecY but may simply shift its position within the complex. As the channel seems to be formed from several Sec61p molecules, more than one copy of Sss1p might also be present, and more complicated models may be required to explain their displacement by a single signal sequence.

Our hypothesis may also provide an explanation for the puzzle of why a small polypeptide chain such as Sss1p/SecE is found in all organisms, is essential for their viability, and has remained distinct from the multispanning Sec61p/SecY component through evolution. It is striking that all other components involved in protein translocation, such as the β-subunit of the Sec61p complex (Sec61β/Sbh1p), the four components of the Sec62/63p complex (Sec62p, Sec63p, Sec71p, and Sec72p), BiP (Kar2p), SecG, SecA, and SecD/F/ydj1 are present in either eukaryotes or in prokaryotes, but not in both. The most basic translocation machinery may thus consist only of a channel-forming multispanning subunit (Sec61p/SecY) and a small, single-spanning polypeptide (Sss1p/SecE), serving as its gate.

Experimental Procedures

Construction of *sec61* Mutant Yeast Strains

Factor Xa insertion derivatives of Sec61p were constructed as described by Wilkinson et al. (1996, 1997). Strains containing the following factor Xa fusions were used in this study: L70-GSIEGRGS-N73 (BWY24, N1/2C); P105-GSIEGRGS-K108 (BWY25, N2/3C); L177-GSIEGRGS-G180 (BWY65, N4/5C); Y265-GSIEGRGS-P268 (BWY66, N6/7C); S351-GSIEGRGS-E354 (BWY73, N7/8C); and G466-GSIEGRGSIEGRGS-T469 (BWY120, N10/C).

The construction of plasmids expressing functional complementary polypeptide fragments of Sec61p has been previously described (Wilkinson et al., 1997). The breakpoints of the complementary fragments are at the same positions as the factor Xa insertions.

Preparation of Microsomes, Purification of Sec Complex, and Reconstitution

Preparation of microsomes from *S. cerevisiae* cells, purification of the Sec complex by immunoaffinity chromatography with Sec62p antibodies, its dissociation into the Sec61p and Sec62/63p subcomplexes, and the reconstitution of complexes into proteoliposomes were done essentially as described (Panzner et al., 1995). The concentration of Sec61p in the final suspension of proteoliposomes ranged from 100 to 500 pmol per microliter.

In Vitro Mutagenesis and In Vitro Transcription

cDNA coding for wild-type ppαF was cloned into the vector pAlter (Promega). All lysine codons in wild-type ppαF were altered to arginine codons (76, 84, 96, 103, 117, 124, 138, 145, and 159), and single lysines were introduced using appropiate oligonucleotides. Codons at positions 10 to 14 of K5 ppαF were deleted to produce the signal sequence deletion mutant K5Δ10-14 ppαF. Stop codons (TAG) were introduced into wild-type ppαF at positions 8 to 19. Transcription was carried out from the pAlter vector with T7 RNA polymerase after linearization of the plasmid with SalI. Transcripts coding for

the M2-signal sequence mutant of ppαF (M2 ppαF) were obtained as described (Panzner et al., 1995).

Translation, Binding of ppαF to Sec Complex, and Photo-Cross-Linking

In vitro translation was carried out in the reticulocyte lysate system for 25 min at 30°C in the presence of ^{35}S-methionine and either suppressor tRNA carrying a modified phenylalanine (TmD-Phe) (Martoglio et al., 1995) or trifluoromethyl-diazirino-benzoic acid (TDBA)-lysyl-tRNA (Mothes et al., 1998). Translation was stopped by addition of 2 mM cycloheximide, and the ribosomes were removed by centrifugation for 10 min at 100,000 rpm in a Beckman TL100 rotor.

Binding of ppαF to reconstituted proteoliposomes was done essentially as described (Matlack et al., 1997). Briefly, a typical binding reaction contained 10 μl of reconstituted proteoliposomes and 1–5 μl of ppαF translation mixture. Potassium acetate and HEPES were adjusted to final concentrations of 150 mM and 50 mM, respectively. After incubation at 30°C for 20 min the samples were irradiated for 15 min on ice. For binding of ppαF to yeast microsomes, 6 μl of yeast membranes was added to 1–5 μl of ppαF translation mixture preincubated with 0.1 U/μl hexokinase and 15 mM glucose.

Immunoprecipitation and Factor Xa Cleavage

Coimmunoprecipitaion of bound and cross-linked ppαF with the Sec complex in digitonin was performed with Sec62p antibodies essentially as described (Matlack et al., 1997). For denaturing immunoprecipitations of the cross-linked products, SDS was added to 2%, and the samples were incubated for 10 min at 50°C. The mixture was adjusted to 0.1% SDS by dilution into immunoprecipitation buffer (50 mM Tris/HCl [pH 7.5], 150 mM sodium chloride, 1% Triton X-100). Affinity-purified antibodies against Sec72p, Sec62p, or the C terminus of Sec61p (Panzner et al., 1995) were added for 1 hr and collected with protein A-Sepharose. The efficiency of immunoprecipitations was 80%–100%. Binding to concanavalin A-Sepharose (ConA) was performed after denaturation in SDS.

The mapping of cross-linking sites with Sec61p-Xa mutants was performed as follows. The denatured cross-linked products were immunoprecipitated with antibodies against the C terminus of Sec61p, and the protein A-Sepharose was washed in Xa buffer (50 mM Tris/HCl [pH 8.0], 100 mM sodium chloride, 0.2% Triton X-100, 2 mM calcium chloride). The samples were divided and in one half bound material was eluted with SDS-sample buffer. The other half was incubated in a final volume of 50 μl with 4 μg of factor Xa (New England Biolabs) for 90 min at 22°C. For mapping of cross-linking sites with mutant N6/7C, two-thirds of the irradiated samples were first treated with the enzyme in Xa buffer, then divided into two parts and immunoprecipitated with antibodies against the C or N terminus of Sec61p after denaturation in SDS. One-third of the sample was subjected directly to immunoprecipitaion with antibodies against the C terminus of Sec61p. The efficiency of immunoprecipitation with antibodies against the N terminus of Sec61p was 40%–70%. This antibody was raised against an N-terminal peptide of Sec61p with an additional cysteine (MSSNRVLDLFKPFESC) and affinity-purified as described (Panzner et al., 1995).

Cross-Linking in the Mammalian System

ppαF containing the first 86 amino acids was synthesized in the wheat germ system in the presence of SRP and canine pancreatic microsomes, and cross-linking and subsequent immunoprecipitation with affinity-purified antibodies against mammalian Sec61α and TRAM were performed as described (Mothes et al., 1998).

Miscellaneous

Extraction of proteoliposomes with alkali was carried out by the addition of 20 vol of 0.1 M sodium carbonate, adjusted to pH 12.5. Cleavage of cross-links to lipids with phospholipase A2 was performed as described (Martoglio et al., 1995). Analysis of the samples was performed by SDS-PAGE with 7.5%–17.5% linear acrylamide gels, followed by quantitation with a Fuji Phosphoimager BAS1000 and autoradiography.

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