Tail-anchored and Signal-anchored Proteins Utilize Overlapping Pathways during Membrane Insertion*

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Membrane protein insertion at the mammalian ER occurs most commonly via the cotranslational pathway, in which a hydrophobic signal sequence emerges from the ribosome and is recognized by the signal recognition particle (SRP)† (1). The ribosome-nascent chain-SRP complex is then targeted to the ER membrane via an association with a cognate receptor complex (1, 2). Upon its arrival at the ER, the nascent membrane protein is delivered to the Sec61 translocon. This comprises multiple Sec61 heterotrimers, composed of α, β, and γ subunits, and it functions as the ER membrane insertion site for precursors delivered via the SRP-dependent targeting route (1–4). It is noteworthy that in higher eukaryotes, the membrane insertion of proteins targeted via the SRP-dependent pathway appears to be principally cotranslational, with the ribosome remaining closely associated with the Sec61 translocon during membrane integration (5, 6).

In contrast to higher eukaryotes, SRP-independent, post-translational translocation plays a significant role in the yeast Saccharomyces cerevisiae (7, 8). In this instance the precursors use cytosolic chaperones to maintain translocation competence (9), and signal sequence recognition occurs at the Sec complex of the ER membrane (10, 11). This Sec complex is made up of the heterotrimeric Sec61 complex together with four other membrane-associated components, namely Sec62p, Sec63p, Sec71p and Sec72p, as well as the ER luminal chaperone Kar2p (the S. cerevisiae equivalent of BiP) (12–14). It has become apparent that Sec62p and Sec63p are not restricted to S. cerevisiae, and similar proteins have been identified in mammals, although their precise function is unknown (15, 16). Specific examples of post-translational translocation have been identified in higher eukaryotes, although these tend to be the exception rather than the rule. In the case of very short presecretory proteins, such as prepromelittin, the N-terminal signal sequence does not have an opportunity to interact with SRP before translation is terminated (17, 18). Hence, prepromelittin translocation is independent of SRP but dependent upon cytosolic component(s) and ATP, presumably to maintain the polypeptide in a “translocation-competent” state (18).

Tail-anchored proteins form a distinct class of integral membrane proteins, possessing a single membrane insertion sequence at their C terminus and displaying their remaining N-terminal portion in the cytosol. The majority of tail-anchored proteins become integrated at the ER membrane (19–21, but see also Ref. 22), and members of this class carry out a range of important cellular functions, such as ER translocation (Sec61β and Sec61γ), vesicle recognition (soluble N-ethylmaleimide-sensitive factor attachment protein receptor, or SNARE proteins), and electron transfer (cytochrome b2) (for reviews, see Refs. 23 and 24). In the case of synaptobrevin 2 (Syb2), the authentic membrane insertion sequence can be replaced by a polyethylene span with a minimum length of 12 residues, demonstrating the lack of any specific sequence requirements for this region (25).

The membrane insertion sequence of tail-anchored proteins acts as an ER targeting signal, and its relative position dictates that membrane insertion is post-translational and hence likely to be SRP-independent (9, 26). To date, only general characteristics regarding the targeting and membrane insertion of tail-anchored proteins are known. The process is ATP-dependent, consistent with a role for cytosolic chaperones in maintaining
the polypeptides in an integration-competent state (19, 27–29). Furthermore, the membrane insertion of tail-anchored proteins includes a membrane binding step that is saturable (29), and this process is also sensitive to prior treatment of the membranes with protease (19). Although both of these observations suggest that the membrane insertion of tail-anchored polypeptides is mediated by proteins, the ER components responsible for this process have remained unidentified.

We set out to identify ER components that may mediate the membrane insertion of tail-anchored proteins using a defined cross-linking approach to identify proteins that are transiently associated with the newly made membrane-integrated polypeptides. We report a defined sequence of associations between newly made tail-anchored proteins and both the Sec61 translocon and Sec61 translocon-associated components. Most significantly, we observe a similar sequence of events with a comparable, cotranslationally inserted, signal-anchor protein. We conclude that the biosynthesis of tail-anchored and signal-anchor proteins is mediated by a similar complement of ER components.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antibodies were raised against specific peptides representing Sec61α, Sec61β, Sec62, B. Dobberstein) (EC). Section 62p, and Sec63p. The anti-Syb2 antibody was a mouse monoclonal recognizing an epitope at the N terminus of Syb2 (from R. Jahn). cDNA Constructs and Transcription—An NcoI fragment incorporating the coding region of the human Sec61α cDNA (30) was subcloned into the pSPUTK vector (Stratagene). The DNA template for its transcription was made by cleavage of the Sec61p pSPUT plasmid with EcoRI. The cDNA for rat Syb2 in pBluescript was a gift from R. Scheller (Calbiochem). The cyanine mutant of synthetophore (Syb2m) was created by changing a leucine (Leu-63) to a cysteine using the QuickChange site-directed mutagenesis kit (Stratagene). The following sense primer was used: 5'-GACCAGAAGCTATCGGAGAGCAAGATGAG-3' and the resulting plasmid was linearized for sense primer was used: 5'-ACCTTGAGCAGATCTACATGGGGACTGG-GCCGAGATGCCC-3'. This PCR product was cut with BgII and ligated into the BgII and Hpal sites of pSPUTK (Stratagene). The resulting plasmid was linearized for transcription using XbaI. The cDNA for rat Syn1A was a gift from Dr. Sabine Hilliker (University of Manchester). The coding region was amplified by PCR, introducing a cysteine using primer 5'-ACCTTGAGCAGATCTACATGGGGACTGGGCC-3' and an XbaI site after the termination codon using primer 5'-GACCAGAAGCTATCGGAGAGCAAGATGAG-3' and the resulting plasmid was linearized for transcription using XbaI. The DNA coding region of invariant chain (Ii) was changed to a methionine using the QuickChange kit with the sense primer 5'-CCGGAGAGCACGAGGATGAG-GTCCGAGAGCTGT-3'. Transcription templates for II derivatives were prepared by PCR using this variant of Ii as a template. The 5'-primer recognized a 150 bp 5' to the SP6 promoter 5'-CCGGAATTC-AGAAGGTTCG-3', whereas the 3' primers were 5'-ACCTTGAGCAGATCTACATGGGGACTGGGCC-3' for Ii1 and 5'-ACCTTGAGCAGATCTACATGGGGACTGGGCC-3'. The authenticity of all PCR-derived constructs was confirmed by DNA sequencing. Transcripts were synthesized using T3 RNA polymerase for Syb2m, or SP6 RNA polymerase for all the other templates, according to the manufacturer’s instructions (New England Biolabs).

Translation and Membrane Insertion—Proteins were synthesized using rabbit reticulocyte lysate (Promega) that had been preincubated at 20°C for 10 min to remove any contaminating membrane fractions. Incubations were performed at 30°C in the presence of both [35S]methionine and canine pancreatic microsomes according to the manufacturer’s instructions. Microsomes were prepared from canine pancreas as described by Walter and Blobel (31) and added to in vitro translations at 1.5–2.0 A260/ml. A time course was performed to establish the amount of Sec61β that was integrated into canine pancreatic microsomes under the experimental conditions that we were using. This analysis was carried out across a 60-min period, and the percentage integration was defined as the proportion of the total protein synthesized at a particular time point which was found to be membrane-associated and resistant to extraction with alkaline sodium carbonate solution (23, 32).

Control experiments showed that in the absence of added microsomes, or when using a protein that lacked its tail anchor sequence, less than 2% of the total protein synthesized in 30 min was recovered by this assay (data not shown). In contrast, >30% of authentic Sec61β was recovered in the membrane fraction using the same assay (see Fig. 2A). On the basis of this analysis, translation reactions were initially carried out for 30 min by which point significant membrane integration was observed (see Fig. 2A). In some subsequent experiments, shorter incubation periods were used to optimize transient associations. In these cases, specific details are indicated in the accompanying figure legend. All puromycin treatments were performed by the addition of 1 mm puromycin and subsequent incubation at 30°C for 5 min.

Cross-linking and Immunoprecipitation—Microsomes were isolated for cross-linking analysis by layering them over HSC buffer (250 mm sucrose, 500 mm KOAc, 5 mm Mg(OAc)2, 50 mm Hepes-KOH pH 7.9) and spinning at 100,000 g for 10 min to yield a membrane pellet. Membrane pellets were resuspended in LSC buffer (250 mm sucrose, 100 mm KOAc, 5 mm Mg(OAc)2, 50 mm Hepes-KOH pH 7.9) and normally incubated at 30°C for 10 min with either bismaleimidothianesulfonic acid (BMH) (0.5 mm final concentration unless otherwise stated), diluted from a 20 mm stock dissolved in dimethyl sulfoxide, or an equivalent dimethyl sulfoxide control. Where time course experiments were carried out, the sample was resuspended in LSC buffer and incubated at 30°C for 0–120 min before cross-linking as above. In one case a parallel sample was incubated at 0°C for 60 min before adding BMH. Cross-linking was stopped by the addition of 10 mM 2-mercaptoethanol to quench any unreacted maleimide groups, and a fraction of the sample was removed for direct analysis. The remainder of the samples were denatured at 70°C for 10 min in the presence of 1% (w/v) SDS and diluted with 4 volumes of Triton X-100 immunoprecipitation buffer (1% (w/v) Triton X-100, 140 mm NaCl, 1 mm EDTA, 10 mm Tris-HCl, pH 7.5). Samples were precleared by the addition of 10% volume Pansorbin (Calbiochem) and incubated at 4°C for 1 h, followed by centrifugation at 15,000 × g for 5 min. The resulting supernatants were subjected to immunoprecipitation by the addition of antiserum at 1:100 (v/v) and incubation at 4°C for 16 h with mixing. Protein A-Sepharose beads and bound material were pelleted in a microfuge and washed several times with Triton X-100 immunoprecipitation buffer. The resulting beads were heated to 70°C for 10 min in SDS-PAGE sample buffer, and unless otherwise stated, the solubilized material was resolved on 16% polyacrylamide Tris-glycine gels run under denaturing conditions.

RESULTS

Discrete Cross-linking Products Are Observed with Newly Synthesized Sec61β—We chose to investigate the association of newly synthesized tail-anchored proteins with ER components using the bifunctional cross-linking reagent BMH, which is highly specific for cysteine residues. This approach had proven very efficient in previous studies of cotranslational membrane protein biosynthesis (32, 33). Furthermore, our preliminary analysis had shown that the membrane insertion of the tail-anchored proteins into ER-derived microsomes could be prevented by prior treatment of the membranes with N-ethylmaleimide, which modifies the free sulfhydryl groups of cysteine residues (data not shown). Hence, BMH was ideally suited to cross-link newly synthesized tail-anchored proteins containing one or more cysteine residues (see Fig. 1) to the N-ethylmaleimide-sensitive, cysteine-containing ER proteins that facilitate their membrane insertion.

Given our interest in the biogenesis of the ER translocon (32), we chose Sec61β, one of the tail-anchored subunits of the Sec61 complex, for our initial studies. The β and γ subunits of the Sec61 complex both have amino acid sequences that are characteristic of tail-anchored proteins (30) and, like Syb2 (19), are capable of authentic post-translational membrane integra-
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The Majority of the Sec61β Cross-linking Products Reflect Transient Associations—Given that Sec61β forms part of the heterotrimeric Sec61 complex, we reasoned that newly synthesized, membrane-inserted Sec61β would probably display two types of associations with endogenous ER components. These would most likely be transient associations, indicative of a biosynthetic pathway, and stable associations, indicative of assembly into the Sec61 complex.

We therefore carried out a time course experiment where membrane-associated Sec61β was isolated from the translation reaction and incubated for increasing periods of time before cross-linking was initiated. From this time course experiment, it was immediately obvious that all of the major cross-linking products except the Sec61α adduct reflected a transient association between the newly synthesized Sec61β polypeptides and ER-associated components (Fig. 2C, lanes 1–5; cf. Fig. 2B, lane 3). Thus, the pattern of BMH-dependent cross-linking products was the same as observed previously when cross-linking was carried out immediately after the isolation of the membrane fraction (Fig. 2B, lane 2, and Fig. 2C, lane 1). In contrast, when the membrane fraction was incubated for 10 min at the translation temperature before cross-linking, only the prominent Sec61α adduct remained (Fig. 2C, lane 2, product indicated by the filled circle in lane 6). The sample could be incubated at 30 °C for up to 120 min before adding the cross-linking reagent, and a strong adduct with Sec61α was still observed, whereas none of the other major products seen at the zero time point was detected (Fig. 2C, lanes 1–5).

Interestingly, the “release” of newly synthesized Sec61β from its transient association with a discrete set of cellular components could be prevented by incubating the samples on ice rather than at 30 °C (Fig. 2C, lanes 4 and 6). This observation indicates that the release of Sec61β from these transient associations is prevented at low temperature. We found that adduct formation with Sec61α was largely unaffected by prolonged incubation before initiating BMH-dependent cross-linking, confirming the stability of reduced thiol groups under the experimental conditions we have used. The prolonged association of Sec61β with Sec61α which we can detect by cross-linking presumably reflects the in vitro formation of Sec61-derived complexes.

We did observe some reduction in the overall intensity of the Sec61β/Sec61α adduct across a 120-min time course, although the radiolabeled Sec61β chains were stable during this period (Fig. 2C, lanes 1–5). In contrast, the Sec61β/Sec61α adduct was unaffected by a 60-min incubation at 0 °C (Fig. 2C, lanes 4 and 6). We believe that Sec61β can be cross-linked to Sec61α both during its biosynthesis, where the association is transient, and during subsequent complex formation, where the association is stable. In the case of complex formation, the radiolabeled Sec61β synthesized in vitro must compete for binding with endogenous unlabelled Sec61β present in the canine pancreatic microsomes. Such competition would limit the proportion of radiolabeled Sec61β that could be cross-linked to Sec61α, and presumably a “steady state” is reached. This is also consistent with our observation that between 30 and 120 min the efficiency of cross-linking to Sec61α is relatively constant (Fig. 2C, lanes 3–5). On the basis of our cross-linking analysis with Sec61β, we concluded that BMH-dependent cross-linking could be exploited successfully to reveal transient associations between newly synthesized tail-anchored proteins and cellular components.

Syb2 and Syn1A Associate with ER Membrane Components during Membrane Integration—To focus our analysis on potential biosynthetic interactions, we next analyzed a tail-anchored protein that would not be expected to form any stable complexes with endogenous ER components. For this reason we chose Syb2 as a previously well characterized tail-anchored protein that is membrane-integrated at the ER and then transported to post-ER vesicular structures (19). When wild type Syb2 was analyzed no BMH-dependent adducts were observed, indicating that the single naturally occurring cysteine residue
To assess how general the associations of newly synthesized Sec61β and Syb2cm with specific ER components were, we repeated a similar experiment using a third tail-anchored protein, Syn1A. This has only one cysteine present in its cytosolic domain (Syn1A, see Experimental Procedures). Given our success with the cross-linking analysis of Sec61β, we prepared a mutant form of Syb2cm with a cysteine residue at an equivalent location within its cytosolic domain (Syb2cm, see Fig. 1). We confirmed that Syb2cm behaved as an authentic tail-anchored protein by showing that it could integrate post-translationally into canine pancreatic microsomes in the same way as the wild type protein (cf. Ref. 19 and data not shown).

As we had seen previously with Sec61β (Fig. 2, B and C), Syb2cm yielded several BMH-dependent cross-linking products when synthesized in the presence of ER-derived microsomal membranes (see Fig. 3A, lanes 1 and 2). A parallel control experiment showed that no such BMH-dependent adducts were obtained with a version of Syb2cm which lacked its transmembrane domain (data not shown). All of the BMH-dependent adducts could be immunoprecipitated with an anti-Syb2 antibody confirming their origin (Fig. 3A, lane 3). By screening a number of antisera recognizing known ER components, we were able to identify specific cross-linking of Syb2cm with Sec61β (~10-kDa partner, Fig. 3A, lane 5, *) and SPC25 (~22-kDa partner, Fig. 3A, lane 6, †). The ~38-kDa product observed in the absence of BMH (Fig. 3A, lane 1, thin arrow) is distinct from the SPC25 adduct (Fig. 3A, lane 6) and is likely an SDS-resistant dimer of Syb2cm. A weaker ~80-kDa adduct with Sec62p (Fig. 3A, lane 4, cf. lanes 7 and 8) could also be observed after immunoprecipitation. All three of these components are known to be either a part of, or closely associated with, the Sec61 translocon. Despite this, we could detect no cross-linking of Syb2cm to Sec61α, the core subunit of the Sec61 translocon (Fig. 3A, lane 4). This is in contrast to the behavior of newly synthesized Sec61β, which exhibited strong cross-linking to Sec61α which generated a discrete ~50-kDa adduct (see Fig. 2B). The identity of at least three other major Syb2cm cross-linking products remains to be determined (see “Discussion”).

To assess how general the associations of newly synthesized Sec61β and Syb2cm with specific ER components were, we repeated a similar experiment using a third tail-anchored protein, Syn1A. Syn1A has only one cysteine present in its cytosolic domain, and this is located 121 residues from the presumptive transmembrane region (Fig. 1). Nevertheless, like the other two proteins analyzed, Syn1A also yielded multiple BMH-dependent cross-linking products (Fig. 3B, lane 1). As with Syb2cm, specific adducts of Syn1A with Sec61β (Fig. 3B, lane 3, *), SPC25 (Fig. 3B, lane 4, †), and Sec62p (Fig. 3B, lane 5, ~90 kDa product) were identified. At least two other major adducts were detected (Fig. 3B, lane 1; Fig. 4, lane 7), but treated with or without BMH as indicated. Total products (lanes 1 and 2) or products immunoprecipitated by specific antisera (lanes 3–8) were then analyzed by SDS-PAGE as indicated. A filled arrowhead marks the position of the Sec61β precursor protein. Adducts with specific proteins are indicated with the following symbols: •, Sec61α; †, SPC25; and §, Sec63p. The mobility of the Sec61α adduct is distorted in lanes 3 and 4 by the presence of comigrating IgG heavy chain. Unidentified adducts of ~22 (○) and ~40 kDa (×) are also indicated. C, newly synthesized, membrane-associated Sec61β was incubated at 30 °C for increasing lengths of time (0–120 min) before cross-linking with 1 mM BMH (lanes 1–5). In one case, a sample of the membrane-associated Sec61β was placed on ice immediately and incubated at 0 °C for 60 min before cross-linking with 1 mM BMH (lane 6). After quenching, the samples were extracted with alkaline sodium carbonate solution, and the membrane fraction was reisolated and analyzed by SDS-PAGE on a 15% polyacrylamide gel. Major cross-linking products are indicated as in B. A filled arrowhead indicates the location of the Sec61β precursor.
these could not be identified by immunoprecipitation (see “Discussion”).

An additional product was brought down nonspecifically during the immunosolubilization of the Sec61β, SPC25, and Sec62p adducts (Fig. 3B, lanes 3, 4 and 5, open arrowhead). However, in other experiments this product was not associated with these adducts under immunoprecipitation (cf. Fig. 4). Once again, Syn1A showed no evidence of cross-linking to Sec61α (Fig. 3B, lane 2). The authenticity of the Syn1A adducts with Sec61β, SPC25, and Sec62p was confirmed further by showing that no such products could be detected when control immunoprecipitations were carried out in the absence of BMH-dependent cross-linking (data not shown).

On the basis of the data outlined above, we concluded that newly synthesized tail-anchored proteins associate with several generic ER components including the β subunit of the Sec61 complex and two Sec61-associated components (SPC25 and Sec62p). In contrast to many previous studies that had investigated cotranslationally inserted membrane proteins (see Ref. 2 and references therein), no cross-linking of the tail-anchored precursors to Sec61α was observed. We reasoned that this may be the result of any one of several factors: 1) Sec61α does not associate with tail-anchored proteins during their membrane integration; 2) Sec61α associates at an earlier stage of the process than we had analyzed; 3) the cysteines present in Sec61α were unsuitable for BMH-dependent cross-linking to the tail-anchored proteins we had used in this study. Our previous success with BMH-dependent cross-linking (32, 33), coupled with our ability to cross-link Sec61β to Sec61α in the context of complex formation (see Fig. 2, B and C), suggested that the third possibility was unlikely. We therefore investigated whether the ER components that are adjacent to newly synthesized tail-anchored proteins are in any way dependent upon the relative time at which cross-linking is carried out.

Syn1A Displays an Early and Transient Association with Sec61α—To address the possibility that tail-anchored proteins may associate with distinct ER components at different stages during their membrane insertion, we investigated the cross-linking partners of Syn1A with respect to the time elapsed from the start of protein synthesis. Syn1A was translated in the presence of microsomal membranes for only 5 min. Samples were then either placed on ice to stabilize transient associations (Fig. 4, lanes 1–6; cf. Fig. 2C, lane 6) or “chased” at the translation temperature for a further 15 min (Fig. 4, lanes 7–12), prior to initiating BMH-dependent cross-linking. The most telling result of this kinetic analysis of Syn1A cross-linking products was our observation that the association between the newly synthesized polypeptide and adjacent ER components is dynamic. Hence, we found significant changes in the cross-linking partners between the two time points studied. Most significantly, after a short 5-min incubation with no chase, cross-linking of Syn1A to Sec61α was detected (Fig. 4, lane 2, ●). The other characteristic of this “early” stage of Syn1A integration was a very large adduct that included mammalian Sec63p (Fig. 4, lane 6, §). The difference between the predicted (120 kDa), and apparent (180 kDa) size of this adduct (Fig. 4, lane 6, §) suggests that either its mobility is aberrant, or the adduct contains one or more components in addition to Syn1A and Sec63p (cf. Ref. 15). At this early stage, weak adducts with Sec61β, SPC25, and Sec62p were also detected (Fig. 4, lanes 3–5).

Following a 15-min chase, during which no further protein synthesis occurred, cross-linking to Sec61α and Sec63p was lost (Fig. 4, lanes 8 and 12), whereas the other adducts were retained and/or showed an increase in intensity (Fig. 4, lanes 2–6).
9–11). The presence of Syn1A adducts with Sec62p and SPC25 even after only 5 min of protein synthesis most likely reflects our inability to generate artificially trapped “integration intermediates” of the tail-anchored proteins studied (cf. Ref. 33). Thus, even after a relatively short period of synthesis, a spread of molecules representing different stages of the membrane integration process will be present in the reaction mixture. Given this experimental limitation, the simplest interpretation of these results is that Syn1A is transiently associated with Sec61α and Sec63p at an early stage of the membrane insertion process. Adducts of Syn1A with Sec61β are also observed at this early stage of synthesis; however, in contrast to Sec61α and Sec63p this association is more prolonged (Fig. 4, cf. lanes 4 and 6). The proximity of Syn1A to SPC25 and Sec62p occurred predominantly at “later” stages of the membrane integration process, i.e. after a 15-min chase (Fig. 4, cf. lanes 4 and 5 with lanes 10 and 11).

The early association observed between Syn1A and Sec61α suggests that Syn1A may be associated with the Sec61 translocon at an early stage of its membrane insertion. This conclusion is supported by our observation of downstream interactions between Syn1A and Sec61-associated components, specifically Sec62p and Sec61β/SPC 25 (see Ref. 35, cf. Refs. 15 and 16). Given that no other integral membrane proteins had previously been shown to associate with Sec62p and Sec63p during membrane insertion, it was possible that these components were specific for tail-anchored proteins.

**Tail-anchored and Signal-anchored Membrane Proteins Associate with a Common Subset of ER Components**—To facilitate a direct comparison between the post-translational membrane insertion of a tail-anchored protein and the cotranslational insertion of a signal-anchored protein, we chose to study the Ii of the human class II major histocompatibility complex. Ii displays a short hydrophilic N-terminal domain on the cytosolic side of the ER membrane (see Fig. 1), and its single transmembrane domain acts as the signal anchor sequence that promotes its cotranslational insertion into the ER membrane (36). To study the cotranslational insertion of Ii via its signal anchor sequence, a transcript encoding the first 81 residues of Ii, and lacking a stop codon (Fig. 1, Ii81), was translated in the presence of ER derived microsomes. This leads to the resulting ribosome-bound nascent chain being trapped at the ER insertion site pending the puromycin-dependent release of the ribosome from the nascent chain (37, 38).

The Ii was converted into a tail-anchored protein by introducing a stop codon immediately after the end of the predicted transmembrane region (Fig. 1, IiTA). Subsequent analysis of IiTA showed that it is indeed capable of authentic post-translational membrane insertion and hence behaves as a tail-anchored protein (data not shown). For both Ii81 and IiTA the single naturally occurring cysteine residue close to the cytosolic side of the predicted transmembrane region was substituted for a methionine residue. To facilitate BMH-dependent cross-linking, a single cysteine residue was then introduced into the cytosolic domains of these polypeptides at a point 28 residues from the start of the predicted transmembrane domain and hence at a location comparable with the cysteine probe present in Sec61β and Syb2cm (see Fig. 1).

As expected from previous studies (37, 38), the trapped form of Ii81 was efficiently cross-linked to Sec61α (Fig. 5, lane 2, filled circle). In addition, we observed adducts of Ii81 with Sec61β, SPC25, and Sec63p (Fig. 5, lanes 3, 4, and 6), none of which had been identified during previous studies that employed alternative cross-linking strategies (cf. Refs. 37 and 38). When samples were treated with puromycin before cross-linking, the adducts with Sec61α and Sec63p were almost completely lost (Fig. 5, cf. lanes 2, 6, 8, and 12). Cross-linking to Sec61β was also diminished (Fig. 5, lane 9), whereas adducts with both SPC25 and Sec62p were enhanced (Fig. 5, lanes 10 and 11).

The cross-linking analysis of IiTA revealed a pattern of adducts which resembled a combination of the results obtained
with Ii81 in its trapped and released forms (see Fig. 5), and adducts of IiTA with Sec61α, Sec61β, SPC25, Sec62p, and Sec63p could be observed (Fig. 5, lanes 14–18). Hence, the tail-anchored form of Ii associates with the same group of ER components as the authentic tail-anchored proteins used during this study (cf. Figs. 2–5). More significantly, the ER components that we have shown to associate with three tail-anchored membrane proteins during their membrane insertion are identical to those associated with a well characterized signal-anchored protein that is delivered to the ER membrane via the cotranslational pathway.

If the cross-linking of newly membrane-inserted IiTA to adjacent ER components is acting to report a biosynthetic pathway faithfully, then one would expect such associations to be transient as we have observed for the majority of adducts detected with Sec61β (cf. Fig. 2C). We therefore carried out a detailed time course study of the cross-linking of IiTA to known ER components. This analysis showed that association of IiTA with all five of the ER proteins that we could identify by immunoprecipitation was transient and that the proximity of IiTA to Sec61α was particularly short lived (Fig. 6A). Our studies of Sec61β integration had shown that the efficiency of its cysteine-mediated cross-linking to Sec61α was not reduced dramatically by prolonged incubation times and not reduced at all if samples were held at 0 °C (cf. Fig. 2C). These data indicated that the inactivation of cysteine residues by oxidation was not responsible for the time-dependent loss of cross-linking which we observed with IiTA. Nevertheless, we also addressed this issue with Ii by taking advantage of its cotranslationally inserted form (Ii81). When a ribosome-bound integration intermediate of Ii81 was generated and treated with BMH across a time course identical to that used to study IiTA, we found that adduct formation was relatively stable in all cases (Fig. 6B). Thus, we can conclude that the loss of cross-linking to IiTA which we observe across the same time course cannot be solely the result of the loss of available cysteine residues.

The reduction in cross-linking efficiency which we do observe with ribosome-bound Ii81 across a 60-min time course (Fig. 6B) is most likely caused by the loss of the ribosomes from a proportion of the Ii81 nascent chains during this incubation period (see Ref. 39). As expected, if we released all of the chains from the ribosome at the start of the time course by puromycin treatment, the nascent chains were rapidly released from their association with the Sec61α and Sec61β subunits of the core ER translocon (Fig. 6C). In contrast, the puromycin-released chains remained adjacent to Sec62p and SPC25 for a longer period, although this association was still clearly transient (Fig. 6C). We therefore conclude that the co- and post-translational pathways for the insertion of newly synthesized membrane proteins at the ER utilize a similar subset of ER components.

DISCUSSION

During this study we have identified a discrete subset of cellular components that are transiently associated with newly membrane-inserted tail-anchored proteins. Among these, we have identified five ER proteins that are either a part of the core ER translocon or closely associated with it. None of these ER components is specifically associated with tail-anchored proteins, and the same set of components is adjacent to a newly integrated signal-anchored membrane protein. Taken together, our data indicate that the pathway that facilitates the membrane insertion of tail-anchored proteins displays substantial overlap with the “classical” pathway that mediates this process for most other types of membrane proteins.

Newly Made Membrane Proteins Are in Transient Proximity to a Specific Subset of ER Components—All of the tail-anchored proteins that we have studied display a transient association with a defined subset of cellular components, as determined by site-specific chemical cross-linking. A number of control experiments confirmed that the time-dependent alterations in adduct formation which we observe are a true reflection of changes in the local environment of the newly integrated membrane proteins and not simply a loss of their capacity to generate BMH-dependent adducts. We conclude that, during their membrane insertion, newly synthesized tail-anchored proteins move from a restricted environment with a high effective concentration of specific ER proteins into a predominantly lipid environment that reflects complete membrane integration (see Fig. 7).

Several cross-linking partners of the newly membrane integrated tail-anchored proteins that we detect in our assay remain to be identified. It is worth noting that at least some of these unidentified components may represent transient interactions with cytosolic components. Interactions between posttranslationally translocated, secretory protein precursors and cytosolic chaperones have previously been detected in a rabbit reticulocyte lysate system (9). Furthermore, in the case of tail-anchored membrane proteins the bulk of the polypeptide remains on the cytosolic side of the membrane where it would be available for such interactions even after membrane integration. Interestingly, we also detect a far more complex pattern of adducts with authentic tail-anchored proteins that have a significant cytoplasmic segment than with our artificial tail-anchored protein, IiTA, which does not (cf. Fig. 1).

On the basis of our cross-linking analysis we have been able to identify several ER proteins that are transiently associated with newly membrane-inserted tail-anchored proteins. In fact,
by carrying out a careful time course study of Syn1A integration, we could define transient associations with two different sets of ER components that we believe reflect different stages during the membrane insertion process. We found that both wild type (Syn1A) and artificial (IiTA) tail-anchored proteins are adjacent to Sec61α and Sec63p at an early stage during their membrane insertion. The proximity of the newly membrane-inserted chains to these components is brief and is lost after a short chase period. In contrast, the cross-linking products that we observed with SPC25 and Sec62p were more prevalent at later time points consistent with associations at a later stage of the membrane insertion process. As already indicated, our inability to generate integration intermediates of tail-anchored membrane proteins undoubtedly limits the precision of this type of “stage specific” cross-linking analysis. However, we found that a comparable cotranslationally inserted membrane protein (Ii81) associated with the same ER components and showed a similar time dependence when re-

**Fig. 6.** Time course of Ii81 and IiTA cross-linking to membrane components. A, IiTA was translated in the presence of microsomes for 10 min and treated with puromycin for 10 min. The microsomes were isolated, then incubated at 30 °C for an additional 0, 20, or 50 min, and finally treated with BMH and immunoprecipitated with various antibodies. The resulting adducts were separated by SDS-PAGE and quantified such that each adduct was 100% at its highest value over the time course. The time scale begins at the addition of puromycin. B, Ii81 was translated in the presence of microsomes for 10 min. The microsomes were isolated, then incubated at 30 °C for an additional 0 or 60 min, and finally treated with BMH and immunoprecipitated with various antibodies. The resulting adducts were separated by SDS-PAGE and quantified such that each adduct was 100% before incubation. The time scale indicates the post-translation incubation. C, Ii81 was translated in the presence of microsomes for 10 min and treated with puromycin for 10 min. The microsomes were isolated, then incubated at 30 °C for an additional 0, 20, or 50 min, and finally treated with BMH and immunoprecipitated with various antibodies. The resulting adducts were separated by SDS-PAGE and quantified such that each adduct was 100% at its highest value over the time course. The time scale begins at the addition of puromycin, and the value at time zero was taken from B, therefore representing the ribosome-arrested state.
circles represent the proposed pathway taken by a newly inserted tail-anchored protein during its integration into the phospholipid bilayer. Tail-anchored proteins interact early and transiently with Sec61α, the major component of the Sec61 complex, and Sec62p is also implicated at an early stage during integration. Interactions with Sec61β occur at both early and late stages, whereas SPC25 and Sec62p are principally involved at later stages of integration. Newly synthesized proteins with a signal anchor sequence display similar associations, although the interaction with Sec61α is maintained during synthesis of the ER luminal domain and can be artificially stabilized by using ribosome-bound integration intermediates. Clearly, this model does not preclude a role for additional, as yet unidentified, components during the membrane integration of tail-anchored proteins.

Roles of Sec61 Complex—The Sec61 complex is known to play a central role during the integration of a variety of cotranslationally inserted membrane proteins (40–42) and acts as the principal gateway for protein traffic across the ER membrane (43). We observe an early association of tail-anchored proteins with both Sec61α and Sec61β, suggesting that the Sec61 complex might play some role during the membrane insertion of tail-anchored proteins (see Fig. 7). This proposal is consistent with the role of Sec61 in the post-translational translocation of small secretory proteins in higher eukaryotes (44). It is noteworthy that the purified Sec61 complex alone is incapable of supporting the membrane insertion of tail-anchored proteins (19). Our cross-linking data identify additional components including Sec62p and Sec63p, and such proteins may represent essential upstream elements of the pathway for tail-anchored membrane protein integration which were lacking from the purified system previously used by Kutay et al. (19). At present we can only speculate about the role played by the Sec61 translocon during the biosynthesis of tail-anchored proteins. The simplest model to explain our data is that the Sec61 complex is the actual site of tail-anchored protein insertion into the ER membrane. Alternatively, the interaction of newly made tail-anchored proteins with translocon-associated components such as Sec62p or Sec63p may account for their transient proximity to the Sec61 complex.

Roles of Sec62p and Sec63p—In this study, we show for the first time that both Sec62p and Sec63p can be cross-linked to newly synthesized membrane proteins in a mammalian cell free system. We also find that the nature of the ER membrane insertion sequence, i.e. tail-anchored or signal-anchored, has no influence upon the transient proximity of a precursor to these components. Sec62p and Sec63p are the mammalian equivalents of proteins that were first identified in the yeast S. cerevisiae (15, 16). Yeast Sec62p and Sec63p have long been associated with the post-translational translocation of secretory proteins across the ER membrane (12–14), although recent data suggest that Sec63p also functions during cotranslational translocation (45). Consistent with this latter proposal, we observe cross-linking to Sec63p at an early stage during the cotranslational integration of a signal-anchored membrane protein. Likewise, the interaction of tail-anchored proteins with mammalian Sec63p is seen at an early time point during membrane insertion.

In yeast, Sec63p provides a binding site for the BiP ortholog, Kar2p, and has been proposed to promote post-translational translocation in addition to “gating” the Sec61 translocon at its luminal side (13, 14, 46). The functions of mammalian Sec62p and Sec63p remain to be established (15, 16), but the proteins are present at roughly equimolar amounts with respect to the Sec61α monomer. Consistent with our cross-linking data, a fraction of Sec62p and Sec63p is clearly associated with the Sec61 complex (15, 16). We find membrane proteins are adjacent to Sec63p at an early stage during their insertion (Fig. 7), and this is consistent with a role for Sec63p, perhaps together with BiP, in regulating or priming the ER translocation site (13, 16, 47). In contrast, we find that newly made polypeptides are adjacent to Sec62p at a late stage of the membrane integration process. Thus, Sec62p presumably facilitates a different stage of the process, for example the transition of the transmembrane domain from the Sec61 complex into the phospholipid bilayer (see also Ref. 11).

Proximity to SPC25—The SPC25 subunit was identified as a major cross-linking partner of all of the newly inserted membrane proteins investigated during this study and was particularly apparent at later stages of the membrane integration process. SPC25 is a subunit of the mammalian signal peptidase complex, and it has been proposed to link the SPC complex to the Sec61 complex via the Sec61β subunit (35). The physical proximity between newly synthesized tail-anchored proteins and the signal peptidase complex which we detect by cross-linking is in close agreement with recent evidence for a functional interaction (48). Thus, when a consensus site for signal peptidase cleavage is introduced at the C terminus of Syb2, derivatives with a sufficiently long transmembrane spanning region are authentically processed by the signal peptidase complex (48). The fact that we identify SPC25 as a major cross-linking partner of all newly made membrane proteins analyzed, both tail-anchored and signal-anchored, indicates that such polypeptides may be scanned by the signal-peptidase complex during their exit from the ER membrane insertion site. This process would ensure the efficient recognition and processing of cleavable ER targeting signals where present (see Fig. 7 and Ref. 2).

In summary, our cross-linking analysis has identified ER components that are associated with tail-anchored proteins during their membrane insertion and shown that they closely mirror those associated with the well characterized cotranslational pathway for membrane insertion. We conclude that the pathways for the integration of tail-anchored and signal-anchored proteins are principally involved at later stages of integration. Newly synthesized proteins, including Sec62p and Sec63p, remain to be established (15, 16), but the proteins are present at roughly equimolar amounts with respect to the Sec61α monomer. Consistent with our cross-linking data, a fraction of Sec62p and Sec63p is clearly associated with the Sec61 complex (15, 16). We find membrane proteins are adjacent to Sec63p at an early stage during their insertion (Fig. 7), and this is consistent with a role for Sec63p, perhaps together with BiP, in regulating or priming the ER translocation site (13, 16, 47). In contrast, we find that newly made polypeptides are adjacent to Sec62p at a late stage of the membrane integration process. Thus, Sec62p presumably facilitates a different stage of the process, for example the transition of the transmembrane domain from the Sec61 complex into the phospholipid bilayer (see also Ref. 11).
chored membrane proteins at the ER exhibit a substantial degree of overlap, and we believe that this reflects similarities between the co- and post-translational pathways for membrane insertion. The precise function of specific ER components during the membrane insertion of tail-anchored proteins is the focus of our current work.

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