A trans-membrane segment inside the ribosome exit tunnel triggers RAMP4 recruitment to the Sec61p translocase

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Membrane protein integration occurs predominantly at the endoplasmic reticulum and is mediated by the translocon, which is formed by the Sec61p complex. The translocon binds to the ribosome at the polypeptide exit site such that integration occurs in a cotranslational manner. Ribosomal protein Rpl17 is positioned such that it contacts both the ribosome exit tunnel and the surface of the ribosome near the exit site, where it is intimately associated with the translocon. The presence of a trans-membrane (TM) segment inside the ribosomal exit tunnel leads to the recruitment of RAMP4 to the translocon at a site adjacent to Rpl17. This suggests a signaling function for Rpl17 such that it can recognize a TM segment inside the ribosome and triggers rearrangements of the translocon, priming it for subsequent TM segment integration.

Introduction

The ER represents the major site of membrane protein biogenesis in eukaryotic cells. Membrane proteins begin their biogenesis in a similar manner to secretory proteins, being targeted cotranslationally by the signal recognition particle (SRP) and its cognate receptor to the translocation channel formed by the Sec61 complex (Rapoport et al., 2004; Rapoport, 2007). The translocon is able to bind to the ribosome such that translocation, like targeting, occurs cotranslationally. Not only does the translocon form an aqueous pore across the membrane through which the nascent chain can pass, but in response to a transmembrane (TM) segment, the channel can open laterally, allowing the TM segment to exit into the lipid bilayer (Martoglio et al., 1995).

The translocon is formed by multiple copies of the Sec61p complex: a heterotrimer of Sec61α, -β, and -γ (Görlich and Rapoport, 1993). The x-ray structure of a dimer of Sec61 heterotrimers from archaebacteria (SecYEβ) has been determined in the absence of ribosomes (Van den Berg et al., 2004). A single heterotrimer forms an hourglass structure reminiscent of a closed channel. The 10 TM segments of SecY (Sec61α homologue) are arranged with pseudo twofold symmetry forming a clam shape. The single TM segment of SecE (Sec61γ homologue) serves as a clamp forming a hinge. Sec61β is located more peripherally, making limited contact with SecY. TM2 of SecY is distorted such that it blocks the pore and has been proposed to act as a plug, which can open the channel in response to its interaction with a signal sequence (Van den Berg et al., 2004). The clam shape also suggests a mechanism to facilitate lateral exit of TM segments from the translocon into the lipid bilayer.

Based upon this structure, it has been proposed that only one of the Sec61 heterotrimers bound to the ribosome actually forms the translocation pore (Van den Berg et al., 2004). It is not clear what function, if any, the other heterotrimers play in the active ribosome–translocon complex (Dobberstein and Sinning, 2004). However, this view has been challenged; a Cryo-EM structure of the bacterial translocon bound to the ribosome predicts that the active channel may be formed by two heterotrimers arranged with the lateral openings facing one another such that a contiguous channel may be formed (Mitra et al., 2005).

Several other proteins associated with the translocon, including the TRAM (translocating nascent chain–associated membrane protein) and TRAP (translocon-associated protein) complex, which facilitate the translocation of most substrates

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Abbreviations used in this paper: DSG, disuccinimidyl glutarate; EKRM, EDTA HS-stripped RM; eq, equivalent; HS, high salt; MBS, maleimidobenzoyl-*N*-hydroxy succinimide; pPL, preprolactin; pPV, pPL–VSV-G fusion; PTC, peptidyl transferase center; RAMP, ribosome-associated membrane protein; RM, rough microsome; rRNA, ribosomal RNA; SRP, signal recognition particle; TM, trans-membrane; VSV-G, vesicular stomatitis viral glycoprotein.

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(Görlich et al., 1992a; Görlich and Rapoport, 1993; Fons et al., 2003; Snapp et al., 2004). Features of the signal sequence appear to play important roles in determining the requirement for these accessory proteins (Voigt et al., 1996; Fons et al., 2003). A small protein, RAMP4, is also tightly associated with the active ribosome–translocon complex (Görlich et al., 1992a) and has been implicated in stabilizing newly synthesized membrane proteins regulating N-linked glycosylation and is suggested to be involved in the ER stress response (Schröder et al., 1999; Yamaguchi et al., 1999; Lee et al., 2003). However, its precise molecular function is poorly understood.

Cryo-EM reconstructions of the ribosome–Sec61p complex have implicated components of the ribosome located around the polypeptide exit site on the 60S subunit, which interact with Sec61p. These include ribosomal proteins Rpl23a, Rpl35, Rpl19, and Rpl26 together with elements of the 28S ribosomal RNA (rRNA; Beckmann et al., 2001; Menetret et al., 2005).

A more active role of the ribosome has been implicated by studies of membrane protein integration (Liao et al., 1997; Haigh & Johnson, 2002). The ribosome–translocon complex is able to respond to a TM segment while it is still deep inside the ribosomal exit tunnel, an ∼100-Å-long aqueous channel, which conveys the nascent chain from the peptidyl transferase center (PTC) to the exit site (Liao et al., 1997; Nissen et al., 2000). Using fluorescent probes incorporated into the nascent chain, translocon rearrangements have been detected in response to the presence of a TM segment in the nascent chain (Liao et al., 1997). Once the TM segment reaches a specific point inside the exit tunnel, the lumenal side of the translocon appears to become sealed, which is most likely caused by the binding of BiP (Hamman et al., 1998; Haigh and Johnson, 2002). Further movement of the TM segment along the exit channel leads to alterations at the ribosome–translocon junction on the cytosolic side of the membrane (Liao et al., 1997). These changes are suggested to prime the translocon for the imminent arrival of the TM segment and permit its subsequent lateral exit into the lipid bilayer (Liao et al., 1997).

Ribosomal proteins Rpl17, Rpl4, and Rpl39 line the ribosomal exit channel (Nissen et al., 2000). Rpl17 and Rpl4 form a restriction of the channel, which has been suggested to interact with the nascent chain (Nissen et al., 2000). Recent studies have shown that the rearrangements of the translocon are triggered precisely when the TM segment is adjacent to ribosomal protein Rpl17 (Liao et al., 1997; Woolhead et al., 2004). Moreover, cross-linking data indicate a much stronger interaction between a TM segment and Rpl17 as compared with a hydrophilic region of nascent chain (Woolhead et al., 2004), suggesting a specific molecular recognition occurring between Rpl17 and the TM segment. Furthermore, the bacterial homologue of Rpl17, L22, has also been shown to recognize the SecM nascent chain while it is still inside the ribosome, causing translational pausing (Nakatogawa and Ito, 2002).

Rpl17 is composed of a compact globular domain and an extended β-hairpin, which extends deep inside the ribosome (Unge et al., 1998; Nissen et al., 2000). A restriction in the exit channel is formed by the tip of the β-hairpin together with regions of Rpl4 (Nissen et al., 2000). Mutations of L22, which lead to defects in recognition of the SecM nascent chain, map to the tip of the β-hairpin (Nakatogawa and Ito, 2002).

The globular region of the protein is exposed on the surface of the ribosome close to the exit site, where it could potentially interact with the translocon (Beckmann et al., 2001). This study addresses this possibility by using cross-linking to probe the molecular environment of Rpl17 at the ribosome surface. The results indicate that Rpl17 is in proximity to the Sec61β component of the translocon. Strikingly, the presence of a TM segment inside the exit tunnel alters the environment of Rpl17 at the surface such that it now also contacts the small translocon-associated protein RAMP4.

Results

Rpl17 is protected from protease by membrane components

To investigate the relationship between Rpl17 and the ER membrane, the sensitivity of Rpl17 in free and membrane-bound ribosomes to limiting concentrations of V8 protease was compared. Immunoblotting using an antibody to the extreme N terminus of Rpl17 revealed that treatment of free ribosomes with V8 protease led to the removal of an ∼2-kD fragment from the C terminus. This clipping was detectable with 2.5 μg/ml protease and was quantitative at a concentration of 25 μg/ml. The remaining fragment of Rpl17 appears to be relatively protease resistant, as even at a concentration of 1 mg/ml, no further degradation was observed. In contrast, in the membrane-bound ribosomes, removal of the C-terminal fragment was only observed at much higher protease concentrations (Fig. 1 A); no degradation was seen with 2.5 μg/ml protease, and even with 1 mg/ml protease, only partial degradation was observed. This indicates that the presence of the membrane alters the sensitivity of Rpl17 to protease either by sterically blocking access of the protease or altering the conformation of Rpl17.

A trivial explanation of these results could be that the presence of the membrane reduces or inactivates V8 protease. As a control, the protease sensitivity of Rpl18 and Rpl23a in free and membrane-bound ribosomes was compared (Fig. 1 A). Rpl18, which is located close to the central protuberance on the ribosome, was resistant to 1 mg/ml protease in both free and membrane-associated ribosomes. This reflects the fact that Rpl18 is tightly folded with only limited exposure to the surface of the ribosome.

Rpl23a, which is located close to the exit site, was equally sensitive to protease in both ribosome fractions and was completely cleaved with 25 μg/ml protease. These controls indicate that the altered sensitivity of Rpl17 to protease is not an indirect effect of the membrane on the activity of the protease.

Next, the question as to whether the protease protection of Rpl17 was caused by the presence of the membrane bilayer by itself or caused by membrane proteins intimately associated with the ribosome was addressed. Therefore, protease sensitivity of Rpl17 in the membrane-bound ribosomes in the presence and absence of the detergent digitonin, which solubilizes the membrane but preserves the interaction between the ribosome, the translocon, and other translocon-associated proteins, was compared

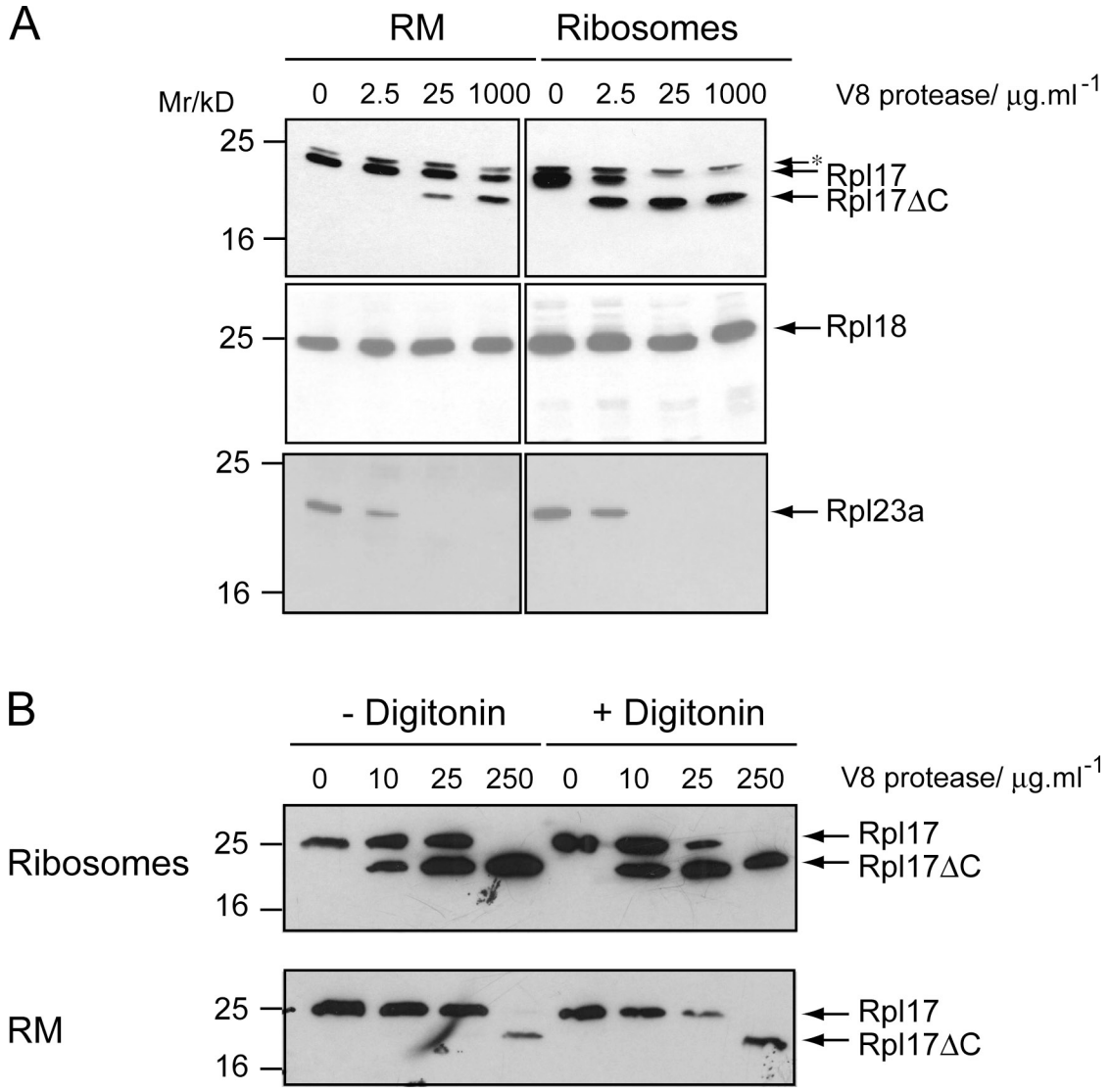

Figure 1. **Membrane components protect Rpl17 from proteolytic digestion.** (A) Free ribosomes and RMs were treated with increasing concentrations of V8 protease and analyzed by SDS-PAGE and immunoblotting with Rpl17$^{N\text{-}trm}$, Rpl18, and Rpl23a antibodies. The position of a stable Rpl17 degradation product, which lacks the C terminus (Rpl17ΔC), is indicated. The antibody weakly cross reacts with another ribosomal protein (*). (B) RMs were solubilized with 2% digitonin, the membranes were subjected to limited digestion with V8 protease, and analyzed as described in A. A mock-treated sample (−digitonin) was treated in an identical manner but with no digitonin present.

(Görlich et al., 1992b). Even in the presence of digitonin, Rpl17 in the membrane-associated fraction was considerably more resistant to V8 cleavage than in the free ribosome fraction (Fig. 1 B). This suggests that it is the membrane proteins associated with the ribosome that are responsible for the protease protection.

There are subtle differences between the proteolysis profiles of Rpl17 in the absence of detergent between the experiments in Fig. 1 (A and B). This is a result of differences in the ionic strength between the low salt buffer used in Fig. 1A and solubilization buffer used in Fig. 1 B (unpublished data).

Rpl17 can be cross-linked to ER membrane proteins

To further investigate the proteinaceous environment of Rpl17 when associated with the membrane, a cross-linking approach was used. Membrane-bound ribosomes were treated with three different bifunctional chemical cross-linkers: maleimidobenzoyl-*N*-hydroxy succinimide (MBS), disuccinimidyl glutarate (DSG), and DFDNB (1,5-difluoro-2,4-dinitrobenzene) with spacer arms of 9.9 Å, 7 Å, and 3 Å, respectively.

Cross-link products were visualized by SDS-PAGE followed by immunoblotting for Rpl17 (Fig. 2 A). A cross-link product of ~30 kD was generated with all three reagents, although the efficiency was considerably lower with DFDNB. An additional product of ~32 kD was seen with MBS and DSG, and finally an ~37-kD cross-link species was seen only with DSG. The latter most likely corresponds to a cross-link between Rpl17 and another ribosomal protein, as it was also observed when cross-linking was performed using free ribosomes (unpublished data).

To ascertain whether the 30- and 32-kD cross-link species, which correspond to cross-link adducts of 10 and 12 kD, are components of the membrane, rough microsomes (RMs) were first

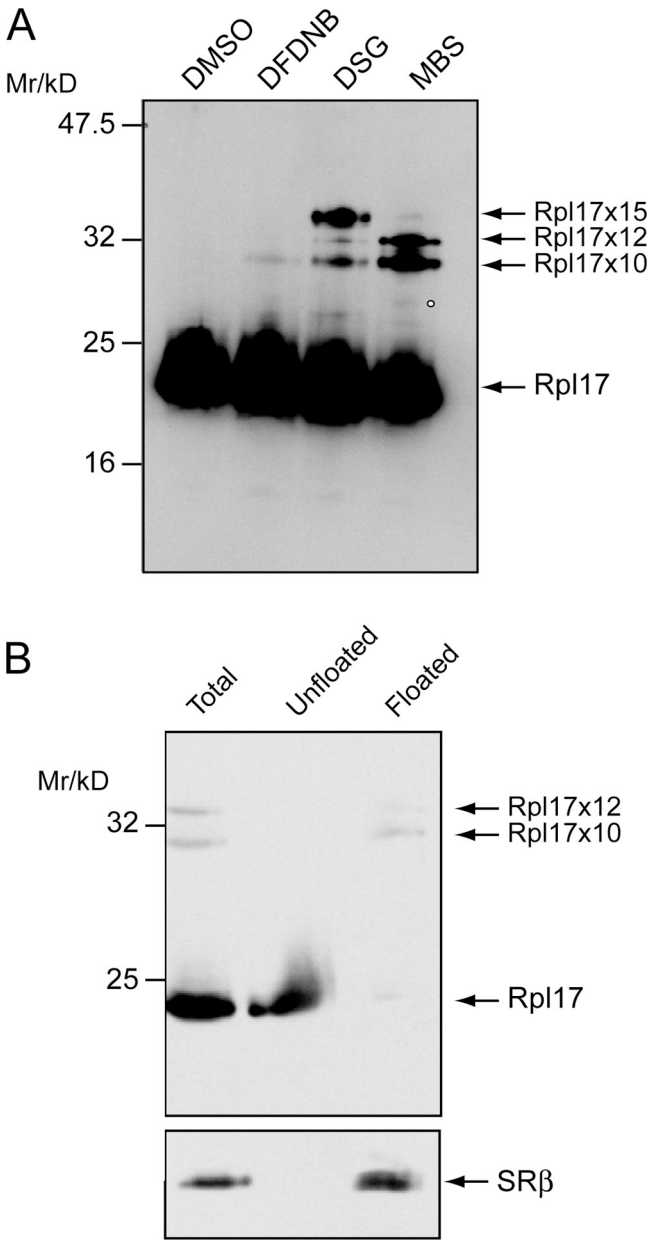

Figure 2. **Cross-linking reveals that Rpl17 is adjacent to membrane components.** (A) RMs were treated with either DMSO or the cross-linkers DFDNB (25 μM), DSG (200 μM), and MBS (200 μM). Reactions were analyzed by SDS-PAGE and immunoblotting with Rpl17$^{C\text{-}trm}$ antibodies. Major cross-links to adducts of the approximate indicated sizes are labeled. An additional, weak 7-kD cross-link adduct (o) was also reproducibly observed with MBS. (B) RMs were treated with 200 μM MBS to induce cross-linking. The microsomes were treated with 1 M LiCl to extract ribosomal proteins from the rRNA and separated from the ribosomal remnants by floatation through a Nycodenz gradient. The floated and nonfloated material were recovered by TCA precipitation and analyzed by SDS-PAGE and immunoblotting with Rpl17$^{C\text{-}trm}$ antibodies. To control for complete floatation of the microsomes, the blot was also probed with antibodies against SRβ, an integral ER membrane protein.

treated with MBS and followed by LiCl, which extracts most ribosomal proteins from the rRNA (Reboud et al., 1980). After extraction, the membranes were floated through a Nycodenz cushion to separate membrane-bound proteins from ribosomal fragments. As expected, the uncross-linked Rpl17 was present exclusively in the nonfloated fraction. In contrast, the 10- and 12-kD cross-link adducts were present exclusively in the floated fractions (Fig. 2 B). Blotting for the integral membrane protein SRβ confirmed the efficient floatation of the membranes. This indicated that the 10- and 12-kD cross-links were to membrane components and not other ribosomal proteins.

Rpl17 is in proximity to Sec61β in the ribosome-translocon complex

To identify the 10- and 12-kD cross-link partners, scaled up cross-link reactions were solubilized with Triton X-100 under high salt (HS) conditions to release membrane-associated proteins from the ribosomes. Ribosomes were reisolated by centrifugation together with any membrane proteins cross-linked to the ribosomal proteins. The ribosomal proteins were then extracted from the rRNA with LiCl, and the extract was then used for immunoprecipitation with Rpl17 antisera. Analysis with SDS-PAGE and staining with Coomassie revealed a discrete band of ∼23 kD present in the lanes corresponding to immunoprecipitation from membrane extracts but not from a mock extract (Fig. 3 A). Analysis of this band by mass spectrometry after tryptic digestion identified six peptides corresponding to Rpl17, confirming its identity (QCVPFR, QWGWTGGR, SAEFLLHMLK, GLDVDSLVIEHIQVNK, INPYMSSPCHIEMILTEK, and PEEEVAQK). Two weakly staining bands of 30 and 32 kD were only present in the cross-linked extract, which is consistent with the immunoblot results (Fig. 2 A). Mass spectrometry of the lower of these two bands revealed several peptides from Rpl17 and two peptides from a subunit of the translocon Sec61β (PGPTPSGTNVGSSGR and FYTEDSPGLK). This indicates that the 30-kD band corresponds to a cross-link between Rpl17 and Sec61β and is in reasonable agreement with the sizes of the two individual proteins, 20 kD and 12 kD, respectively.

To confirm this result, Triton X-100/LiCl extracts from cross-linked membrane-bound ribosomes were immunoprecipitated with Rpl17 and Sec61β antisera and immunoblotted with Rpl17 antibodies (Fig. 3 B). The 30-kD cross-link product could indeed be immunoprecipitated by both Rpl17 and Sec61β antisera. This confirmed that the lower cross-link species is Sec61β. Moreover, the slightly larger 32-kD cross-link product was also immunoprecipitated by Sec61β antibodies and must therefore also contain Rpl17 and Sec61β. Most likely, this corresponds to cross-linking between different residues of the two proteins resulting in forms, which migrate differently in SDS-PAGE.

When cross-linking reactions were probed with Sec61β antibodies, as expected, two strong cross-links of 30 and 32 kD were present, and these could both be immunoprecipitated with Rpl17 antibodies (Fig. 3 B). In addition to the two cross-links to Rpl17, treatment with MBS leads to the formation of a strong cross-link adduct of 9 kD (Fig. 3 B), which corresponds to an unidentified 60S ribosomal protein (unpublished data).

Treatment of the cross-link reactions with V8 protease, which does not cleave Sec61β (Fig. S1), indicates a digestion pattern of the Rpl17xSec61β cross-links consistent with cross-linking of Sec61β to the N- and C-terminal proteolytic fragments of Rpl17 characterized in Fig. 1. This again rationalizes the presence of two distinct cross-link species.

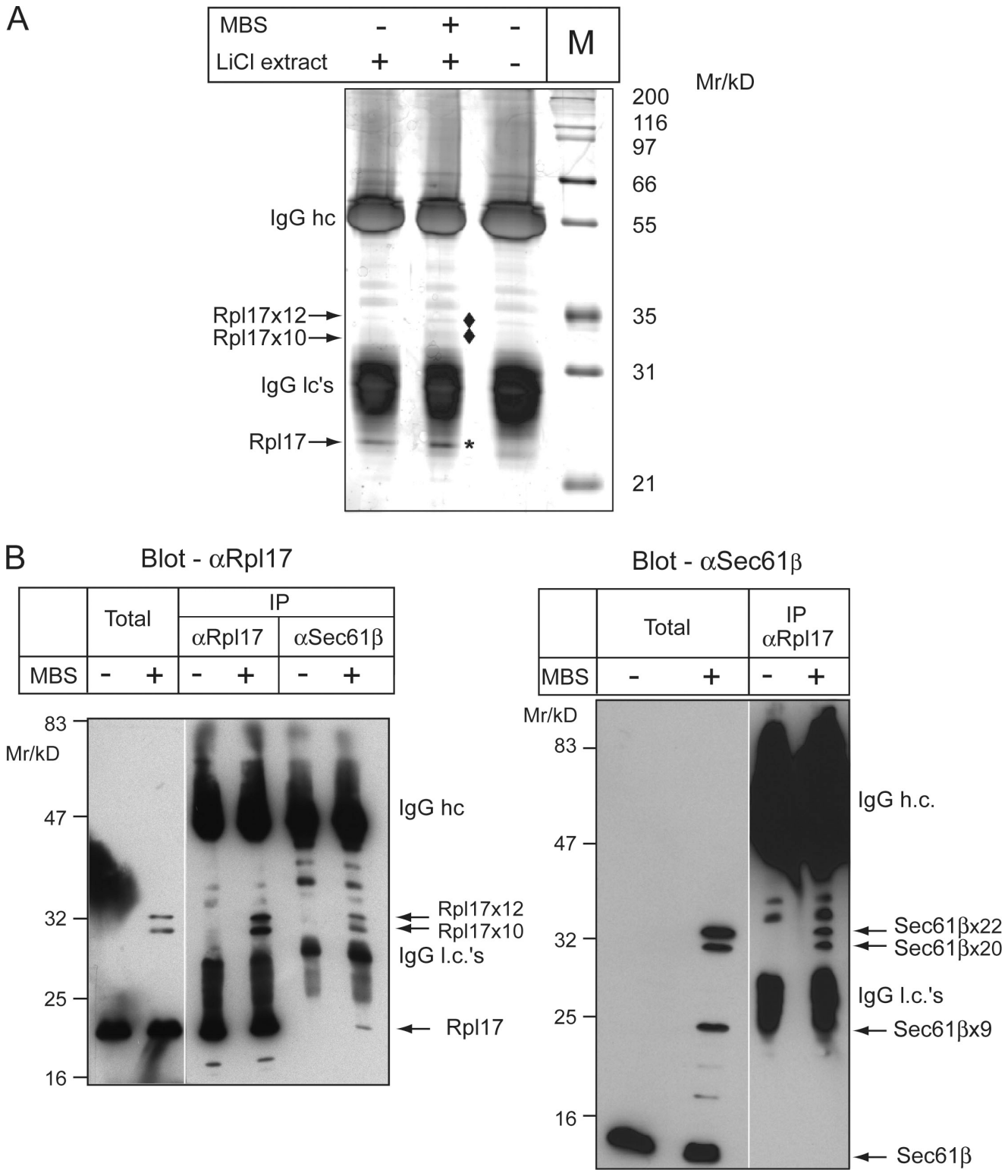

Figure 3. **Rpl17 can be cross-linked to Sec61β.** (A) RMs (900 eq) were cross-linked where indicated with 200 μM MBS. After solubilization with digitonin, ribosomes were reisolated, ribosomal proteins were extracted with LiCl, and the extract was immunoprecipitated with anti-Rpl17$^{C\text{-}trm}$ antibodies. Bound protein was eluted with SDS and analyzed by SDS-PAGE and staining with Coomassie brilliant blue. A mock immunoprecipitation performed in the absence of a microsomal extract was also performed to identify bands arising from the antiserum. The prominent band at 21 kD (*) was excised along with the 30-kD band that was exclusive to the LiCl extract from MBS-treated RM (◆) and analyzed by mass spectrometry after in-gel tryptic digestion. M, molecular weight marker. (B) Denaturing immunoprecipitation (IP) of cross-linking reactions (100 eq of RM) was performed as above either using anti-Rpl17$^{C\text{-}trm}$ or anti-Sec61β antiserum. The samples were analyzed by SDS-PAGE and immunoblotting using Rpl17$^{C\text{-}trm}$ antiserum (left) or Sec61β antiserum (right). Position of IgG heavy chain (hc) and light chains (lc) are indicated. White lines indicate that intervening lanes have been spliced out.

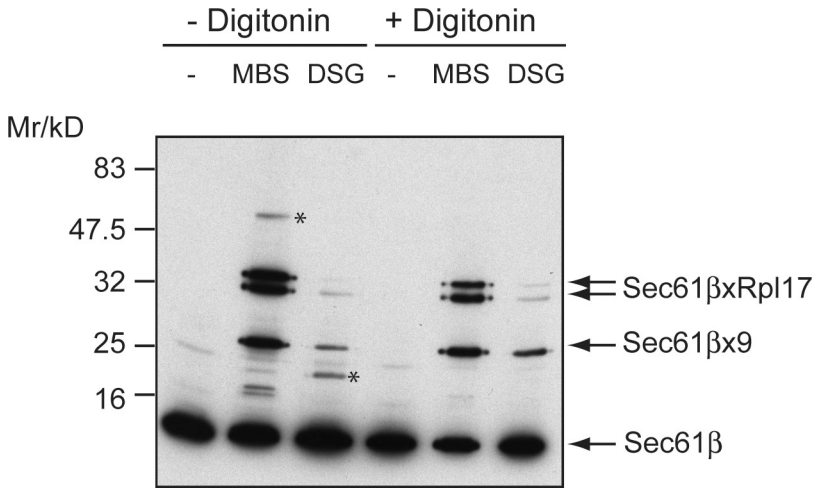

Figure 4. **Sec61β and Rpl17 remain in proximity after membrane solubilization.** RMs were resuspended in solubilization buffer and where indicated solubilized with 2% digitonin. Cross-linking was induced with either MBS or DSG. The reactions were analyzed by SDS-PAGE and immunoblotting with Sec61β antisera. Two major cross-link species, indicated by asterisks, were only present in intact membranes and were lost after detergent treatment.

Collectively, these data indicate that Rpl17 is located in proximity to Sec61β when the ribosome is bound to the membrane. The fact that this cross-link adduct is also formed albeit weakly with DFDNB, which has a spacer arm of 3Å, indicates that these proteins are indeed very close and may even interact directly.

Rpl17 is still protected from protease in the presence of digitonin. Therefore, cross-link formation between Sec61β and Rpl17 after solubilization of microsomes with digitonin was assessed. Microsomes were either mock treated or solubilized by digitonin, and then cross-linking was induced with MBS or DSG. Samples were then analyzed by SDS-PAGE and immunoblotting for Sec61β (Fig. 4). The major Sec61β cross-link products observed with MBS were all still present after digitonin treatment, including the 30- and 32-kD cross-links to Rpl17, which is consistent with the protease protection data. Interestingly, an ∼47-kD MBS cross-link product and an ∼16-kD DSG cross-link product were both specifically lost after digitonin treatment, indicating that membrane solubilization perturbs the positioning of Sec61β with respect to these partners.

The presence of a TM segment in the ribosomal exit tunnel triggers changes in Rpl17 cross-linking

RMs represent a snapshot of ribosomes translocating a whole spectrum of secretory and membrane proteins and therefore reflect a wide range of different functional states. To look at more defined states of the ribosome, translocation or integration intermediates were generated with specific nascent chains of specific lengths.

As Rpl17 has been strongly implicated in the recognition of TM segments while they are still deep inside the exit tunnel of the ribosome (Liao et al., 1997; Woolhead et al., 2004), it was decided to look at specific integration intermediates. Specifically, to assess whether the environment of Rpl17 at the ribosome–translocon interface changes in response to the presence of a TM segment in the nascent chain inside the ribosome. A construct analogous to that used in previous studies (Liao et al., 1997; Woolhead et al., 2004), comprising the N terminus of preprolactin (pPL), including the signal sequence, TM segment, and C-terminal cytosolic domain of the vesicular stomatitis viral glycoprotein (VSV-G) termed pPL–VSV-G fusion (pPV), was used (Fig. 5 A). This permitted the generation of integration intermediates with the VSV-G TM segment located at different distances from the PTC. This places identical residues adjacent to Rpl17, as in the previous studies, allowing a direct comparison (Liao et al., 1997; Woolhead et al., 2004).

It has been shown previously that in integration intermediates where the VSV-G TM segment is located in the nascent chain, less than four residues from the PTC, no interaction between the TM segment and Rpl17 can be detected by cross-linking (Woolhead et al., 2004). Furthermore, the ribosome–translocon junction exists in a closed conformation, as detected by fluorescent-quenching experiments, and the lumenal face of translocon is open (Liao et al., 1997). However, once the TM segment is nine residues from the PTC, cross-linking between Rpl17 and the TM segment is now observed (Woolhead et al., 2004), correlating with sealing of the lumenal face of the translocon (Liao et al., 1997). Extension of the chain by a further two residues is sufficient to open the seal on the cytosolic face of the translocon. Therefore, pPV nascent chains were generated with a length of 87 residues, which positions the TM segment 4 residues from the PTC where it cannot yet interact with Rpl17, and of 92 and 94 residues, which positions the TM segment 9 and 11 residues, respectively, from the PTC, such that it can interact with Rpl17 and trigger changes in the translocon.

The integration intermediates were produced by translating the corresponding truncated mRNAs in rabbit reticulocyte lysate in the presence of purified SRP and EDTA HS-stripped RMs (EKRM), which lack endogenous ribosomes. Translation of these different constructs in the presence of microsomes gave products of the expected size (Fig. 5 B). Processing of the nascent chain by signal peptidase was strongly dependent on puromycin treatment. This indicates that the nascent chains are still bound to the ribosome unless treated with puromycin. The nascent chains are too short to access signal peptidase in the absence of puromycin. As expected, a construct with a stop codon present was cleaved even in the absence of puromycin. To look solely at the behavior of Rpl17 in programmed ribosomes, translocation intermediates were assembled, and the membranes floated through a HS Nycodenz step gradient to remove nonprogrammed

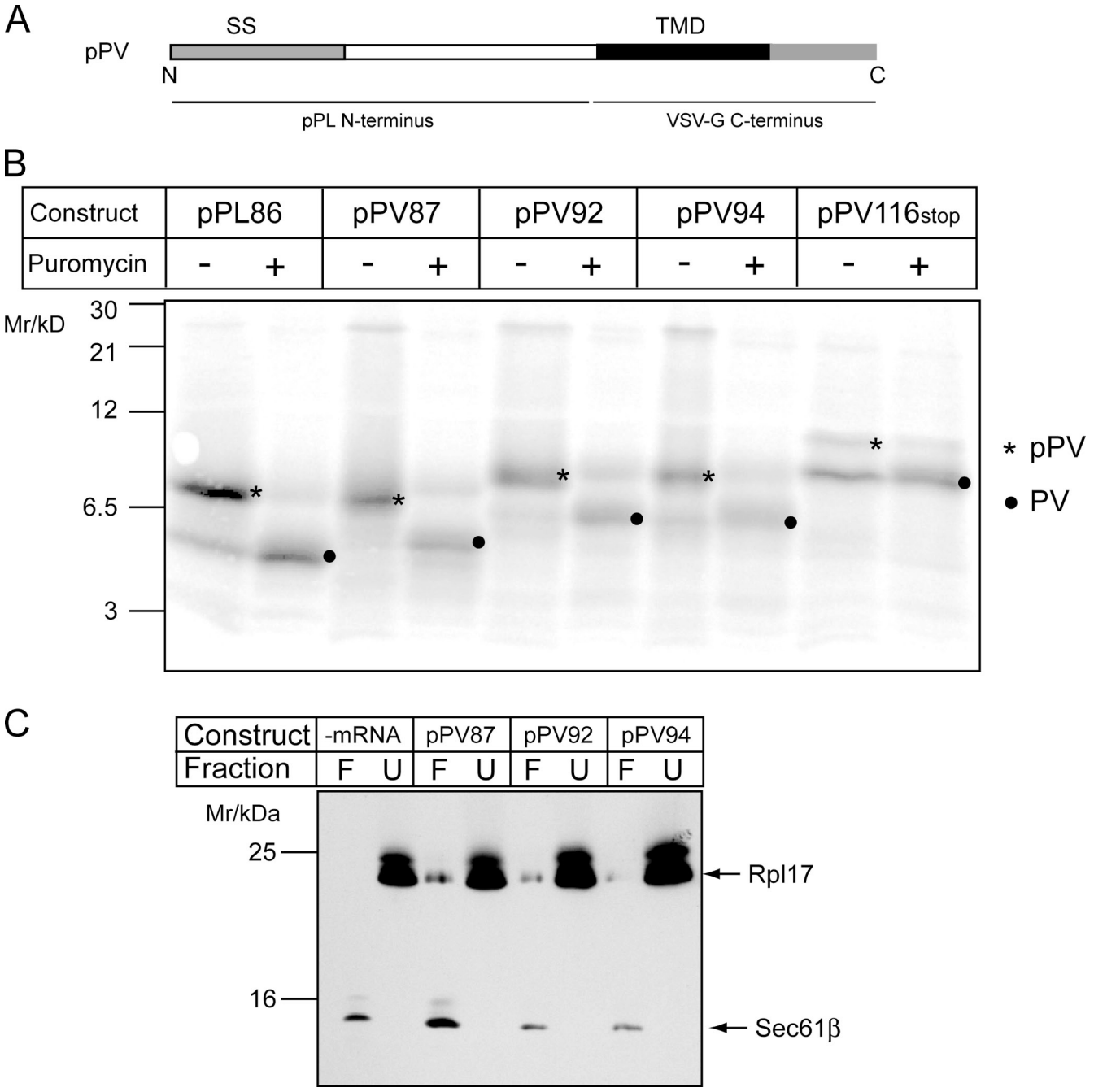

Figure 5. **Generation and purification of pPV integration intermediates.** (A) Schematic representation of pPV fusion protein, consisting of the N terminus of pPL, including the signal sequence (SS), fused to the TM domain (TMD) segment and C-terminal cytosolic domain of VSV-G. (B) mRNAs encoding pPL and pPV fusion proteins of defined lengths, as indicated, and lacking a stop codon together with full-length pPV with an intact stop codon (pPV116STOP) were translated in rabbit reticulocyte lysate in the presence of EKRM and purified SRP. Where indicated, the reactions were treated with puromycin to release the nascent chain from the ribosome. The samples were precipitated and analyzed by SDS-PAGE and phosphorimaging. The unprocessed pPL/pPV (*) and signal sequence–processed PL/PV (•) forms are indicated. (C) Where indicated, insertion reactions were programmed with pPV mRNAs of the indicated length lacking a stop codon. The resulting translocation intermediates were stabilized with cycloheximide and adjusted to 500 mM KOAc. The membranes were reisolated by floatation through an HS Nycodenz gradient, and the floated (F) and unfloated (U) fractions were collected. Fractions were analyzed by SDS-PAGE and immunoblotting with $Rpl17^{N\text{-}trm}$ and Sec61β antisera.

ribosomes. As shown in Fig. 5 C, in the absence of mRNA, no Rpl17 was present in the floated fraction. In contrast, when pPV mRNAs were present, a small but detectable amount of Rpl17 was detected in the floated fraction.

These reactions were scaled up fivefold so that the floated material could be treated with MBS and Rpl17 cross-link products detected (Fig. 6 A). When cross-linking was performed with pPV87, a strong cross-link doublet was observed around 30 kD, corresponding to the Rpl17xSec61β adduct. Interestingly, the lower cross-link product was much stronger than the upper product as compared with RMs. An additional but much weaker cross-link product was also observed. With longer

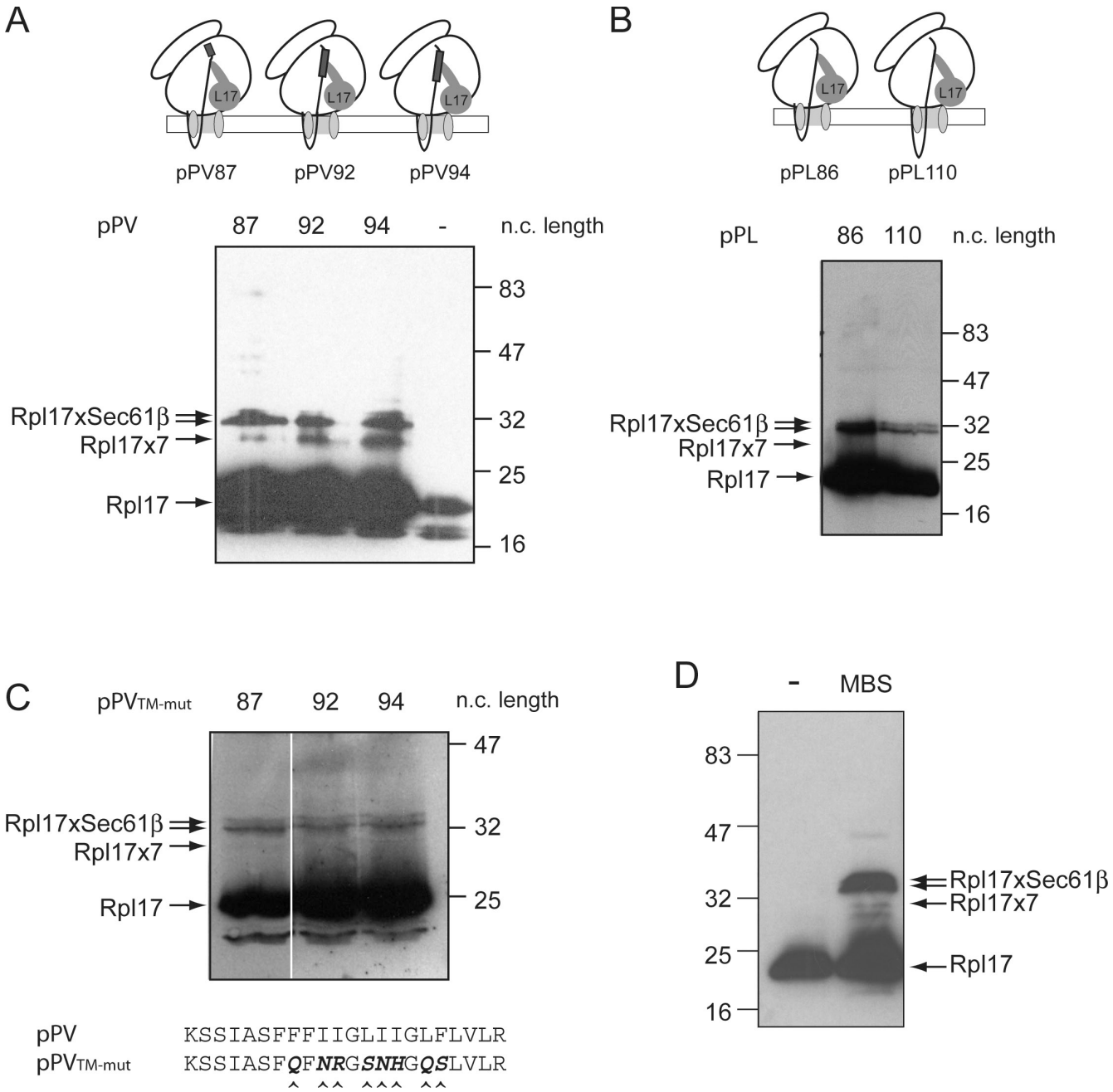

Figure 6. **Cross-linking of Rpl17 in pPV integration intermediates.** (A) Insertion reactions were performed using pPV mRNAs of defined nascent chain (nc) lengths. As a control, a mock insertion reaction lacking exogenous mRNA was also performed (−). The resulting integration intermediates were purified by floatation through an HS gradient (as described in Fig. 5 C) and treated with 200 µM MBS before analysis by SDS-PAGE and immunoblotting for Rpl17$^{C\text{-}trm}$. (B) Translocation intermediates were generated as in A using pPL86mer or 110mer, and these were purified and treated with MBS. (C) Translocation intermediates were generated and treated with MBS as in A using a mutant of pPV in which seven of the hydrophobic residues within the TM segment were mutated to polar residues as indicated (^). (D) RM were treated either with DMSO (−) or 200 µM MBS and analyzed by SDS-PAGE and immunoblotting for Rpl17$^{C\text{-}trm}$. A long exposure of the immunoblot reveals, in addition to the two Sec61β cross-link products, two weaker cross-link products of ∼6 and ∼7 kD. White line indicates that intervening lanes have been spliced out.

constructs, which position the TM segment at locations where it is known to make contact with Rpl17, changes in the cross-link pattern were observed. The cross-link to Sec61β remained unchanged; however, the 7-kD cross-link adduct, which was rather faint in the pPV87 intermediate, now became more prominent, particularly with the pPV92 construct and even more so in the pPV94 intermediate. This suggests that when Rpl17 becomes exposed to the TM segment inside the channel, Rpl17 remains close to Sec61β; however, the protein is now also in proximity to an additional 7-kD protein.

It is possible that the change in the cross-link profile is not caused by the presence of the TM segment but rather an increase in the distance between the signal sequence and the PTC. Therefore, purified translocation intermediates with pPL nascent chains of 86 and 110 residues were generated and the cross-link analysis repeated. As expected, the pattern for pPL86

closely resembled that of pPV87. However, in contrast to the longer pPV constructs, the pPL110 intermediate led to a cross-link pattern identical to that obtained with pPL86 (Fig. 6 B). Thus, the fact that extending the pPL nascent chain to 110 causes no change in the cross-link profile indicates that it is properties of the nascent chain not merely the chain length that triggers the changes.

As a further control, intermediates of 87, 92, and 94 residues were generated using a mutant of pPV where seven of the hydrophobic residues within the TM segment had been changed to polar or charged residues (TM-mut; Fig. 6 C), leading to a predicted change in ΔG_{app} for Sec61-mediated TM segment insertion from −2.0 to +9.8 kcal/mol^{-1} (Hessa et al., 2007). In contrast to pPV, $pPV_{TM\text{-}mut}$ intermediates produced identical cross-linking patterns at all three chain lengths, and no enhanced cross-link to a 7-kD component was observed (Fig. 6 C). Thus, it is clearly the properties of the nascent chain and specifically features of the TM segment that triggers the change in cross-linking rather than changes in chain length.

The initial cross-linking experiments performed with RMs were revisited to see whether a 7-kD cross-link adduct was visible. On longer exposures, two weaker Rpl17 cross-link products could indeed be discerned (Fig. 2 A and Fig. 6 D), the upper one of which comigrates with the 7-kD cross-link.

The presence of a TM segment in the ribosomal exit tunnel correlates with cross-linking of RAMP4

The next step was to establish the identity of the 7-kD cross-link partner. The cross-link is unlikely to be the nascent chain itself, as experiments using radiolabeled nascent chains failed to detect cross-links to Rpl17 with MBS (unpublished data). RAMP4 is a small single-spanning membrane protein known to be tightly associated with the translocon (Görlich et al., 1992b; Schröder et al., 1999). To test whether the 7-kD protein could be RAMP4, MBS cross-linking was performed with either RMs or RMs treated with EDTA to dismantle ribosomes and blotted for Rpl17, Sec61β, and RAMP4. As expected, strong cross-links were observed between Rpl17 and Sec61β, which were lost upon EDTA treatment (Fig. 7 A). RAMP4 gave a rather complex cross-linking pattern; however, several bands were absent in the EDTA-treated microsomes. One of these adducts comigrated precisely with a 7-kD Rpl17 cross-link adduct, which is consistent with cross-linking between RAMP4 and Rpl17.

To confirm that this cross-link was indeed between RAMP4 and Rpl17, RMs were treated with MBS, and denaturing immunoprecipitation was performed with Rpl17 antibodies and the eluted material probed with RAMP4 antibodies (Fig. 7 B). A band corresponding in size to the 27-kD Rpl17 cross-link product was detectable above the background smear of antibody-specific bands in the immunoprecipitation eluate only in the reaction treated with MBS. When the immunoprecipitation was performed in reverse (immunoprecipitation with RAMP4 and blot with Rpl17 antibodies), the 7-kD cross-link to Rpl17p could be immunoprecipitated but not the larger Sec61β cross-links.

To verify that the 7-kD cross-link observed in the integration intermediates contains RAMP4, cross-linking of pPV integration intermediates was performed with MBS and blotted for RAMP4 (Fig. 7 C). Most of the RAMP4 cross-links remained unchanged between the different integration intermediates. However, the 27-kD cross-linking product showed a pronounced increase in intensity in the pPV92 and pPV94 intermediates as compared with pPV87, which is in good agreement with the behavior of the 7-kD Rpl17 cross-link adduct (Fig. 6 A). Collectively, these data indicate that the 7-kD Rpl17 cross-link adduct is RAMP4.

RAMP4 is recruited to the ribosome–Sec61 complex

The increase in RAMP4 cross-link product could reflect a recruitment of RAMP4 to the translocon or repositioning of Rpl17 relative to RAMP4 that is already bound to the translocon. If the former scenario is this case, a pool of RAMP4 that is not associated with translocon must exist. To test whether this is the case, RMs were solubilized with digitonin, and the ribosomes pelleted. The degree of association of Sec61β and RAMP4 with ribosomes was then compared (Fig. 8 A). Around 80% of the Sec61β protein pelleted with ribosomes as has been reported previously (Görlich and Rapoport, 1993). In contrast, only around 10–20% of RAMP4 was in the ribosome pellet. This indicates that only a small fraction of RAMP4 is tightly associated with the translocon and that the rest is either not translocon associated or only weekly associated. This is in agreement with the previous observation that the amount of RAMP4 in the ribosome-associated membrane protein (RAMP) fraction is clearly much lower as compared with Sec61β and Sec61γ (Görlich and Rapoport, 1993).

To assess whether the cross-link between RAMP4 and Rpl17 corresponds to the pool of RAMP4, which is tightly associated with the translocon, cross-link formation was monitored before and after solubilization with digitonin (Fig. 8 B). As observed previously (Fig. 4), the Sec61β-Rpl17 cross-link was resistant to detergent treatment, and likewise, the 7-kD Rpl17-RAMP4 cross-link adduct was also insensitive to membrane solubilization. Thus, the cross-link between Rpl17 and RAMP4 corresponds to the small pool of RAMP4, which is tightly associated with the translocon.

To test more directly whether the presence of a TM segment adjacent to RPL17 leads to recruitment of RAMP4 to the translocon, we prepared a RAMP fraction from targeting intermediates generated with pPV87 and pPV94, which produced differential cross-linking between Rpl17 and RAMP4. Preliminary experiments indicated that ∼5% of the Sec61 complexes from the input PKRM (puromycin HS-stripped RM) formed productive ribosome-associated integration intermediates (Fig. 8 C). When the relative amount of RAMP4 and Sec61β in the RAMP fraction was compared (Fig. 8 C), it was evident that RAMP4 was enriched in the pPV94 intermediate in contrast to pPV87, which is similar to that observed with RM.

Discussion

Determination of the structure of the large ribosomal subunit revealed that ribosomal proteins L22/Rpl17 and L4 form a narrow restriction in the exit tunnel, which conveys the nascent

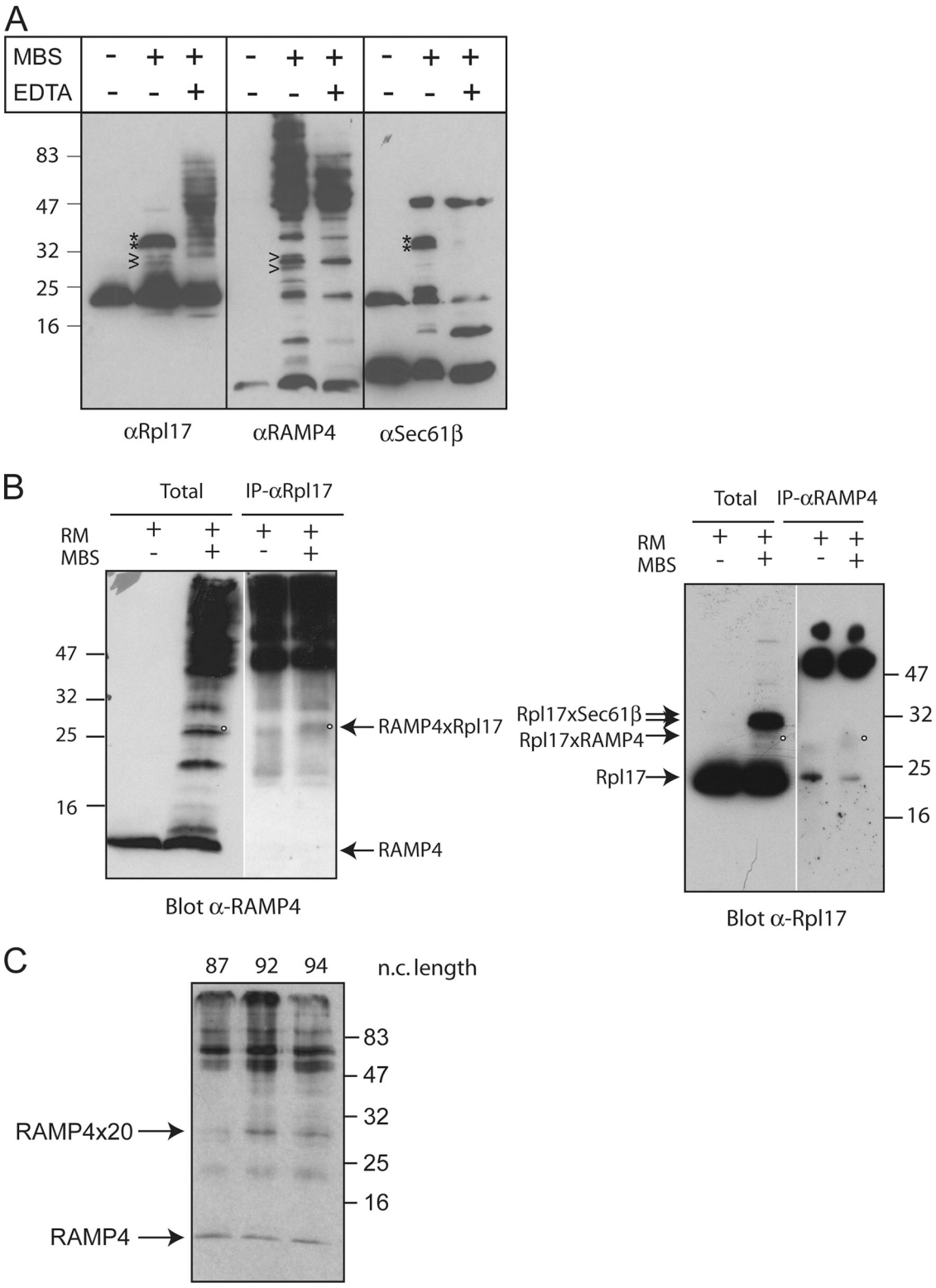

Figure 7. **The presence of a TM segment leads to differential cross-linking between Rpl17 and RAMP4.** (A) RMs were either mock treated or treated with 25 mM EDTA before cross-linking with 200 μM MBS. The reactions were separated on the same SDS-PAGE gel and immunoblotted for Rpl17$^{C\text{-}trm}$, RAMP4, and Sec61β. Positions of the Rpl17-Sec61β cross-links are indicated (*) together with the 7-kD Rpl17 cross-link, which comigrates with a 20-kD RAMP4 cross-link (>). (B) Denaturing immunoprecipitation (IP) of cross-linking reactions (100 eq of RM) was performed using either anti-Rpl17$^{C\text{-}trm}$ or anti-RAMP4 antiserum. The samples were analyzed by SDS-PAGE and immunoblotting using either anti-RAMP4 or anti-Rpl17$^{C\text{-}trm}$ antibodies. Total and immunoprecipitation fractions were lanes from the same gel with exposure times of 30 s and 3 min, respectively. The position of 27-kD Rpl17-RAMP4 cross-link product is indicated (o). (C) pPV translocation intermediates of defined nascent chain (nc) lengths (residues) were generated, purified, and treated with MBS as described in Fig. 6 A.The reactions were analyzed by SDS-PAGE and immunoblotting with RAMP4 antisera. The 27-kD Rpl17-RAMP4 cross-link product is again indicated and shows a strong dependence on the length of the pPV nascent chain. Values on blots are shown in kilodaltons. White lines indicate that intervening lanes have been spliced out.

chain from the PTC to the exit site (Ban et al., 2000). During membrane protein integration, it has been shown that the presence of a TM segment inside the exit tunnel leads to rearrangement of the translocon as detected by altered accessibility to probes incorporated into the nascent chain (Liao et al., 1997). This rearrangement occurs when the TM segment is adjacent to Rpl17 and correlates with strong cross-linking between the TM segment and Rpl17 (Woolhead et al., 2004). Thus, Rpl17 is strongly implicated in the molecular recognition of the TM segment by the ribosome. Furthermore, in bacterial ribosomes, the Rpl17 homologue L22 is involved in recognition of stall sequences within the SecM nascent chain (Nakatogawa and Ito, 2002).

In this study, it was shown that as well as contacting the nascent chain inside the tunnel, Rpl17 is intimately associated with components of the translocation machinery at the surface of the ribosome. Binding of ribosomes to the membrane leads to protection of Rpl17 from proteolysis; specifically, access of V8 protease to a site close to the C terminus is strongly sensitive to the presence of the membrane. Based upon the x-ray structure of L22 in the *Haloarcula marismortui* 50S subunit and the size of the protected fragment, this site is predicted to be a cluster of acidic residues (^{152}EKE^{154}) located at the base of the β-hairpin of Rpl17, which in the archaeal homologue is solvent exposed (Ban et al., 2000). Protease protection is maintained after solubilization of the membrane with digitonin, which removes lipid but preserves the interaction of the ribosome with the translocon. This indicates that protease protection is largely dependent on RAMPs.

Consistent with this data, cross-linking experiments reveal that Rpl17 is in proximity to Sec61β when ribosomes are bound to the membrane (Woolhead et al., 2004). Cross-links were formed with reagents with a spacer arm of 7–10 Å but could even be detected with a spacer arm as short as 3 Å. Proteolysis of the Rpl17-Sec61β cross-link products yields two fragments containing Sec61β, one adduct corresponding to the N-terminal 21-kD fragment of Rpl17 and a 1-kD adduct, which is derived from the C terminus but is not recognized by the C-terminal Rpl17 antibody. This suggests that the C-terminal 2-kD fragment is cleaved in two, and indeed, there is a cluster of three glutamates from residues 162–164. Thus, the 1-kD adduct most likely corresponds to a fragment generated by cleavage at residues ^{152}EKE^{154} and ^{162}EEE^{164}. The entire C terminus of Rpl17 lacks cysteine residues, and the 1-kD fragment contains two lysine residues (K153 or K159). Thus, as MBS reacts with cysteine and lysine residues, this cross-link would be predicted to be formed between either of the lysines in the 1-kD fragment of Rpl17 and the single cysteine in Sec61β.

The cross-linking data indicates that Sec61β must be very close to Rpl17 and may even make a direct contact. A 15-Å cryo-EM map of the ribosome–translocon complex reveals a contact proposed to be associated with helix 24 of the 28S rRNA, which is directly associated with Rpl17 (Beckmann et al., 2001; Menetret et al., 2005). This contact is also seen in the bacterial ribosome–SecYEG complex (Mitra et al., 2005).

The resolution of the cryo-EM reconstructions is presently too low to assign which if any of the contacts between the Sec61 complex and the ribosome involve Sec61β. Although most of the cytosolic domain of Sec61β is absent from the x-ray structure of the SecYEβ complex (Van den Berg et al., 2004), this region has been shown to bind ribosomes with moderate affinity and can block the association of ribosomes with stripped membranes (Levy et al., 2001). However, it is unlikely that Sec61β forms the primary binding site, as Sec61p complexes

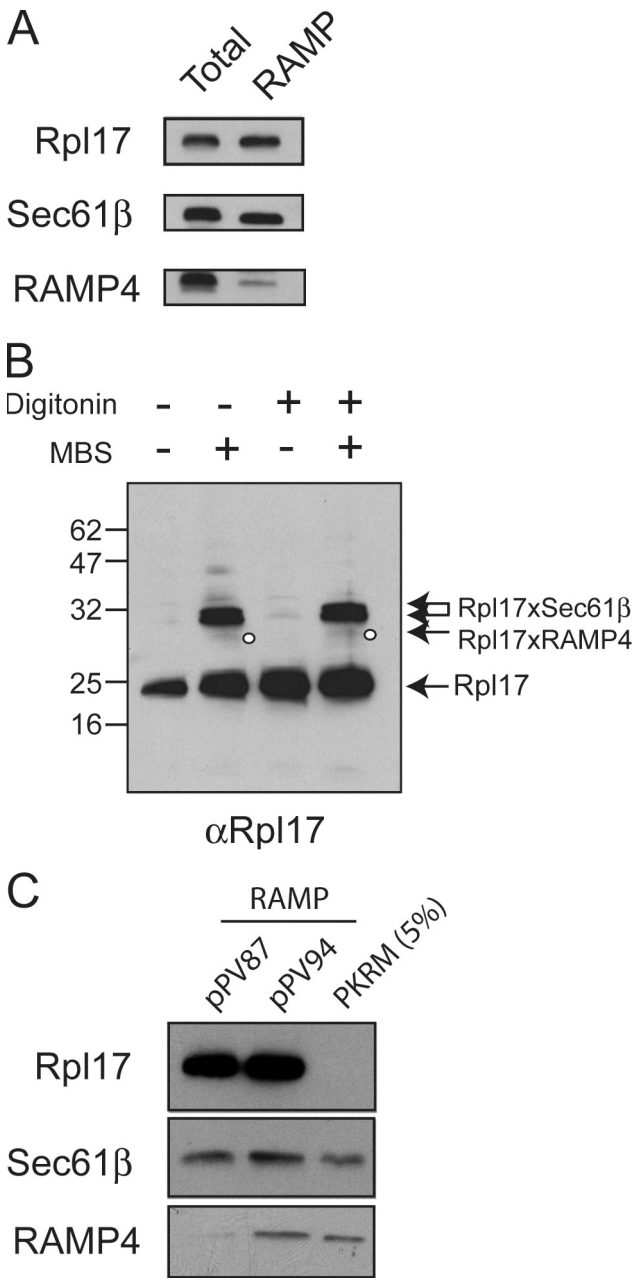

Figure 8. **RAMP4 is recruited to the ribosome–translocon complex.** (A) RMs were solubilized with digitonin and centrifuged through a sucrose cushion to yield a ribosomal pellet that contains RAMPs. Equal amounts of the total and RAMP fraction were analyzed by SDS-PAGE and immunoblotting with Rpl17, Sec61β, and RAMP4 antisera. (B) Cross-linking of microsomes with MBS before and after digitonin treatment was performed as described in Fig. 4. Reactions were analyzed by immunoblotting with Rpl17$^{C\text{-}trm}$ antiserum. Position of the 27-kD Rpl17xRAMP4 cross-link species is indicated (o). Values on blot are shown in kilodaltons. (C) pPV translocation intermediates with chain lengths of 87 and 94 were generated and purified as described in Fig. 6 A, and they were solubilized with digitonin and the RAMP fraction prepared as in A. Fractions were analyzed, alongside 5% of the input PKRM used to generate the translocation intermediates, by immunoblotting with Rpl17, Sec61β, and RAMP4 antisera.

lacking Sec61β still bind ribosomes with high affinity (Kalies et al., 1998). In contrast, both the C-terminal tail and loop 8 of Sec61α have been shown to be important for high affinity ribosome binding (Raden et al., 2000; Cheng et al., 2005).

Sec61β has been implicated in an early step of the translocation reaction involving the SRP receptor, indicating that Sec61β most likely facilitates the transfer of the nascent chain from SRP to the translocon (Kalies et al., 1998; Jiang et al., 2008). Furthermore, evidence suggests that substrates contact Sec61β very early in the translocation process. Cotransin/CAM471, small molecule inhibitors of VCAM-1 translocation, lead to accumulation of the substrate in proximity to Sec61β (Besemer et al., 2005; Garrison et al., 2005). Likewise, during membrane protein integration, TM segments initially locate adjacent to Sec61β and Sec61α before moving to a distinct site distal to Sec61β before lateral exit (Ismail et al., 2006). It is quite possible that an interaction of Sec61β with Rpl17 could function to guide the signal sequence into the translocon after its release from SRP. Consistent with this, Sec61β can be cross-linked from probes predicted to be located extremely close to the exit site (Meacock et al., 2002).

Cross-linking of Rpl17 in integration intermediates revealed a striking change in cross-link pattern when a TM segment lies adjacent to Rpl17 inside the exit tunnel. Cross-linking to Sec61β persisted, but an additional cross-link to a 7-kD protein, RAMP4, was now present. This change correlates precisely with reorganization of the translocon as indicated by previous fluorescent experiments using almost identical nascent chains (Liao et al., 1997). Rpl17 contacts both the signal (TM segment) and the downstream effector, the translocon, and thus could act as a signal relay to transmit the signal resulting from TM segment recognition in the tunnel to the translocation machinery. The bacterial homologue of Rpl17, L22, has been shown to recognize the SecM nascent chain and trigger long-range rearrangements of the 60S subunit, which cause translational pausing (Woolhead et al., 2006). Another bacterial ribosomal protein, L23, has been shown to respond to nascent chains inside the exit tunnel, increasing the affinity of SRP for the ribosome (Bornemann et al., 2008). Thus, there are strong precedents for this mode of signaling. TM recognition by the β-hairpin of Rpl17 may trigger a conformational change of the globular domain adjacent to the translocon-inducing rearrangements either directly or by repositioning of helix 24, which is thought to contact both Rpl17 and the translocon (Ban et al., 2000; Menetret et al., 2005). Consistent with this model, a recent study indicates that helix 24 has considerable conformational flexibility within the ribosome (Petrone et al., 2008).

RAMP4 was originally identified as a small 7-kD protein, which cofractionated with ribosomes after solubilization with digitonin and HS in the so-called RAMP fraction (Görlich and Rapoport, 1993). However, the levels of RAMP4 in the RAMP fraction were clearly lower than that of translocon components Sec61α, Sec61β, and Sec61γ. This suggests that RAMP4 is only associated with a subset of translocons. Consistent with this concept, cross-linking experiments have identified interactions between the nascent chain and RAMP4 in only a subset of translocation intermediates (Schröder et al., 1999). This study now shows that a large pool of RAMP4 is either not associated with the translocon or is only very peripherally associated. This is in good agreement with blue native PAGE analysis of RAMP4 after ribosome dissociation, which revealed only a minor fraction of RAMP4 and Sec61p comigrating as a complex (Wang and Dobberstein, 1999).

The cross-link observed between Rpl17 and RAMP4 correlates with the fraction of RAMP4, which is tightly associated with the translocon. A dramatic increase in the cross-link between Rpl17 and RAMP4 was observed when a TM segment is present in the ribosomal exit tunnel. Thus, the simplest explanation is that under these conditions, recruitment of RAMP4 to the ribosome–translocon complex is induced. This scenario is supported by the enrichment of RAMP4 in the RAMP fraction of pPV94 integration intermediates.

The recruitment of RAMP4 to the translocon before the arrival of the TM segment correlates with translocon remodeling, which has been proposed to prime the switch from a translocation to integration mode. It is unlikely that RAMP4 is essential for membrane protein integration by itself, as animals lacking RAMP4 are viable as are yeast cells lacking *YSY6*, the RAMP4 homologue (Winzeler et al., 1999; Hori et al., 2006). Furthermore, proteoliposomes containing only trace amounts of RAMP4 are functional for membrane protein integration, and additional RAMP4 is not stimulatory (Görlich and Rapoport, 1993).

Animals lacking RAMP4 show induction of the unfolded protein response in tissues with high secretory activity such as the pancreas and pituitary gland (Hori et al., 2006). This suggests that at high levels of secretion, RAMP4 becomes critical for efficient folding of newly synthesized proteins. Likewise, *YSY6*-null strains also show an altered ER stress response (Giaever et al., 2002). Moreover, expression of both RAMP4 and *YSY6* is strongly induced by the unfolded protein–responsive transcription factors XBP1 and Hac1p, respectively. This again suggests that the function of RAMP4 becomes more critical under ER stress conditions. Importantly, it has been shown that under conditions of ER stress, a newly synthesized membrane protein becomes targeted for ubiquitination and degradation, suggesting that the protein is either misfolded or misintegrated. Up-regulation of RAMP4 rescues this effect again, indicating a role in membrane protein biogenesis, which becomes critical under ER stress.

Thus, the finding that RAMP4 is recruited to translocons before the arrival of a TM segment is entirely consistent with a role in facilitating correct integration and/or folding of newly synthesized membrane proteins. Considering the small size of the RAMP4 protein, it is unlikely to function in isolation and is perhaps more likely to act by modulating the translocon itself or by driving recruitment of additional downstream components. In this regard, it is noteworthy that RAMP4 has been reported to interact with the ER chaperone calnexin (Yamaguchi et al., 1999) and has also been implicated in regulating N-linked glycosylation (Schröder et al., 1999).

In conclusion, the data are consistent with a model whereby Rpl17 is positioned in the ribosome such that it contacts the wall of the exit tunnel and the translocon. Rpl17 can sense the presence of a TM segment in the exit tunnel, triggering rearrangements that lead to the recruitment of RAMP4 to the translocon, priming it for subsequent membrane protein integration.

Materials and methods

Antibodies

Peptides MVKYSTDPANPTKSAC$_{CONH2}$, CKKISQKKLKKQKLMARE$_{COOH}$, and CERARGRRASRG-YKN$_{COOH}$ corresponding to the N and C termini of human Rpl17 and Rpl18, respectively, were coupled to keyhole limpet hemocyanin using sulfo-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Thermo Fisher Scientific) and immunized into rabbits using standard techniques (Harlow and Lane, 1988). Antibodies against Rpl23a (Pool et al., 2002), Sec61β (Görlich and Rapoport, 1993), SRβ (Bacher et al., 1999), and RAMP4 (Schröder et al., 1999) have been described previously.

Plasmid construction

A fragment including the TM segment and C-terminal region of VSV-G TM was amplified by PCR from the plasmid pGEM4-VSVG (which comprises the complete VSV-G ORF inserted into pGEM4 under control of the SP6 promoter) using the primer 5′-TCCATGACCTCTCCTCGATGAAAAGCTC-TATTGCCT-3′ together with T7 primer. The resulting 220-bp fragment was digested with BseRI and EcoRI and ligated into pGEM3-PPLMM (Lyko et al., 1995), which had been digested with the same enzymes to yield pGEM-pPV. pGEM-pPV$_{TM\text{-}mut}$ was generated by mutagenizing pGEM-pPV using the Quikchange protocol (Agilent Technologies) in conjunction with the primers 5′-GCCTCTTTTCACTTTAACAGGGGTTCAAACCATGGACAAT-CCTTGGTTCTCCG-3′ and 5′-CGGAGAACCAAGGATTGTCCATGGTTT-GAACCCCTGTTAAAGTGAAAAGAGGC-3′.

Preparation of ribosomes and microsomes

RMs, EKRMs, free ribosomes, and SRP were prepared from dog pancreas as described previously (Walter and Blobel, 1983; Martoglio et al., 1997; Fulga et al., 2001). Membranes were resuspended in RM buffer (25 mM Hepes-KOH, pH 7.6, 25 mM KOAc, 2 mM Mg(OAc)$_2$, 250 mM sucrose, and 2 mM DTT) at a concentration of four equivalents (eqs; Walter and Blobel, 1983) per microliter. Ribosomes were resuspended in RM buffer at a concentration of 190 A_{260} U/ml.

Transcription, translation, and integration reactions

pPV transcription templates were generated from the pGEM-pPV plasmid by PCR using *Pwo* polymerase (Roche) with SP6 primer and suitable 3′ reverse primers. The templates were purified using a Reaction clean-up kit (QIAGEN) and transcribed with SP6 polymerase (New England Biolabs, Inc.) in the presence of Cap analogue (New England Biolabs, Inc.), and the resulting mRNAs were desalted using microspin columns (G-25; GE Healthcare). pPL templates were generated from pGEM3-PPLMM either by digestion with *PvuII* to generate pPL86mer or by PCR to generate pPL110mer.

20-µl integration assays were assembled using 14 µl nuclease-treated rabbit reticulocyte lysate (Promega), capped mRNA, 10 µCi [^{35}S]-L methionine (MP Biomedicals), 20 µM unlabeled amino acids (−methionine), four eq EKRM, and 20 nM SRP. The reactions were incubated for 15 min at 30°C, split in two, and treated with either 1 mM cycloheximide on ice or 2 mM puromycin for 10 min at 30°C. Membranes were then reisolated by centrifugation through a 100-µl 500-mM sucrose cushion in HS buffer (25 mM Hepes-KOH, pH 7.6, 500 mM KOAc, 10 mM Mg(OAc)$_2$, 2 mM DTT, and 250 µM cycloheximide) in the TLA45 rotor (45,000 rpm for 20 min at 4°C; Beckman Coulter). The pellets were resuspended in SDS-PAGE sample buffer and analyzed on 16.5% tricine gels (Schägger and von Jagow, 1987) followed by phosphorimaging using a phosphorimager (FLA-3000; Fujifilm). Images were subsequently processed using Aida software (Raytest).

Purification and cross-linking of integration intermediates

50-µl integration reactions were assembled using cold methionine. Reactions were incubated for 15 min at 30°C and treated with 1 mM cycloheximide and 500 mM KOAc. Membranes were reisolated by centrifugation through a HS cushion (45,000 rpm for 20 min at 4°C; TLA45 rotor). The pellet was resuspended in 60 µl 50% (wt/vol) Nycodenz solution (Nycomed) in HS buffer and layered under 100 µl 45% Nycodenz solution (in HS buffer). Finally, the 45% solution was overlayed with 60 µl 10% Nycodenz solution (in HS buffer), and the tubes were centrifuged in the TLA100 rotor (100,000 rpm for 1 h at 4°C; Beckman Coulter). Floated microsomes were recovered in 100 µl from the 45:10% interface. The unfloated material was also collected in 100 µl. For direct analysis, both fractions were precipitated with TCA. For cross-linking, the floated fraction was diluted with 1 ml of ice-cold HS buffer, centrifuged in the TLA100.3 rotor (70,000 rpm for 30 min at 4°C; Beckman Coulter), and the microsomes were resuspended in 20 µl of RM buffer (lacking DTT). Cross-linking was then performed with MBS as described for RM.

Limited proteolysis of ribosomes

Proteolysis was performed in 10-µl reactions in RM buffer containing either 10 eq of RM or an eq amount of ribosomes (1.0 A_{260} U/ml). Endoproteinase Glu-C from *Staphylococcus aureus* V8 (Roche) was added to a final concentration between 2.5 and 1,000 µg/ml. Reactions were incubated for 1 h at 37°C and terminated by precipitation with ice-cold 10% (wt/vol) TCA.

For proteolysis in the presence of digitonin, RM and ribosomes were resuspended in solubilization buffer (25 mM Hepes-KOH, pH 7.6, 250 mM KOAc, 5 mM Mg(OAc)$_2$, 10% (wt/vol) glycerol, and 2 mM DTT) at a concentration of 1 eq/µl and either mock treated or treated with 2% (wt/vol) digitonin for 30 min on ice. Insoluble material was removed from the detergent-treated samples by brief centrifugation (16,000 *g* for 10 min at 4°C). Proteolysis was performed with V8 proteinase followed by TCA/acetone precipitation.

Identification of cross-link products by mass spectrometry

Cross-link reactions were performed with 900 eq of RM in 900 µl RM buffer (without DTT) with 200 µM MBS for 20 min at 25°C before quenching with 10 mM ethanolamine-HCl, pH 8.0, and 10 mM 2-mercapto-ethanol. Reactions were treated with 900 µl 2% (vol/vol) Triton X-100, 0.5 M KOAc, and solubilized for 15 min on ice. Ribosomes were recovered by centrifugation in the TLA100.2 rotor (100,000 rpm for 30 min at 4°C; Beckman Coulter), resuspended in 200 µl 10 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$, 1.5 M LiCl, 1% (vol/vol) Triton X-100, and 0.5 mM PMSF, and held on ice for 15 min to disassemble ribosomes. rRNA together with any aggregates was removed by centrifugation in the TLA 100.3 rotor (70,000 rpm for 60 min at 4°C). The supernatant was diluted with 1 ml 10 mM Tris-HCl, pH 7.6, and incubated overnight at 4°C with 20 µl anti-Rpl17$^{C\text{-}trm}$, affinity purified, and coupled to protein A–Sepharose (~1 mg antibody/ml resin; GE Healthcare; Harlow and Lane, 1988). The resin was collected and washed four times with 25 mM Tris-HCl, pH 7.6, 500 mM NaCl, 1 mM EDTA, and 0.4% (vol/vol) NP-40 and once with 10 mM Tris-HCl, pH 7.6, before elution with SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE, and staining was performed with colloidal Coomassie brilliant blue. Tryptic in-gel digestion and nanoelectrospray ionization quadrupole time of flight mass spectrometry was performed as described previously (Opitz et al., 2002).

Online supplemental material

Fig. S1 shows the sensitivity of the two Rpl17-Sec61β cross-link species to V8 protease and reveals that the two cross-link adducts correspond to Sec61β cross-linked to the N- and C-terminal regions of Rpl17, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200807066/DC1.

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