Regulation of RibosomeDetachment from the Mammalian Endoplasmic Reticulum Membrane*

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In current models, protein translation in the endoplasmic reticulum (ER) occurs in the context of two cycles, the signal recognition particle (SRP) cycle and the ribosome cycle. Both SRP and ribosomes bind to the ER membrane as a consequence of the targeting process of translocation. Whereas SRP release from the ER membrane is regulated by the GTPase activities of SRP and the SRP receptor, ribosome release from the ER membrane is thought to occur in response to the termination of protein synthesis. We report that ER-bound ribosomes remain membrane-bound following the termination of protein synthesis and in the bound state can initiate the translation of secretory and cytoplasmic proteins. Two principal observations are reported. 1) Membrane-bound ribosomes engaged in the synthesis of proteins lacking a signal sequence are released from the ER membrane as ribosome-nascent polypeptide complexes. 2) Membrane-bound ribosomes translating secretory proteins can access the translocon in an SRP receptor-independent manner. We propose that ribosome release from the ER membrane occurs in the context of protein translation, with release occurring by default in the absence of productive nascent polypeptide-membrane interactions.

In mammalian cells, cytoplasmic ribosomes engaged in the synthesis of secretory or membrane protein precursors are selected for targeting to the endoplasmic reticulum (ER)1 membrane. While free in the cytoplasm, such ribosome-nascent polypeptide complexes (RNCs) are recognized by the signal recognition particle (SRP) and targeted to the ER membrane via interaction with the SRP receptor (1–3). The targeting reaction culminates in the association of RNCs with the translocon, the cohort of ER membrane components that mediates protein translocation, and transfer of the nascent polypeptide across the ER membrane ensues (3–5). For translocation to proceed, the signal sequence must engage components of the ER membrane, fulfilling a postulated second signal sequence recognition event (6–8).

Via the targeting phase of the translocation cycle, SRP and biosynthetically active ribosomes associate with the ER membrane. How are these components recycled back to the cytoplasm to complete an exchange cycle? It is now established that SRP release from the ER membrane occurs through the combined GTP binding and GTPase activities of SRP and the SRP receptor complex (9, 10). In contrast, little is known regarding the fate of the membrane-bound ribosome following the termination of protein translation. Many models depict that upon termination, the ribosome dissociates into its component 60 S and 40 S subunits and subsequently detaches from the ER membrane (11, 12). In these models, the release of ribosomal subunits from the ER membrane supports a ribosome cycle in which ribosomes engaged in the synthesis of signal sequence-bearing nascent polypeptides are selected from a free cytoplasmic pool, trafficked to the ER membrane, and subsequently to protein translocation, recycled back to the cytoplasm. This series of reactions provides a rationale for the segregation of ribosomes into free and membrane-bound pools. Although ribosome release from the ER membrane represents a fundamental element of the protein translocation cycle, it remains to be determined experimentally how ribosome release from the ER membrane is coupled to the termination of protein translation.

In vitro studies with rat liver rough microsomes (RM) have demonstrated that following treatment with puromycin, free 40 S ribosomal subunits are capable of exchanging with membrane-bound 40 S subunits, whereas free 60 S subunits do not participate in exchange reactions with membrane-bound 60 S subunits (13). These data suggest that following puromycin-induced termination, the 60 S subunits remain in stable association with the ER membrane in the absence of protein synthesis. Indeed, in tissue culture cells it has been proposed that the release of 60 S ribosomal subunits from the ER membrane is kinetically delayed from the termination of protein synthesis (14, 48). Furthermore, experiments in which RM are solubilized with digitonin and subsequently treated with puromycin indicate that membrane-bound ribosomes remain associated with the translocon (15). These data suggest that ribosome release from the ER membrane may be subject to as yet undefined regulatory influences and further emphasize the need for experimental analysis of the regulation of ribosome dissociation from the ER membrane.

Our studies utilized the well established in vitro translation/translocation system consisting of rabbit reticulocyte lysate and canine pancreas rough microsomes. In examining the distribution of ribosomes during the elongation and termination stages of protein synthesis, we report that ribosome release from the ER membrane does not accompany termination. Rather, ribosome release was observed to occur in response to the synthesis of a cytosolic protein on a membrane-bound ribosome. In addition, we report that secretory precursors whose
synthesis is initiated on membrane-bound ribosomes can access the translocon in an SRP receptor-independent manner.

**EXPERIMENTAL PROCEDURES**

**Preparation of RM and Ribosome-free Reticulocyte Lysate—Canine pancreas RM were isolated and treated with *Staphylococcus aureus* nuclease (Calbiochem) as described (16). Nuclease treatment was required to degrade microsome-associated mRNAs, thereby increasing the translation of exogenously added mRNA by membrane-bound ribosomes. However, this treatment decreased the overall translational capacity of the RM, presumably by stranding ribosomes on cleaved mRNA fragments, as reported previously for free reticulocyte lysate ribosomes (17).

To remove any contaminating free ribosomes from RM preparations, 100 equivalents (eq; as defined in Ref. 16) of RM were diluted 5-fold with 2.5 mM sucrose, 25 mM K-HEPES (pH 7.2), 350 mM KOAc, 5 mM Mg(OAc)_2, and 1 mM DTT. This solution is then overlaid with 750 μl of 1.9 M sucrose, 25 mM K-HEPES (pH 7.2), 350 mM KOAc, 5 mM Mg(OAc)_2, 1 mM DTT, and 200 μl of the same buffer lacking sucrose. The sample was centrifuged in an SW55 rotor (Beckman Instruments, Palo Alto, CA) at 55,000 rpm for 4 hr at 4 °C. Under these conditions, the RM float, whereas any contaminating free ribosomes sediment to yield a pellet. After 400 μl of supernatant was collected, the centrifugation was repeated upon a 100-mM sucrose cushion in the same buffer. Membranes were pelleted by centrifugation at 40,000 rpm for 5 min at 4 °C in a TLA100 rotor and then resuspended in the same buffer for 10 min at 4 °C in a TLA100.2 rotor (Beckman Instruments). The membrane pellet was then resuspended in RM buffer.

For some experiments, SRP receptor was inactivated by mild proteolysis prior to flotation of the RM. 100 eq of RM were diluted 20-fold with RM buffer, and chymotrypsin ( Worthington) was then added to a final concentration of 5 μg/ml. The sample was placed on ice for 30 min; the digestion was subsequently quenched by addition of phenylmethylsulfonyl fluoride to 1 mM, and membranes were pelleted by centrifugation at 60,000 rpm for 10 min at 4 °C in a TLA100.2 rotor. Pellets were resuspended in 100 μl of RM buffer and processed for flotation as described above.

Ribosomes were removed from reticulocyte lysate (Promega, Madison, WI) by centrifugation at 80,000 rpm for 30 min at 4 °C in a TLA100 rotor (Beckman Instruments). The supernatant was removed and then centrifuged again under identical conditions to ensure that ribosomes and ribosomal subunits were quantitatively depleted. The resulting ribosome-free post-ribosomal supernatant (PRS) was aliquoted and frozen in liquid nitrogen, and the pelleted ribosomes were resuspended in RM buffer.

**Plasmid Construction—**To fuse N-terminal luciferase extensions with pRL, the pRL-encoding plasmid pGEMBP1 (18) was first modified with pPL, the pPL-encoding plasmid pGEMBP1 (18) was first modified with pPL86 were generated by linearizing pGEMBP1 with *Pvu* I site was created at the extreme N terminus of the pPL coding region, using the primers 5'-ACA-ATG-GCC-CTC-AAC-AGC-3' (sense primer) and either 5'-GAG-ACA-CCA-TGG-TAA-ACC-ATG-GAC-GAG-GGC-CAT-TGT-AAT-GAA-CCC-TTT-GCC-3' (antisense primer for Rluc40), or 5'-GAG-ACA-CCA-TGG-TAA-ACC-ATG-GAC-GAG-GGC-CAT-TGT-AAT-GAA-CCC-TTT-GCC-3' (antisense primer for Rluc94). Rluc polymerase chain reaction fragments and the modified pGEMBP1 vector were digested with *Nco*I and ligated together. Ligation products were transformed into the *Escherichia coli* strain DH5α, and positive clones were selected from *Ncol* and ligated together. Ligation products were sequenced (Wisconsin Genetics Analysis Package) and confirmed by restriction analysis.

**In Vitro Translation and Ribosome—**Transcripts coding for pPL86 were generated by linearizing pGEMBP1 with *Pvu*II. For pPL55, the plasmid was linearized with *FokI*. Transcripts coding for Rluc94 were generated by linearizing pRL-null with *Bcl*I. For Rluc56, the plasmid was linearized using *EcoR*I, and for Rluc33, the plasmid was linearized with *Vsp*I. GFP146 was created by linearizing the GFP-containing plasmid (provided by T. Meyer) with *Xho*I. All restriction enzymes were obtained from either New England Biolabs (Beverly, MA) or Promega. Transcription was carried out using a MEGAscript T7 kit (Ambion, Austin, TX). Translations were carried out as described (19).

Where indicated, ribosome-free reticulocyte lysate was substituted for unfractonated lysate, and RM were used as the source of ribosomes. Translations were conducted at 25 °C for 30 min using "S"-labeled Pro-Mix (Amersham Pharmacia Biotech).

**RNA Complex with Membranes—**Completed translation mixtures (20 μl) were diluted to 100 μl with 110 mM KOAc, 25 mM K-HEPES (pH 7.2), and 2.5 mM Mg(OAc)_2, and then were layered upon a 100-μl 5 μM sucrose cushion in the same buffer. Membranes were pelleted by centrifugation at 40,000 rpm for 5 min at 4 °C in a TLA100 rotor and then resuspended directly into SDS-PAGE sample buffer (0.5 μl Tris, 5% SDS, 100 mM β-mercaptoethanol). The supernatants, containing unbound ribosomes, were precipitated by addition of saturated ammonium sulfate to a final concentration of 66% and were washed once with 10% trichloroacetic acid before being resuspended in sample buffer. Samples were then processed for SDS-PAGE. Gels were visualized using a Fujix MacBAS1000 PhosphorImager, and digital images were prepared using Adobe Photoshop 4.0. All quantification of the PhosphorImager output was performed using Fujix MacBAS version 2.0 software.

Alternatively, RNC association with membranes was determined by flotation centrifugation. Completed translation mixtures (40 μl) containing free ribosomes were placed on ice, and cycloheximide was added to 1 mM to ensure that further translation could not occur. 4 μg of carrier RM were then added, and samples were incubated 10 min on ice, followed by 20 min at 25 °C. Completed translation mixtures using RM-bound ribosomes as the translation source were treated similarly, except additional RM were not added following the 30-min translation. Samples were then diluted to 100 μl with RM buffer and were mixed with 400 μl of 2.5 mM sucrose, 150 mM KOAc, 25 mM K-HEPES (pH 7.2), 2.5 mM Mg(OAc)_2, and 1 mM DTT. Samples were overlaid with 110 mM KOAc, 25 mM K-HEPES (pH 7.2), 2.5 mM Mg(OAc)_2, and 1 mM DTT, and were centrifuged in an SW55 rotor at 55,000 rpm for 4 hr at 4 °C. For some samples, KOAc concentrations were adjusted to 0.5 M and were incubated for 15 min on ice immediately prior to flotation centrifugation. Sucrose solutions containing 0.5 M KOAc were then used for flotation centrifugation of these samples. Following centrifugation, all samples were frozen in liquid nitrogen, cut into thirds using a sharp knife, and prepared for SDS-PAGE as described above.

Release of polypeptides from ribosomes was conducted by diluting completed translation mixtures (20 μl) to 50 μl with 110 mM KOAc, 25 mM K-HEPES (pH 7.2), and 2.5 mM Mg(OAc)_2 and then adding puromycin to 1 μM. Samples were incubated 10 min at 25 °C for 15 min. To analyze polypeptide accessibility to exogenous protease, proteinase K was added to translation mixtures to a final concentration of 100 μg/ml, and samples were incubated on ice for 30 min. Where indicated, CHAPS was included at a final concentration of 0.5%. Immunoprecipitation of pPL nascent polypeptides using anti-pPL polyclonal antisera (U.S. Biochemical Corp.) was conducted as described (20).

**Nascent Polypeptide Association with Ribosomes—**Samples were layered upon 10–30% sucrose gradients containing 25 mM K-HEPES (pH 7.2), 150 mM KOAc, 5 mM Mg(OAc)_2 and 1 mM cycloheximide. Samples were centrifuged at 40,000 rpm for 2.5 hr at 4 °C in an SW41 rotor (Beckman Instruments). Gradients were manually fractionated by puncturing the tube bottoms, and fractions were trichloroacetic acid-precipitated and analyzed by SDS-PAGE.

**RESULTS**

**De Novo Protein Translation by Membrane-bound Ribosomes—**As noted in the Introduction, little is known regarding the mechanism by which ribosomes and/or ribosomal subunits dissociate from the ER membrane following the termination of protein synthesis. The central role of SRP in regulating the association of biosynthetically active ribosomes with the ER membrane is, however, well established. To investigate the mechanism of ribosome dissociation from the ER membrane, we utilized an experimental system that in prior studies was used to establish the mechanism of SRP-dependent ribosome-membrane association (21). Necessarily, it was essential to these studies that the analysis be strictly limited to membrane-bound ribosomes, and so the canine pancreas microsome frac-
programmed with the indicated mRNA and either lacking RM (lanes 1 and 5) or presence of 0.5% CHAPS (lane 3) and were separated by SDS-PAGE. B, RM were preincubated with either RM buffer or under run-off translation conditions (PRS + 1 nM amino acids) for 30 min at 25 °C. Translation reactions, programmed with the indicated mRNA and either lacking RM (lanes 1 and 5) or containing 2 eq RM as the sole source of ribosomes (lanes 2 and 6), were subsequently separated by SDS-PAGE. In lanes 3, 4, 7, and 8, RM were pelleted after the indicated preincubation, and the pellet (P) and supernatant (S) fractions were assayed independently for translation activity. The weak band present in all lanes corresponds to globin, and its presence was insensitive to translation inhibitors.

The Fate of Membrane-bound RNCs—The experiments depicted in Fig. 1 indicate that ribosomes engaged in the synthesis of endogenous mRNAs did not dissociate from the ER membrane following run-off translation. That such ribosomes were clearly capable of de novo protein synthesis suggests, but does not prove, that run-off translation had occurred. Because of the difficulties in unequivocally ascertaining the translation status of the endogenous mRNAs, we focused our analysis on those ribosomes engaged in the de novo synthesis of proteins from exogenous mRNAs. To determine the fates of ribosome-nascent polypeptide complexes following the de novo initiation of protein synthesis on membrane-bound ribosomes, a series of fractionation experiments was performed. In control experiments, proteins were synthesized by free reticulocyte lysate ribosomes in the presence or absence of RM and were then subjected to centrifugation to separate membrane-associated from free RNCs (Fig. 2A, lanes 1–4). When synthesized in the absence of RM, the majority of all RNCs were recovered in the supernatant fraction (Fig. 2A, lanes 1 and 2). Minor amounts of RNCs were recovered in the pellet fraction in the absence of membranes, as is commonly observed in such experiments (23, 24). When synthesis on free ribosomes was performed in the presence of RM, only RNCs engaged in the synthesis of signal sequence-bearing nascent polypeptides (pPL86) bound to the membranes; RNCs engaged in the synthesis of Rluc94 and GFP146 (146 amino acids) to the ribosome-free reticulocyte lysate PRS, in the absence of RM, did not yield protein translation (Fig. 1B, lane 2). In contrast, when salt-washed, purified RM were present, translation of pPL86, Rluc94, and GFP146 was observed (Fig. 1B, lane 2). The faint band apparent in all lanes represents the non-enzymatic labeling of globin and was observed in the presence or absence of cycloheximide (data not shown). To more rigorously determine whether the observed protein translation activity was derived from the membrane-bound ribosomes, the purified RM were pelleted using centrifugation conditions that retain free ribosomes and/or ribosomal subunits in the supernatant and allow recovery of the RM in the pellet fraction. The pellet and supernatant fractions from such centrifugations were then separately assayed for translation activity. As shown in Fig. 1B, lanes 3 and 4, the translation of exogenous mRNA was wholly initiated by membrane-bound ribosomes (Fig. 1B, lanes 3 and 4).

The observation that membrane-bound ribosomes could initiate new rounds of translation was consistent with either of two models. 1) In the presence of PRS, membrane-bound ribosomes undergo run-off protein translation, yielding the release of the membrane-bound ribosomal subunits into a free ribosomal subunit pool and the subsequent initiation of protein synthesis on free ribosomes. 2) The ribosomes/ribosomal subunits remain in association with the ER membrane following termination, with initiation occurring on the bound ribosomes. To differentiate between these two possibilities, RM were preincubated under run-off translation conditions to allow the bound ribosomes to undergo termination reactions and were subsequently fractionated, as described above. As shown in Fig. 1B, lanes 7 and 8, translation activity was again solely recovered in the membrane-bound ribosomal population, indicating that run-off translation does not result in the detachment of translationally active ribosomes from the ER membrane. These results were further corroborated by immunoblots for ribosomal proteins L3/L4, wherein we were unable to identify the release of ribosomes into the supernatant fraction (data not shown). Significantly, the data presented in Fig. 1 indicate that membrane-bound ribosomes can initiate translation of mRNA regardless of whether the encoded protein possesses (pPL86) or lacks (Rluc94 and GFP146) an ER signal sequence.

The data in Fig. 1A indicate, membrane-bound ribosomes retain protein translation activity. Given the presence of active, stably bound ribosomes on the membrane fraction, it was possible to investigate experimentally the process by which the membrane detachment of such ribosomes was regulated. To do so, the ability of the membrane-bound ribosomes to initiate translation of exogenously added mRNA was evaluated. As a control experiment, the addition of mRNA encoding truncated forms of preprolactin (pPL86; 86 amino acids), Renilla luciferase (Rluc94; 94 amino acids), or green fluorescent protein (GFP146; 146 amino acids) to the ribosome-free reticulocyte lysate PRS, in the absence of RM, did not yield protein translation (Fig. 1B, lanes 1 and 5).
some-associated. To determine whether Rluc94 and GFP146 translation products were released from the membrane in the context of intact RNCs, supernatant fractions, obtained by centrifugation of translation reactions conducted with membrane-bound ribosomes, were centrifuged through 10–30% sucrose gradients, and the distribution of the translation products was determined. An immunoblot for ribosomal proteins L3/L4 indicates the migration of 80 S ribosomes in the gradients (Fig. 2B, upper panel). In comparing the relative migration of the ribosomal peak and the radiolabeled nascent polypeptides, it is clear that the majority of the Rluc94 and GFP146 nascent polypeptides had been released from the membrane as intact RNCs (Fig. 2B, middle and lower panels). Thus, translation of proteins lacking a signal sequence on membrane-bound ribosomes yields the detachment of intact RNCs from the ER membrane.

SRP Receptor-independent Targeting of pPL86 RNCs—Following the translation of pPL86 on membrane-bound ribosomes, completed pPL86 nascent polypeptides sedimented in association with the ER membrane (Fig. 2A, lanes 5 and 6). However, the mechanism by which membrane association was conferred was not clear. Did the pPL86 RNCs remain membrane-bound throughout the translation period? Alternatively, did the RNCs release from the membrane early in synthesis, only to re-target by the SRP-dependent pathway? To address these questions, the following hypothesis was examined; if membrane-bound ribosomes engaged in the de novo protein synthesis of secretory precursors dissociate from the membrane early in synthesis, such RNCs would require the SRP receptor activity for re-targeting to the membrane. A stringent test of this hypothesis was performed by inhibiting SRP receptor activity. It is well established that the cytoplasmic domain of the SRP receptor can be inactivated by mild proteolysis, under conditions where the remainder of the translocation machinery is intact (25–29). Therefore, RM were treated at a low concentration of chymotrypsin (5 µg/ml) to degrade the SRP receptor (SRα), while leaving the translocon component Sec61α intact (Fig. 3A). From the data presented in Fig. 3A, a conservative estimate indicates that 10 eq of proteolyzed RM may contain that quantity of SRα present in 0.5 eq of native RM (~20-fold reduction). To assess the functional consequences of the proteolysis-dependent loss of SRα, the association of pPL86 RNCs with native and proteolyzed membranes was determined by flotation centrifugation (Fig. 3B). A number of aspects of these experiments warrant mention. First, the experiments were performed with a truncated secretory precursor, pPL86. Previous studies have unequivocally demonstrated that this precursor remains in association with the ribosome and retains post-translational targeting and translocation competence (18, 19). Thus, any functional SRα present in the proteolyzed RM fraction would be expected to be accessible to pPL86 RNCs and would display activity in the targeting assay. Second, as noted above, under standard reaction conditions, the total translation activity of the lysate-derived free ribosomes is approximately 10-fold higher than the membrane-bound ribosomes. To ensure that these experiments accurately depicted the activities of the free and membrane-bound ribosomes, the ribosome content of the lysate-derived free ribosomes was the sole source of ribosomes. Again, nascent pPL86 was recovered in association with the membranes; Rluc94 and GFP146 were, however, recovered in the supernatant fraction (Fig. 2A, lanes 5 and 6).

In the experiments described above, Rluc94 and GFP146 were synthesized on artificially truncated mRNAs lacking termination codons and thus should remain predominantly ribo-

![Fig. 2. Association of free and membrane-bound RNCs with RM. A, free ribosomes were used to translate the indicated mRNA in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 2 eq RM. In lanes 5 and 6, RM-bound ribosomes served as the sole source of translation activity. Completed reactions were centrifuged, and pelleted RM and supernatants were analyzed by SDS-PAGE. B, supernatant fractions, as shown in A, lane 6, were layered upon 10–30% sucrose gradients and centrifuged. Fractions were collected and analyzed by SDS-PAGE. An immunoblot for large ribosomal proteins L3/L4 is displayed as a control for 80 S ribosomal migration through the gradients. GFP146 were recovered in the supernatant fraction (Fig. 2A, lanes 3 and 4). These data are entirely consistent with previous studies in demonstrating that ribosomes engaged in the synthesis of secretory precursors associate with the ER membrane, whereas ribosomes engaged in the synthesis of cytosolic proteins remain in the supernatant (cytosol) fraction. As an additional note, in these and related experiments, protein translation can occur on both free and membrane-bound ribosomes. To determine the relative activities of the two modes of translation, the ribosome content and thus translation activity of the reticulocyte lysate fraction were titrated to define the point of translation equivalence with the membrane-bound ribosome fraction. The results of these experiments indicate that under standard reaction conditions (2 eq of RM (16) in 20 µl of translation medium) lysate-derived free ribosomes provide 10-fold greater translation activity versus membrane-bound ribosomes (data not shown). Thus, translation occurs predominantly on free ribosomes. Similar experiments were then performed under conditions where membrane-bound ribosomes were the sole source of ribosomes. Again, nascent pPL86 was recovered in association with the membranes; Rluc94 and GFP146 were, however, recovered in the supernatant fraction (Fig. 2A, lanes 5 and 6).]
Membrane-bound ribosomes synthesizing a secretory precursor access the translocon by an SRP receptor-independent mechanism. A, RM were untreated or were proteolyzed with 5 μg/ml chymotrypsin. Proteolysis was stopped by addition of trichloroacetic acid to 10%, and the indicated amounts of RM (lanes 1–5) or proteolyzed RM (lanes 6–10) were subjected to SDS-PAGE and immuno blot analysis. B, free pPL86 RNCs (lanes 1–4) or membrane-bound pPL86 RNCs (lanes 5–7) were incubated either without RM (lane 1), with RM (lanes 2 and 5), or with proteolyzed RM in the presence of low salt (0.15 M KOAc; lanes 3 and 6) or high salt (0.5 M KOAc; lanes 4 and 7). Samples were then subjected to flotation centrifugation, with RM and bound RNCs migrating into the top fractions. Fractions were then analyzed by SDS-PAGE, and the percentages of pPL86 in the top fractions were quantified. C, analyzed by SDS-PAGE, and the percentages of pPL86 in the top fractions were quantified. Fractions were then analyzed by SDS-PAGE, and the percentages of pPL86 in the top fractions were quantified. Fractions were then analyzed by SDS-PAGE, and the percentages of pPL86 in the top fractions were quantified.

As depicted in Fig. 3B, native, unproteolyzed RM bound essentially all free pPL86 RNCs, yet membranes that had been pretreated with chymotrypsin displayed a markedly reduced ability to support free RNC targeting, as assayed at low or high salt concentrations (Fig. 3B, lane 2 versus lanes 3 and 4). These data confirm the established requirement for intact SRP receptor.

Membrane detachment of the pPL55 RNCs was unexpected, as we have previously demonstrated that membrane-bound ribosomes that initiate translation of shorter proteins (<60 amino acids). By using shorter versions of the pPL and luciferase precursors, it was observed that in the absence of membranes, all reticulocyte lysate-derived free RNCs remained in the unbound, supernatant fractions (Fig. 4, lanes 1 and 2). Upon addition of RM, the free RNCs again remained in the supernatant, unable to interact productively with the membranes (Fig. 4, lanes 3 and 4). For the short luciferase polypeptides that lack signal sequences (Rluc56 and Rluc33), this was expected. However, the inability of free RNCs carrying the pPL55 nascent polypeptide to bind to membranes can be traced to the incomplete emergence of the signal sequence from the ribosomal polypeptide exit site (6, 23). Membrane-bound ribosomes were then used to initiate translation of these short nascent polypeptides. Surprisingly, all RNCs detached from the membrane during the 30-min time frame of the experiment (Fig. 4, lanes 3 and 6).

Membrane detachment of the pPL55 RNCs was unexpected, as we have previously demonstrated that membrane-bound ribosomes that initiate translation of a longer nascent polypeptide, pPL86, are able to insert the nascent polypeptide into the translocon while continually remaining membrane-bound (Fig. 3). Thus, the release of the pPL55 RNCs from the membrane must not occur during the actual time required for synthesis of the 55 amino acids, but rather after a longer period. To examine this conclusion, we constructed mRNAs encoding pPL86 with N-terminal luciferase extensions of varying length (Fig. 5A). Provided that the extensions did not drastically interfere.
with signal sequence function, we reasoned that translation of these mRNAs would yield an increase in the period prior to the complete emergence of a signal sequence from the ribosome, allowing for kinetic dissection of the release process. Thus, the modified pPL86 nascent polypeptides mimic nascent integral membrane proteins, which possess internal topogenic signals. Free RNCs possessing pPL86 with N-terminal extensions were able to target to native RM, although the efficiency of targeting decreased slightly as the length of the extensions increased (Fig. 5B, lane 2). The targeted RNCs were bound to the membranes in a physiologically relevant manner, as treatment with proteinase K digested only the N-terminal extensions and left the pPL86 portion of the nascent polypeptide intact, consistent with previous models of a looped signal sequence orientation in the membrane (31) (data not shown). Inactivation of the SRP receptor by proteolysis inhibited targeting of these free RNCs (Fig. 5B, lane 3). In contrast, membrane-bound RNCs possessing pPL86 with the N-terminal luciferase extensions were able to remain membrane-bound continually, regardless of the presence of functional SRP receptor (Fig. 5B, lanes 4 and 5). These results indicate that increasing the time required for a membrane-bound ribosome to expose a complete signal sequence from ∼65 (pPL86) to ∼159 amino acids (Rluc94 + pPL86) does not adversely affect SRP-independent targeting of such ribosomes. Similar to the conclusions reached using the pPL55 nascent polypeptide, these results indicate that the membrane detachment of the Rluc94 RNCs reported in Fig. 2 does not occur during the actual synthesis of the 94 amino acids. In conclusion, the presence of an exposed signal sequence, which is a requirement for the binding of free RNCs to the ER membrane, is also a prerequisite for the continued association of translating ribosomes with the membrane. However, a significant time window exists for membrane-bound ribosomes to participate in protein translocation, several conclusions can be drawn and are schematically illustrated in Fig. 6. 1) Membrane-bound ribosomes are indiscriminate in their selection of mRNA and thus can initiate the synthesis of cytosolic as well as secretory protein precursors. 2) Membrane-bound ribosomes that initiate synthesis of a protein lacking an EK signal sequence are released from the membrane as ribosome-nascent polypeptide complexes. 3) Membrane-bound ribosomes that initiate translation of a signal sequence-bearing protein remain attached to the membrane throughout protein synthesis. 4) Nascent secretory protein precursors whose synthesis is initiated on membrane-bound ribosomes can target to the translocon and be translocated in the absence of a functional SRP receptor. 5) For a translationally active membrane-bound ribosome to maintain a prolonged association with the membrane, emergence of a topogenic signal from the ribosomal exit site must occur.

Of the conclusions noted above, points 1 and 2 were obtained by direct analysis of the distribution of RNCs following de novo synthesis of precursor proteins on membrane-bound ribosomes. Other than removal of the ribosomes from the reticulocyte lysate by centrifugation, very little experimental modifications of the established in vitro translation/translocation system were performed. In contrast, experiments evaluating the requirement for SRP receptor activity for targeting of secretory precursors whose synthesis was initiated on membrane-bound ribosomes required additional experimental manipulation, in the form of mild proteolysis. Although this is an established method for inactivation of the SRP receptor (25–29), it could be argued that a small fraction of SRP receptor
molecules was proteolytically protected by bound ribosomes and thus could be later utilized by bound ribosomes in the targeting of precursor proteins to the translocon. Although there is no literature to support this hypothesis, two aspects of the experimental design directly address this concern. First, the experiments were performed with a truncated secretory precursor protein that is post-translationally competent for targeting and translocation. By using an experimental system in which protein translation is kinetically uncoupled from the SRP receptor-dependent targeting reaction, any kinetic limitation imposed upon the targeting process, as would accompany a vast reduction in SRP receptor concentration, would be minimized. Second, in these experiments the ribosome content and thus the translation activity of the reticulocyte lysate were titrated to ensure that identical quantities of nascent polypeptides were synthesized by both the membrane-bound and free populations of ribosomes. Importantly then, the stoichiometric relationship between the number of nascent polypeptides synthesized on membrane-bound or free ribosomes and the quantity of SRP receptor was identical. Yet, the data in Fig. 3 thus the translation activity of the reticulocyte lysate were titrated to ensure that identical quantities of nascent polypeptides whose synthesis was initiated on membrane-bound ribosomes were released from the membrane as RNCs, and as free RNCs were then re-targeted to the membrane via the SRP-SRP receptor pathway. Given that proteolysis disabled the targeting of free RNCs, it can be concluded that such a release and re-targeting event did not occur.

Ribosome-nascent polypeptide association with the translocon in the absence of SRP-mediated targeting has been observed in other experimental systems (6, 32–36). These studies made use of the intrinsic ability of salt-washed ribosomes to bind to the translocon in the absence of cytosolic factors, such as the nascent polypeptide-associated complex (NAC). While its role in RNC targeting is controversial, NAC is postulated to occupy the membrane attachment site (M-site) on ribosomes and can be displaced by SRP upon emergence of a signal sequence from the ribosome, allowing membrane binding of the RNC to occur (33, 36). The experimental observations presented in this report have focused on RNC detachment from the ER membrane, and although NAC may be involved in these processes, any such role remains as yet uncharacterized.

On the basis of the data presented herein, we propose that the conformation of the biosynthetically active ribosome dictates its own membrane attachment. In this model, the ribosome accesses a number of different conformational states as it segues through cycles of elongation, and it is these conformational changes that have functional consequences for binding to the ER membrane (37–39, 47). We postulate that upon undergoing a transition from an inactive to an active translational state, the membrane-bound ribosome, specifically the large subunit, assumes conformations that have reduced affinity for the ER membrane. If the ribosome is engaged in the synthesis of a cytoplasmic protein, and thus fails to expose a signal sequence within a given time frame, release from the ER membrane is predicted to occur, with protein synthesis continuing in the cytosol. Conversely, synthesis of a signal sequence-bear-
ing protein leads to the long lived association of translationally active ribosomes with the ER membrane via interactions of the translocating nascent polypeptide with components of the ER. These interactions of the nascent polypeptide with the ER, initially with the translocon (via the signal sequence) and subsequently with luminal components of the ER (via the mature domain), contribute to continued ribosome binding (22, 30, 40). Such interactions between the nascent polypeptide and components of the ER would becomplemented by short-lived direct interactions between the ribosome and components of the ER membrane, as has been recently proposed (41). Upon termination, the ribosome would no longer be conformationally active and thus, at least with respect to the large ribosomal subunit, could remain membrane-bound indefinitely. Although a mechanism for the regulation of ribosome-membrane association that evokes ribosomal conformation as a primary regulatory input is highly speculative, there is ample evidence indicating that translationally active ribosomes undergo remarkable conformational excursions (37–39, 47).

It should be noted that although ribosome release from the ER membrane in response to synthesis of a cytosolic protein can be readily detected, we have as yet been unable to define the precise kinetics of the ribosome release process. It is anticipated that the release process is relatively slow, but because of technical limitations surrounding the fractionation of ribosomes and microsomal membranes with an appropriate temporal resolution, this critical parameter remains undefined. The identification of the rate constants for release is an essential element in the characterization of this novel process and is the subject of current studies.

Of what significance are the current findings to the field of protein trafficking? The data presented herein provide a clear explanation for the necessary segregation of mRNA molecules encoding cytosolic, secretory, or membrane protein precursors between free and membrane-bound pools, particularly when it is appreciated that the ribosome itself does not select for either pool of mRNA molecules (42–44). By the proposed mechanism, should the demand for synthesis of cytosolic proteins exceed the translational capacity of free ribosomes, membrane-bound ribosomes could initiate the synthesis of such proteins and could detach from the membrane. In this way, ribosomes could be recruited from the membrane-bound pool to the cytoplasmic free pool, thus providing an efficient way to support the synthesis of cytoplasmic proteins without an immediate need for increased ribosome biogenesis, a task which is, on a relative basis, slow and can monopolize a large portion of the cellular synthesis machinery (45, 46). Conversely, should the demand for the synthesis of secretory proteins rapidly increase, an efficient mechanism has evolved whereby membrane-bound ribosomes can undergo repetitive cycles of translation while remaining on the membrane, bypassing the need for detachment followed by re-targeting to the ER during each cycle. Finally, the mechanism by which ribosomes are released from the ER membrane, as part of the cycle of ribosome attachment and release that is thought to accompany protein translocation, has until now not been understood. The studies reported herein and in the accompanying manuscript (48) represent initial experimental analyses of this fundamental and ubiquitous process and identify the regulation of the trafficking dynamics of the ribosome as an important area for future study.

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