Endoplasmic Reticulum-bound Ribosomes Reside in Stable Association with the Translocon following Termination of Protein Synthesis*

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In current views, translation-coupled ribosome binding to the endoplasmic reticulum (ER) membrane is transient, with association occurring via the signal recognition particle pathway and dissociation occurring upon the termination of protein synthesis. Recent studies indicate, however, that ribosomal subunits remain membrane-bound following the termination of protein synthesis. To define the mechanism of post-termination ribosome association with the ER membrane, membrane-bound ribosomes were detergent-solubilized from tissue culture cells at different stages of the protein synthesis cycle, and the composition of the ribosome-associated membrane protein fraction was determined. We report that ribosomes reside in stable association with the Sec61α-translocon following the termination stage of protein synthesis. Additionally, in vitro experiments revealed that solubilized, gradient-purified ribosome-translocon complexes were able to initiate the translation of secretory and cytosolic proteins and were functional in assays of signal sequence recognition. Using this experimental system, synthesis of signal sequence-bearing polypeptides yielded a tight ribosome-translocon junction; synthesis of nascent polypeptides lacking a signal sequence resulted in a disruption of this junction. On the basis of these data, we propose that in situ, ribosomes reside in association with the translocon throughout the cycle of protein synthesis, with membrane release occurring upon translation of proteins lacking topogenic signals.

In mammalian cells, ribosomes comprise a common pool that is distributed between the cytosol and the endoplasmic reticulum (ER) membrane (1). Ribosome binding to the ER membrane occurs following recognition of the nascent polypeptide by the signal recognition particle (SRP), interaction of SRP with its receptor at the ER membrane, and subsequent transfer of the ribosome-nascent polypeptide complex (RNC) to the ER translocation machinery, referred to as the translocon (2–4). The translocon then performs additional nascent polypeptide recognition functions necessary for the formation of a tight ribosome-translocon junction and subsequent protein translocation (5–8).

The experimental studies leading to the identification of SRP provided clear evidence that secretory protein synthesis could be initiated on free, cytosolic ribosomes and the resulting RNC complexes transferred to the ER membrane, yielding a membrane-bound ribosome fraction (9–11). With the SRP pathway functioning as the input pathway for ribosome association with the ER, it has long been presumed that the termination of protein synthesis yields the release of ribosomal subunits from the ER membrane to the cytosol, thereby completing a cycle of ribosome binding and release (12, 13). However, in contrast to the very well documented mechanism of SRP-mediated RNC targeting, there is a paucity of experimental evidence in support of termination-elicited ribosome release from the ER. In fact, recent reports have indicated that a substantial fraction of the membrane-associated ribosome pool remains bound following the termination of translation (14, 15). Since membrane-bound and cytosolic ribosomes are known to comprise a common pool, how then is ribosome release from the ER membrane effected? We have previously demonstrated in in vitro studies that membrane-bound ribosomes are capable of reinitiating translation and have proposed that topogenic signals in the nascent polypeptide determine the compartmental fate of these ribosomes (15). For example, membrane-bound ribosomes engaged in the synthesis of cytosolic proteins are released from the membrane, whereas ribosomes translating secretory proteins remain in association with the membrane and can access translocation sites in an SRP receptor-independent manner (15). It is unclear from these studies, however, whether upon termination ribosomes remain in direct association with the translocon or are transferred to a distinct binding site, from which they can later reaccess the translocation machinery.

In view of findings demonstrating that bound ribosomes can regulate translocon gating (6, 16), that the nascent polypeptide exit site on the ribosome directly abuts the translocon (17–19) and more recent findings that the translocon serves as the site for malfolded protein export from the ER (20), it is clear that insights into the regulation of ribosome-translocon interactions are crucial to the understanding of both ribosome exchange and bidirectional protein traffic across the ER. Toward this end, we report that in intact cells membrane-bound ribosomes remain in direct association with the Sec61α-translocon throughout termination and reinitiation of protein translation. Evidence is also provided demonstrating that the disassembly of the ribosome-translocon junction occurs upon synthesis of protein lacking a topogenic signal. These results indicate that the physiological process of ribosome exchange on the ER membrane is driven by the compartmental fate of the translated protein and thus is not mechanistically coupled to the termination of protein synthesis.

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The abbreviations used are: ER, endoplasmic reticulum; RM, rough microsomes; SRP, signal recognition particle; RNC, ribosome-nascent polypeptide complex; RAMP, ribosome-associated membrane protein; pPPL, preprolactin; Rhc, Renilla luciferase; DHPC, diheptanoyl-sn-phosphatidylcholine; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
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EXPERIMENTAL PROCEDURES

Reagents—[35S]Pro-Mix and [32P]Orthophosphate were obtained from Amersham Biosciences. Pactamycin was from Upjohn (Kalamazoo, MI). Puromycin, cycloheximide, Triton X-100, and Triton X-114 were from Sigma. Digitonin and dodecylmaltoside were from Calbiochem. DHPC was obtained from Avanti Polar Lipids (Alabaster, AL). Nuclease-treated reticulocyte lysate was from Promega (Madison, WI). Cell culture reagents and proteinase K were from Invitrogen.

Cell Culture and Labeling—Human Jurkat T cells were cultured in RPMI supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37° C with 5% CO2. Cells were kept within a density range of 0.3–1 × 10⁶ cells/ml by subculturing every 2–3 days. For labeling with [35S]orthophosphate, cells at a density of 0.5 × 10⁶ cells/ml were starved in serum- and phosphate-free RPMI for 40 min at 37° C. [35S]Orthophosphate was added to a final concentration of 100 μCi/ml, and cells were labeled for 40 min.

Optimization of Cell Lysis Conditions—Jurkat cells were sedimented, washed with ice-cold phosphate-buffered saline, and resuspended at 1 × 10⁶ cells/ml in lysis buffer containing 2% detergent (digitonin, dodecylmaltoside, or DHPC), 400 mM KOAc, 25 mM K-HEPES (pH 7.2), 5 mM Mg(OAc)₂, 0.2 mM cycloheximide, 400 units/ml RNasin, and 1 mM phenylmethylsulfonyl fluoride. Samples were incubated for 30 min at 25° C and were then centrifuged at 10,000 rpm for 10 min at 4° C. The precipitated supernatants were washed in acetone and resuspended in SDS-PAGE sample buffer (5% SDS, 0.5 M Tris, and 100 mM β-mercaptoethanol), whereas 150 μl of supernatants were layered above 50-μl sucrose cushions (0.5 M sucrose, 400 mM KOAc, 25 mM K-HEPES (pH 7.2), 5 mM Mg(OAc)₂) in TLA100 tubes. To sediment ribosomes, samples were centrifuged at 80,000 rpm (250,000 × g) for 30 min at 4° C in a TLA100 rotor (Beckman). Pellets were resuspended in SDS-PAGE sample buffer (5% SDS, 0.5 M Tris, and 100 mM β-mercaptoethanol), whereas 150 μl of supernatants were layered above 50-μl sucrose cushions (0.5 M sucrose, 400 mM KOAc, 25 mM K-HEPES (pH 7.2), 5 mM Mg(OAc)₂) in TLA100 tubes. To sediment ribosomes, samples were centrifuged at 35,000 rpm (90,000 × g) for 1.5 hr at 4° C in a SW28 rotor (Beckman). Fractions comprising the ribosome peak were pooled and diluted 1:1 with 25 mM K-HEPES (pH 7.2), 5 mM Mg(OAc)₂, and 0.1% digitonin. Gradients were centrifuged at 26,000 rpm for 4 hr at 4° C in a SW28 rotor (Beckman).

RESULTS

Translation-independent Association of Ribosomes with the Translocon—The present study was performed to define the physiological basis of ribosome exchange on the ER membrane, as occurs in the intact cell, and to determine the membrane binding site for ER-associated ribosomal subunits following the termination of protein synthesis. In order to identify the binding partners of membrane-bound ribosomes present in intact cells, both prior to and after the termination of protein synthesis, it was necessary to utilize an experimental system in which actively translating ribosomes could be readily distinguished from inactive ribosomes. Thus, these studies were conducted in tissue culture cells (Jurkat), whose monosome and polysome populations are clearly distinguishable upon velocity sedimentation. The use of tissue culture cells necessitated the development of methodologies that would allow efficient solubilization of the ER membrane without disruption of the binding interactions between ribosomes and ribosome-associated membrane proteins (RAMPs) (24). The relative abilities of three detergents (digitonin, dodecylmaltoside, and DHPC) to fulfill this criterion are described in Fig. 1. When added to cells, all of the indicated detergents were capable of solubilizing the ER membrane, as determined by the liberation of ER components into the supernatant following low speed (7500 × g) centrifugation (Fig. 1A). As expected, following a high speed (250,000 × g) centrifugation step in which ribosomes are sedimented, the ER luminal chaperone GRP94 was recovered, whereas RAMPs were employed (Fig. 1B). However, translocon components Sec61α and ribophorin I, proteins that have been designated as RAMPs in previous studies (24, 25), were separated from ribosomes to different degrees and in a detergent-specific manner. In the presence of DHPC, ribosome-RAMP interactions were greatly disrupted (Fig. 1B), whereas in the presence of dodecylmaltoside, ribosome-Sec61α interactions were stable, al-
through ribosome-ribophorin I interactions were disrupted (Fig. 1B). In contrast, digitonin was able to maintain the majority of ribosome-RAMP interactions and thus was utilized in subsequent studies.

We next employed experimental conditions in which cellular, ER-associated ribosomes could be identified as either actively translating polysomes or as post-termination monosomes. In actively metabolizing cells, most membrane-bound ribosomes are active in protein translation and reside within polysomes (Fig. 2A). In examining the RAMP fraction derived from ER-associated cellular polysomes, we observed that the majority of the Sec61α was recovered in association with these polysomes, whereas a significant fraction (approximately 30%) of cellular ribophorin I and the entirety of GRP94 were found at the top of the gradient, not associated with ribosomes (Fig. 2A). To obtain post-termination monosomes, cells were treated under a variety of experimental conditions to promote polysome breakdown. Jurkat cells undergo apoptosis upon prolonged serum starvation, with the induction of apoptosis resulting in an inhibition of the initiation stage of protein translation (26, 27). We verified these prior findings in demonstrations that 24 h of serum starvation caused a decrease in the levels of translationally active polysomes and an accompanying increase in the levels of inactive 80 S monosomes (Fig. 2B). Surprisingly, following apoptosis-elicited polysome breakdown, Sec61α, the primary site of ribosome binding, remained in association with the monosome fraction (Fig. 2B). If, following termination, membrane-bound ribosomes had diffused away from the translocon, the resulting “vacant” Sec61 complexes would have been recovered in a free form at the top of the gradient (Fig. 2, A and B). Interestingly, a slightly smaller form of Sec61α was observed following serum deprivation, suggesting that the induction of apoptosis is accompanied by the proteolysis of Sec61α.

Treatment of cells with pharmacological inhibitors of translation, such as puromycin, a compound that promotes premature termination, or pactamycin, an inhibitor of the initiation stage of translation, efficiently elicited polysome breakdown (Fig. 2, C and D). Consistent with the results obtained following serum deprivation, Sec61α was found in association with monosomes arising from pharmacological inhibition of protein synthesis. These results corroborate earlier in vitro findings obtained in isolated rough microsomes and thus support the hypothesis that upon termination, membrane-bound ribosomes remain in association with the translocon (28). Significantly, these data comprise the first experimental evidence that this phenomenon occurs in intact cells.

Analysis of Translocon Composition as a Function of Ribosome Translation State—Having demonstrated that upon termination, membrane-bound ribosomes remain in association with Sec61α, we next examined whether the total complement of RAMPs associated with translationally active polysomes and post-termination monosomes varied as a function of translation. In these experiments, polysomes from control cells and 80

![Figure 1. Detergent-specific solubilization of endoplasmic reticulum proteins.](image)

![Figure 2. Translocon components remain ribosome-associated throughout the protein translation cycle.](image)
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S monosomes from pactamycin-treated cells were selected as highly enriched populations of translationally active and post-termination ribosomes, respectively. Equivalent amounts of ribosomes from each population were purified and subsequently extracted with Triton X-114 to enrich for the bound integral membrane protein fraction. As shown in Fig. 3A, the composition of the ribosome-associated membrane protein fraction displayed little variation with the translation state of the associated ribosome. It should also be noted that the post-termination ribosomes recovered from the pactamycin-treated cells lack nascent polypeptides, and thus the nascent polypeptide does not appear to perform a function in regulating translocon composition. As can be readily seen from the data presented in Fig. 3A, the three main heterooligomeric complexes of the translocon, the Sec61 complex (Sec61α, -β, and -γ), the oligosaccharyltransferase complex (ribophorin I, ribophorin II, and OST48), and the TRAP complex (TRAPα, -β, -γ, and -δ) all are present at nearly equivalent levels in the two ribosome populations.

Previous studies have indicated that several translocon components are phosphoproteins and that phosphorylation state can regulate their interactions with ribosomes (29–31). Furthermore, phosphorylation of RM has been demonstrated to have functional consequences for protein translocation (30). To determine whether the phosphorylation state of translocon components varies as a function of the translation state of associated ribosomes, cells were labeled with [32P]orthophosphate, and polysome- and monosome-associated membrane protein fractions were isolated. In these analyses, we observed that two components of the RAMP fraction, TRAPα and Sec61γ, were recovered as phosphoproteins and, furthermore, that their phosphorylation state did not vary with the ribosome translation state (Fig. 3B). Hence, it appears that phosphorylation of translocon components does not serve a regulatory role in coupling translocon composition to the ribosome translation state.

Functional Analysis of Soluble Ribosome-Translocon Complexes—Thus far, our analyses have focused on the characterization of membrane proteins that remain bound to active ribosomes (polysomes) and post-termination ribosomes (monosomes) following solubilization with digitonin. However, there are proteins that participate in the translocation reaction, such as TRAM and the signal peptidase complex (32, 33), that are not found in association with ribosomes following solubilization of the ER with digitonin (24, 25). We therefore wanted to determine whether, following the reinitiation of translation by membrane-bound ribosomes, the core RAMPs identified in Fig. 3A were responsible for the nascent polypeptide recognition events that contribute to the regulation of ribosome-membrane interactions (15, 28). To address this question, ribosome-translocon complexes were purified from canine RM, as depicted in Fig. 4. We chose this experimental system to ensure purity of the final ribosome-translocon preparation. By initially floating the RM though high salt-containing (350 mM KOAc) sucrose solutions and then pelleting the membranes in a subsequent centrifugation step, any free ribosomes present in the RM fraction were removed. The purified RM were then solubilized with digitonin, and the ribosome-translocon complexes were subsequently purified by velocity sedimentation on linear sucrose gradients. Importantly, the complement of RAMPs bound to these RM-derived ribosomes was identical to that bound to...
Jurkat cell-derived ribosomes (compare Figs. 3A and 4). These ribosome-translocon complexes were then used in \textit{in vitro} translation reactions with mRNAs encoding truncated polypeptides either possessing (preprolactin) or lacking (luciferase) an ER signal sequence. The ability of the soluble, ribosome-bound translocon to perform the essential nascent polypeptide recognition event was then assessed using a protease protection assay. We hypothesized that if recognition occurred, the soluble translocon would remain bound to the ribosome and thereby yield protection of the nascent polypeptide from exogenous proteases; conversely, if recognition did not occur, the translocon would detach from the ribosome, and the nascent polypeptide would become accessible to protease. This experimental approach has previously been validated in studies demonstrating that digitonin-soluble translocons can bind to free RNCs bearing secretory polypeptides to protect them from proteolytic degradation (19, 34). While assays would be preferred that directly assess whether translocons remained bound to ribosomes following translation, such analyses were hindered by the low percentage of translationally active ribosomes present in the purified preparations (data not shown).

We first assessed the topology of nascent pPL86 polypeptides within the translocon to verify that in our experimental system, the addition of protease to the luminal side of the translocon would not degrade a nascent polypeptide contained within. Toward this end, mRNAs were prepared that encoded for pPL86 with N-terminal luciferase extensions of varying length. Free ribosomes carrying these nascent polypeptides have previously been shown to target to the ER membrane in an SRP-dependent manner (15). \textit{In vitro} translation in the presence of RM, pPL86-mers lacking extensions were protected from protease, in agreement with numerous reports (Fig. 5A, lanes 1 and 2) (7, 35). However, while the nascent pPL86 polypeptides possessing N-terminal extensions displayed protease sensitivity, the digestion products were of the same approximate size as pPL86 (Fig. 5A, lanes 3–8, \textit{light arrows}). Thus, the topology of pPL86 within the translocon appears to be a hairpin, with the N-terminal extensions breaching the ribosome-translocon junction and accessing the cytosol. This result is similar to the previously described hairpin topology of nascent vesicular stomatitis virus glycoprotein upon RNC targeting to the ER (36).

We next tested the nascent polypeptide recognition properties of the translocon in several different conditions. When synthesized on free ribosomes in the absence of RM, both pPL86 and Rluc94 were protease-sensitive (Fig. 5B, lanes 1 and 2). In contrast, the post-translational addition of RM yielded protection of pPL86, but not Rluc94, consistent with the notion that the SRP targeting machinery selectively directs free, signal sequence-bearing RNCs to the translocon (Fig. 5B, lane 3 versus lane 4). When translation was initiated using membrane-bound ribosomes, pPL86 was again resistant to protease, as the RNC continually remained membrane-bound and accessed the translocon in an SRP-independent manner (Fig. 5B, lane 5 versus lane 6) (15). In contrast, when the synthesis of a cytosolic protein (Rluc94) lacking a signal sequence is initiated on membrane-bound ribosomes, the RNCs detach from the membrane, allowing protease access to the nascent polypeptide (Fig. 5B, lanes 5 and 6) (15). Importantly, similar results were obtained using the soluble, gradient-purified ribosome-translocon complexes as the source of translation activity (Fig. 5B, lanes 7 and 8). Using this system, the nascent pPL86 was inaccessible to protease, indicating that the soluble translocon recognizes the signal sequence and remains bound to the ribosome. However, upon translation of Rluc94 on soluble ribosome-translocon complexes, the nascent polypeptide was found to be wholly protease-accessible (Fig. 5B, lanes 7 and 8). This result suggests that the translocon had failed to interact productively with the Rluc94 nascent polypeptide and the ribosome-translocon junction had become disrupted, yielding protease sensitivity. As a control, and to allow unequivocal identification of any limit digestion products, translations were conducted using the soluble ribosome-translocon complexes in the absence of exogenously added mRNA. A number of high molecular weight proteins were detected (\textit{arrowheads}) but were degraded to a single diffuse band (\textit{asterisk}) following proteolysis.

**Fig. 5.** Soluble, ribosome-bound translocons can perform nascent polypeptide recognition functions. \textbf{A}, topology of nascent polypeptides within the translocation channel was assessed using mRNAs encoding pPL86 (lanes 1 and 2) on pPL86 with N-terminal luciferase extensions of varying length. Free ribosomes carrying these nascent polypeptides have previously been shown to target to the ER membrane in an SRP-dependent manner (15). \textit{In vitro} translation in the presence of RM, pPL86-mers lacking extensions were protected from protease, in agreement with numerous reports (Fig. 5A, lanes 1 and 2) (7, 35). However, while the nascent pPL86 polypeptides possessing N-terminal extensions displayed protease sensitivity, the digestion products were of the same approximate size as pPL86 (Fig. 5A, lanes 3–8, \textit{light arrows}). Thus, the topology of pPL86 within the translocon appears to be a hairpin, with the N-terminal extensions breaching the ribosome-translocon junction and accessing the cytosol. This result is similar to the previously described hairpin topology of nascent vesicular stomatitis virus glycoprotein upon RNC targeting to the ER (36).
in the absence of added mRNA, yielded several higher molecular weight radiolabeled proteins representing the residual presence of endogenous mRNAs in the ribosome-translocon preparations. Following proteolysis, these proteins were degraded to give a diffuse band of -40–60 amino acids (asterisk). The radiolabeled band, indicated by the asterisk in Fig. 5B, lane 8, thus does not represent specific degradation products of either pPL86 or Rluc94. We propose, then, that the translocon components identified in Fig. 3A are functional in nascent polypeptide recognition and determine whether, upon reinitiation of translation, a membrane-bound ribosome remains in association with the translocon or alternatively is released from the membrane to continue translation in the cytosol.

**DISCUSSION**

In the present study, we have investigated the interactions of ER-associated ribosomes with components of the membrane during the initiation, elongation, and termination stages of translation and report the following conclusions. 1) Following termination, ribosomes remain in stable association with the translocon. 2) The protein composition of the translocon does not vary when bound ribosomes transition between translationally active and post-termination states. 3) The translocon performs nascent polypeptide recognition functions necessary for determining the compartmental fate of translationally active membrane-bound ribosomes. On the basis of the data contained herein, we propose that in the intact cell, membrane-bound ribosomes remain in association with the translocon throughout the cycle of initiation, elongation and termination. Consistent with previous results, membrane-bound ribosomes engaged in the synthesis of cytosolic proteins are released from the translocon. This finding is in agreement with our previous suggestion that during the elongation cycle of protein synthesis, a stable ribosome-translocon junction requires productive translocation of the nascent polypeptide (15, 28).

The results obtained by in vivo analysis of membrane components interacting with post-termination ribosomes are complementary to several previous observations. In an early experiment defining the ribosome-binding characteristics of Sec61a, in vitro treatment of RM with puromycin at physiological salt concentrations did not yield disruption of the ribosome-Sec61a interaction (24). Although puromycin treatment does not replicate physiological termination, these data suggested the existence of a stable ribosome-translocon interaction. This concept is supported by experiments investigating ER membrane permeability to small molecules. Upon treatment of permeabilized cells or rough microsomes with puromycin, an increase in the passage of small molecules across the ER membrane was observed, but it was diminished upon the release of ribosomes from the membrane with increasing salt concentrations (16, 37). These data support the proposal that following termination, ribosomes remain directly bound to the translocon and prevent the pore from adopting a conformation closed to small molecules; removal of ribosomes from the translocon complex then restores membrane impermeability through closure of the pore and/or translocon disassembly, as has previously been observed (38).

Recent studies using cryoelectron microscopy have defined the interactions between ribosomes and the Sec61 complex at the atomic level (17–19). The composite structures of ribosomes and attached Sec61 complexes are similar under conditions in which the ribosome is stalled in the process of translocation or lacks a nascent chain. However, we have demonstrated that translocon-associated ribosomes can initiate translation of cytoplasmic proteins and as such will eventually dissociate from the translocon (Fig. 5B). To accommodate these two observations, we suggest that in the absence of binding interactions between the nascent polypeptide and components of the translocation machinery, ribosomal conformation changes that accompany the elongation cycle of protein translation lead to the eventual dissociation of the ribosome-translocon complex. From current atomic structure models, it can be suggested that movement of ribosomal expansion segment 27 may contribute to this release process (19). As the complex between the translocon and a ribosome translating a cytoplasmic protein exists only transiently (15), capturing the complex to allow identification of its structural details will prove challenging.

An important role for the translocon in mediating the process of protein export, or retrotranslocation, from the ER has emerged in recent years (20). This process, a component of ER-associated degradation, requires that malfolded protein substrates be selected for export and discharged through the translocon to the cytoplasm for proteosomal degradation (39, 40). However, our results indicate that in the cell, translocons are occupied by ribosomes under steady-state conditions, and termination of translation does not yield ribosome dissociation from the translocon. How then do translocons become available to participate in retrotranslocation? Forward and reverse translocation of protein substrates through the translocon does not occur simultaneously, since microsomes saturated with bound RNAs have been shown to be retrotranslocation-incompetent (40). Perhaps the onset of retrotranslocation is accompanied by structural transitions within the translocon, having the effect of both displacing inactive ribosomes from the cytoplasmic face of the translocon and preventing free ribosomes from targeting to such a translocon. Irrespective of the mechanism, the data presented herein indicate that accessing a vacant translocon for the purpose of retrotranslocation may be more complex than implied by classical ribosome exchange models.

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