Cell, Vol. 92, 621–631, March 6, 1998, Copyright ©1998 by Cell Press

TRAM Regulates the Exposure of Nascent Secretory Proteins to the Cytosol during Translocation into the Endoplasmic Reticulum

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Summary

Translocational pausing is a mechanism used by certain specialized secretory proteins whereby discrete domains of a nascent chain destined for the endoplasmic reticulum lumen are transiently exposed to the cytosol. Proteoliposomes reconstituted from total endoplasmic reticulum proteins properly assemble translocationally paused intermediates. The capacity of the translocon to correctly pause the nascent chain is dependent on a glycoprotein fraction whose active component is TRAM. In the absence of TRAM, the normally sealed ribosome-membrane junction still opens in response to a pause transfer sequence. However, nascent chain domains that are not exposed to the cytosol in the presence of TRAM are so exposed in its absence. Thus, TRAM regulates which domains of the nascent chain are visible to the cytosol during a translocational pause.

Introduction

A fundamental phase in the biogenesis of most secretory and membrane proteins is their proper translocation across or insertion into the endoplasmic reticulum (ER) membrane (reviewed by Andrews and Johnson, 1996; Rapoport et al., 1996). In mammalian cells, these events occur cotranslationally at sites termed translocons, aqueous translocation channels that span the lipid bilayer. The reconstitution into liposomes of only three protein complexes, the receptor for the signal recognition particle (SRP), Sec61 complex, and the translocating chain-associated membrane protein (TRAM), is sufficient to reproduce both the translocation and membrane integration of all proteins tested thus far (Görlich and Rapoport, 1993; Oliver et al., 1995; Voigt et al., 1996). Of these proteins, the Sec61p complex (composed of α, β, and γ subunits) was found to be the sole component absolutely required for translocation subsequent to membrane targeting (Görlich and Rapoport, 1993; Jungnickel and Rapoport, 1995). Thus, although a wide variety of proteins at or near the site of translocation are generally considered part of the translocon (Walter and Lingappa, 1986), the minimal machinery required to facilitate polypeptide transport appears to be remarkably simple.

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In addition to transport across the membrane, a variety of other reactions, both covalent and noncovalent, are crucial for proper functional maturation. These include cleavage of signal sequences, glycosylation, disulfide bond formation, intrachain folding, and oligomerization with other proteins (see for example, Bulleid, 1993; Chen et al., 1995; Silberstein and Gilmore, 1996). Many of these modifications can only occur during translocation, a unique time when regions of the nascent protein that later in biogenesis would be inaccessible are unfolded and accessible to various enzymes. For this reason, it may be important for the maturation of certain proteins that the rate of the translocation process be modulated to accommodate complexities in protein biogenesis. Thus, the translocation machinery may need to provide regulatory functions beyond simply serving as an aqueous conduit through which nascent chains can pass.

To explore the possibility of regulated translocation, we had previously examined the early translocation of apolipoprotein B (apo B). This unusually large and hydrophobic secretory protein must be cotranslationally assembled with lipids (among other modifications) prior to its secretion (reviewed by Dixon and Ginsberg, 1993; Yao and McLeod, 1994). Interestingly, the secretion of apo B containing lipoproteins appears to be regulated entirely posttranslationally (Yeung et al., 1996; Fisher et al., 1997). These features of apo B biogenesis suggested the possibility that its translocation might be regulated.

Indeed, the translocation of apo B was demonstrated to be unusual in that it was not continuous. Instead, nascent apo B was found to stop and then restart its translocation at several discrete points during chain growth (Chuck et al., 1990). These points of translocational pausing are directed by specific topogenic sequences, termed pause transfer sequences (Chuck and Lingappa, 1992). A total of 23 translocational pauses were subsequently identified and found to be distributed asymmetrically in three clusters along the length of the 500 kDa apo B molecule (Kivlen et al., 1997). While the various responses to each and every pause transfer sequence in apo B remains to be elucidated, a mechanistic role has been identified for at least one. A dramatic structural change was demonstrated to occur at the interface between the ribosome and the membrane upon engagement of an apo B pause transfer sequence (Hegde and Lingappa, 1996). This change allowed a discrete, over 70 amino acid region of the nascent chain to be temporarily accessible to the cytosol, an environment not normally encountered by secretory proteins. Thus, pausing is a means by which at least the rate of translocation, the environment of the nascent chain, and the conformational state of the translocation apparatus (e.g., the ribosome-membrane junction) can be regulated.

In this study, we have sought to identify the regulatory component(s) of the translocon that affect translocational pausing. Using proteoliposomes reconstituted from fractionated components of the ER, we identify TRAM as a necessary component required for properly

assembling a translocationally paused nascent chain. Furthermore, we demonstrate that TRAM serves to limit the cytosolic exposure of paused secretory proteins to specified domains, preventing other regions of the nascent chain from being inappropriately revealed to the cytoplasm. These data identify a previously unappreciated feature of translocational pausing and demonstrate a novel regulatory role for TRAM. Thus, components of the translocon can serve the role of a translocational accessory factor to significantly modulate aspects of protein biogenesis distinct from translocation. The implications of regulated translocation and the potential consequences of its misregulation are discussed.

Results

Pausing Can Be Reconstituted

In order to identify putative regulatory components involved in translocational pausing, our first aim was to reconstitute some or all of these events in a system tractable to fractionation. We therefore examined whether pausing would occur in proteoliposomes reconstituted from solubilized total ER membrane proteins, an approach that was used to reconstitute translocation (Nicchitta and Blobel, 1990). Rough microsomal membranes (RMs) were washed with EDTA and high salt to remove peripheral membrane proteins (resulting in EDTA-extracted, O.5 M potassium chloride–washed rough microsomes [EKRMs]) and extracted with 0.8% cholate. The solubilized proteins were then reincorporated into vesicles by the removal of detergent to yield reconstituted microsomal membranes (rRMs). Assays of preprolactin (pPL) translocation across each of these membranes demonstrated that the rRMs, like RMs and EKRMs, were active in supporting transport into the lumen of the vesicles (Figure 1A). However, not all of the translocated chains in the rRMs underwent signal sequence cleavage, reflecting some incompleteness of this reaction in reconstituted membranes, as has been previously observed (Nicchitta and Blobel, 1990; Görlich et al., 1992).

To assay pausing in this same set of membranes, we examined translocation intermediates of PrI-pause, a construct in which a pause transfer sequence from apo B has been inserted into preprolactin (Hegde and Lingappa, 1996). In RMs and EKRMs, the majority of the PrI-pause translocation intermediate was accessible to proteinase K (PK), resulting in the digestion to a specific lower molecular weight fragment (species d; see Figure 1B). This indicated that these nascent chains had stopped translocation into a protease-protected environment at a discrete point, the ribosome-membrane junction apparently opened, and the remainder of the chain was synthesized into an environment that is accessible to PK. Similar results were observed with the rRMs, demonstrating the presence of paused nascent chains. In this case, two proteolytic fragments are observed following PK digestion, representing the signal sequence–cleaved and –noncleaved paused nascent chains (labeled species d and b, respectively), again reflecting the incomplete action of signal peptidase. Furthermore, because the size of the signal-cleaved protected fragment (species d) after PK digestion is the same in both rRMs and RMs, we conclude that the pause occurred at the same point in both membranes, causing the same domains to be accessible to the cytosol.

When translocation intermediates are treated with EDTA, the ribosome-nascent chain-translocon complex is disassembled. Because the paused nascent chain is situated in the translocon, straddling the cytosol and ER lumen, disruption of the translocation apparatus forces the nascent chain into one or the other compartment (Chuck and Lingappa, 1992). In RMs, a substantial number of the paused nascent chains restart translocation into the lumen following EDTA treatment (Figure 1B). By contrast, treatment of chains paused in EKRMs or rRMs with EDTA results in their movement to a compartment totally accessible to PK digestion (Figure 1B). Thus, although EDTA is able to abolish the translocationally paused state of the nascent chain in all three membrane preparations, only in RMs did they functionally restart translocation into the lumen.

This defect in forward translocation into the lumen of rRMs was found to be restored if lumenal proteins were incorporated into the vesicles during the reconstitution (data not shown; see Figure 1C). Furthermore, the paused chains that did not restart translocation into the lumen (in the absence of lumenal proteins) were found upon sedimentation of membrane to reside free in the cytosol (data not shown). Each of these findings demonstrating that pausing and restarting could be reconstituted in rRMs was verified on a second substrate in which we have observed translocational pausing. Rat BiP, truncated at codon 366, produces a translocation intermediate that is paused at a specific point in translocation (Figure 1C, lanes 1 and 2). Thus, proteolysis of this translocation intermediate yields a smaller protected fragment (species d), indicating that these chains were spanning the membrane and accessible to protease on the cytosolic side. However, because the chains are fully translocated into the lumen upon treatment with EDTA (Figure 1C, lane 3), these membrane-spanning chains must not have been integrated into the bilayer permanantly. Rather, they had paused translocation at a particular point, with subsequent domains synthesized into the cytosol. Assay of this substrate in rRMs lacking or containing lumenal proteins demonstrated that the pause could be reproduced in both membrane preparations, while the EDTA-mediated restarting only occurred in the presence of lumenal proteins (Figure 1C, lanes 4–9).

We conclude from the above data that translocational pausing and restarting are able to be reproduced in membranes reconstituted from fractionated components of the ER. Furthermore, it appears that different components (membrane proteins versus lumenal proteins) are required for these two steps. Not only do these results provide a manipulable experimental system in which to study translocational pausing, but they also provide a functional assay for one role of lumenal proteins in specialized aspects of regulated protein translocation. Whether lumenal proteins are involved in a broader range of functions in translocation remains unclear (Görlich and Rapoport, 1993; Nicchitta and Blobel, 1993; Brodsky et al., 1995). A schematic representation of translocational pausing showing the origin of the various species (a–d) generated after PK treatment of a

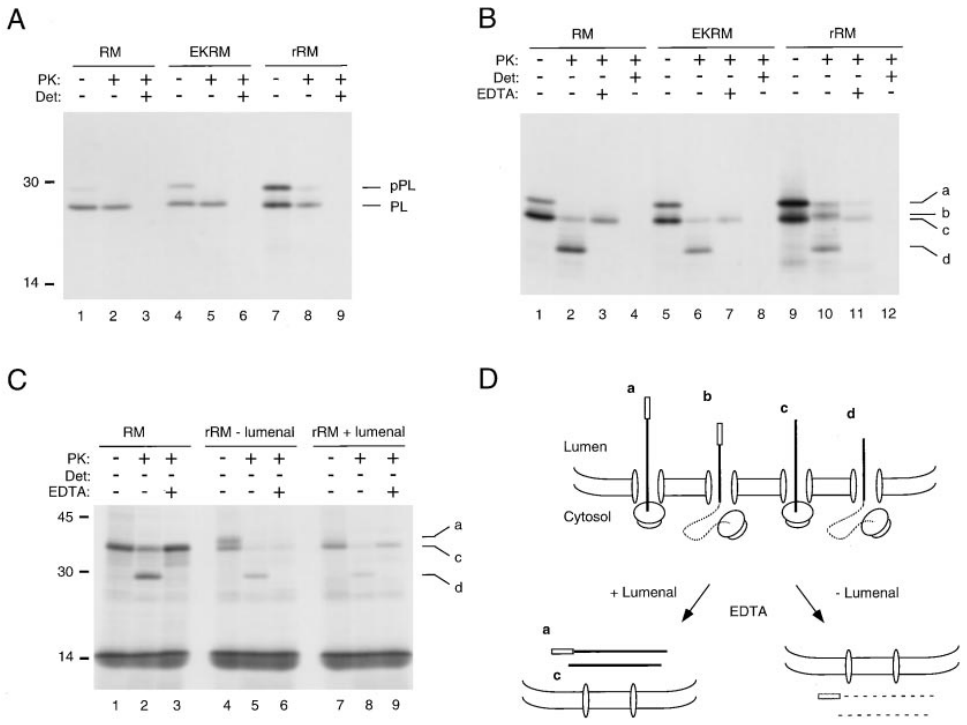

Figure 1. Translocational Pausing in Reconstituted Membranes

RM, EKRM, and rRM (prepared from a 0.8% cholate extract) were each used to assay translocation and pausing.

(A) Proteolysis assay for translocation of full-length prolactin in each membrane preparation. The positions of precursor (pPL) and processed prolactin (PL) are indicated.

(B) Assay for translocational pausing of Prl-pause. Following synthesis and assembly of the Prl-pause translocation intermediate in each membrane preparation, samples were divided and either left untreated or treated with 10 mM EDTA for 10 min at 25°C. They were subsequently analyzed by a protease protection assay to assess cytosolic exposure of portions of the nascent chain. The position of the major species remaining after PK digestion of the rRM sample are indicated by the letters a through d.

(C) Translocational pausing in BiP, truncated at codon 366, was assayed in RM or rRM prepared without or with lumenal proteins. Methodology and labeling of the figure was as in (B).

(D) The interpretation of the data in (B) and (C) is schematized, with the species a-d indicated. The signal sequence is arbitrarily drawn in the lumen for simplicity, although it may well reside in the translocon in the "loop" orientation. Species b and d are products after digestion of the cytosolically exposed portion (indicated by the dotted line) of a paused nascent chain that does and does not contain a signal sequence (open box). After EDTA treatment, chains are no longer paused and reside either in the lumen (if lumenal proteins were present in the membranes) or cytosol (where they are accessible to PK digestion).

paused intermediate is shown in Figure 1D. In subsequent experiments we focused on the Prl-pause substrate, with paused nascent chains being defined as those which, after PK treatment, yield species b or d.

To optimize the pausing activity, rRMs were reconstituted from membrane proteins extracted from EKRMs with varying concentrations of cholate from 0.35% to 0.8%. These reconstituted membranes were then assayed for pPL translocation and translocational pausing of Prl-pause. Optimal pausing was found in a different membrane preparation (made from the 0.8% detergent extract) than in the membrane preparation (made from the 0.5% detergent extract) that demonstrated optimal translocation of pPL (data not shown). This observation suggested to us that factor(s) in addition to the minimal components required for pPL translocation might be needed for reconstitution of pausing.

Uncoupling Pausing from Translocation

In order to more definitively determine whether translocational pausing requires factors in addition to Sec61 complex and SRP-receptor, we took advantage of the fact that neither of these components is glycosylated. This allows the preparation of reconstituted membranes that lack a significant proportion of proteins (i.e., glycoproteins) but still contain the minimal translocation components and thus support translocation (Görlich et al., 1992). Reconstituted membranes were prepared that either contained the full complement of proteins (rRMs), were depleted of Con A-binding glycoproteins (cRMs), or depleted but subsequently replenished with the glycoprotein fraction (cRMs + gp). Each of these membranes was then assayed for pausing and translocation.

As expected, all of the membrane preparations supported the translocation of pPL (Figure 2A). By contrast, the cRMs failed to translocate βL efficiently, presumably reflecting the requirement for the glycoprotein TRAM (Figure 2B). The cRMs also demonstrated a defect in the behavior of the Prl-pause substrate. The percent of synthesized chains that were found to be paused diminished significantly from ~16% to <3%. Thus, a substantial lesion in pausing is detected in membranes

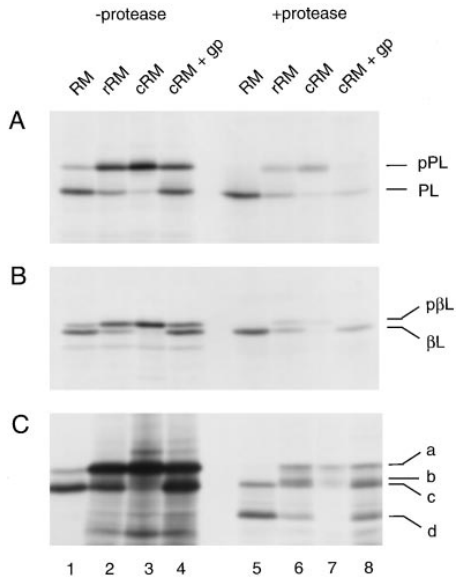

Figure 2. Effect of Glycoprotein Depletion on Translocational Pausing

A 0.75% deoxyBigCHAP extract was prepared, and a portion of it was depleted of glycoproteins. A portion of this glycoprotein-depleted extract was replenished with the glycoprotein fraction, and all three samples were reconstituted into proteoliposomes (to yield rRM, cRM, and cRM + gp, respectively). Each of these membrane preparations, along with RM, were assayed for pPL and βL translocation ([A] and [B], respectively), and pausing activity of Prl-pause (C). The positions of the precursor and processed products of prolactin and βL are indicated to the right of the gels, as are the positions of species a to d of the Prl-pause analysis.

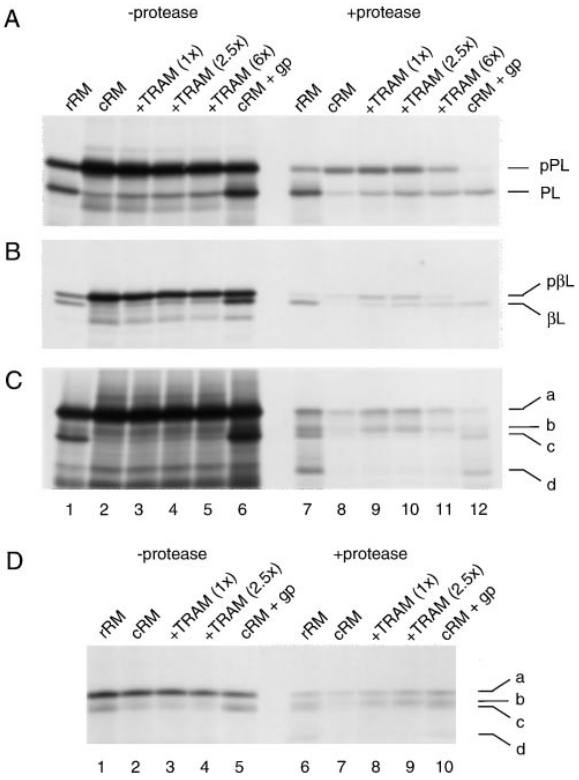

Figure 3. The Defect in Pausing Due to Glycoprotein Depletion Is Restored by TRAM

A 0.75% deoxyBigCHAP extract was used to prepare rRM, cRM, and cRM + gp as in Figure 2. In addition, portions of the glycoprotein depleted extract were replenished with purified TRAM at a concentration of 1, 2.5, or 6 equivalents per μl of extract (which was at 1 equivalent per μl) prior to reconstitution of proteoliposomes. Each of these membranes were then used to assay pPL and βL translocation ([A] and [B], respectively) and pausing activity of Prl-pause (C) exactly as in Figure 2. In (D) the membranes indicated were assayed for pausing activity as in (C), except that prior to proteolysis the membranes were isolated by sedimentation. This allowed the examination of only those chains which had targeted to the membrane, excluding the remainder of the chains from the analysis.

which still retain translocation activity (of at least pPL), indicating that certain aspects of translocational pausing require component(s) in addition to the minimal translocon.

TRAM Is Required to Assemble a Paused Nascent Chain

Initial efforts at characterizing the fractionation properties of the translocational pausing activity showed it to consistently cofractionate with TRAM. For example, the detergent extraction (data not shown) and glycoprotein fractionation properties (Figure 2) of pausing activity paralleled the translocation activity of a TRAM-dependent protein. Similar results were obtained with other detergents (DeoxybigCHAP and BigCHAP; data not shown). Furthermore, TRAM appears to play a role in facilitating the formation of a tight ribosome-membrane junction early in translocation (Voigt et al., 1996). Thus, we thought it may similarly be involved in some aspect of pausing, which also involves modulation of the ribosome-membrane junction.

To test this hypothesis directly, we replenished the cRMs with purified TRAM and determined whether the defect in pausing was restored. Figure 3C demonstrates that replenishment of cRMs with TRAM increases the percent of paused chains to a level similar to replenishment with total glycoproteins. In the case of TRAM, the paused chains all contain a signal sequence (reflected in an increase in species b, see lanes 9–11 of Figure 3C). By comparison, replenishment with total glycoproteins restored pausing as well as signal sequence cleavage, resulting in an increase of species d (Figure 3C, lane 12). Notwithstanding these differences in signal sequence cleavage, the extent of replenishment of pausing again paralleled the extent of translocation activity of βL (compare Figure 3B to Figure 3C). These data suggest that TRAM is the main or only component of the glycoprotein fraction that was involved in assembling a paused nascent chain at the translocon. However, it remains possible that other glycoprotein components may enhance or otherwise modulate pausing in subtle ways not detected by the assays employed.

In these reconstituted membranes, a relatively small proportion (~10%–15%) of the total synthesized chains actually are accounted for following protease digestion. This was likely due to the saturation of the functional translocation sites on these membranes with the substrate. The excess chains would then remain in the cytosol, having never engaged the translocation machinery.

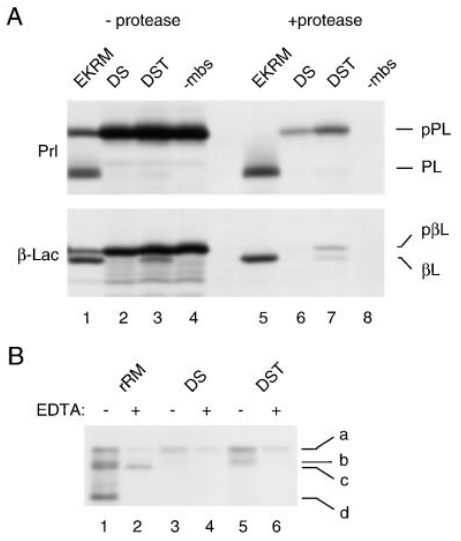

Figure 4. Translocational Pausing in Proteoliposomes Containing Purified Components

Purified SRP-receptor and Sec61 complex, without (DS membranes) or with TRAM (DST membranes) were reconstituted with pure phospholipids into proteoliposomes. These were compared to rRMs with respect to pPL and βL translocation (A). Aliquots of each sample before and after protease digestion were analyzed separately. The "−protease" lanes demonstrate that the amount of translation in each sample was equivalent, while the "+protease" lanes allow a comparison of the relative translocation efficiencies between samples. Note that because the "−protease" and "+protease" samples were analyzed separately, they are not directly comparable. In (B) the same membranes were tested for pausing activity. In addition, a portion of each Prl-pause translocation product was treated with EDTA prior to proteolysis (as in Figure 1B) to verify that the pause would be disrupted.

This was confirmed directly by isolating the properly targeted chains by sedimentation of the microsomal membranes and performing the proteolysis assay for pausing on this sample. As expected, identical results were obtained when only the membrane-targeted chains were examined (Figure 3D) as when the total translation reaction was analyzed (Figure 3C). Thus, although total translocation efficiency is diminished in the reconstituted membranes, these results clearly establish a functional role for TRAM in assembling a paused nascent chain.

Of the nonglycoproteins in the rRMs, only Sec61 complex and SRP-receptor are required for translocation. We next asked whether these are also the minimal requirements for pausing (in conjunction with TRAM) or if there might be other requirements. For these experiments, purified components of the ER membrane were reconstituted with pure phospholipids into proteoliposomes (Görlich and Rapoport, 1993) that were then tested for translocation and pausing activity. As demonstrated previously, proteoliposomes containing only SRP-receptor, also termed docking protein, and Sec61 complex (DS membranes) were able to translocate pPL, but not βL (Figure 4A). The additional inclusion of TRAM in the proteoliposomes (DST membranes) largely restored translocation of βL and modestly stimulated pPL translocation.

Assays of translocational pausing in these membranes demonstrated that DST, but not DS, membranes were able to assemble paused nascent chains (Figure 4B). The size of the PK protected fragment of paused chains (species b) suggests that the DST membranes assemble paused nascent chains indistinguishable from those assembled in rRMs (with the exception of signal sequence cleavage, which does not occur in DST membranes due to absence of the signal peptidase complex). In order to further verify that the membrane-spanning chains were paused, an aliquot was treated with EDTA. As expected for paused chains, they were no longer found spanning the membrane. Rather, they appeared to have moved to a location in the cytosol, where they are fully suceptible to subsequent PK digestion. Thus, it appears that TRAM is the only component, in addition to the minimal translocation machinery, that is absolutely required for pausing. By contrast, restarting of a pause into the ER lumen likely requires additional factor(s) of the ER lumen (see Figure 1C). The identity of the specific lumenal proteins involved and their role in the biogenesis of pause-containing proteins remain to be determined.

Cross-Linking of TRAM to Paused Nascent Chains

Until these studies, the only functional role for TRAM during secretory protein biogenesis was at an early stage of translocation (Görlich et al., 1992), when it is required for certain proteins to be inserted properly at the translocon (Voigt et al., 1996). This was supported by cross-linking studies which demonstrated that TRAM was adjacent to the nascent chain early in translocation (Görlich et al., 1992; High et al., 1993; Mothes et al., 1994). At later points during secretory protein translocation, cross-links to Sec61α, but not TRAM, were observed, suggesting that TRAM had left the immediate proximity of the nascent chain (Mothes et al., 1994). Given that we had now demonstrated a functional role for TRAM during pausing later in translocation, we wondered whether a physical proximity to the nascent chain could also be detected.

Nascent chain cross-linking, followed by immunoprecipitation with antibodies to either Sec61α or TRAM, was used to monitor the environment of a translocating chain at various stages of its biogenesis (Figure 5). We examined three points during the biogenesis of the Prl-pause protein: (i) early in translocation, when a role for TRAM in signal sequence–dependent insertion of a nascent chain into the translocon had been demonstrated (Voigt et al., 1996); (ii) a subsequent point at which the initial interaction with TRAM was completed, but before synthesis of the pause transfer sequence; and (iii) a point at which the chain was translocationally paused. Figure 5A shows a schematic depiction of these points of truncation used in the cross-linking experiment.

We found that at point (i), the majority of cross-links were to TRAM and not Sec61α (Figure 5B, lanes 1 and 2). At point (ii), the cross-links to TRAM had largely disappeared, with concomitant appearance of cross-links to Sec61α (Figure 5B, lanes 3 and 4). Finally, at point (iii), substantial cross-links to both Sec61α and

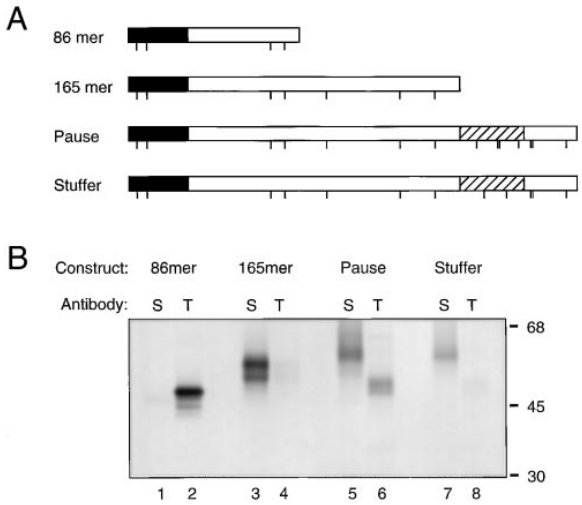

Figure 5. Cross-Linking of Paused Nascent Chains to TRAM

(A) The pPL-86-mer, pPL-165-mer, Prl-pause, and Prl-stuffer are diagrammed. The black bar denotes the signal sequence and the striped bar represents the pause or stuffer sequence. The hatch marks show the relative positions of the lysine residues that can contain the nascent chain cross-linker.

(B) Nascent chain translocation intermediates of pPL-86-mer, pPL-165-mer, Prl-pause, and Prl-stuffer were prepared with the TDBA-lysyl nascent chain cross-linker. Following cross-linking with UV irradiation, samples were divided into two equal aliquots and immunoprecipitated with affinity purified antibodies against Sec61α (S) or TRAM (T), as indicated above each lane, prior to analysis by SDS-PAGE and autoradiography. Equal amounts of translation products were analyzed in each lane, although the amount of translated substrate was about 4-fold higher for the 86-mer and 165-mer than the pause and stuffer substrates. The heterogeneity of migration seen in some of the cross-links is likely to be due to cross-links from different positions in the nascent chain.

TRAM were observed (Figure 5B, lanes 5 and 6). The cross-links to TRAM, but not Sec61α, were specific to the pause, since the TRAM cross-links largely disappeared (Figure 5B, lanes 7 and 8) when the pause transfer sequence was replaced by an irrelevant stuffer (and thereby abolishing pausing activity; Hegde and Lingappa, 1996). Thus, the TRAM protein is both functionally required during translocational pausing and is in close proximity to the paused nascent chain. Furthermore, TRAM is not always found adjacent to a translocating chain but moves to a position near the translocating chain only when a pause transfer sequence is engaged.

TRAM Regulates Nascent Chain Exposure to the Cytosol

As noted above, we consistently observed that loss of pausing activity upon glycoprotein depletion resulted in both a decrease in the number of paused chains as well as a decrease in the total number of protease-protected nascent chains (i.e., the sum of species a–d). This apparent "loss" in translocation efficiency of Prl-pause appeared to be of approximately the same magnitude as the loss of translocation efficiency of βL (compare Figure 2B to Figure 2C). The defect in βL translocation has been shown to be due to the absence of the glycoprotein TRAM, which is required in a signal sequence–dependent manner early in translocation (Voigt et al., 1996). However, the defect in Prl-pause translocation was at first puzzling, given that the pPL signal sequence is not TRAM-dependent (Görlich et al., 1992), and pPL translocation did not diminish significantly in these same membranes (Figure 2A).

It appeared that chains that were paused in the presence of TRAM were simply unaccounted for in its absence and not reflected in an increase of nonpaused chains (see for example, Figure 2C, lane 6 versus lane 7). Thus, in the absence of TRAM, the ribosome-membrane junction did not simply remain sealed upon emergence of a pause transfer sequence (which would manifest as all chains being fully protected from PK digestion). Rather, the chains that would have been paused in the presence of TRAM were now in a location that was exposed to cytosolically disposed PK.

One possible explanation was that, in the absence of TRAM, the ribosome-nascent chain complex of a paused substrate might dissociate from the membrane and thus not be scored as translocated. This seemed plausible, since it was shown that the ribosome-membrane junction is significantly altered during a translocational pause (Hegde and Lingappa, 1996); if TRAM served some role in maintaining ribosome binding, then its absence might have this effect. Alternatively, perhaps the junction still opens in the absence of TRAM, but instead of a limited, discrete portion of the nascent chain becoming exposed to the cytosol, the exposure is flexible and more extensive. In this case, PK treatment would not generate a discrete protected fragment, but a rather heterogeneous set of products that might be difficult to detect by SDS-PAGE. Thus, it would appear as if the number of paused chains had decreased, but they would not be accounted for by an increase in nonpaused chains.

In order to distinguish between these possibilities, we first determined whether the unaccounted chains in cRMs had detached from the membrane or were still tightly bound to the translocon. Following the assembly of Prl-pause translocation intermediates in various reconstituted membranes, one aliquot was subjected to PK digestion as above, while an equal aliquot was analyzed for membrane binding of the nascent chain intermediates. The number of chains that could be accounted for following PK digestion was compared to the number that were found tightly docked at the translocon (Figure 6A). For a nonpaused translocation intermediate (the 86-mer of pPL), no discrepancy was observed between the proteolysis and membrane binding data (Figure 6A, striped bars). By contrast, analysis of the Prl-pause translocation intermediate (Figure 6A, black bars) revealed that in cRMs only about 50% of the membrane-bound nascent chains could be accounted for as protected from proteolysis. In membranes that contained TRAM (RMs, rRMs, and cRMs + TRAM) and thus did not show a defect in pausing, a discrepancy between proteolysis and membrane binding was not observed.

These data suggested that, in the absence of TRAM, chains that should be paused are still docked at the membrane but accessible to protease in such a way as to not generate the signature protease protected fragment of a properly paused nascent chain. Instead

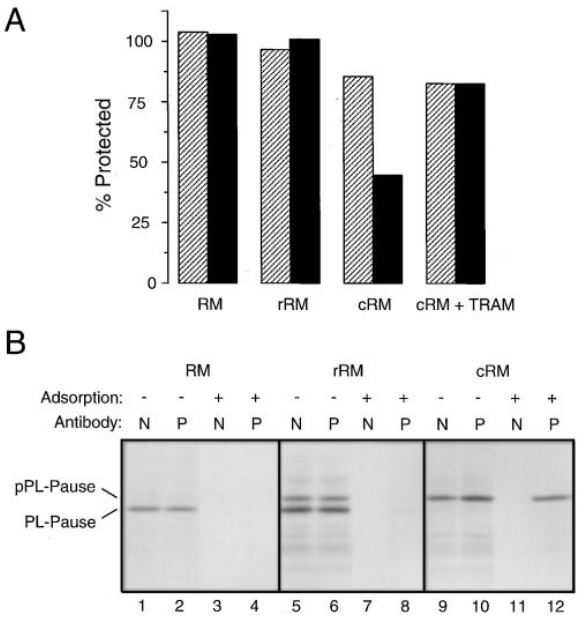

Figure 6. Inappropriate Exposure of Paused Nascent Chains to the Cytosol

(A) Translocation intermediates of pPL-86-mer (striped bars) and Prl-pause (black bars) were assembled in each of the membrane preparations indicated below the graph and divided into two equal aliquots. One was assayed for translocation by protease protection (as in previous figures) and the other for stable membrane binding as judged by flotation with the membranes in the presence of high salt. The ratio of the number of chains accounted for after digestion by PK to the number floated with the membranes, multiplied by 100, is graphed. For the Prl-pause substrate, the number of protease protected chains was determined by summing the amount in each of species a–d (see Figure 1D). The percent of translated chains that floated with the membranes differed between the membrane preparations and in the experiment shown above were approximately 80%, 50%, 20%, and 20% for RM, rRM, cRM, and cRM + TRAM, respectively. The remainder of chains presumably did not target to the membrane.

(B) Translocation intermediates of Prl-pause were assembled in each of the membrane preparations indicated above the gels and incubated with either antibodies to prolactin (P) or nonspecific antibodies (N). The membranes were then isolated by floatation through a high salt sucrose cushion and divided into unequal aliquots. One-twentieth of the sample was analyzed directly ("−" adsorption), while the remainder was solubilized under nondenaturing conditions and immune complexes captured with immobilized Protein A ("+" adsorption). Only the cRMs incubated with specific antibodies to prolactin resulted in substantial immunoadsorption of the translocation intermediate (lane 12), demonstrating that the prolactin domain was exposed to the cytosol in these membranes.

of a discrete domain of the chain being exposed to the cytosol, other portion(s) that should be protected from protease are available for digestion. To demonstrate this directly, we determined whether regions of a properly paused nascent chain that are not exposed to antibodies in the cytosol become exposed in the absence of TRAM. When the Prl-pause substrate is translocationally paused, the entire N-terminal prolactin domain is protected from PK digestion (see Figure 1) and, as expected, is not accessible to antibodies in the cytosol (Figure 6B, lanes 1–4 and 5–8). However, in cRMs this domain is found to be inappropriately accessible to cytosolic anti-prolactin antibodies (Figure 6B, lanes 9–12). Thus, the defect in pausing in these membranes is not that the ribosome-membrane junction fails to open, but at a subsequent step, instead of a discrete domain of the nascent chain becoming exposed to the cytosol, the exposure is excessive and unregulated.

Discussion

Our previous studies demonstrated that translocational pausing involves structural reorganization of the ribosome-membrane junction and perhaps the translocation channel itself (Hegde and Lingappa, 1996). The consequences of these changes for the nascent chain were shown to be significant, allowing large but discrete domains of the chain to be temporarily exposed to the cytosol. These data not only illustrated that the translocon was a dynamic structure but suggested that at least some aspects of secretory protein translocation may be amenable to regulation. The present studies were undertaken to define particular stages of translocational pausing that are regulated by the translocon and to identify the specific components that mediate this regulation.

We have now identified a requirement for the membrane glycoprotein TRAM in regulating the proper translocational pausing of a secretory protein. Surprisingly, the point of regulation mediated by TRAM is not one that had been anticipated. Rather than governing the opening of the ribosome-membrane junction, TRAM is recruited to the translocon at a subsequent stage to modulate the extent of nascent chain exposure to the cytosol. The data indicate that one role of TRAM may be to prevent cytosolic exposure of already translocated domains of the nascent chain. Thus, in the absence of TRAM, translocational pausing becomes a far more promiscuous event than would be the case otherwise.

The Many Faces of TRAM

Prior to these studies, two different roles for the TRAM protein, one in translocation and another in membrane integration, had been proposed. Functional studies employing proteoliposomes containing defined components of the ER membrane had implicated TRAM as being necessary for the translocation of some, but not other, proteins (Görlich et al., 1992). Subsequently, it was shown that this requirement was dependent on the structure of the signal sequence of the substrate (Voigt et al., 1996). Proteins containing TRAM-dependent signal sequences did not become properly inserted into the translocation site in the absence of TRAM. Cross-linking studies that showed that nascent chains, and more specifically the N-terminal domain of signal sequences, contacted TRAM only during the early phases of translocation supported the conclusions of the functional experiments (High et al., 1993; Mothes et al., 1994).

The second proposed role of TRAM was based on experiments demonstrating that TRAM could be cross-linked to a transmembrane segment during multiple discernible steps of its integration into the bilayer (Do et al., 1996). Although a functional requirement in this process has not yet been demonstrated, it was speculated that TRAM may serve to position or temporarily retain a

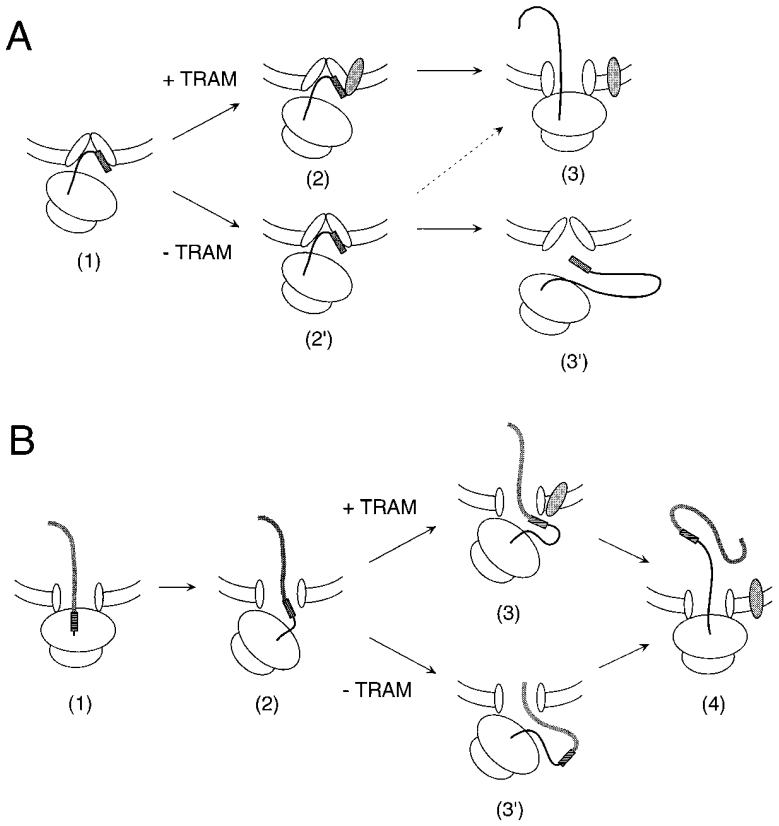

Figure 7. Model of TRAM Action during Translocation

Comparison of the suggested roles of TRAM during the early (A) and later (B) stages of translocation across the ER membrane. The cartoons depict the series of events occurring in the presence and absence of TRAM (represented by the shaded oval in the membrane). Sec61 complex is represented by the white ovals in the membrane. The signal sequence (in [A]) and pause transfer sequence (in [B]) are represented by the shaded box in the nascent chain. In (B) the domain that normally is not exposed to the cytosol (in the presence of TRAM) is represented by the thickened, gray portion of the nascent chain. See text for details.

membrane segment within specific sites inside the translocon. In conjunction with the Sec61 complex, TRAM may thus facilitate a concerted movement of transmembrane segments from an aqueous to a hydrophobic environment. The current studies provide an altogether different role for TRAM in regulating secretory protein exposure to the cytosol during translocational pausing. While each of these putative functions seems disparate, features shared between the currently proposed role in pausing with each of the previously suggested roles suggest some common mechanisms.

Figure 7 shows models depicting the proposed roles of TRAM at the signal sequence–dependent (A) and pause transfer sequence–dependent (B) phases of translocation. In both scenarios, a topogenic sequence causes the recruitment of TRAM to the site of translocation (Figures 7A[2] and 7B[2]). For the signal sequence–dependent role of TRAM, a transition to a tightly docked ribosome-nascent chain-translocon complex is somehow facilitated (Figure 7A[3]). In the absence of TRAM (Figure 7A[2′]), this productive insertion is usually not achieved, resulting in the failure of the chain to initiate transfer across the membrane (Figure 7A[3′]). During translocational pausing, the absence of TRAM results in an analogous situation where the nascent chain is not properly situated with respect to the translocon (Figure 7B[3] versus Figure 7B[3′]). Instead of failing to be translocated, however, the consequence at this later stage of translocation is the excess exposure of the nascent chain. It should be noted that the unregulated exposure of the nascent chain to the cytosol that occurs in the absence of TRAM may well be dependent upon the simultaneous absence of lumenal proteins. That is, in the absence of TRAM, the nascent chain might be prevented from dislocation by lumenal proteins. Regardless of this caveat, it seems clear that TRAM facilitates some aspect of proper chain positioning at the translocon. This model is analogous to the proposed role of TRAM in integration, namely one of holding or positioning a topogenic sequence (in this case a transmembrane segment) at a particular site within the translocon.

A second event, translocon gating, may be common to each of the proposed roles of TRAM. In the signal sequence–dependent phase, proper docking of the ribosome-nascent chain complex onto the translocon (facilitated by TRAM) results in the subsequent opening of a lumenal gate (Crowley et al., 1994). In the case of membrane integration, both the lumenal and cytosolic gates of the translocon are tightly regulated during the integration process (Liao et al., 1997). And finally, during pausing, TRAM serves to prevent domains that have already translocated from having access to the cytosol once the ribosome-membrane junction (which is presumably the cytosolic gate) has been opened. Taken together, it is tempting to suggest that TRAM may regulate or be a component of the translocon gate(s) that prevent mixing of lumenal and cytosolic contents during each of these crucial phases of translocation. Perhaps TRAM serves such a function by modulating translocon pore size or architecture. In the absence of TRAM, the translocon may be unable to precisely position certain domains of a nascent chain with respect to the lumen or cytosol. Further studies monitoring the gating events or translocon size in reconstituted proteoliposomes will be required to address these issues of TRAM function.

The Possibilities for Translocational Regulation and Disregulation

A priori, the extent of cytosolic exposure of the nascent chain during translocational pausing could have been determined entirely by sequences encoded within the substrate. Thus, once the ribosome-membrane junction opened, features of the subsequently translated domain, such as secondary structure folding, would determine what became exposed. While this still may be the case in some instances, the observation in this study that domain exposure can be modulated without changing the sequence of the translating substrate suggests a different scenario. In this model, the initial event would be the recognition of certain sequence features of the nascent chain (such as the pause transfer sequence) by particular accessory components of the translocon (such as TRAM). However, the ensuing events would not be fixed. Instead, a *combination* of parameters, which might include sequences within the nascent chain or rate of activity of accessory translocon components, would determine the eventual outcome of a translocational pause.

This model allows significantly more flexibility than one in which all of the information is "hard wired" into the sequence of the substrate. One advantage of this flexibility is that the sequence of the segment to be exposed is not constrained in any way. This would allow for numerous *types* of domains to be exposed for unrelated purposes. Furthermore, various parameters, such as extent of nascent chain exposure or length of time a domain is exposed, could be modulated in *trans* by the action of machinery. The machinery, in this case TRAM, might be subject to changes in its activity depending on the metabolic state of the cell.

How might secretory proteins use this type of regulation in their biogenesis? In the case of apo B, a protein whose expression is regulated almost exclusively posttranslationally (Borchardt and Davis, 1987), the possibilities are many and varied. This unusual molecule is enormous, undergoes poorly understood modifications, and is assembled into lipoprotein particles, features which are likely to be carefully orchestrated in the cell (Dixon and Ginsberg, 1993; Yao and McLeod, 1994). While it is interesting to speculate generally about the role of pausing in the numerous aspects of apo B biogenesis, it is perhaps more instructive to consider the possibilities of how one specific event might be regulated.

The secretion of apo B containing lipoprotein particles appears to largely involve the ER degradation pathway utilizing the cytosolic proteasome (Yeung et al., 1996; Fisher et al., 1997). This pathway of degradation is likely to employ components of the translocation machinery, as has been suggested for other substrates (Wiertz et al., 1996; Pilon et al., 1997). Thus, it is intriguing that one of the regulated events in apo B biogenesis involves transient exposure of the nascent chain to the cytosol during translocation. If the extent of this exposure were regulated in vivo (perhaps by modulating the activity of TRAM), as we were able to achieve biochemically in vitro, one might envisage this event as a key regulatory step in apo B secretion.

Under physiologic conditions requiring efficient lipoprotein secretion, TRAM activity may be increased to limit any cytosolic exposure and thereby preventing significant accessibility to the proteasome machinery. Conversely, conditions warranting depressed secretion of apo B may work by ultimately inhibiting TRAM activity and thereby exaggerating the translocational pause. This might allow some domains of the nascent chain to reside in the cytosol for extended periods, thereby resulting in its eventual degradation. Such notions are supported by the observation that metabolic conditions under which apo B is being actively degraded by the cell are accompanied by finding regions of this molecule accessible in the cytosol (Rusinol et al., 1993; Du et al., 1996). The significance of such a correlation, and its relationship to the translocational regulation mediated by TRAM, remain to be determined.

Experimental Procedures

Materials

Rabbit reticulocyte lysate (RRL) and dog pancreatic rough microsomal membranes were prepared and used as described previously (Hegde and Lingappa, 1996, and references therein). Polyclonal antibodies to prolactin were from United States Biochemical. Affinity purified antibodies to Sec61α and TRAM were prepared and used as previously described (Mothes et al., 1994). PK was from Merck and was prepared as a 10 mg/ml stock in 10 mM Tris (pH 8), predigested for 10 min at 37°C, and stored at −80°C. The cholate and deoxyBigCHAP used in the preparation of detergent extracts from EKRMs were from Sigma and Calbiochem, respectively, and prepared as 10% w/v stocks. Immobilized Conconavalin A (Con A) was from Pharmacia. All other reagents were of the highest quality available commercially.

Plasmids, Transcription, Translation, and Proteolysis

Bovine pPL, βL, Prl-pause, and Prl-stuffer constructs have been described previously (Hegde and Lingappa, 1996). Prl-pause and Prl-stuffer constructs encode the first 165 amino acids of pPL, followed by a 30 amino acid pause transfer sequence from apo B (amino acids 261–290) or an irrelevant 30 amino acid stuffer, and ending with 26 amino acids from apo B (amino acids 304–329), after which is a Stu1 site. Both of these constructs were truncated at Stu1 before transcription to generate mRNA used to assemble the paused and nonpaused translocation intermediates. To generate message coding for pPL-86-mer and pPL-165-mer, the Prl-pause construct was truncated at Pvu2 and EcoR1, respectively, prior to transcription. The Rat BiP coding region (Munro and Pelham, 1986), engineered into the sp64 vector, was truncated at codon 366 with EcoR1 prior to transcription. Transcription by SP6 polymerase, and translation in RRL in the presence of microsomal membranes and [^{35}S]methionine (ICN) was as previously described (Hegde and Lingappa, 1996). All translation reactions were performed at 25°C for 40 min, and proteolysis was with PK (0.5 mg/ml) at 0°C for 30 min.

Membranes and Proteoliposomes

Reconstitutions from crude detergent extracts were performed by minor modifications of previously described procedures (Nicchitta and Blobel, 1990; Görlich et al., 1992). EKRMs (prepared as in Walter and Blobel, 1983), were resuspended in extraction buffer (350 mM KAc, 50 mM HEPES, 12 mM $MgAc_2$, 15% glycerol [v/v], and 5 mM 2-mercaptoethanol) at 1 equivalent per μl (see Walter and Blobel, 1983). 10% w/v detergent (either cholate or deoxyBigCHAP) was added to the appropriate final concentrations as described in the figure legends. After 15–30 min on ice, particles larger than 30S were sedimented and the soluble proteins, representing the detergent extract, were reserved on ice. In some experiments, glycoproteins were depleted by incubation at 4°C, with gentle mixing, for 12 hr with 0.2 vol of packed Con A sepharose. For glycoprotein replenishment, elution from Con A was carried out at room temperature by incubation for 12 hr with EB containing 0.5 M methyl-α-D-mannopyranoside. The eluted glycoproteins were precipitated by addition

of polyethylene glycol (PEG 6000) to 15% w/v, sedimented in a microcentrifuge for 10 min, and the precipitated proteins dissolved in the glycoprotein depleted detergent extract. In other experiments, lumenal proteins were prepared by adding digitonin to 0.25% w/v to RMs (resuspended at 1 equivalent per μl in 50 mM HEPES [pH 7.4], 0.25 M sucrose, and 1 mM DTT). Following incubation on ice for 15 min, the membranes were sedimented and the supernatant, containing the lumenal proteins, was adjusted to 15% w/v PEG 6000. The PEG-precipitated lumenal proteins were sedimented in a microcentrifuge for 10 min and dissolved in one-fifth the original volume of a cholate extract containing membrane proteins. Reconstitution was achieved by incubation with Biobeads SM2 (30–40 μg per 100 μl of cholate extract or 50–75 μg per 100 μl of deoxyBigCHAP extracts) for 12 hr at 4°C, after which the fluid phase was separated and diluted 5-fold with ice-cold distilled water, and the proteoliposomes sedimented by centrifugation at 75,000 rpm for 15 min in the TL100.3 rotor. They were resuspended at approximately five times the initial concentration in 100 KAc, 50 mM HEPES (pH 7.4), 1.5 mM $MgAc_2$, 0.25 M sucrose, and 1 mM DTT. Reconstitutions from purified components (Figure 4) were performed exactly as described previously (Görlich and Rapoport, 1993).

Membrane Flotation Studies

Following translation, aliquots of the samples were adjusted to 2 M sucrose, 500 mM KAc, 50 mM HEPES (pH 7.6), and 5 mM $MgAc_2$ (final volume of 50 μl) and overlayered with 50 μl of 1.8 M sucrose and 50 μl 0.25 M sucrose in the same buffer. Following centrifugation at 100,000 rpm for 60 min in a TLA100 rotor, 80 μl was removed from the top (containing the floated membranes) and an aliquot analyzed by SDS-PAGE and autoradiography.

Immunoadsorption of Nascent Chains

Following translation, the samples were incubated with the appropriate antibodies for 60 min at 4°C. The microsomal membranes were then isolated by flotation as described above (but scaled up 5-fold and centrifuged in the TL100.2 rotor at 100,000 rpm for 100 min). Each sample was supplemented with 1 ml TXSWB (1% Triton X-100, 100 NaCl, 50 mM Tris (pH 8.0), 10 mM EDTA) and 10 ml immobilized protein A (BioRad) and incubated for 60 min with overhead mixing. The beads were washed four times with TXSWB and the immune complexes solubilized in 1% SDS and 0.1 M Tris (pH 8.9) prior to analysis by SDS-PAGE.

Nascent Chain Cross-Linking

Synthesis of TDBA-lysyl incorporated nascent chains and subsequent UV cross-linking was performed as described before (Jungnickel and Rapoport, 1995) with the following modifications. Translations were performed in reticulocyte lysate, and microsomal membranes were isolated by centrifugation and resuspended in the original translation volume of 0.25 M sucrose, 50 mM HEPES (pH 7.6), and 1 mM DTT prior to UV irradiation for 5 min on ice. Samples were then denatured by adjusting to 1% SDS and heating to 100°C prior to subsequent immunoprecipitation analysis.

Miscellaneous

SDS-PAGE was performed using 15% acrylamide gels that were either dried directly or fluorographed with Enhance (Dupont) as directed by the manufacturer, prior to visualizing the radioactive proteins by autoradiography. Immunoprecipitations were done as described previously (Mothes et al., 1994). Quantitation of autoradiograms was performed following the digitization of the image using an AGFA flatbed scanner and Adobe Photoshop software. Bands were occasionally excised from the gel and the radioactivity quantitated by liquid scintillation counting to verify that quantitation of the computer image was accurate and linear.

Acknowledgments

We would like to thank W. Mothes for preparation of the Sec61α and TRAM antibody reagents and advice regarding their use. This work was supported by grants HL 45480 and AG 02132 to V. R. L. and a Medical Scientist Training Program grant to R. S. H.

Received November 14, 1997; revised January 23, 1998.

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