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Sequence-Specific Alteration of the Ribosome–Membrane Junction Exposes Nascent Secretory Proteins to the Cytosol

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Summary

Tight docking of the ribosome at the translocation channel ensures that nascent secretory proteins are shielded from the cytoplasm during transfer into the endoplasmic reticulum. Discrete pause transfer sequences mediate the transient stopping of translocation in certain proteins. Here we show that during a translocational pause, the junction between the ribosome and translocation channel is opened, exposing the nascent chain to the cytosol. While transient, this opening is sufficient to demonstrate macromolecular interactions between the translocating chain and molecules added to the cytosol, such as antibodies and site-specific proteases. Moreover, this opening is accompanied by alterations in the proteins that neighbor the nascent chain. These results demonstrate that specific sequences within a translocating nascent chain can elicit dramatic and reversible structural changes in the translocation machinery. Thus, the translocon is dynamic and can be regulated.

Introduction

The general mechanisms by which the simplest secretory proteins are targeted to and translocated across the mammalian endoplasmic reticulum (ER) membrane have been elucidated to a large degree (reviewed by Rapoport, 1992; Siegel, 1995). Once the ribosome is targeted and docked securely at the aqueous translocation channel, the nascent chain is cotranslationally translocated across the ER membrane. Cross-linking studies have demonstrated that nascent secretory chains are in the proximity of several integral membrane proteins (Wiedmann et al., 1987; Kellaris et al., 1991; Görlich et al., 1992). The purification and subsequent reconstitution of these membrane proteins into lipid vesicles has demonstrated crucial roles for a subset of these proteins in translocation.

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). In contrast, the translocating chain-associated membrane protein was required or stimulatory for the translocation of some but not other secretory proteins (Görlich et al., 1992; Görlich and Rapoport, 1993). Still other membrane proteins, such as the translocon-associated protein complex, can be cross-linked to nascent secretory proteins, but their role in translocation remains to be elucidated (Wiedmann et al., 1987). In addition to these membrane proteins, several other protein complexes are in close proximity to, and in many cases interact with, nascent secretory chains. These include the signal peptidase complex (Evans et al., 1986), glycosyltransferase complex (Kelleher et al., 1992), and various resident ER proteins (e.g., BiP, GRP94, protein disulfide isomerase, and calnexin) thought to act as molecular chaperones (reviewed by Gething and Sambrook, 1992; Bergeron et al., 1994). These proteins and protein complexes, present at one time or another at the site of translocation, are proposed to be members of a large structure generally termed the translocon.

The architectural organization of the various proteins in the translocon and their juxtaposition with the machinery of protein synthesis remain to be fully elucidated. Sec61α is thought to be the major component of the aqueous channel that forms the central core of the translocon (Mothes et al., 1994). Furthermore, the inability of iodide ions supplied from the cytosolic side to quench fluorophores incorporated into secretory nascent chains in the translocation channel argues strongly for the presence of a tight seal between the ribosome and the translocon (Crowley et al., 1994). This interpretation is supported by the observations that Sec61α is protected from proteolysis when ribosomes are bound to the membrane (Kalies et al., 1994), and that the C-terminal 70–90 amino acid residues of nascent secretory proteins are protected from proteases by the ribosome and translocation channel in detergent-solubilized rough microsomes (Matlack and Walter, 1995). Thus, it is thought that nascent secretory proteins occupy a continuous channel that spans from the peptidyl transferase center within the ribosome to the ER lumen, and that they are completely shielded from molecules in the cytosolic environment. However, these conclusions were mainly drawn from data studying early translocation intermediates of simple model secretory proteins. It remained to be determined whether during the biogenesis of more complex secretory proteins, the translocon was more dynamic; for example, to accommodate cotranslational modifications or folding.

Previously, the biogenesis of apolipoprotein B (apo B) was demonstrated to be unusual in that its translocation 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 (PT) sequences (Chuck and Lingappa, 1992). However, it has not been clear whether translocational pausing is due to *cis*-acting effects of PT sequences on the nascent chain (e.g., primarily affecting nascent chain folding but not altering the translocation machinery per se), or whether PT sequences, like signal sequences, interact with the translocation machinery to cause translocational pausing.

In this study, we have examined both the architectural organization of the ribosome–translocon junction as well as the environment surrounding the nascent chain during the translocation of PT sequence–containing proteins. We find that during translocational pausing, dramatic structural changes occur at the interface between

the ribosome and the membrane. These changes allow large regions of the nascent secretory protein to be temporarily accessible to the cytosolic environment. This is in marked contrast with the translocation of a simple secretory protein, which is always well shielded from the cytosol. Furthermore, a translocationally paused nascent chain can be cross-linked to membrane proteins that are not seen to cross-link with a matched but nonpaused nascent chain. Taken together, our data support a model in which the translocon is dynamic both in its interaction with the translating ribosome and with respect to the proteins adjacent to the nascent chain.

Results

Experimental Design

Fully assembled translocation intermediates containing nascent secretory proteins of defined lengths can be generated by programming in vitro translation reactions with mRNAs truncated within the coding region (Gilmore and Blobel, 1985) and thus lacking an in-frame stop codon. Intermediates of secretory proteins formed in this manner appear to maintain the correct architecture of the translocon and ribosome around the nascent chain and thus can be studied and manipulated (Crowley et al., 1993, 1994). Here we use several independent probes to examine the disposition of the translocon for a series of translocation intermediates generated as described above.

Accessibility of Paused Nascent Chains to Proteases

Early translocation intermediates of the secretory protein prolactin have previously been shown to be inaccessible to proteases from the cytosolic side of the membrane (Connolly et al., 1989). Furthermore, these same intermediates were shown to reside in a tunnel from the ribosome to the ER lumen such that the nascent chain is inaccessible even to small molecules like iodide ions from the cytosolic side, implying the presence of a tight seal between the ribosome and translocon (Crowley et al., 1993, 1994). However, some have suggested that longer intermediates may not contain a tight ribosome–membrane junction, rendering the nascent chain partially accessible to proteases (Connolly et al., 1989), while other studies have implied that the junction is tight for a variety of chain lengths (Matlack and Walter, 1995).

We therefore first examined the accessibility to proteases of several apo B translocation intermediates and, as a control, prolactin translocation intermediates. Since some of the apo B truncations are much longer than full-length prolactin, we also engineered multiple prolactin coding regions in tandem following a single signal sequence (see Experimental Procedures). These allowed us to assess directly whether simple chain length had any effect on protease accessibility or the stability of the ribosome–membrane junction. Figure 1A demonstrates that translocation intermediates of prolactin, regardless of length, are well protected from proteinase K (PK). These nascent chains are still tethered to the ribosome as peptidyl-tRNAs, as evidenced by their cosedimentation with polysomes on sucrose gradients and their ability to be precipitated by the detergent cetyltrimethylammonium bromide (CTABr, data not shown), which selectively precipitates tRNA (Gilmore and Blobel, 1985). Thus, although the C-terminal region of the translocation intermediates is in the cytosol (albeit within the ribosome), it is not accessible to PK, implying that the junction between the ribosome and translocon is tightly sealed. This is consistent with the results of Crowley et al. (1993, 1994) and Matlack and Walter (1995), who also concluded that the junction between the ribosome and membrane is a tight seal.

In marked contrast, three of the six apo B translocation intermediates were accessible to PK (Figure 1B). In the cases in which they were accessible to protease, a substantial fraction of the chains were degraded to yield a smaller protected fragment (Figure 1B, arrowheads), which was reactive to an antibody directed at the N-terminus of apo B (data not shown). We interpreted these data to indicate that the protease had access to the chain at either the ribosome–membrane junction or within the ribosome, following digestion of ribosomal proteins.

In those truncations that demonstrated a substantial accessibility to protease digestion, we also noted that a proportion of chains remained undigested. This subset of fully protected chains is one that has in large part spontaneously released from the ribosome and is no longer tethered by peptidyl-tRNAs. This conclusion is supported by the fact that these chains are not efficiently precipitated by CTABr, whereas prior to protease digestion, approximately the same percentage of chains is precipitated by CTABr regardless of point of truncation (data not shown). We therefore conclude that for those truncations that display significant accessibility to proteases, the majority of chains that are not accessible to protease are no longer translocation intermediates.

The points at which apo B is accessible to PK are regions we have previously defined as sites of translocational pausing (Chuck and Lingappa, 1992; Kivlen et al., unpublished data). That is, the nascent chain has temporarily stopped translocation into a protease-protected environment at a discrete point, while the remainder of the chain is synthesized into an environment that is at least partially accessible to PK. For example, the 329 amino acid translocation intermediate shown in Figure 1B has approximately 32 kDa of chain translocated into the ER lumen and protected from protease, with the remainder accessible to PK digestion. At the subsequent 402 amino acid truncation point, the chain is still paused at the position that generates the 32 kDa protected domain. However, still later in chain growth, at 472 amino acids, the translocational pause has restarted, and the chain is fully protected from PK digestion. The events occurring during the translocation of this region of apo B were the focus of our subsequent experiments.

Since PK is a general and relatively aggressive protease, it has at times been thought to be the source of potential artifacts in the assessment of topology. To rule out artifactual proteolysis as a source of apo B accessibility to PK, we used the highly specific protease,

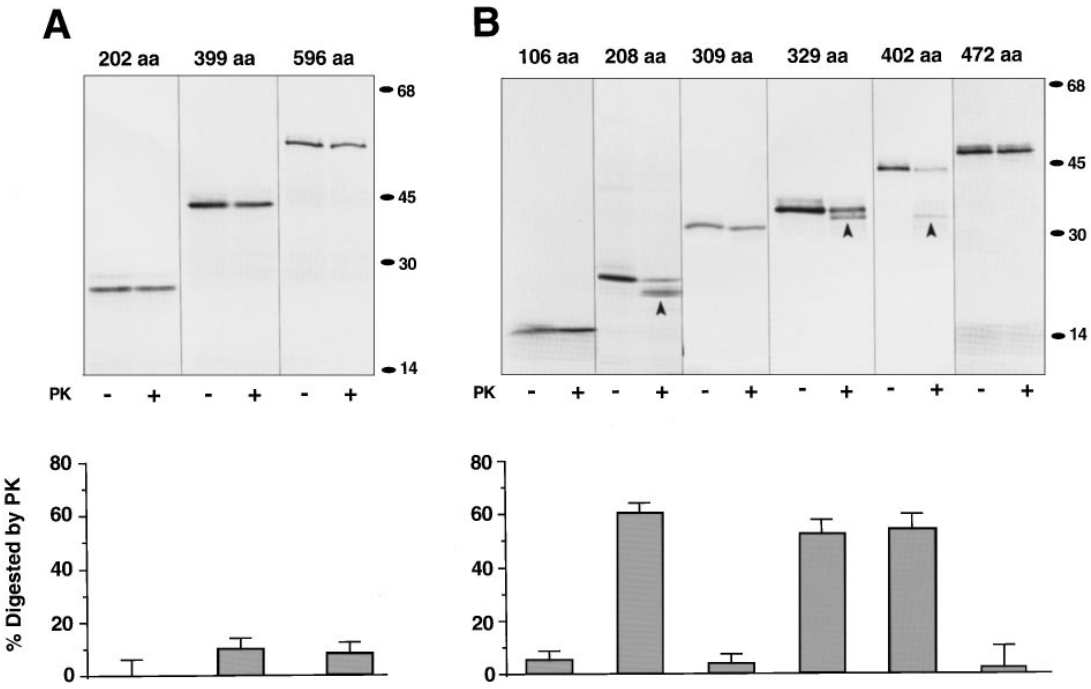

Figure 1. Access of Nascent Translocation Intermediates to Proteinase K

(A) Plasmids encoding one, two, or three prolactin coding regions in tandem were truncated before the termination codon and translated to generate translocation intermediates of the lengths (in amino acids) indicated above each panel. Shown are the autoradiograms from a representative experiment for each of the translation reactions before (minus) and after (plus) digestion with PK. The percent of translation products that was digested by PK was determined by quantitation of the autoradiogram from three independent experiments, averaged, and graphed below each panel. The standard error is represented by the error bar.

(B) Truncations coding for the N-terminal length of mature apo B indicated above each panel were analyzed exactly as described in (A). In each instance in which a significant proportion of translation product was accessible to digestion by PK, a smaller N-terminal fragment was generated and is indicated with an upward-pointing arrowhead.

Factor Xa (FXa), to reexamine an apo B translocation intermediate that was found to be paused and accessible to PK. For this experiment, we first ascertained that there were no intrinsic sites for FXa cleavage within apo B (data not shown). Then we engineered the 4 amino acid FXa recognition sequence (Ile-Glu-Gly-Arg), followed by the myc epitope, into apo B at amino acid 329, a site 72 amino acids before the point of truncation used to generate a paused translocation intermediate (Figure 2A). Thus, if the ribosome–membrane junction is tightly sealed, the cleavage site would be predicted to have exited the ribosome (which shields the C-terminal 20–40 amino acids; Malkin and Rich, 1967) and to reside somewhere within the translocon or ER lumen. In this case, it should not be accessible to FXa from the cytosolic side of the membrane. If, however, the ribosome–membrane junction is open, and domains of the nascent chain are indeed exposed to the cytosol during a translocational pause, the cleavage site may be accessible to FXa.

Figure 2 demonstrates that FXa has access to a paused nascent chain from the cytosolic side of the membrane. Digestion of the apo B translocation intermediate results in the digestion of approximately 50% of the chains to yield a large N-terminus fragment (Figure 2B, lane 2, upward-pointing arrowhead) and a small C-terminus fragment (downward-pointing arrowhead). Digestion with PK following FXa cleavage revealed that the small C-terminus fragment is in the cytosol, where it can be degraded by PK, in contrast with the larger N-terminus fragment, which is not further degraded by PK because it is in the ER lumen (data not shown). Furthermore, CTABr precipitation of the FXa-digested material shows that the small C-terminal fragment still contains the tRNA, as expected, and is not derived from spontaneously released nascent chains (data not shown). Thus, we conclude that those chains that are accessible to FXa cleavage are genuine translocation intermediates that are still tethered to the ribosome.

Treatment of the paused translocation intermediates with EDTA, which allows paused nascent chains to restart translocation (Chuck and Lingappa, 1992), resulted in the chains no longer being accessible to FXa cleavage (Figure 2B). Likewise, truncation at a later point or translation of full-length apo B, by which time translocation had restarted and hence the pause had been abolished, rendered the chain largely inaccessible to FXa cleavage (Figure 2C). These results are consistent with the earlier findings on PK accessibility of serial apo B truncations (see Figure 1B). All the above results strongly suggest that the paused nascent chain is directly and transiently exposed to the cytosolic environment.

An independent method of examining the ribosome–membrane junction was developed by Matlack and Walter (1995), in which they examined nascent chains in microsomes that had first been solubilized with detergents before proteolysis. They found that approximately

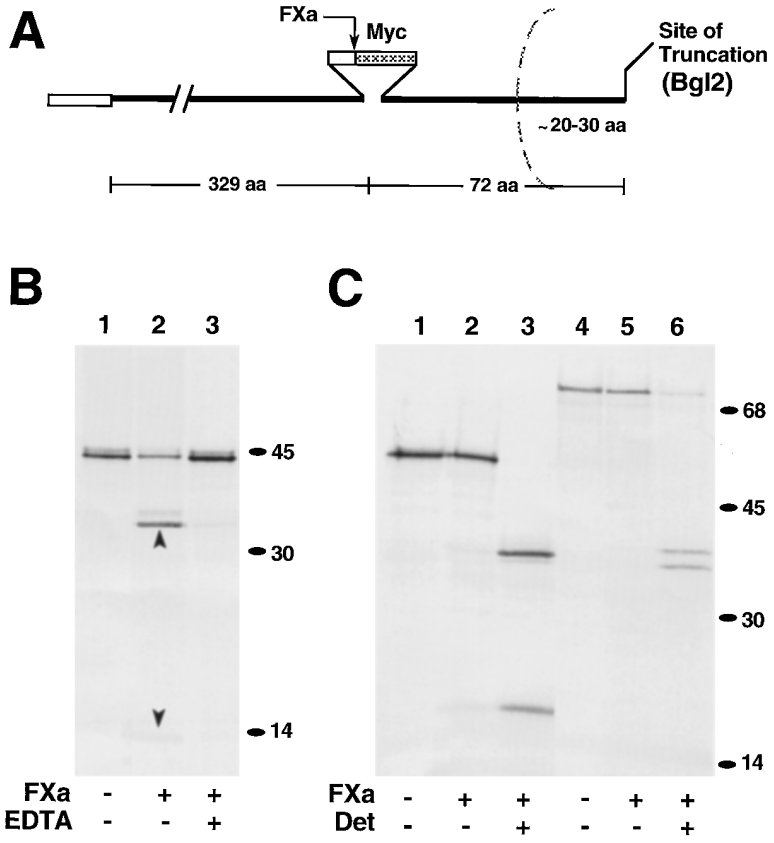

Figure 2. Access of Paused Translocation Intermediates to Factor Xa

(A) Construct ApoB–FXa. The signal sequence (open box), FXa cleavage site, and myc epitope (stippled box) are indicated. The ribosome (curved dotted line) is expected to shield the C-terminal 20–30 amino acids of the chain.

(B) ApoB–FXa was truncated at BglII, translated, the sample divided, and one aliquot incubated for 10 min at 25°C with 10 mM EDTA (lane 3). The microsomes were isolated and digested with FXa (lanes 2 and 3) as indicated. The N- and C-terminal fragments resulting from FXa digestion are indicated by the upward- and downward-pointing arrowheads, respectively.

(C) ApoB–FXa was truncated at NcoI (lanes 1–3) or left untruncated (lanes 4–6), translated, and digested with FXa as in (B). Where indicated (lanes 3, 6), 0.5% Triton-X 100 was included during FXa digestion.

70 amino acids were protected from proteolysis due to shielding from the ribosome and translocation channel, whereas in the absence of microsomes, only 40 amino acids were protected by the ribosome alone. When we analyzed paused and nonpaused nascent chains by this method, we found that only the 30–40 C-terminal amino acids of paused nascent chains were protected, but of nonpaused controls, 70–100 amino acids were protected (data not shown). This observation is consistent with the results of both the PK and FXa digestion experiments presented above. Taken together, these findings argue that a paused nascent chain is exposed to the cytosol at a point between its exit from the ribosome and its entry into the translocation channel, whereas a nonpaused translocating nascent chain is not.

Accessibility of Paused Nascent Chains to Antibodies

We next determined how much chain was actually exposed to the cytosol and whether this was sufficient to be recognized and bound by specific antibodies. This determination would serve several purposes: first, it would provide independent and very strong corroboration that the ribosome–membrane junction is open during translocational pausing; second, it would demonstrate that the cytosolically exposed domains of the nascent chain are large enough to allow their interaction with macromolecules in the cytosol; third, it would allow us to map the extent of the cytosolically accessible region of the paused nascent chain; and fourth, it would potentially provide a means of trapping paused chains even in the presence of conditions that would otherwise lead to restarting of translocation.

First, we engineered an epitope tag into the region of apo B that is predicted (based on the above results with FXa) to be exposed to the cytosol. We inserted a 13 amino acid myc epitope into two sites, 72 and 19 amino acids away from the point of truncation that yields a paused nascent chain (termed ApoB–Myc1 and ApoB–Myc2, respectively; Figure 3A). By determining which, if either, of these epitopes is accessible to antibodies from the cytosolic side of the membrane, we should be able to determine how much of the nascent chain is exposed to the cytosol. Furthermore, because the epitope in construct ApoB–Myc2 is predicted to have just emerged from the ribosome (Figure 3A), we should be able to ascertain definitively whether the junction between the ribosome and translocon is open to the cytosol.

ApoB–Myc2 was truncated and translated in the presence of microsomal membranes to yield paused translocation intermediates. As expected, the epitope-tagged apo B translocation intermediate was found to be paused and accessible to PK digestion similar to wild-type apo B, yielding a proteolytic fragment of approximately 32 kDa (Figure 3B, compare with the 402 amino acid intermediate in Figure 1B). Furthermore, the paused nascent chains could be made to restart translocation into the ER lumen with EDTA treatment, now rendering them inaccessible to PK. The paused versus restarted

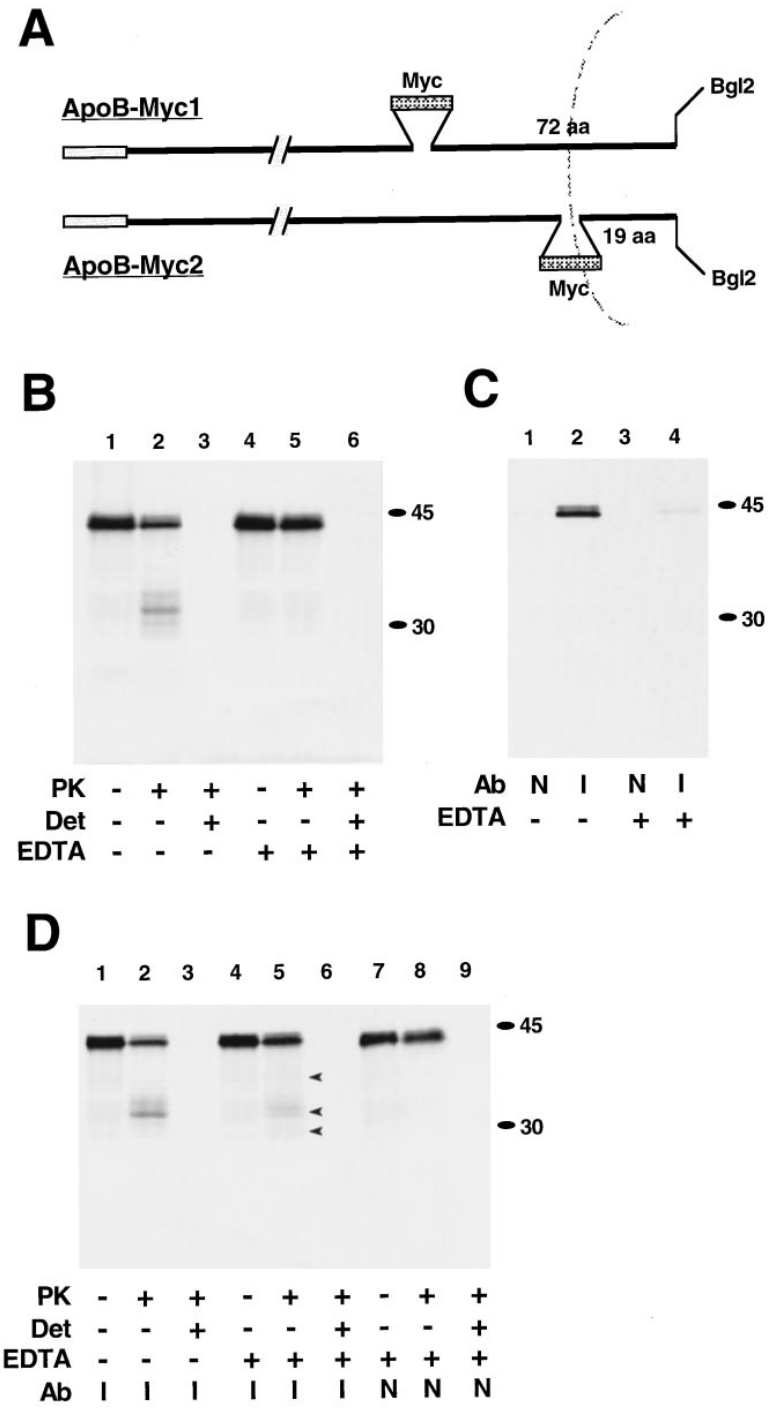

Figure 3. Access of Paused Translocation Intermediates to Antibodies

(A) Constructs ApoB–Myc1 and ApoB–Myc2.

(B) ApoB–Myc2 was truncated at BglII, translated, and one aliquot treated with 10 mM EDTA, as in Figure 2B. Microsomal membranes were isolated and divided for immunoadsorption (see [C]) or digestion with PK. Where indicated, 0.5% Triton X-100 (Det) was included during protease digestion.

(C) The intact isolated microsomes from (B) were immunoadsorbed using α-myc antibodies (I) or nonspecific antibodies (N), as described in Experimental Procedures.

(D) ApoB–Myc1 was truncated at BglII, translated, and the microsomal membranes isolated. The sample was then divided and incubated for 60 min with either α-myc antibodies (I) or nonspecific antibodies (N). Samples were then adjusted to 10 mM EDTA where indicated, incubated at 25°C for 10 min, and subjected to PK digestion. The arrowheads in lane 5 indicate fragments generated by digestion of the sample with PK.

nascent chains were then examined for the accessibility of the myc epitope to cytosolically added antibodies (Figure 3C). We found that when apo B nascent chains were paused (as assessed by their accessibility to PK, and under the same conditions that rendered them accessible to FXa when they contained an FXa recognition site), the myc epitope in ApoB–Myc2 was immunoadsorbed by cytosolically added α-myc antibody. Restarted nascent chains were not immunoadsorbed by the α-myc antibody, and an irrelevant antibody was unable to recognize a paused nascent chain (Figure 3C). Identical results were obtained with the construct ApoB–Myc1 (data not shown). Furthermore, translocation intermediates of prolactin into which a myc epitope was engineered were not immunoadsorbed by the α-myc antibodies, nor were paused chains lacking the engineered myc epitope (data not shown). Because the myc epitope is accessible in the cytosol in both the ApoB–

Myc1 and ApoB–Myc2 constructs, we interpolate that at least a 57 amino acid domain of the paused nascent chain is exposed between its emergence from the ribosome and its entrance into the protected environment of the translocon/ER lumen.

We next determined whether an antibody bound to a paused nascent chain intermediate would prevent its restarting translocation into the ER lumen. Paused nascent chain intermediates of ApoB–Myc1 were generated, the antibodies were bound, and the samples subsequently treated with EDTA to disassemble the ribosome and restart translocation into the ER. At each stage of the experiment, the topology of the nascent chain was monitored by digestion of the samples with PK. The generation of proteolytic fragments of approximately 32 kDa was used as an indicator that some of the nascent chains were accessible to the cytosol, while the lack of such fragments implied complete translocation into the ER lumen. The inclusion of antibodies in the cytosol had no effect on the proteolytic accessibility of a paused nascent chain (Figure 3D, lanes 1–3). However, we found that specific antibodies against the nascent chain prevented it from translocating into the ER lumen upon EDTA treatment, as evidenced by accessibility to PK (Figure 3D, lane 5, arrowheads). The inclusion of nonspecific antibodies had no effect on restarting translocation of a paused chain, as indicated by the lack of PK accessibility following EDTA treatment (Figure 3D, lanes 7–9). These data indicate that a bound antibody (approximately 150 kDa) is able to prevent a nascent chain from translocating through the translocon into the ER lumen. This is consistent with other studies which concluded that large folded domains are unable to transit efficiently across the ER (Ooi and Weiss, 1992).

Trapping of Nascent Chains in "Real Time" Using Antibodies in the Cytosol

To detect the transient opening of the ribosome–membrane junction during ongoing translation, we took advantage of the observation that antibodies bound to the nascent chain prevented its further translocation into the ER (Figure 3D). We reasoned that α-myc antibodies in the cytosol during the translation of ApoB–Myc1 should bind to at least some nascent chains during the brief window of time when the myc epitope is exposed to the cytosol (i.e., when the ribosome–membrane junction is opened during the translocational pause). Once bound, the antibody–nascent chain complex would be prevented from further translocation into the lumen, and the antibody–antigen complexes could be adsorbed from the cytosolic side of the microsomes (Figure 4A). Since the interaction between antibody and nascent chain is not at equilibrium, the number of chains bound will be small, determined predominantly by the rate constant of the antibody–antigen binding reaction and the amount of time the chain is paused. Higher rate constants and longer times of pause would result in more nascent chains being captured. As a negative control, if the antibody is added to the translation reaction after translocation has taken place, the newly synthesized chains should not be adsorbed, as they are inaccessible in the lumen of the microsomal membranes.

Four different translation reactions were performed in the presence of rough microsomal membranes: first, ApoB–Myc1 was translated in the presence of an irrelevant monoclonal antibody; second, ApoB–Myc1 was translated in the presence of α-myc antibodies; third, ApoB–Myc1 was translated in the absence of antibodies, and α-myc antibodies were added posttranslationally; and fourth, apo B (lacking the myc epitope) was translated in the presence of α-myc antibodies. An aliquot of each reaction was analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) to demonstrate that the presence or absence of antibodies did not affect translational efficiency (Figure 4B; lanes 1–4 correspond to the conditions above). The remainder of each sample was subjected to immunoadsorption as follows (see Experimental Procedures for details). The microsomal membranes were isolated by centrifugation, washed to remove any free antibodies, and resuspended. At this point, the only antibodies still in the sample must be bound to the microsomal membrane, either nonspecifically or via specific antigen–antibody interaction. The antibody complexes were then collected with immobilized protein G, following solubilization of the sample under nondenaturing conditions. These samples were analyzed by SDS–PAGE and autoradiography (Figure 4C). We found that the only conditions that resulted in significant immunoadsorption of the nascent chain were those in which the epitope was present in the translated protein and those in which the antibodies were specific to the epitope; and, most importantly, only those in which the antibodies were added cotranslationally. If the antibodies were added posttranslationally, significantly less nascent chain was immunoadsorbed (Figure 4C, compare lanes 2 and 3). We interpret these results to indicate that the antibodies in the cytosol have access to the nascent chain *only* during the course of translocation and not afterwards. This indicates that the ribosome–membrane junction was indeed opened *during* translocation, and the region of the nascent chain predicted to be exposed was available to the cytosolically added antibodies.

To validate further the interpretation of the data in Figure 4C, we determined the topology of the population of chains trapped by antibody binding. For this experiment, we immunoadsorbed intact vesicles containing antibody-trapped nascent chains and subjected them to proteolysis to demonstrate that they are indeed spanning the membrane (Figure 4A). Furthermore, release of these immunoadsorbed chains by a peptide encoding the myc epitope should allow the trapped nascent chain to translocate into the vesicle lumen and thus be protected from protease digestion. The finding that adsorbed chains, but not adsorbed and peptide-released chains, are accessible to protease digestion (Figure 4D), strongly argues that the apoB chains are spanning the membrane, prevented from complete translocation into the lumen only by the bound antibody.

Probing the Environment of Paused Nascent Chains Using Cross-Linking

In view of the dramatic changes in the translocation apparatus during translocational pausing, we reasoned

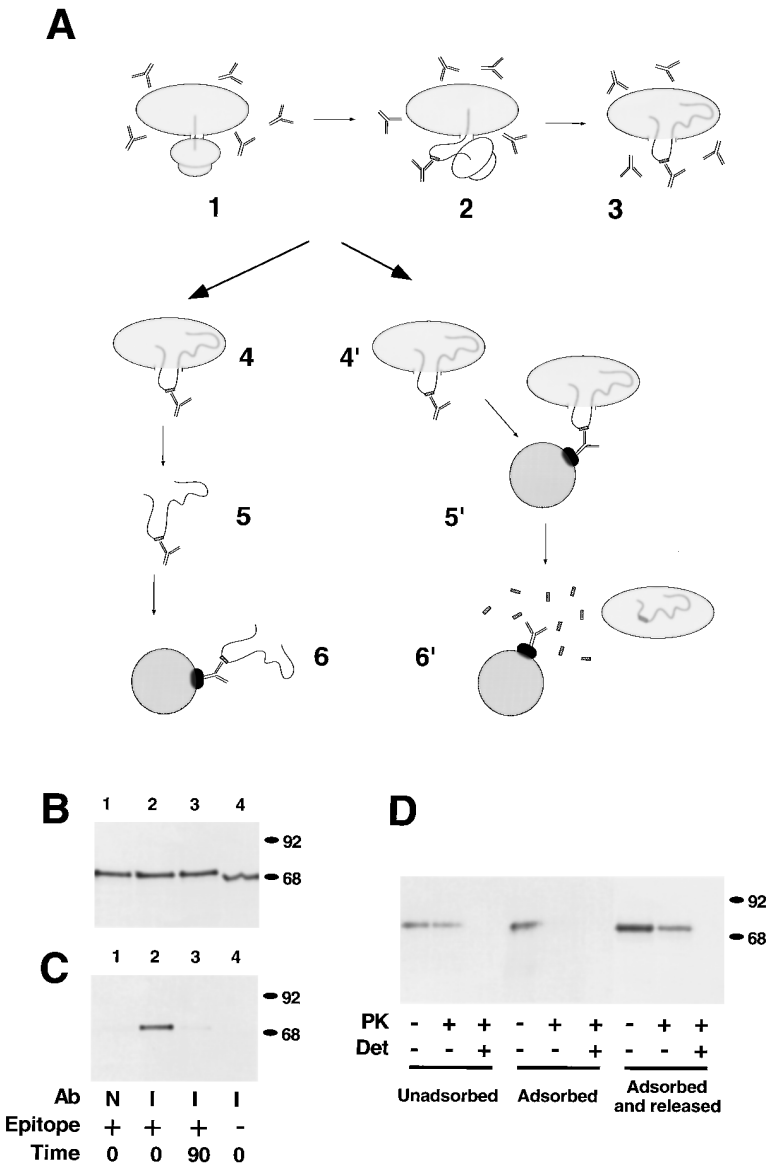

Figure 4. Capture of Translocating Nascent Chains with Antibodies in the Cytosol

(A) The experimental design for (C) (diagrams 1–6) and (D) (diagrams 1–6'). The untruncated ApoB–Myc1 plasmid is translated in the presence of antibodies (1), which, when the epitope is exposed to the cytosol, binds to some of the nascent chains (2) and prevents its subsequent translocation into the ER lumen (3). The free antibodies are removed (4) and the antibody–antigen complexes are collected by immobilized protein G (6). Alternatively, intact vesicles are adsorbed using immobilized protein G (5′) and subsequently released by a peptide encoding the myc epitope (6′).

(B) and (C) ApoB15 (minus epitope) and ApoB–Myc1 (plus epitope) were translated for 90 min in the presence of microsomal membranes and either nonspecific antibodies (N) or α-myc antibodies (I) included at 25 μg/ml. To one sample, α-myc antibodies were added posttranslationally (at 90 min, lane 3). An equal aliquot (1 μl) of each of the samples was analyzed directly (B), while the remainder (49 μl) was subjected to immunoadsorption as described in Experimental Procedures (C).

(D) Following translation of ApoB–Myc1 in the presence of α-myc antibodies, microsomal membranes were isolated by gel filtration and antibody-bound vesicles adsorbed by immobilized protein G. An aliquot of the adsorbed material was treated with 10 μM myc peptide. The unadsorbed material (lanes 1–3), the adsorbed material (lanes 4–6), and the adsorbed and peptide-released material (lanes 7–9) were subjected to PK digestion as indicated below the gel.

that the subset of translocon proteins that are adjacent to the paused nascent chain might also be different. Using a cross-linking approach, it has been shown that a conventional secretory protein such as prolactin is adjacent to Sec61α, and sometimes to translocating chain-associated membrane protein, during its entire transit across the ER (Mothes et al., 1994). We wondered whether other translocon proteins, either known or previously unidentified, were adjacent and therefore cross-linkable to a *paused* nascent chain.

To determine whether PT-specific changes in the nascent chain environment occur, we compared the pattern of cross-linked proteins for a paused versus matched nonpaused nascent chain. We chose to use prolactin as the coding region into which we inserted the PT and nonpausing control sequences, since the translocation and cross-linking properties of this protein have been extensively characterized (Wiedmann et al., 1987; Görlich et al., 1992; Mothes et al., 1994). Two constructs were generated that were identical to each other except that one contained a PT sequence from apo B (Prl-pause), and the other contained an irrelevant stuffer sequence (Prl-stuffer, Figure 5A). We first verified that Prl-pause and Prl-stuffer translocation intermediates were and were not translocationally paused, respectively, by evaluating their accessibility to PK digestion from the cytosolic side of the membrane (Figure 5B). As expected, the Prl-pause nascent chain was accessible to PK digestion, yielding a smaller proteolytic fragment, while the Prl-stuffer chain was not digested by PK. Furthermore, upon EDTA treatment, both the Prl-pause and Prl-stuffer nascent chains were inaccessible to PK digestion (data not shown). Next, these same nascent chains, both before and after EDTA treatment, were subjected to cross-linking, using various concentrations of the bifunctional cross-linker disuccinimidyl suberate. We found that while most of the cross-linked proteins are the same for both Prl-pause and Prl-stuffer

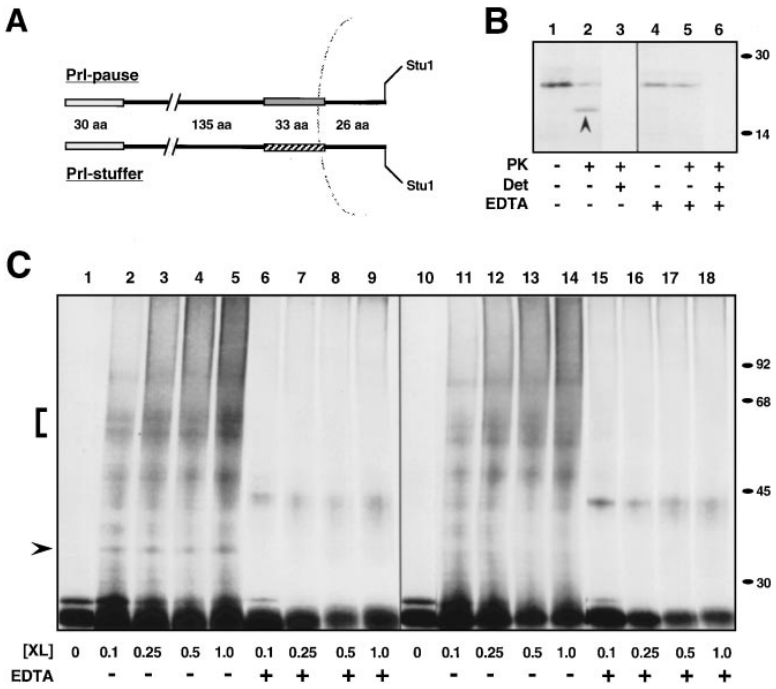

Figure 5. Paused Nascent Chains Are Adjacent to an 11 kDa Membrane Protein

(A) Prl-pause and Prl-stuffer constructs. The N-terminal 165 amino acids are from bovine pre-prolactin and the C-terminal 26 amino acids before the point of truncation (at StuI) are residues 304–329 from mature apo B. The insert between these two domains is either a PT sequence (stippled box, representing amino acids 261–290 of mature apo B) or an irrelevant stuffer sequence (striped box) resulting from the nucleic acid sequence of the pause inserted in the reverse orientation.

(B) The plasmids Prl-pause (lanes 1–3) and Prl-stuffer (lanes 4–6) were truncated at StuI and analyzed as in Figure 3B. The upward-pointing arrowhead in lane 2 indicates the fragment generated from proteolysis of the Prl-pause translocation intermediate.

(C) Prl-pause (lanes 1–9) and Prl-stuffer (lanes 10–18) translocation intermediates were cross-linked to adjacent proteins at various cross-linker concentrations as described in Experimental Procedures. Lanes 1 and 10 are samples that were not treated with cross-linker. The bracket at the left indicates the position of cross-links to Sec61α, as confirmed by immunoprecipitation (data not shown). The arrowhead indicates the position of a cross-link to an approximately 11 kDa protein that is specific to a paused nascent chain intermediate.

nascent chains, only the paused nascent chains are cross-linked to a protein of approximately 11 kDa in size (Figure 5C, arrowhead). As expected, both Prl-pause and Prl-stuffer nascent chains were cross-linked with equal efficiency to Sec61α, indicating that they were in the translocation channel.

Characterization of the 11 kDa cross-linking partner indicate that it is unlikely to be either Sec61β or ribosome-associated membrane protein 4 (Görlich and Rapoport, 1993), known membrane proteins of approximately the same size, since antibodies against these proteins failed to recognize the cross-linked adduct in immunoprecipitation experiments (data not shown). We found that the 11 kDa protein is likely to be an integral membrane protein, as it was not extracted from the membrane at pH 11.5 (Figure 6B) and still seen to cross-link in membranes stripped of peripheral membrane proteins (Figure 6A). Furthermore, the unchanged migration of the cross-linked adduct upon endoglycosidase-H treatment (Figure 6C) and the inability of the adduct to bind ConA (Figure 6D) argue that the 11 kDa protein is not glycosylated. Furthermore, the 11 kDa protein appears to be stably associated with either the nascent chain or translocon, since it was found to cosediment with the nascent chain on sucrose gradients following detergent solubilization of the microsomal membranes. That is, ribosome–nascent chain complexes isolated by velocity sedimentation were seen to cross-link to the 11 kDa protein (Figure 6A).

These results demonstrate that when a nascent chain is translocationally paused, the proteins adjacent to it have changed: a new protein of 11 kDa moves into a position where it can be cross-linked to the nascent chain residing within the translocation channel. By contrast, the matched nonpaused nascent chain, although residing in the translocation channel, is not adjacent to the 11 kDa protein. Likewise, when the pause is abolished and translocation of the chain has restarted, this cross-linked adduct is lost. These data underscore the conclusion that the translocon is dynamic and may indicate that previously unidentified proteins are involved in events related to translocation and, more specifically, translocational pausing.

Discussion

In this work, we have demonstrated that nascent secretory proteins containing PT sequences are transiently exposed to the cytosolic environment during their cotranslational translocation across the ER membrane. This exposure of the nascent chain to the cytosol is facilitated by an opening of the normally tight junction between the ribosome and the translocation channel. This dynamic change in the ribosome–membrane junction is not stochastic but, rather, appears to be mediated by the PT sequence encoded within the nascent chain. We initially detected these changes in the translocation apparatus through the use of truncated mRNAs to generate translocation intermediates that could be easily studied (Figures 1–3). We then demonstrated opening of the ribosome–membrane junction in real time, during the ongoing translation and translocation of a PT sequence containing secretory protein (Figure 4). Finally, we show that during a translocational pause, coordinate changes occur in the protein machinery with which the nascent chain is associated (Figures 5 and 6).

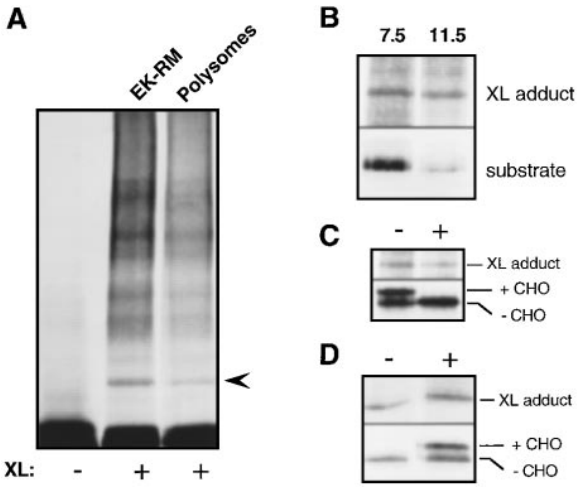

Figure 6. Characterization of the 11 kDa Cross-Linking Protein

(A) The plasmid PrI-pause was truncated at StuI and translated using EDTA-salt washed rough microsomes (EK-RM). The microsomal membranes were isolated and either subjected to cross-linking directly (lane 2) or solubilized in 1% digitonin, the ribosome-bound nascent chains isolated by sucrose gradient sedimentation and subsequently treated with cross-linker (lane 3). The arrowhead indicates the position of the 11 kDa cross-link described in Figure 5C.

(B) StuI-truncated PrI-pause was translated, the microsomal membranes isolated, and the sample treated with 0.2 mM cross-linker. The sample was then either diluted in 10-fold excess 0.1 M Tris (pH 7.5) or 0.1 M $NaCO_3$ (pH 11.5). The microsomal membranes were isolated by centrifugation and analyzed by SDS–PAGE. Shown are the cross-linked adduct, which was not extracted from the membrane under pH 11.5 conditions, and the un-cross-linked substrate (from a shorter exposure of the same gel), which was largely extracted under pH 11.5 but not pH 7.5 conditions.

(C) Cross-linking products generated as above were incubated in the absence (minus) or presence (plus) of endoglycosidase H prior to analysis by SDS–PAGE and autoradiography. As a control, a mixture of glycosylated (plus CHO) and unglycosylated (minus CHO) apoB15 translation product was also treated in parallel.

(D) Cross-linking products or the control apoB15 translation product, as in (C), were passed over a ConA column in the absence (minus) or presence (plus) of 1 M α-methyl-mannopyranoside and the flow-through fraction analyzed by SDS–PAGE and autoradiography.

The Translocon Is More Than Just a Channel

Our results support a model in which the translocon plays an active role beyond the simple transfer of nascent chains across the ER membrane (Lingappa, 1991) and in which specific components of the translocation machinery may be regulated by sequences within the particular protein being translocated (Figure 7). Data from several sources (Crowley et al., 1993, 1994; Kalies et al., 1994; Matlack and Walter, 1995), including this work (Figure 1A), indicate that early translocation intermediates of simple secretory proteins are well shielded from the cytosol by a tight ribosome–membrane junction (Figure 7A). However, upon translation and translocation of PT sequences, we show here that the junction between the ribosome and membrane is opened, and translocation of the nascent chain temporarily stops (Figure 7B). During this time, the chain appears to be adjacent or, possibly, bound to proteins in a manner unique to the translocationally paused state (Figure 7, stippled oval). At least in the instance studied in this paper, further translation during the window of time when the chain is translocationally paused results in the expelling of the newly synthesized protein domain into the cytosol (Figure 7C). At a later time during chain growth, the translocational pause is relieved, translocation of the cytosolically disposed regions of the nascent chain resumes (Figure 7D), and the tight ribosome–membrane junction is reestablished (Figure 7E).

Translocational Pausing Alters the Translocational Machinery

The current studies were motivated by initial observations that the translocation of certain proteins such as apo B appeared to be different from the well-studied model proteins such as prolactin. Apo B had been shown to stop translocation transiently, without integrating into the membrane, before being fully translocated into the ER, due to the action of PT sequences (Chuck et al., 1990; Chuck and Lingappa, 1992). In those earlier studies, the mechanism by which translocational pausing rendered regions of the nascent chain accessible to cytosolic proteases was not understood. In principle, the proteolytically sensitive domains could have resided in the ribosomal tunnel, in the translocon, or even partially in the lumen of the ER. Because digestion with PK from the cytosolic side might significantly disrupt the architecture of the ribosome and translocation channel, it was not possible to determine whether translocational pausing involved alterations in the translocation machinery and the ribosome–membrane junction or simply changes in folding of the nascent chain that rendered it more accessible to PK digestion. The studies presented here demonstrate that translocational pausing transiently alters the organization of the ribosome–membrane junction and translocon in such a way as to render the chain directly accessible to the cytosol. Such changes might be due to the ribosome being docked differently at the membrane, conformational changes in the ribosome, or changes in the binding of nascent polypeptide-associated complex, which can shield nascent chains in the ribosomal tunnel (Wang et al., 1995). These changes are not observed in the cases of either a simple secretory protein or a PT-containing protein subsequent to restarting of translocation. At this point, we cannot rule out other subtle changes in the interaction between the ribosome and the membrane, e.g., those which may occur as a simple function of chain length (Connolly et al., 1989). However, given that we find nonpaused nascent chains of various lengths to be fully protected from proteases and other probes, any such changes would appear to be on a much smaller scale than the ones accompanying sequence-specific translocational pausing that are the subject of this study.

Timing a Translocational Pause

For how long are the translocationally paused domains of a nascent chain exposed to the cytosol? Our data on antibody binding to paused chains in real time allow us to make such an estimate to a first approximation. Over 140 amino acids exist between the truncation point (StuI at amino acid 329, Figure 1B), which first reveals the

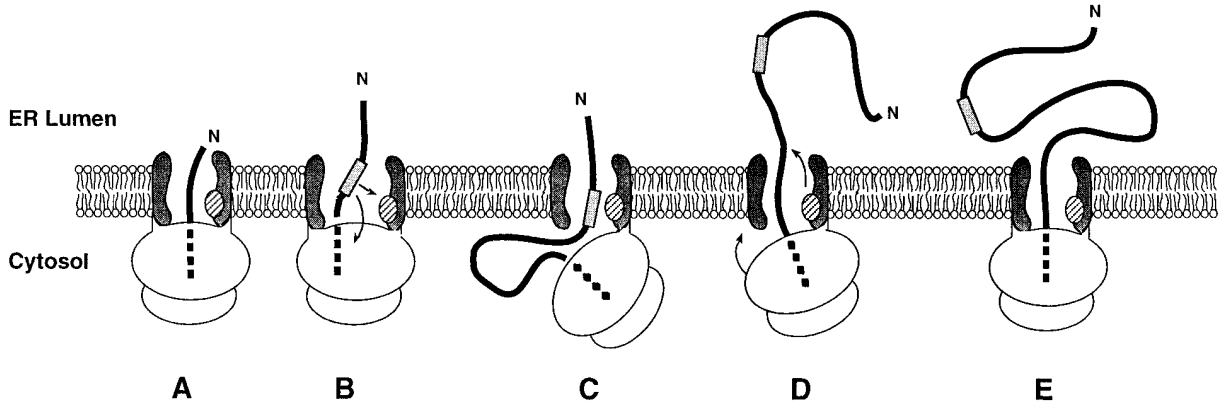

Figure 7. Model of a Dynamic Translocation Channel

The diagrams indicate the successive events proposed to occur at the translocon during the translocation of PT-containing proteins. The stippled box within the translocating nascent chain represents the PT sequence. The stippled membrane proteins comprise the translocation channel, which is thought to be composed predominantly of the Sec61p complex. The striped oval in the translocon represents pause-specific membrane protein(s). Arrows designate dynamic events occurring during translocation. See text for details.

cytosolically accessible domain and a later point at which the chain is no longer accessible (NcoI at amino acid 472). Thus, we infer that the myc epitope that was inserted at the StuI site in the construct ApoB–Myc1 was accessible for at least the length of time it takes to synthesize 60–80 residues, and, potentially, significantly longer. Given a translation rate of approximately 60–70 residues per minute in reticulocyte lysate at 25°C, (R. S. H., unpublished data; Frydman et al., 1994), the window of time would be approximately 50–80 s. Extrapolation of our data to in vivo translation conditions, where rates of translation can be 5–10 times faster than in reticulocyte lysate, one would still expect the nascent chain to be exposed for several seconds or longer. These values, while subject to variation as a function of the rate of protein synthesis, indicate that there is sufficient time for interactions between a translocationally paused nascent chain and macromolecules in the cytoplasm. In principle, this could allow translocationally paused secretory or membrane proteins to achieve particular events in folding or modification. Candidates for such modifiers might include cytosolic chaperones such as members of the *hsp70* family or peptidyl–prolyl isomerases. Although the length of time that the chain is exposed to the cytosol is brief, it is significant. The rate of antibody binding to its antigen is not unlike other common cellular reactions, such as chaperone binding or kinase recognition of their substrates. Nascent chain exposure times in the range of several seconds is sufficient for interactions with cytosolic components, especially in vivo, where diffusion is usually not limiting.

A Specific PT Sequence–Associated Protein

In addition to evidence in favor of an opening of the ribosome–membrane junction, we have presented an independent line of evidence that the translocation channel itself changes its conformation in response to translocational pausing. Cross-linking experiments probing the environment surrounding the nascent chain demonstrated a difference in the set of neighboring proteins (Figure 5C). An 11 kDa membrane protein that was most likely not Sec61β or ribosome-associated membrane protein 4 was found to cross-link specifically to paused nascent chains. Although the presence of this cross-link demonstrates that the translocon has changed with respect to the nascent chain, the role of this protein in translocation or PT sequence action is currently not clear. It is possible that it is a protein involved in directing translocational pausing by direct interaction with the nascent chain, serving as a PT sequence receptor. Consistent with this notion, we have observed cross-links to an 11 kDa protein using an independent PT sequence in a different chimeric context as the substrate (R. S. H. and V. R. L., unpublished data). Likewise, the 11 kDa protein could potentially be a currently unidentified member of the translocation apparatus. Because it appears to be adjacent to the nascent chain transiently and only during sequence-specific translocational pausing, it may have escaped obvious detection in previous studies.

The work of Görlich and Rapoport (1993), in which translocation was reconstituted from purified components, demonstrated the requirement for a surprisingly simple minimal translocation apparatus consisting only of the Sec61p complex and, in some cases, translocating chain-associated membrane protein. However, it remains entirely possible that various other membrane proteins are involved in aspects of the maturation of nascent chains that are not monitored by the assays used, or which are not rate-limiting in the in vitro translocation assays. Consistent with this notion is the existence of several membrane proteins such as the translocon-associated protein complex (Wiedmann et al., 1987; Görlich et al., 1992) and the 11 kDa protein in this study, which are found in the proximity of the nascent translocating chain but whose functions remain obscure. The answers to these questions will require the identification of this protein followed by depletion and reconstitution studies, as has been done for other components of the

translocation apparatus (Nicchitta and Blobel, 1990; Görlich et al., 1992; Görlich and Rapoport, 1993).

Regulation of Nascent Chain Translocation across the ER Membrane

Three features of the findings described here suggest a system amenable to cellular regulation. First, the change in disposition of the chain upon translocational pausing does not solely involve *cis*-acting events. Rather, the change in translocation status of the chain is associated with substantial changes in the protein machinery of translocation, including the ribosome–membrane junction and the translocation channel. Second, the changes observed are selective for a subset of translocation substrates, those containing PT sequences. Third, the changes observed upon translocational pausing are transient, with some pauses apparently lasting longer than others.

It is tempting to view translocational pausing as a general mechanism by which a region of chain could be isolated during translocation and subjected to cotranslational modifications that might not otherwise be possible. Pausing might facilitate these events in one or more of the following ways. The chain could be made accessible to a compartment in which the enzymes that catalyze specific reactions are present (e.g., the cytosol or the plane of the membrane). Alternatively, and by analogy to the role of signal recognition particle-mediated elongation arrest in facilitating targeting, pausing may make modification events kinetically more favorable as a result of delayed chain translocation. Finally, by allowing transient rearrangement of translocon components, pausing may allow a single translocon protein to serve multiple functional roles. Protein disulfide isomerase, a known translocon component, appears to be a functional subunit in a number of different enzyme complexes and provides a precedent for such a rationale (Koivu et al., 1987; Wetterau et al., 1990). Hence, translocational pausing, while being specific for complex translocating chains, as opposed to the simplest secretory proteins, may set the stage for an extremely diverse array of events in protein biogenesis.

Experimental Procedures

Materials

Rabbit reticulocyte lysate, dog pancreatic microsomal membranes, and EDTA-salt washed microsomes were prepared and used as described previously (Chuck and Lingappa, 1992; Walter and Blobel, 1983). Antibodies to the myc epitope (clone 9E10) and the myc peptide were the gift of the laboratory of A. Murray or purchased from Oncogene Science (Uniondale, NY). Control antibodies were against the HA epitope tag and were the gift of the laboratory of I. Herskowitz. Antibodies were purified using a protein G affinity column (Harlow and Lane, 1988). Antibodies against Sec61α were a gift from the laboratory of P. Walter. Antibodies against Sec61β and ribosome-associated membrane protein 4 were a gift from T. Rapoport. All other reagents were of the highest quality available commercially.

Plasmid Constructions

All manipulations of nucleic acids were done by standard techniques (Sambrook et al., 1989). All constructions are derived from pSP64 (Promega, Madison, WI), into which the 5′ untranslated region of Xenopus globin is inserted at the HindIII site. pSP SP1, a plasmid encoding the signal sequence of bovine prolactin, was cut with XbaI and ligated to a polymerase chain reaction (PCR) fragment of bovine prolactin encoding amino acids 4–227 of the mature protein to create the plasmid 1Prl. The plasmids 2Prl and 3Prl were created by successively inserting the PCR product described above into 1Prl or 2Prl, respectively, digested with XbaI. These plasmids were cut at XbaI prior to their use in Figure 1. ApoB–Myc1 and ApoB–Myc2 were made by inserting oligonucleotides encoding the myc epitope tag (GTEQKLISEEDLA) into the StuI and BstE2 sites, respectively, of ApoB15 (Chuck et al., 1990). ApoB–FXa was made by inserting oligos encoding amino acids TIEGRM into the KpnI site of ApoB–Myc1. An EcoRI linker was inserted at the MscI site of bovine prolactin to generate the plasmid BPI-EcoRI. A PCR fragment encoding amino acids 264–406 of mature apo B was then inserted into this EcoRI site to generate the plasmid BPI(B7–10)@MscI. A second PCR fragment encoding the amino acids 234–263 was inserted in both the forward and reverse orientation into the SacI site of BPI(B7–10)@MscI to generate the plasmids BPI(B6–10)@MscI and BPI(B7–10stuffer)@MscI, respectively. These two plasmids were digested with SpeI and PstI, treated with Klenow fragment, and recircularized to make Prl-pause and Prl-stuffer.

Cell-Free Translation and Proteolysis

Transcription, translation in reticulocyte lysate, and proteolysis with PK was as described (Chuck and Lingappa, 1992), with minor modifications as noted in the figure legends. Where indicated, microsomal membranes were sedimented by layering the sample onto a 100 μl cushion of 0.5 M sucrose, 100 mM KCl, 50 mM HEPES (pH 7.4), 5 mM $MgOAc_2$, and centrifugation for 4 min at 50,000 rpm in a TLA100 (Beckman). For FXa digestion, the microsomes were resuspended in FXa Buffer (100 mM NaCl, 50 mM Tris [pH 8.0], 5 mM $MgOAc_2$, 2 mM $CaCl_2$, 0.25 M sucrose) prior to addition of FXa to 0.05 mg/ml for 75 min at 22°C. Digestions were terminated by addition of boiling 1% SDS, 0.1 M Tris (pH 8.9).

Immunoadsorption of Nascent Chains

Microsomal membranes from translation reactions were isolated as described above, resuspended in physiological salt buffer (PSB) (100 mM KCl, 50 mM HEPES [pH 7.4], 5 mM MgOAc2, 0.25 M sucrose), and incubated with antibodies for 60 min at 4°C. The microsomes were reisolated by centrifugation, washed once in PSB, resuspended in TXSWB (1% Triton X-100, 100 NaCl, 50 mM Tris [pH 8.0], 10 mM EDTA), and incubated with 10 μl immobilized protein G (Pierce, Rockford, IL) for 60 min with constant mixing. The beads were washed four times with TXSWB prior to analysis by SDS–PAGE. In Figure 4C, immunoadsorption was as above but with the antibodies being included during the translation, as indicated in the figure legend, instead of being added after isolation of the microsomes. In Figure 4D, the microsomal membranes from the translation reaction were isolated by gel filtration through CL-4B (Pharmacia, Uppsala, Sweden) in PSB, divided into two, and incubated with 10 μl of immobilized protein G beads for 60 min at 4°C. The unadsorbed material was removed to another tube, and the beads were washed three times in PSB and resuspended in a total volume of 30 μl PSB with or without 10 μM myc-peptide. Each sample was then divided into three and subjected to proteolysis with PK as described above.

Chemical Cross-Linking

Microsomal membranes from translation reactions were isolated by centrifugation and resuspended in PSB, and the cross-linker disuccinimidyl suberate (freshly dissolved as a 50 mM stock in DMSO) was added to the appropriate concentration. The sample was incubated at 22°C for 30 min before the addition of one-tenth volume of 1 M Tris, 1 M glycine (pH 8.5). After a 15 min incubation on ice, the samples were processed further; carbonate extraction, endoglycosidase H digestion, and ConA chromatography were as described previously (Kellaris et al., 1991). In Figure 5C, samples were adjusted to 1% digitonin prior to cross-linking to reduce nonspecific cross-linking to ER lumenal proteins at the higher cross-linker concentrations. For Figure 6A, lane 3, translation reactions were adjusted to 1% digitonin, layered onto a 10%–50% sucrose gradient in PSB containing 0.2% digitonin, and centrifuged for 60 min at 55,000 rpm in a TLS-55 rotor (Beckman). The polysomal fraction (monitored by absorbance at 260 nm) was subjected to

cross-linking with 0.25 mM disuccinimidyl suberate as described above.

Miscellaneous

SDS–PAGE was performed using either 15% or 12%–17% gradient gels. The gels were either dried directly or fluorographed with En-Hance (Dupont), prior to autoradiography. Immunoprecipitations were as described (Chuck et al., 1990). Precipitation with CTABr was as described (Gilmore and Blobel, 1985). Quantitation of autoradiograms was performed following the digitization of the image, using an AGFA flatbed scanner and Adobe Photoshop software.

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