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THE REGULATION OF PROTEIN TRANSLOCATION AT THE ENDOPLASMIC RETICULUM

by

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DISSERTATION

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for my parents

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Gott allein regelt; Sterbliche können lediglich stimulatorischen oder hemmenden Einfluß ausüben.

Günter Blobel

The Regulation of Protein Translocation at the Endoplasmic Reticulum

Ramanujan S. Hegde

In mammalian cells, secretory and membrane proteins translocate across the endoplasmic reticulum membrane through protein-conducting channels termed translocons. Substantial progress during the past decade has identified the fundamental mechanisms of simple secretory protein translocation. If and how the translocation of more complex substrates is regulated remains obscure. To address this issue, the events of protein translocation have been explored in two such complex substrates, apolipoprotein B and the prion protein.

Studies exploring apolipoprotein B were used to elucidate the molecular basis of its translocational regulation and consequences of its dysregulation. Unlike simple secretory proteins, apolipoprotein B pauses during its translocation across the endoplasmic reticulum membrane. Biochemical dissection of translocational pausing in vitro identified it as a means by which apolipoprotein B can have regulated access to environments, such as the cytosol, that are otherwise shielded from the nascent chain. Subsequent fractionation and reconstitution studies identifed the translocon component TRAM as a necessary protein in mediating translocational pausing. Biochemical manipulation of TRAM revealed a role in regulating the fidelity of nascent chain exposure to the cytosolic environment.

Parallel studies exploring the translocation of the prion protein identified three distinct topological forms synthesized at the endoplasmic reticulum. Mutations within the prion protein were used to manipuliate the relative ratio of synthesis in each of these forms. The histopathological and biochemical analyses of transgenic mice expressing each of these mutations revealed a role

for one of the topologic forms of the prion protein in the development of neruodegenerative disease. Subsequently, this same topologic form of the prion protein was identified as the likely molecular basis for a genetic human neurodegenerative disease.

Together, the studies described herein provide compelling evidence for the existence of regulated events during the biogensis of complex secretory and membrane proteins. The consequences of translocational dysregulation have been demonstrated in vitro and in vivo, and the implications for human disease have been realized. Importantly, specific sites of regulation, mechanism of action, and regulatory machinery have been elucidated. Thus, a rudimentary molecular framework has been provided within which to understand and manipulate regulated protein biogenesis in cell biology and disease.

Peter Walter 5/15/98

Peter Walter
Chairperson, Thesis Committee

Table of Contents

List of Tables

List of Figures

I. Introduction

The secretory pathway

A defining feature of all eukaryotic cells is the remarkably complex assortment of specialized membrane-delimited compartments. These intracellular compartments serve to organize thousands of biochemical activities within the cell. The discipline of modern cell biology arose in part through early efforts to understand the sorting and traffic of proteins within these membrane-bound organelles.

In pioneering work by Palade and others, one of the compartment systems within the cell, the endoplasmic reticulum (ER), was discovered to have bound to it "small ribonucleoprotein particulate components of the cytoplasm", later termed ribosomes (Palade, 1955). The presence of such membrane-bound ribosomes, particularly in the pancreas and liver, was correlated to the synthesis of secretory proteins. By contrast, free ribosomes were usually associated with proteins which were destined to remain in the cytosol.

It was later discovered that secretory proteins, synthesized on membrane bound ribosomes, appeared first in the lumen of the ER. At later times, these proteins were found to reside in other compartments of the cell, such as the Golgi apparatus, prior to secretion from the cell. Such observations led to the definition of a secretory pathway: a system in which proteins synthesized at the ER are transported and sorted by repeated vesicle budding and fusion through various membrane bound compartments prior to secretion from the cell (Palade, 1975).

The signal hypothesis

An important realization of these studies was that the secretory protein traversed the membrane only once, at its site of synthesis on the ER membrane. This raised the puzzling question of how mRNAs for secretory proteins could be selectively synthesized on membrane bound ribosomes. Early hypotheses to

explain this observation included attributing the specificity for the membrane to the translating ribosome, the mRNA itself, or certain regions of the nascent protein. Distinguishing between the models became possible with the development of methods to isolate and manipulate different subcellular fractions in a cell-free system.

Milstein et al. (1972) and others (Swan et al., 1972; Kemper et al., 1974; Blobel and Dobberstein, 1975a) observed that the translation in a cell-free system of mRNA coding for a secretory protein yielded a product that differed from the authentic protein by an amino-terminal extension. Such an extension was not observed upon translation of nonsecretory proteins in the same system, lending support to a model in which this amino-terminal segment of the protein was involved in segregation of proteins to the ER membrane. Subsequent studies demonstrated that in most cases, the amino-terminal extensions of secretory proteins were cleaved to generate authentic forms if the cell-free translation system was supplemented with ER-derived microsomal membrane vesicles (Blobel and Dobberstein, 1975b).

The signal hypothesis, set forth in 1975 by Blobel and Dobberstein, attempted to incorporate these and other experimental observations into a conceptual framework for understanding the mechanism of secretory protein consignment to the ER lumen. It proposed that the amino-terminal peptide extensions of secretory proteins, denoted "signal sequences", directed the targeting of these nascent chains to the ER membrane. This targeting was proposed to be mediated by specific receptors in the ER membrane. Upon engaging these receptors, an aqueous pore would be formed that both spanned the membrane and was continuous with the ribosome. Thus, the nascent secretory protein could be transported directly into the ER lumen concurrent with its synthesis by the membrane bound ribosome.

Initially, there was much debate about the validity of this hypothesis, particularly the proposed requisite role for protein targeting and translocation machinery (von Heijne and Blomberg, 1979; Engelman and Steitz, 1981). However, the two decades following the signal hypothesis have seen the accumulation of substantial evidence in favor of this view. Not only has essentially every secretory protein been found to contain a signal sequence, but the proposed receptors for the signal sequence have been identified, and the translocation channel demonstrated and visualized. It is now well established that an increasingly complex macromolecular machine, termed the translocon (Walter and Lingappa, 1986), is responsible for the proper transport and biogenesis of proteins at the ER membrane.

For secretory proteins, the major role of this translocon is to facilitate the movement of the entire polypeptide across the otherwise impermeable ER membrane. By contrast, membrane proteins demand significantly more. In addition to translocation of some but not other domains, the translocon must also recognize potential membrane-spanning domains, properly orient these domains with respect to themselves as well as the membrane, and facilitate their integration into the lipid bilayer. Because of these and other complexities, most work on the function of the translocon has thus far focused on small secretory proteins as model study systems.

These studies have led to the discovery of protein conducting channels in the ER membrane (Simon and Blobel, 1991; Crowley et al., 1993, 1994), the isolation and functional reconstitution of their main components (Görlich et al., 1992b; Görlich and Rapoport, 1993), and most recently, their direct visualization (Hanein et al., 1996). Although far from complete, a framework for the mechanism of (at least simple) secretory protein biogenesis has emerged. With the tools developed during the elucidation of this framework in hand, increasing

attention is being paid to the molecular mechanisms of more complex events in protein biogenesis. The findings of these recent studies, although often contradictory, may be painting a rudimentary picture of the next frontier in protein translocation.

Secretory vs. membrane proteins: common aspects

In mammalian systems, secretory and membrane proteins are translocated across the ER membrane concurrent with their synthesis by membrane bound ribosomes (Morrison and Lodish, 1975; Palade, 1975). This "cotranslational" translocation begins in the cytosol with the synthesis of the first hydrophobic segment of a nascent polypeptide, either a signal or transmembrane (TM) sequence. Following the emergence of this hydrophobic sequence from the ribosome, it is recognized by the signal recognition particle (SRP), which mediates the targeting of the ribosome-nascent chain-SRP complex to the ER membrane in a GTP-dependent manner (reviewed by Siegel, 1995; Millman and Andrews, 1997). Once this complex is bound to the membrane, the nascent chain is transferred into the aqueous translocation channel, which is subsequently sealed from the cytosolic environment by a tight ribosome-membrane junction (Crowley et al., 1993; Jungnickel and Rapoport, 1995). Up to this point in biogenesis, both secretory and transmembrane proteins use the same SRP-dependent pathway of targeting. Furthermore, the 'generic' translocon in which this early nascent chain resides must be capable of facilitating the subsequent cotranslational biogenesis of both secretory and transmembrane proteins, directed by what is translated next (e.g., McCune et al., 1980; Kehry et al., 1980; Yost et al., 1983).

The molecular components of this 'generic' translocon are numerous and varied. The most recently identified components, the heterotrimeric Sec61

complex (with α, β, and γ subunits) and translocating-chain associated membrane protein (TRAM), turn out to be functionally crucial from the standpoint of translocation (Görlich et al., 1992a; Görlich et al., 1992b; Görlich and Rapoport, 1993). These proteins, when reconstituted with pure lipids and SRP receptor into proteoliposomes, are able to catalyze both the vectorial translocation of secretory proteins into the lumen as well as the integration of membrane proteins in the bilayer (Görlich and Rapoport, 1993; Oliver et al., 1995). Numerous lines of evidence have established that the functional and structural core of the translocon is composed of the heterotrimeric Sec61 complex. This complex has been shown to be adjacent to translocating nascent chains (Mothes et al., 1994), absolutely necessary for translocation (Görlich and Rapoport, 1993), and in some instances sufficient for translocation (Jungnickel and Rapoport, 1995). TRAM, on the other hand, has been shown to be adjacent to secretory and membrane proteins only at certain (poorly defined) stages of their translocation and/or integration (Görlich et al., 1992b; Mothes et al., 1994; Do et al., 1996). Functionally, TRAM was shown to facilitate the translocation of many, but not all proteins by aiding in the initial formation of a tight ribosome-membrane junction at the translocon (Görlich et al., 1992b; Voigt et al., 1996). However, the precise role of TRAM, if any, at later stages of translocation or during membrane integration remains obscure.

In addition to these 'minimal' components, several other protein complexes interact with nascent translocating polypeptides. Signal peptidase (a complex of 5 proteins) and oligosaccharyl transferase (a 3 protein complex) have defined enzymatic activities that are important for the maturation of many nascent chains (Evans et al., 1986; Kelleher et al., 1992). Similarly, many of the ER lumenal proteins (e.g., BiP, GRP94, calnexin, protein disulfide isomerase, and others) have been shown to interact with a variety of nascent chain substrates, and are

thought to act as molecular chaperones to promote proper folding and assembly (e.g., Munro and Pelham, 1986; Ou et al., 1993). Thus, although crucial for proper protein maturation and function, these accessory components of the translocon currently have poorly defined roles in the translocation process. It should be stressed however, that simply because translocation (of model secretory and membrane proteins) is *able* to proceed in the absence of these components, it is hasty to discount them from the translocation process. Given the potentially multiple functions of each of these proteins and the limited number of translocation substrates thus far examined, it is quite likely that in certain situations, one or more of these accessory factors serves an indispensable role in translocation. So how does this translocon, minimally composed of only the Sec61p complex and TRAM, handle the topologically diverse group of proteins encountered at the ER membrane?

The diversity of membrane proteins

Before one considers the question of how a universal translocon handles topologically diverse proteins, it is instructive to first ask what is required of such a translocon. For a secretory protein, one might imagine that the minimal requirement is simply an aqueous channel sufficiently large to accommodate a nascent chain that spans the ER membrane. In this situation, once the ribosome is docked tightly at this channel, continued translation of the message would result in vectorial discharge of the nascent chain into the ER lumen. The Sec61 complex can, at least in the case of the simplest secretory proteins, satisfy these requirements of tight ribosome binding and formation of a protein conducting channel (Görlich and Rapoport, 1993; Jungnickel and Rapoport, 1995, Hanein et al., 1996). However, it is already apparent that even for secretory proteins, the situation is not always this simple. Many secretory proteins require TRAM for

translocation (Görlich et al., 1992b; Voigt et al., 1996), while others do not necessarily maintain a tight ribosome-membrane junction throughout their translocation (Hegde and Lingappa, 1996). While the significance of these additional requirements and events in translocation of secretory proteins is currently unclear, they already hint at a more complex and dynamic translocon than would be necessary for the simplest case.

Membrane proteins complicate matters severely. Even a simple single spanning membrane protein needs to translocate certain domains into the ER lumen, leave others in the cytosol, properly orient the TM segment, and move it from the aqueous translocation channel into the lipid bilayer. Multi-spanning membrane proteins face even further challenges: i) TM segments must be oriented properly relative to each other, ii) in many cases, a subset of the TM segments may need to assemble into a defined structure (such as formation of an ion channel) before the protein is integrated into the bilayer, and iii) some TM segments may need to assemble with TM segments from other membrane proteins. Because many, if not all, of these events occur in an aqueous environment (i.e., before integration of TM segments into the lipid bilayer), the translocon should be adaptable to a variety of situations. It must not only be able to accommodate multiple TM segments at once, but also be capable of releasing some of these segments into the bilayer in a defined sequence, while keeping other regions of the chain in the channel. Furthermore, because TM segments synthesized by a membrane bound ribosome would be expected to enter the translocon with the N-terminal domain facing the lumen, some may need to be reoriented to achieve the final topology of a particular multi-spanning membrane protein. Finally, the translocon may need to allow the nascent chain to have access to TM segments that have already integrated into the bilayer in order to facilitate proper folding or other interactions. Thus, unless all of these TM

segment gymnastics are dealt with entirely post-translocationally, the translocon must either be enormously large, or structurally and functionally flexible. How do the currently available data, and our current views of the translocon compare with these requirements demanded for membrane protein biogenesis?

The classical model

The current view (literally) of the translocon using electron microscopy has shown the Sec61 complex to be in oligomeric rings in the ER membrane (Hanein et al., 1996). These rings, approximately 85-100 Å in diameter, appear to contain between 3-4 copies of Sec61 complex arranged around a central pore of approximately 20 Å in diameter. The large size of this pore clearly fits the requirement of accommodating an extended nascent chain (anhydrous diameter of 5-7 Å, ~11 Å when fully hydrated), and even chains with some secondary structure. Thus, although sufficiently large to house a TM segment in an α-helical conformation (~10-12 Å anhydrous diameter, 15-17 Å if fully hydrated), how might a static structure such as this mediate the biogenesis of multi-spanning membrane proteins?

The classical model to explain this conundrum has been to propose that the ribosome cycles between membrane bound and unbound states (Katz, et al., 1977; Blobel, 1980; see Figure 1). It is bound and docked at the translocon when synthesizing domains that follow a signal sequence or TM segment that is oriented with the N-terminus in the cytosol. These portions of the chain are thus transferred directly into the translocon destined for the ER lumen. Furthermore,

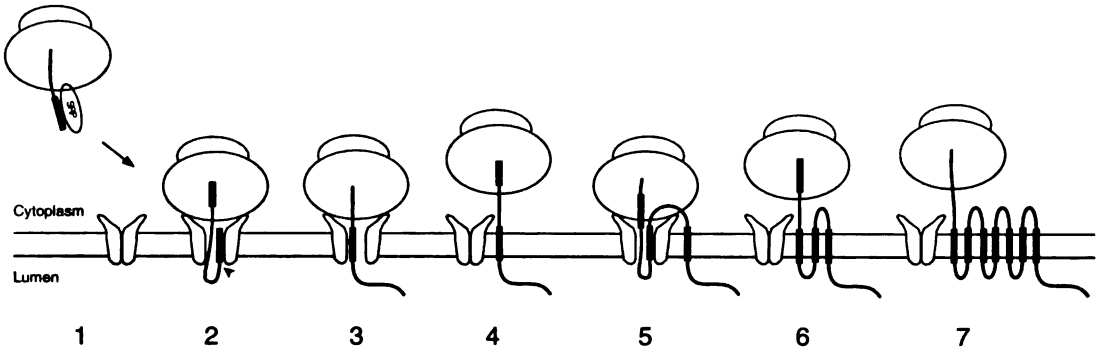


Figure 1. Conventional model of membrane protein biogenesis. In this model, hydrophobic sequences (either signal or TM segments) are recognized by SRP, targeted to the translocation channel, and inserted in the 'loop' orientation with the N-terminus of the segment facing the cytoplasm (steps 1 & 2 or 4 & 5). Subsequent stretches are synthesized by a membrane-bound ribosome and directly enter the translocation channel (e.g., steps 2, 3 and 5). When the next hydrophobic TM segment enters the channel (step 3), the ribosome detaches from the translocon, the TM segment(s) are released into the lipid bilayer, and the translocation channel closes (e.g., steps 4 and 6). This cycle of events repeats until all TM segments are 'spooled' into the membrane (step 7).

the next TM segment would enter the translocon oriented properly (opposite of the previous one) with the N-terminus directed toward the lumen. Upon halting further translocation when this TM segment reaches the translocon, the ribosome would detach and the subsequent domain (which should be cytosolic) would be synthesized directly into the cytosol. The next synthesized TM segment would provide an internal signal sequence for retargeting the ribosome (with or without SRP) to the translocon and positioning of this segment in an N-cytosolic orientation. If each TM segment exited the translocon to the lipid bilayer before the next one entered, the 20 Å size of the translocon would suffice entirely, and the TM segments would simply be sequentially "spooled" into the lipid bilayer in an alternating fashion. Although this model can theoretically explain the biogenesis of a wide variety of membrane and secretory proteins while demanding very little of the translocon, evidence for it is lacking in most cases, and contradictory in others (see Rapoport et al., 1996, and references therein).

The "spooling" model predicts that the ribosome detaches from the translocon when synthesizing cytosolic domains of a membrane protein (e.g., Figure 1, steps 4 and 6). Unfortunately, not only is there little if any indication of such a detachment, but evidence to the contrary has been provided by Mothes et al. (1997). They were able to demonstrate that the cytosolic domain of the nascent chain following a TM segment could be crosslinked to components of the translocon (Sec61α and Sec61β). Furthermore, severing the nascent chain within this cytosolic domain between the TM segment and the ribosome did not release the ribosome from the membrane. Finally, even after severing the nascent chain, the domain of the chain still bound to the ribosome (which itself remained bound to the membrane) could still be crosslinked to the translocon. These experiments indicate that not only is the ribosome still membrane bound

when synthesizing the cytosolic domains of a membrane protein, but both it and the nascent chain are still at the translocon.

A second prediction of the "spooling" model is that TM segments should integrate into the lipid bilayer shortly after they enter the translocation channel (Figure 1, step 4). This allows the ribosome to detach from the translocon with the chain firmly anchored into the membrane, and permits the translocon to prepare for the next TM segment. While it seems clear that the ribosome does not detach from the translocon (see above), TM segments have been shown in some cases to integrate into the bilayer shortly after entering the translocation channel (Mothes et al., 1997). However, the immediate integration of TM segments as they are synthesized does not always occur. With some substrates, it appears that the TM segment remains in the aqueous translocon long after it enters, not integrating until translation of the entire protein has finished (Thrift et al., 1991; Do et al., 1996). In the most dramatic example, it appears that up to six TM segments of some membrane proteins could be synthesized without the protein being integrated into the lipid bilayer (Borel and Simon, 1996). That is, these nascent chains could be extracted from the membrane by treatments which do not extract completed integral membrane proteins. Thus, at a time in biogenesis when (according to the above model) several TM segments should have integrated into the lipid bilayer, the entire nascent chain was still in an aqueous environment.

Finally, the "spooling" model requires that incredibly diverse TM segments (some very hydrophobic while other that are amphipathic or even quite charged) all be recognized by the translocon (and subsequently be moved into the lipid bilayer), while all domains of secretory proteins (some of which can be more hydrophobic than bona fide TM segments) need to be allowed to translocate into the lumen. While this may be possible (albeit energetically unfavorable in some

cases), it makes difficult the formation of intramolecular interactions between TM segments that stabilize the final structure. Not only are such interactions functionally important, but they can allow multiple relatively hydrophilic TM segments to form a larger unit that energetically favors integration into the lipid bilayer. Indeed, during the biogenesis of some membrane proteins, individual TM segments are unable to integrate into the lipid bilayer unless they interact with other specific TM segments, which by themselves also cannot integrate into the bilayer (Skach and Lingappa, 1993; Wilkinson et al., 1996). Thus, while it remains entirely possible that *some* membrane proteins are integrated in the "spooling" fashion (Kuroiwa et al., 1996), other mechanisms are also likely to be involved.

A bigger, more dynamic translocon

It is clear that most alternative mechanisms of membrane protein biogenesis, especially if they allow for multiple TM segments to interact within the aqueous translocation channel, are likely to require a channel that is significantly larger than the 20 Å pore visualized by Hanein et al. (1996). Evidence that these exist comes from two sources (see Figure 2).

First, Hamman et al. (1997) recently utilized a biophysical approach to estimate the pore size of a functioning translocon containing a nascent chain intermediate. Their approach was to prepare translocation intermediates of a secretory protein in which a fluorescent probe was incorporated into the nascent chain and positioned within the translocation channel. These translocation intermediates were then incubated with various quenchers of the fluorescent probe. By determining the maximum size of the quenching molecules that are able to physically enter the translocation channel containing the nascent chain

and effectively quench the fluorescence, the channel was estimated to be 40-60 Å in diameter (Figure 2b).

The second line of evidence that the translocation channel may in some cases be larger than 20 Å is provided indirectly by the data of Borel and Simon (1996). Although they did not address this question directly, they were able to demonstrate that up to six TM segments were able to assemble at the membrane before any of them integrated into the lipid bilayer. Although they did not directly demonstrate that these TM segments were in the translocation channel per se (e.g., by crosslinking studies), it is the most likely aqueous arena in which they were contained. If this were the case, it is quite unlikely that the six TM segments (each in an α-helical conformation with an anhydrous diameter of 11 Å) all fit within a pore of 20 Å diameter.

If the conclusions of both the direct and indirect measurements of translocation channel pore size are taken at face value, it might be concluded that the channel has the potential to modulate its size, maybe by recruiting more copies of Sec61. Indeed, even the direct electron microscopic observations of purified Sec61p complex formed channels (Hanein et al., 1996) revealed significant structural heterogeneity. This was suggested to potentially be due to variable numbers of Sec61 complex per ring or perhaps differences in subunit composition. Furthermore, if all portions of a multi-spanning membrane protein are synthesized by a translocon-bound ribosome (as suggested by Mothes et al., 1997; see above), and several TM segments can actually assemble within this translocon (Borel and Simon, 1996), a unifying model of membrane protein biogenesis begins to emerge.

In order to accommodate several TM segments within the translocon and allow their reorientation (which would be necessary if all of them entered the translocon in the same orientation due to a membrane bound ribosome), the

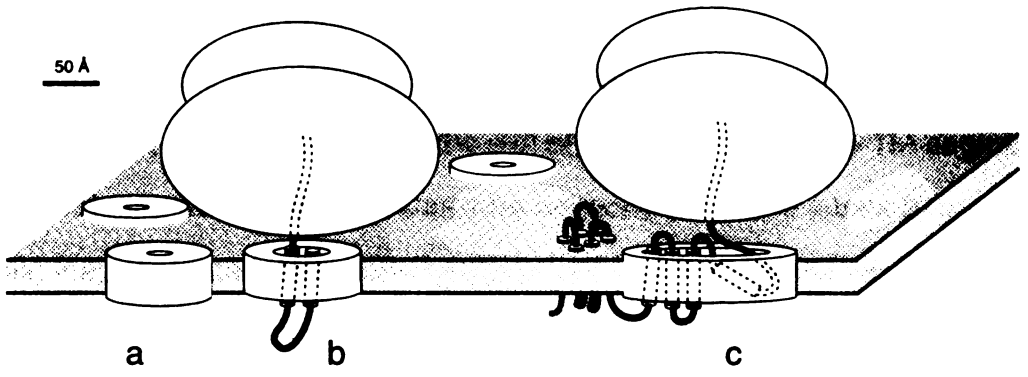


Figure 2. The size of the translocation channel as estimated by various methods. Direct visualization of the structures formed by purified Sec61 complex reveals a channel with a pore of approximately 20 Å in diameter (structure a, Hanein et al., 1996). Biophysical examination of a pore in the process of translocating a secretory protein shows the pore to be significantly larger, at 40-60 Å in diameter (structure b, Hamman et al., 1997). Based on the ability of the translocation pore to house at least six TM segments (suggested by the data in Borel and Simon, 1996), a speculative maximum pore size of ~100 Å is depicted (structure c). The ribosome (~250 Å in diameter) is shown for comparison. The black bar represents 50 Å.

translocation channel may need to expand to 80 Å in diameter or more (see Figure 2c). For example, six TM segments positioned in the translocon perpendicular to the plane of the membrane may require at much as 40-50 Å, assuming that each TM segments is in an alpha-helical conformation with a width of 11 Å. When the next TM segment is synthesized, there should be room to properly orient it, requiring an additional ~30-35 Å (the length of an average TM segment) or more (see Figure 2c).

The translocon, in addition to allowing multiple TM segments to accumulate and assemble, should also permit the exit of some or all of these TM segments at any time during translation. This may be necessary in some cases to prevent certain inappropriate interactions between TM segments, while allowing fully assembled sections of a protein to integrate into the bilayer as appropriate. Consistent with this proposed model of a laterally gated translocon, it has been demonstrated that some TM sequences are adjacent to lipid early in the biogenesis of a protein while sometimes remaining adjacent to translocon components (Martoglio et al., 1995; Mothes et al., 1997). Furthermore, some TM segments can in fact exit to the bilayer before synthesis of subsequent domains (Mothes et al., 1997), while other TM domains remain in an aqueous environment until the completion of translation (Thrift et al., 1991; Do et al., 1996). Together, these data suggest that the translocon is capable of, but not obligated to release TM segment(s) before synthesis of translation, with the decision perhaps determined by sequences within the nascent chain.

Several questions arise with the idea of a dynamic, expandable translocon of such enormous sizes. Is the ribosome-translocon-nascent chain complex capable of such dynamic changes during cotranslational translocation? When and how might the translocon expand and contract in size? How is the permeability barrier of the ER membrane maintained during these changes which

create such an enormous pore? Although definitive answers to these questions require further studies, recent observations suggest some possibilities.

Evidence that the translocon is a dynamic structure has been provided in multiple ways. First, it was demonstrated that early in translocation the translocon is gated on the lumenal side, regulated by the nascent chain (Crowley et al., 1994). The translocation channel is initially closed to the lumenal side, even after nascent proteins are targeted and docked at the cytosolic side. However, upon further elongation of the nascent chain to a length of ~70-80 amino acids, a lumenal "gate" opens and the ER lumen is continuous with the ribosome via the pore of the translocation channel.

At later times in the translocation of a secretory protein, the ribosome membrane junction at the cytosolic side of the translocon was also shown to be gated, again regulated by the nascent chain. In these instances, pauses in translocation are accompanied by an opening of the ribosome-membrane junction that exposes large domains of the nascent chain to the cytosolic environment (Hegde and Lingappa, 1996). Although the significance of these findings remains to be determined, the rearrangements of the ribosome-membrane junction were sufficiently protracted to allow interactions of the nascent chain with macromolecules in the cytosol.

In addition to dynamic events in gating, rearrangements of membrane proteins of the translocation channel have been suggested to occur. Perhaps the most consistently observed variable is the presence of the TRAM protein. By contrast to Sec61α, TRAM is not always found adjacent to a nascent chain positioned in the translocation channel (Mothes et al., 1994). Rather, it appears as if it may be nearby only during specific events in translocation such as tight insertion of some signal sequences into the translocon (Voigt et al., 1996) or integration of some TM segments into the bilayer (Do et al., 1996). Additionally,

changes in the crosslinking pattern during certain points in translocation provide further evidence that the proteins neighboring the nascent chain are malleable (Hegde and Lingappa, 1996; Mothes et al. 1994).

Recently, many of these changes were observed to occur in regulated fashion during the biogenesis of simple model membrane proteins: the lumenal gate was observed to close promptly after synthesis of a TM segment, the ribosome membrane-junction was observed to open shortly thereafter, and crosslinking patterns varied during these events (Liao et al., 1997). Furthermore, since these events were shown to occur while the TM segment was still inside the ribosome, some nascent chain sequences may be first recognized by the ribosome itself. Thus, it appears that components in multiple compartments (the cytosol, membrane, and/or lumen) may recognize regulatory sequences in the nascent chain to elicit reorganization of the translation-translocation machinery.

The observation that the translocon can be quite dynamic is certainly consistent with an ability to expand its size by the recruitment of more Sec61 complex, TRAM, or other components. In fact, the recognition of TM segments by the ribosome, before it reaches the translocon, may facilitate translocon expansion. This early detection system could allow the lumenal gate to close and the translocon to expand before the TM segment arrives, allowing it ample room to be rotated and/or positioned within the translocon. Some of this space may be created by the opening of the ribosome membrane junction. Alternatively, the opening of the junction (as judged by accessibility of the nascent chain to cytosolic probes) may be an epiphenomenon reflecting a growing translocon. The phenomenon of translocational pausing, where the junction is also observed to open (Hegde and Lingappa, 1996), may reflect a similar enlarging of the translocon for the purpose of allowing specialized folding or modifications within the translocon.

Thus, a translocon that is dynamic in both its size and gating in all three dimensions (cytosolic, lumenal, and in the plane of the bilayer) could explain much of the disparate initial findings on the biogenesis of membrane proteins (see Figure 3). Such a translocon would be flexible enough to handle each substrate slightly differently to accommodate subtle variations and requirements crucial to achieving a functional end product. Indeed, the contradicting conclusions resulting from many of the studies which each used different test substrates may reflect this flexibility.

Finally, this model does not necessarily postulate the existence of components in addition to the major ones identified for secretory proteins. Although the lumenal gate has not yet been identified, it could involve conformational changes in the Sec61 complex or one of the several known lumenal proteins. Similarly, a gate in the plane of the bilayer may be composed of either components of the Sec61 complex or TRAM, both of which have been shown to be adjacent to TM segments during particular steps of the integration process (Do et al., 1996). Furthermore, expansion of this structure could be accomplished by simply recruiting more copies of Sec61 complex into the ring. These features, along with the recent possibility that the ribosome may play an active part in the dynamics of the translocon (Liao et al., 1997) provide more than enough players and wobble room for complex events in membrane protein biogenesis.

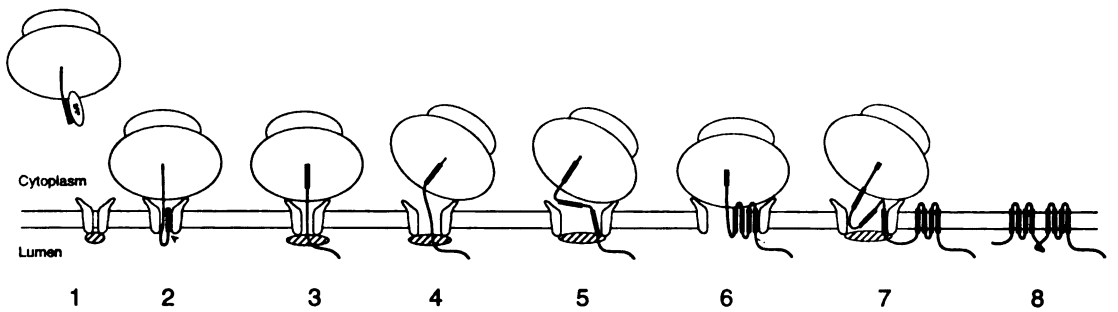


Figure 3. Speculative model of mechanism of membrane protein biogenesis. A lumenal gate (striped oval) is opened upon tight ribosome binding to the translocon, and translocation of the nascent chain ensues (steps 1 & 2). Upon synthesis of a TM segment, the lumenal gate is closed (step 3) and ribosome-membrane junction opened (step 4). At this point, before the TM segment emerges from the ribosome, the translocon is expanded (step 4) to accommodate orientation of multiple TM segments relative to each other (step 5). When a functional unit of TM segments is assembled within the translocation channel (step 6), they are released into the lipid bilayer (step 7) while subsequent TM segments are retained prior to assembly of the next functional unit. In this model, the permeability barrier of the membrane is maintained by a combination of the lumenal gate and ribosome-membrane junction, each of which is modulated by sequences in the nascent chain. TM segments are allowed to re-orient themselves and assemble with other TM segments in the space created by an enlarged translocation channel and/or open ribosome-membrane junction (e.g., see steps 5 and 7). TM segments are allowed to leave the translocation channel prior to completion of protein synthesis, but do not necessarily leave as they enter the translocon.

With Complexity Comes Regulation

The enormous diversity of proteins that transit the secretory pathway demands a mechanistic complexity in biogenesis that has yet to be fathomed. The current understanding of the molecular components that direct the translocation of a limited subset of simple secretory proteins has revealed a remarkably (and probably deceptively) simple picture. As the lessons learned from these studies are being extended to slightly more complex substrates, it is becoming more and more obvious that our understanding is neither complete nor clear. Many substrates, especially membrane proteins, appear to require the translocation machinery to make "decisions" specific to a particular situation or substrate: Which domains of a protein are TM segments? Which TM segments should be held in the translocation channel for purposes of folding or association with distal domains, and which should be integrated immediately into the bilayer? What should the orientation of various TM segments be? With each of these decisions comes the opportunity for regulation.

Although fundamental advances toward answering such questions will undoubtedly require the development of new ideas as well as techniques, a handful of initial studies on complex substrates may suggest the functional regulation of protein biogenesis. As with transcriptional and translational control, the cell may use translocational control as an additional means of generating diversity of gene expression. Indeed, several membrane proteins have been observed to be expressed in multiple topological forms, with the diversity apparently being generated at the time of translocation at the ER membrane (see Levy, 1996 for a review). For example, the protein ductin not only has two orientations (Finbow et al., 1993), but each orientation appears to serve different functions. One of the topological forms is found as a subunit of the vacuolar H^+-ATPase, while the other form is a component of a connexon channel found in

gap junctions. That this diversity originates at the translocation site in the ER was demonstrated by showing that ductin translated and translocated in a cell-free system results in the synthesis in both orientations (Dunlop, et al., 1995).

Similarly, the P-glycoprotein product of the multidrug resistance gene (MDR1) found in various cancer cells is a membrane protein with at least two topological forms (Skach et al., 1993; Zhang et al., 1993). Although predicted to span the membrane 12 times, several of its TM segments apparently can exist in multiple orientations or locations, perhaps regulated by factors in the cytosol (Zhang and Ling, 1995). This type of structural variability appears to be qualitatively different than that observed in ductin, where the entire protein is reversed in orientation with respect to the membrane. However, similar to ductin, MDR1 has been proposed to serve multiple functions in the cell (Pastan and Gottesman, 1991). Whether the different topological forms are recruited to different regions of the cell for specialized functions, as appears to be the case for ductin, remains to be determined.

Finally, some proteins may contain potential TM segments that are not used under all circumstances. For example, the prion protein (PrP), a brain glycoprotein involved in various neurodegenerative diseases (Prusiner, 1996) , contains a hydrophobic domain initially predicted to serve as a TM segment (Bazan et al., 1987). Despite this hydrophobic segment, PrP does not appear to normally span the membrane in vivo, but rather is translocated across the ER membrane, C-terminally glycolipididated, and trafficked to the cell surface (Stahl et al., 1987). By contrast, studies in cell free systems have shown that not only can PrP span the membrane at its putative TM segment, but under some conditions, nearly all of it is found as a transmembrane protein (Hay et al., 1987a). The generation of this topological form is dependent on both hydrophobic and hydrophilic sequences in the PrP molecule (Yost et al., 1990)

and appears to be regulated by cytosolic factors (Lopez et al., 1990). However, just as the normal role of the PrP molecule remains enigmatic, so does the topological regulation of this unusual protein. It will be interesting to see whether the topology of PrP is regulated in vivo by trans-acting cellular factors, and whether dysregulation of these events at the ER plays a role in any of the wide variety of diseases attributed to PrP. If so, it seems likely that a transmembrane form, not being observed in normal brain, is involved in events related to prion disease that are carried out, in part, by as yet unidentified components of the translocon.

The identification and functional reconstitution of the core components of the translocon using simple substrates, have now set the stage for exploring the functional complexity and structural diversity of accessory translocon components in biogenesis of more complex secretory and membrane proteins. The initial studies on membrane proteins have already revealed unappreciated subtleties of the translocation process, pointing to a new site of regulation in the cell. Future work using the tools obtained from past work on model proteins, used to study the biogenesis of currently enigmatic substrates, will surely elucidate new functions for old machinery and new machinery involved in currently mysterious functions.

II. The Translocation of Apolipoprotein B

Sequence-specific alteration of the ribosome-membrane junction exposes nascent secretory proteins to the cytosol.

Abstract

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; Walter and Johnson, 1994; Siegel, 1995). Signal sequences within the nascent secretory protein are first recognized by a cytosolic ribonucleoprotein, signal recognition particle (SRP). Binding of SRP appears to displace from nascent secretory proteins a complex termed the Nascent polypeptide Associated Complex (NAC) which is associated with both secretory and non-secretory nascent chains (Wiedmann et al., 1994). Subsequently, the ribosome-nascent chain-SRP complex is directed to the secretory pathway via an interaction between SRP and its receptor at the ER membrane. The nascent chain is then transferred from SRP, via a GTP dependent step, to binding sites within an aqueous translocation channel (Rapiejko and Gilmore, 1997; reviewed by Millman and Andrews, 1997). Once the ribosome is docked securely at the translocation channel, the nascent chain is co-translationally translocated across the ER membrane.

Several independent approaches have led to the conclusion that the nascent chain is translocated through an aqueous channel. First, it has been demonstrated that partially translocated nascent chains can be extracted from membranes using aqueous perturbants (Gilmore and Blobel, 1985). Second, electrophysiological measurements across rough ER microsomes reveal the appearance of aqueous channels across the membrane upon treatment with puromycin, suggesting that those channels were previously occupied with nascent secretory proteins blocking the passage of ions (Simon and Blobel, 1991). More recently, it was demonstrated that fluorescent probes incorporated

into the nascent chain are in an aqueous environment during their transit across the ER membrane (Crowley et al., 1993, 1994).

Crosslinking studies have demonstrated that nascent secretory chains are in the proximity of several integral membrane proteins (Wiedmann et al., 1987; Krieg et al., 1989; High et al., 1991; Görlich et al., 1992a). However, convincingly demonstrating a role for any of these proteins in the translocation process remained elusive. The means by which functional studies could be performed was facilitated by the pioneering studies of Nicchitta and Blobel (1990). Their studies demonstrated that proteins of the ER membrane could be solubilized and subsequently reconstituted into proteoliposomes that retained the ability to translocate secretory proteins. These techniques allowed the fractionation, purification, and subsequent reconstitution of selected membrane proteins into lipid vesicles (Nicchitta et al., 1991; Migliaccio et al., 1992; Görlich et al., 1992b; Görlich and Rapoport, 1993). As a result of these studies, crucial roles for a subset of ER proteins in translocation have been demonstrated.

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 (TRAM) was required and/or stimulatory for the translocation of some but not other secretory proteins (Görlich et al., 1992b; Görlich and Rapoport, 1993). Still other membrane proteins such as the translocon-associated protein (TRAP) complex can be crosslinked to nascent secretory proteins, but their role in translocation remains to be elucidated (Wiedmann et al., 1987; Görlich et al., 1992b).

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 in 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 (Walter and Lingappa, 1986).

The architectural organization of the various proteins in the translocon, and their juxtaposition with the machinery of protein synthesis remains to be fully elucidated. Sec61α has been demonstrated to be adjacent to the nascent chain at all times during translocation, implicating it as the major component of the aqueous channel that forms the central core of the translocon (Mothes et al., 1994). Furthermore, the Sec61p complex has been shown to strongly bind to ribosomes synthesizing secretory proteins (Kalies et al., 1994), raising the possibility that the putative channel formed by Sec61p is continuous with the ribosomal tunnel from which nascent polypeptides emerge (Malkin and Rich, 1967; Blobel and Sabatini, 1970).

Fluorescent probes incorporated into a nascent secretory protein were used to demonstrate a continuous aqueous channel between the site of protein synthesis in the ribosome (the peptidyl transferase center) and the ER lumen. In these experiments, water-soluble quenching agents (iodide ions) were shown to have access to fluorophores in the nascent chain only if they are supplied from the lumenal side of the membrane (Crowley et al., 1994). Furthermore, the inability of iodide ions supplied from the cytosolic side to quench fluorophores in secretory nascent chains under conditions in which they could be crosslinked to translocon proteins argued strongly for the presence of a tight seal between the ribosome and the translocon (Crowley et al., 1994; Mothes 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 carboxy 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 are completely shielded from molecules in the cytosolic environment. However, these conclusions are 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 is 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). Moreover, even while spanning the membrane, translocationally paused intermediates were found to be extractable with sodium carbonate pH 11.5, indicating that they were not integrated into the lipid bilayer, but presumably, resided in the aqueous translocation channel. While simple secretory proteins such as prolactin appears not to pause during translocation, a variety of proteins besides apo B, whose biogenesis is more complex than that of prolactin, also demonstrate translocational pausing (Nakahara et al., 1994; RSH and VRL, unpublished). These observations suggest that translocational pausing may be a general mechanism involved in

translocation of complex proteins across the membrane of the ER. 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 to the translocation of a simple secretory protein, which is always well shielded from the cytosol. Furthermore, a translocationally paused nascent chain can be crosslinked to membrane proteins that are not seen to crosslink with a matched but non-paused 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 been shown previously 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 to even 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 allow us to directly assess whether simple chain length has any effects on protease accessibility or the stability of the ribosome-membrane junction. Figure 4A 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 co-sedimentation 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 carboxy terminal region of the translocation intermediates is in the cytosol (albeit within the ribosome), it are 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 4B). In the cases where they were accessible to protease, a substantial fraction of the chains were degraded to yield a smaller protected fragment (arrowheads, Figure 4B), which was reactive to an antibody directed at the amino 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 are ones which have in fact largely

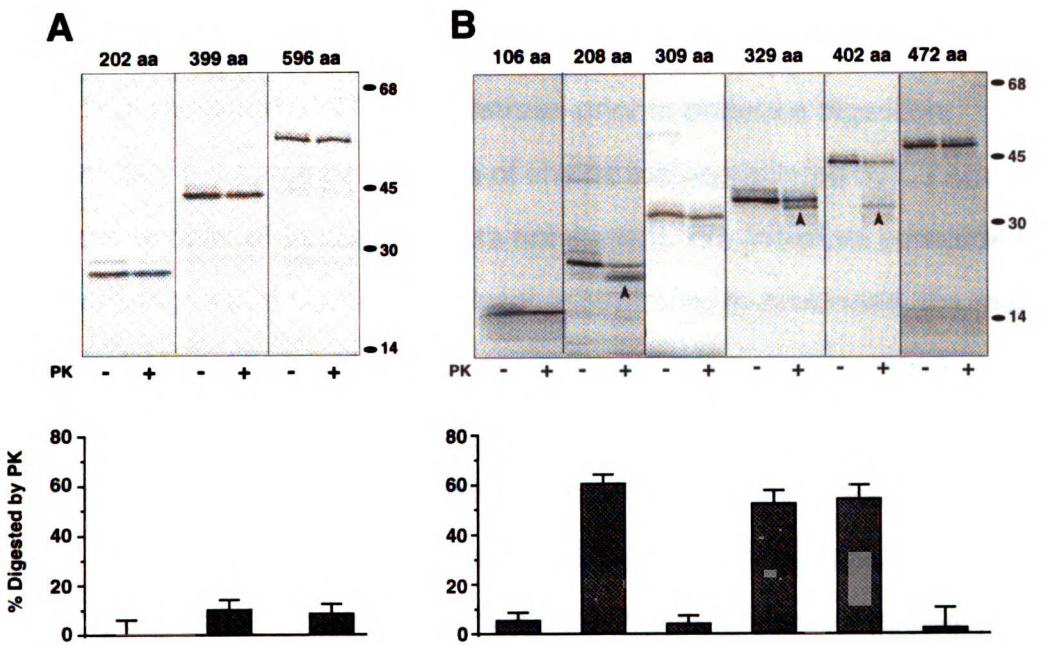


Figure 4 - Access of nascent translocation intermediates to proteinase K.
(A) Plasmids encoding one, two, or three prolactin coding regions in tandem were truncated before the termination codon and translated to generate translocation intermediates of the lengths (in amino acids) indicated above each panel. Shown are the autoradiograms from a representative experiment for each of the translation reactions before (-) and after (+) digestion with proteinase K (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 panel A. In each instance that 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.

spontaneously released from the ribosome, and are 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 are 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., in preparation). 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 aa translocation intermediate shown in Figure 4B has approximately 32 kD of chain translocated into the ER lumen and protected from protease, with the remainder accessible to PK digestion. At the subsequent 402 aa truncation point, the chain is still paused at the position that generates the 32 kD protected domain. However, still later in chain growth, at 472 aa, 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, Factor Xa (FXa), to re-examine 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 four 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 5A). Thus, if the ribosome membrane junction is tightly sealed, the cleavage site would be predicted to have exited the ribosome (which shields the carboxy-terminal 20-40 amino acids; Malkin and Rich, 1967) and 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 5 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 amino terminus fragment (upward arrowhead, Figure 5B, lane 2) and a small carboxy terminus fragment (downward arrowhead). Digestion with PK following FXa cleavage revealed that the small carboxy terminus fragment is in the cytosol where it can be degraded by PK, in constrast to the larger amino 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 carboxy-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,

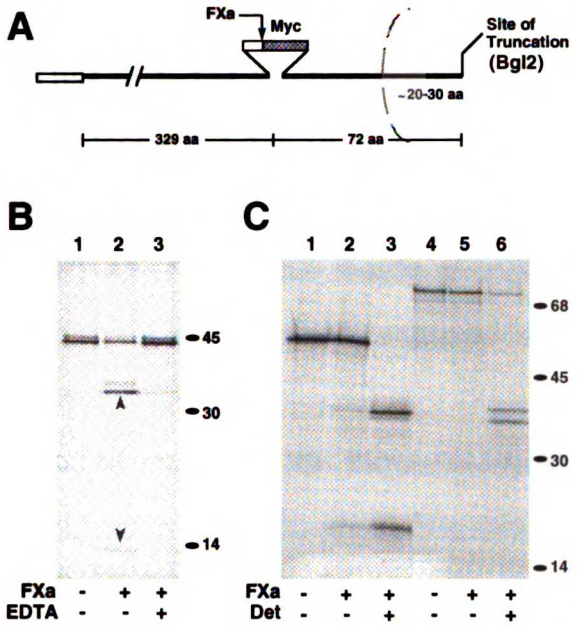


Figure 5 - Access of paused translocation intermediates to Factor Xa.
(A) Construct ApoB-FXa. The signal sequence (open box), Factor Xa cleavage site (FXa), and myc epitope (shaded box) are indicated. The ribosome (curved dotted line) is expected to shield the C-terminal 20-30 amino acids of the chain. (B) ApoB-FXa was truncated at Bgl2, 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, 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 Nco1 (lanes 1-3) or left untruncated (lanes 4-6), translated, and digested with FXa as in panel B. Where indicated (lanes 3, 6), 0.5% Triton-X 100 was included during FXa digestion.

1992), resulted in the chains no longer being accessible to FXa cleavage (Figure 5B). Likewise, truncation at a later point or translation of full length apoB, at which time translocation has restarted and hence the pause has been abolished, renders the chain largely inaccessible to FXa cleavage (Figure 5C). These results are consistent with the earlier findings on PK accessibility of serial apo B truncations (see Figure 4B). All of 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 were first solubilized with detergents before proteolysis. They found that approximately 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 non-paused nascent chains by this method, we found that only the 30-40 carboxy-terminal amino acids of paused nascent chains were protected, but 70-100 amino acids were protected of non-paused controls (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, where a non-paused translocating nascent chain is not.

Accessibility of paused nascent chains to antibodies

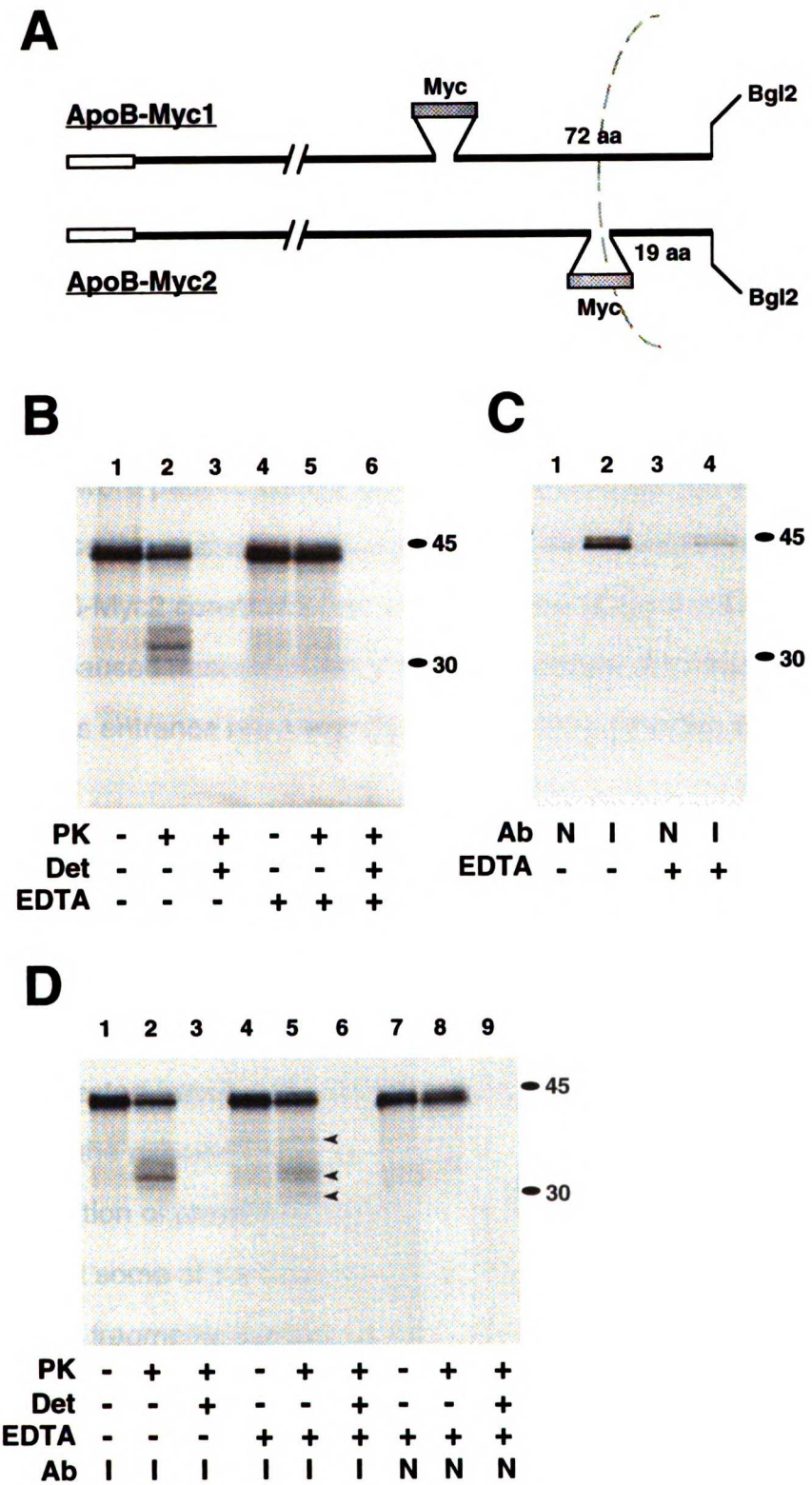
We next determined how much chain was actually exposed to the cytosol, and whether this was sufficient to be recognized and bound by specific antibodies. This determination would serve several purposes: it would a) provide

independent and very strong corroboration that the ribosome-membrane junction is open during translocational pausing, b) demonstrate that the cytosolically exposed domains of the nascent chain are large enough to allow their interaction with macromolecules in the cytosol, c) allow us to map the extent of the cytosolically accessible region of the paused nascent chain, and d) 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; see Figure 6A). 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 (see Figure 6A), we should be able to definitively ascertain 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 kD (Figure 6B, compare to the 402 aa intermediate in Figure 4B). 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 nascent chains were then examined for the accessibility of the myc epitope to cytosolically added

Figure 6 - Access of paused translocation intermediates to antibodies.
(A) Constructs ApoB-Myc1 and ApoB-Myc2. (B) ApoB-Myc2 was truncated at Bgl2, translated, and one aliquot treated with 10 mM EDTA as in Figure 5B. Microsomal membranes were isolated and divided for immunoadsorption (see panel C) or digestion with PK. Where indicated, the 0.5% Triton X-100 (det) was included during protease digestion. (C) The intact, isolated microsomes from panel B were immunoadsorbed using α-myc antibodies (I) or non-specific antibodies (N) as described in Experimental Procedures. (D) ApoB-Myc1 was truncated at Bgl2, translated, and the microsomal membranes isolated. The sample was then divided, and incubated for 60 min with either α-myc antibodies (I) or non-specific 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.


A
ApoB-Myc1
Myc
72 aa
Bgl2
ApoB-Myc2
19 aa
Myc
Bgl2
B
1 2 3 4 5 6
45
30
PK - + + - + +
Det - - + - - +
EDTA - - - + + +
C
1 2 3 4
45
30
Ab N I N I
EDTA - - + +
D
1 2 3 4 5 6 7 8 9
45
30
PK - + + - + + - + +
Det - - + - - + - - +
EDTA - - - + + + + + +
Ab I I I I I I N N N

antibodies (Figure 6C). We found that when apo B nascent chains are paused (as assessed by their accessibility to PK, and under the same conditions that renders them accessible to FXa when they contain a 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 6C). 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 kD were 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 6D, 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 (arrowheads, Figure 6D, lane 5). The inclusion of non-specific antibodies had no effect on restarting translocation of a paused chain, as indicated by the lack of PK accessibility following EDTA treatment (Figure 6D, lanes 7-9). These data indicate that a bound antibody (approximately 150 kD) 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).

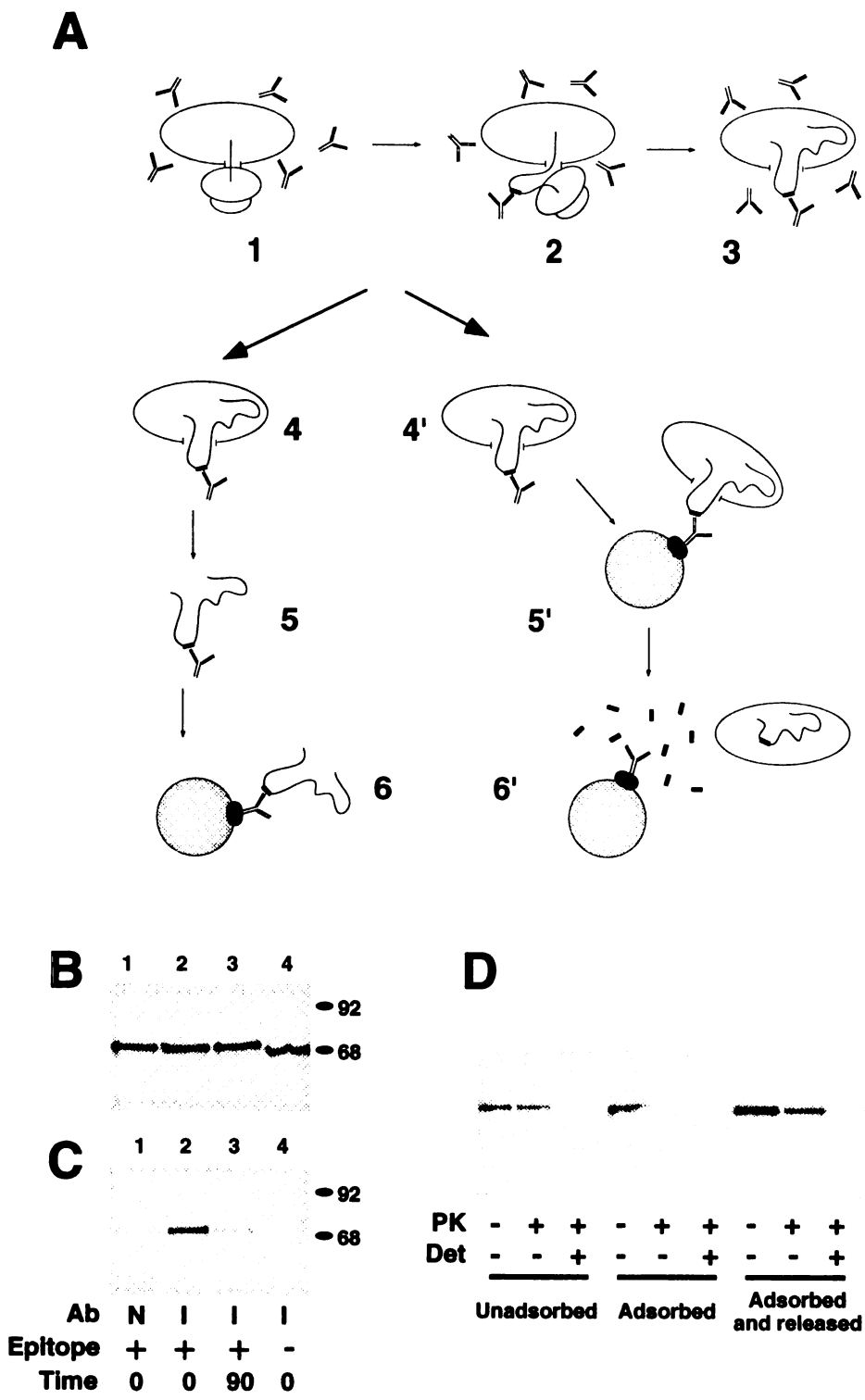
Trapping of nascent chains in "real time"using antibodies in the cytosol

In order 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 (see Figure 6D). 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 can be adsorbed from the cytosolic side of the microsomes (see Figure 7A). 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: a) ApoB-Myc1 was translated in the presence of an irrelevant monoclonal antibody, b) ApoB-Myc1 was translated in the presence of α-myc antibodies, c) ApoB-Myc1 was translated in the in the absence of antibodies, and α-myc antibodies were added post-translationally, and d) apo B (lacking the myc epitope) was translated in the presence of α-myc antibodies. An aliquot of each reaction was analyzed by SDS-PAGE to demonstrate that the presence or absence of antibodies did not affect translational efficiency (Figure 7B; lanes 1-4 correspond to conditions a-d above). The remainder of each sample was subjected to immunoadsorption as follows (see Experimental Procedures for details). The microsomal membranes were isolated by centrifugation and 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 non-specifically, or via specific antigen-antibody interaction. The antibody complexes were then collected with immobilized protein-G following solubilization of the sample under non-denaturing conditions. These samples were analyzed by SDS-PAGE and autoradiography (Figure 7C). We found that the only conditions which resulted in significant immunoadsorption of the nascent chain were when the epitope was present in the translated protein, when the antibodies were specific to the epitope, and most importantly, only if the antibodies were added cotranslationally. If the antibodies were added post-translationally, significantly less nascent chain is immunoadsorbed (compare lanes 2 and 3, Figure 7C). 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

Figure 7 - Capture of translocating nascent chains with antibodies in the cytosol. (A) The experimental design for panel C (diagrams 1-6) and panel 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,C) ApoB15 (- epitope) and ApoB-Myc1 (+ epitope) were translated for 90 min in the presence of microsomal membranes and either non-specific antibodies (N) or α-myc antibodies (I) included at 25 μg/ml. To one sample, α-myc antibodies were added post-translationally (at 90 min, lane 3). An equal aliquot (1 μl) of each of the samples was analyzed directly (Panel B) while the remainder (49 μl) was subjected to immunoadsorption as described in Experimental Procedures (Panel 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.


A
1
2
3
4
4'
5
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6
6'
B
1 2 3 4
92
68
C
1 2 3 4
92
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Ab N I I I
Epitope + + + -
Time 0 0 90 0
D
92
68
PK - + + - + + - + +
Det - - + - - + - - +
Unadsorbed
Adsorbed
Adsorbed and released

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 further validate the interpretation of the data in Figure 7C, 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 in order to demonstrate that they are indeed spanning the membrane (see Figure 7A). 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 7D) strongly argue that the apoB chains were spanning the membrane, prevented from complete translocation into the lumen only by the bound antibody.

Probing the environment of paused nascent chains using crosslinking

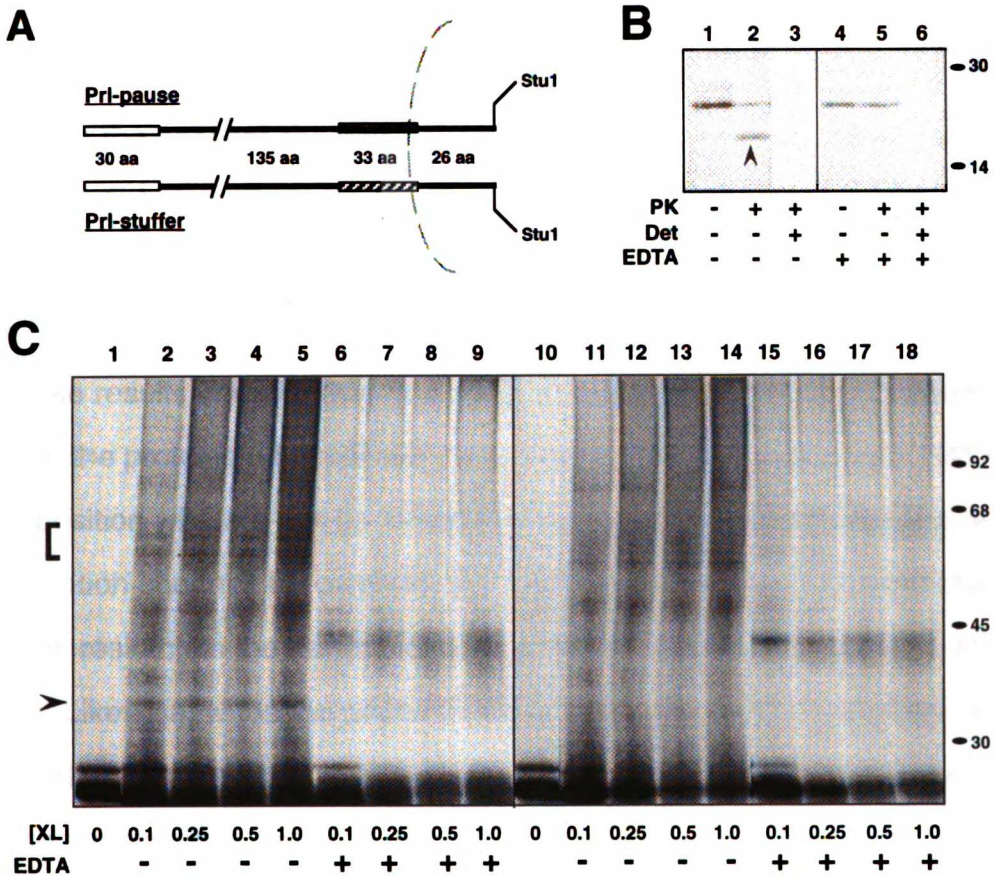
In view of the dramatic changes in the translocation apparatus during translocational pausing, we reasoned that the subset of translocon proteins that are adjacent to the paused nascent chain might also be different. Using a crosslinking approach, it has been shown that a conventional secretory protein such as prolactin is adjacent to Sec61α, and sometimes TRAM, 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 crosslinkable to a *paused* nascent chain.

In order to determine whether pause transfer specific changes in the nascent chain environment occur, we compared the pattern of crosslinked proteins for a

paused versus matched, non-paused nascent chain. We chose to use prolactin as the coding region into which we inserted the pause transfer and non-pausing control sequences, since the translocation and crosslinking properties of this protein have been extensively characterized (Wiedmann et al., 1987; Görlich et al., 1992b; 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, see Figure 8A). 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 8B). 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 crosslinking using various concentrations of the bifunctional crosslinker disuccinimidyl suberate (DSS). We found that while most of the crosslinked proteins are the same for both Prl-pause and Prl-stuffer nascent chains, only the paused nascent chains are crosslinked to a protein of approximately 11 kD in size (arrowhead, Figure 8C). As expected, both Prl-pause and Prl-stuffer nascent chains were crosslinked with equal efficiency to Sec61α, indicating that they were in the translocation channel.

Characterization of the 11 kD crosslinking partner indicate that it is unlikely to be either Sec61β or RAMP4 (Görlich and Rapoport, 1993), known membrane proteins of approximately the same size, as antibodies against these proteins failed to recognize the crosslinked adduct in immunoprecipitation experiments (data not shown). We found that the 11 kD protein is likely to be an integral

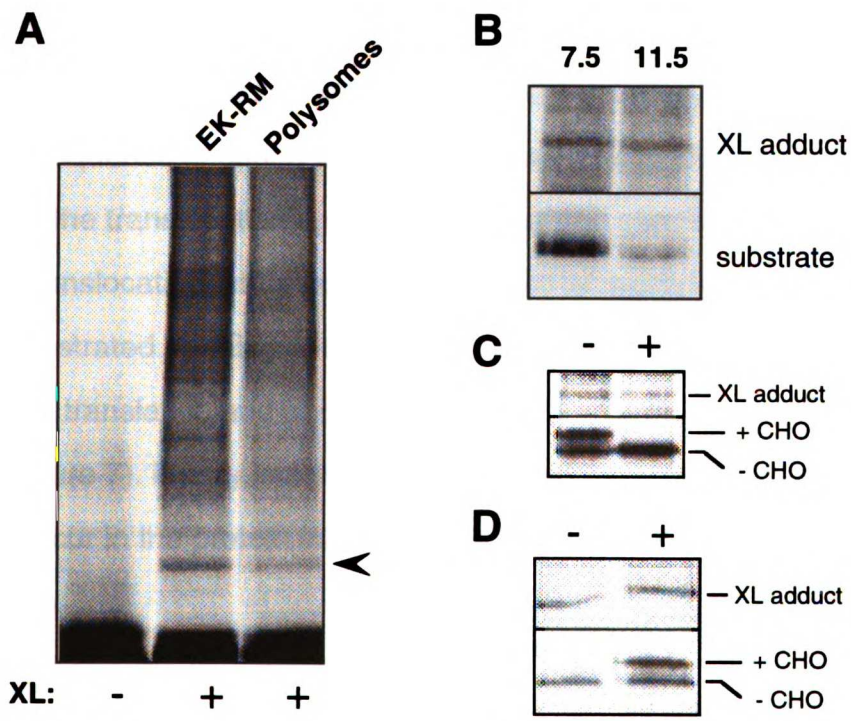
Figure 8 - Paused nascent chains are adjacent to an 11 kD membrane protein. (A) Prl-pause and Prl-stuffer constructs. The N-terminal 165 amino acids are from bovine pre-prolactin and the C-terminal 26 amino acids before the point of truncation (at Stu1) are residues 304-329 from mature apo B. The insert between these two domains is either a PT sequence (shaded box, representing amino acids 261-290 of mature apo B) or an irrelevent 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 Stu1 and analyzed as in Figure 6B. 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 crosslinked to adjacent proteins at various crosslinker concentrations as described in Experimental Procedures. Lanes 1 and 10 are samples that were not treated with crosslinker. The bracket at the left indicates the position of crosslinks to Sec61α, as confirmed by immunoprecipitation (data not shown). The arrowhead indicates the position of a crosslink to an approximately 11 kD protein that is specific to a paused nascent chain intermediate.


A
Pri-pause
Stu1
30 aa
135 aa
33 aa
26 aa
Pri-stuffer
Stu1
B
1 2 3 4 5 6
30
14
PK - + + - + +
Det - - + - - +
EDTA - - - + + +
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
92
68
45
30
[XL] 0 0.1 0.25 0.5 1.0 0.1 0.25 0.5 1.0 0 0.1 0.25 0.5 1.0 0.1 0.25 0.5 1.0
EDTA - - - - + + + + - - - - + + + +

membrane protein, as it was not extracted from the membrane at pH 11.5 (Figure 9B) and still seen to crosslink in membranes stripped of peripheral membrane proteins (Figure 9A). Furthermore, the unchanged migration of the crosslinked adduct upon endoglycosidase H treatment (Figure 9C), and the inability of the adduct to bind Con A (Figure 9D) argue that the 11 kD protein is not glycosylated. Furthermore, the 11 kD protein appears to be stably associated with either the nascent chain or translocon, as 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 crosslink to the 11 kD protein (Figure 9A).

These results demonstrate that when a nascent chain is translocationally paused, the proteins adjacent to it have changed: a new protein of 11 kD moves into a position where it can be crosslinked to the nascent chain residing within the translocation channel. By contrast, the matched, non-paused nascent chain, although residing in the translocation channel, is not adjacent to the 11 kD protein. Likewise, when the pause is abolished and translocation of the chain has restarted, this crosslinked 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.

Figure 9 - Characterization of the 11 kD crosslinking protein.
(A) The plasmid Prl-pause was truncated at Stu1 and translated using EDTA-salt washed rough microsomes (EK-RM). The microsomal membranes were isolated and either subjected to crosslinking directly (lane 2) or solubilized in 1% digitonin, the ribosome-bound nascent chains isolated by sucrose gradient sedimentation, and subsequently treated with crosslinker (lane 3). The arrowhead indicates the position of the 11 kD crosslink described in Figure 8C. (B) Stu1 truncated Prl-pause was translated, the microsomal membranes isolated, and the sample treated with 0.2 mM crosslinker. 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 crossinked adduct, which was not extracted from the membrane under pH 11.5 conditions, and the uncrosslinked substrate (from a shorter exposure of the same gel), which was largely extracted under pH 11.5 but not pH 7.5 conditions. (C) Crosslinking products generated as above were incubated in the absence (-) or presence (+) of endoglycosidase H prior to analysis by SDS-PAGE and autoradiography. As a control, a mixture of gycosylated (+CHO) and unglycosylated (-CHO) apoB15 translation product was also treated in parallel. (D) Crosslinking products or the control apoB15 translation product, as in Panel C, were passed over a Con A column in the absence (-) or presence (+) of 1 M α-methyl-mannopyranoside and the flow-through fraction analyzed by SDS-PAGE and autoradiography.


A
EK-RM
Polysomes
XL: - + +
B
7.5 11.5
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substrate
C
- +
XL adduct
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- CHO
D
- +
XL adduct
+ CHO
- CHO

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 (Figure 4-6). 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 7). Finally, we show that during a translocational pause, coordinate changes occur in the protein machinery with which the nascent chain is associated (Figure 8-9).

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 10). Data from several sources (Crowley et al., 1993, 1994; Kalies et al., 1994; Matlack and Walter, 1995) including this work (see Figure 4A) indicate that early translocation intermediates of simple secretory proteins are well shielded from the cytosol by a tight ribosome-membrane junction (Figure 10A). 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 10B). During this time, the chain appears to be adjacent or, possibly, bound to proteins in a manner unique to the translocationally paused state (shaded oval, Figure 10). 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 10C). At a later time during chain growth, the translocational pause is relieved, translocation of the cytosolically disposed regions of the nascent chain resumes (Figure 10D), and the tight ribosome-membrane junction is reestablished (Figure 10E).

Translocational pausing alters the translocational machinery

The current studies were motivated by initial observations that the translocation of certain proteins such as apolipoprotein B appeared to be different than 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, 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.

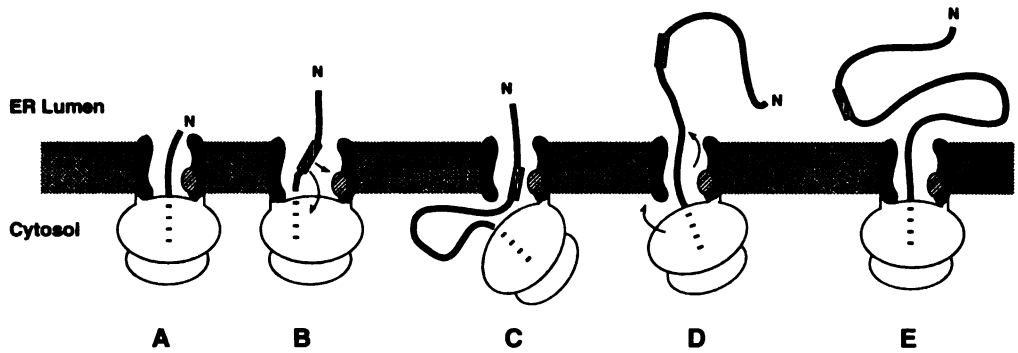
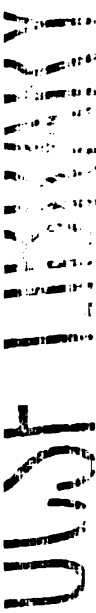


Figure 10 - Model of a dynamic translocation channel.

The diagrams indicate the successive events proposed to occur at the translocon during the translocation of pause transfer containing proteins. The shaded box within the translocating nascent chain represents the PT sequence. The shaded 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.

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 (NAC), which can shield nascent chains in the ribosomal tunnel (Wang et al., 1995). These changes are not observed in the case of either a simple secretory protein or a paused 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. which may occur as a simple function of chain length (Connolly et al., 1989). However, given that we find non-paused 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 (Stu1 at aa 329, see Figure 4B) which first reveals the cytosolically accessible domain and a later point at which the chain is no longer accessible (Nco1 at aa 472). Thus, we infer that the myc epitope that was inserted at the Stu1 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, (RSH, unpublished data;

Frydman et al., 1994), the window of time would be approximately 50-80 seconds.

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 rate 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 diffision 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. Crosslinking experiments probing the environment surrounding the nascent chain demonstrated a difference in the set of neighboring proteins (Figure 8C). An 11 kD membrane protein that is most likely not Sec61β or RAMP4 was found to crosslink specifically to paused nascent chains. Although the presence of this crosslink 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 crosslinks to an 11 kD protein using an independent pause transfer sequence in a different chimeric context as the substrate (RSH and VRL, unpublished results). Likewise, the 11 kD 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, TRAM. However, it remains entirely possible that various other membrane proteins are involved in aspects of the maturation of nascent chains which 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 TRAP complex (Wiedmann et al., 1987; Görlich et al., 1992b) and the 11 kD protein in this study that 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., 1992b; 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 can be isolated during translocation, and subjected to co-translational modifications that might not otherwise be possible. Pausing might facilitate these events in one or more of the following ways. The chain can be made accessible to a compartment in which the enzymes that catalyse specific reactions are present (e.g. the cytosol or the plane of the membrane). Alternatively, and by analogy to the role of SRP-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 (PDI), 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 Andrew Murray's laboratory or purchased from Oncogene Science (Uniondale, NY). Control antibodies were against the HA epitope tag, and were the gift of Ira Herskowitz's laboratory. Antibodies were purified using a protein G affinity column (Harlow and Lane, 1988). Antibodies against Sec61α were a gift from Peter Walter's laboratory. Antibodies against Sec61β and RAMP4 were a gift from Tom 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) into which the 5' untranslated region of Xenopus globin is inserted at the Hind3 site. pSP SP1, a plasmid encoding the signal sequence of bovine prolactin was cut with Xba1 and ligated to a PCR fragment of bovine prolactin encoding amino acids 4 to 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 digested with Xba1, respectively. These plasmids were cut at Xba1 prior to their use in Figure 4. ApoB-Myc1 and ApoB-Myc2 were made by inserting oligonucleotides encoding the myc epitope tag (GTEQKLISEEDLA) into the Stu1 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 EcoR1 linker was inserted at the

Msc1 site of bovine prolactin to generate the plasmid BPI-EcoR1. A PCR fragment encoding amino acids 264 to 406 of mature apo B was then inserted into this EcoR1 site to generate the plasmid BPI(B7-10)@Msc1. A second PCR fragment encoding the amino acids 234 to 263 was inserted in both the forward and reverse orientation into the Sac1 site of BPI(B7-10)@Msc1 to generate the plasmids BPI(B6-10)@Msc1 and BPI(B7-10stuffer)@Msc1, respectively. These two plasmids were digested with Spe1 and Pst1, 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 $MgOAc_2$, 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 7C, 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 7D, the microsomal membranes from the translation reaction were isolated by gel filtration through CL-4B (Pharmacia, Uppsula, 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 3 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 Crosslinking - Microsomal membranes from translation reactions were isolated by centrifugation, resuspended in PSB, and the crosslinker DSS (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 Con A chromatography were as described previously (Kellaris et al., 1991). In Figure 8C samples were adjusted to 1% Digitonin prior to crosslinking to reduce non-specific crosslinking to ER lumenal proteins at the higher crosslinker concentrations. For Figure 9A, 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 crosslinking with 0.25 mM DSS 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 EnHance (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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III. The Regulation of Translocational Pausing

TRAM regulates the exposure of nascent secretory proteins to the cytosol during translocation across the endoplasmic reticulum membrane.

Abstract

Transient pausing during translocation across the endoplasmic reticulum (ER) is a means by which certain secretory proteins can have access to environments, such as the cytosol, that are otherwise shielded from the nascent chain. In this study, we have used proteoliposomes reconstituted from components of the ER membrane to explore the biochemical mechanism of a translocational pause. Proteoliposomes reconstituted from total ER proteins were capable of properly assembling paused translocation intermediates such that only selected domains of the nascent chain were exposed to the cytosol. This capacity of the translocation apparatus was shown to be dependent on a glycoprotein fraction, the active component of which was identified as TRAM. In the absence of TRAM, the normally sealed ribosome-membrane junction still opens in response to a pause transfer sequence. However, regions of the nascent chain that are not exposed to the cytosol in the presence of TRAM are found accessible to the cytosol in its absence. Thus, TRAM regulates which domains of a nascent chain are visible to the cytosol during a translocational pause.

Introduction

In eukaryotic cells, the endoplasmic reticulum (ER) is the birthplace for the vast majority of secretory and membrane proteins. A fundamental phase in the biogenesis of these proteins is their proper translocation across or insertion into the ER membrane (reviewed by Rapoport et al., 1996; Andrews and Johnson, 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.

Biogenesis of secretory and membrane proteins entails more than just appropriate segregation into the ER lumen or membrane. A variety of other reactions are crucial for proper functional maturation. These include both covalent modifications such as cleavage of signal sequences, glycosylation, and disulfide bond formation, as well as non-covalent events such as intrachain folding and oligomerization with other proteins (see for example, Bulleid, 1993; Chen et al., 1995; Silberstein and Gilmore, 1996). Because many of these

reactions vary from protein to protein and occur either during or soon after the translocation process, the spatial and temporal coordination of all of the events presents the cell with a significant challenge. Although certain individual reactions, such as signal cleavage and glycosylation, have been studied in detail (Meyer and Hartmann, 1997; Silberstein and Gilmore, 1996), little is understood about the mechanisms involved in orchestrating these individual events during the biogenesis of secretory or membrane proteins.

Many of the covalent and non-covalent 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 post-translationally, apparently at the level of ER degradation (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 kD apo B molecule (Kivlen et al., 1997). While the various roles of each and every pause transfer sequence in apo B remains to be elucidated, a mechanistic role has been demonstrated 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

Experimental Design and Assay for Pausing

The minimum translocon components required to reconstitute polypeptide transport are the Sec61 complex and SRP-receptor (Görlich and Rapoport, 1993). The model secretory protein prolactin is one of a few substrates that is translocated efficiently by this minimum translocon. For this reason, we chose as the pause-containing test substrate a version of prolactin into which a pause transfer sequence from apo B has been engineered (termed Prl-pause; see Hegde and Lingappa, 1996). This substrate allows one to specifically evaluate any consequence of additional machinery on translocational pausing without significant confounding effects on other aspects of translocation (e.g., TRAM-dependence of certain signal sequences).

In addition to choice of substrates, several methods are available to detect and examine translocational pausing. In most approaches, fully assembled translocation intermediates containing nascent proteins of a defined length are generated by programming an *in vitro* translation reaction with mRNA truncated within the coding region (Gilmore and Blobel, 1985), and thus lacking an in frame stop codon. These translocation intermediates can then be examined to ask: (i) whether the ribosome-membrane junction has opened [e.g. by using collisional quenching of fluorescent probes on the nascent chain (Liao et al., 1997)], (ii) at what point has the nascent chain stopped or paused (e.g., by using proteolysis), and (iii) how much of the nascent chain is exposed to the cytosol [e.g., by using immunoadsorption with antibodies in the cytosol (Hegde and Lingappa, 1996)].

In principle, these parameters are not mutually linked and may vary independently of each other. Furthermore, the optimal method to discern each of the above parameters can differ depending on the sensitivity and specificity

required. One method, digestion of translocation intermediates with proteinase K (PK), offers multiple advantages. First, any chains that are not properly targeted to the translocon will be completely digested, greatly reducing background. Second, the opening of the ribosome-membrane junction can be assayed. Although the possibility exists that PK can gain access to the nascent chain as a result of overdigestion of a tight ribosome-membrane junction, this has been shown not to be the case for the specific assay conditions employed here (Jungnickel and Rapoport, 1995; Hegde and Lingappa, 1996; Mothes et al., 1997). Finally, the point at which a chain has stopped translocation into the lumen can be determined by PK digestion followed by examination of the size of the protected fragment, thereby inferring which domains were exposed (and digested). For these reasons PK digestion was routinely employed to assay all of these aspects of translocational pausing.

Pausing can be reconstituted

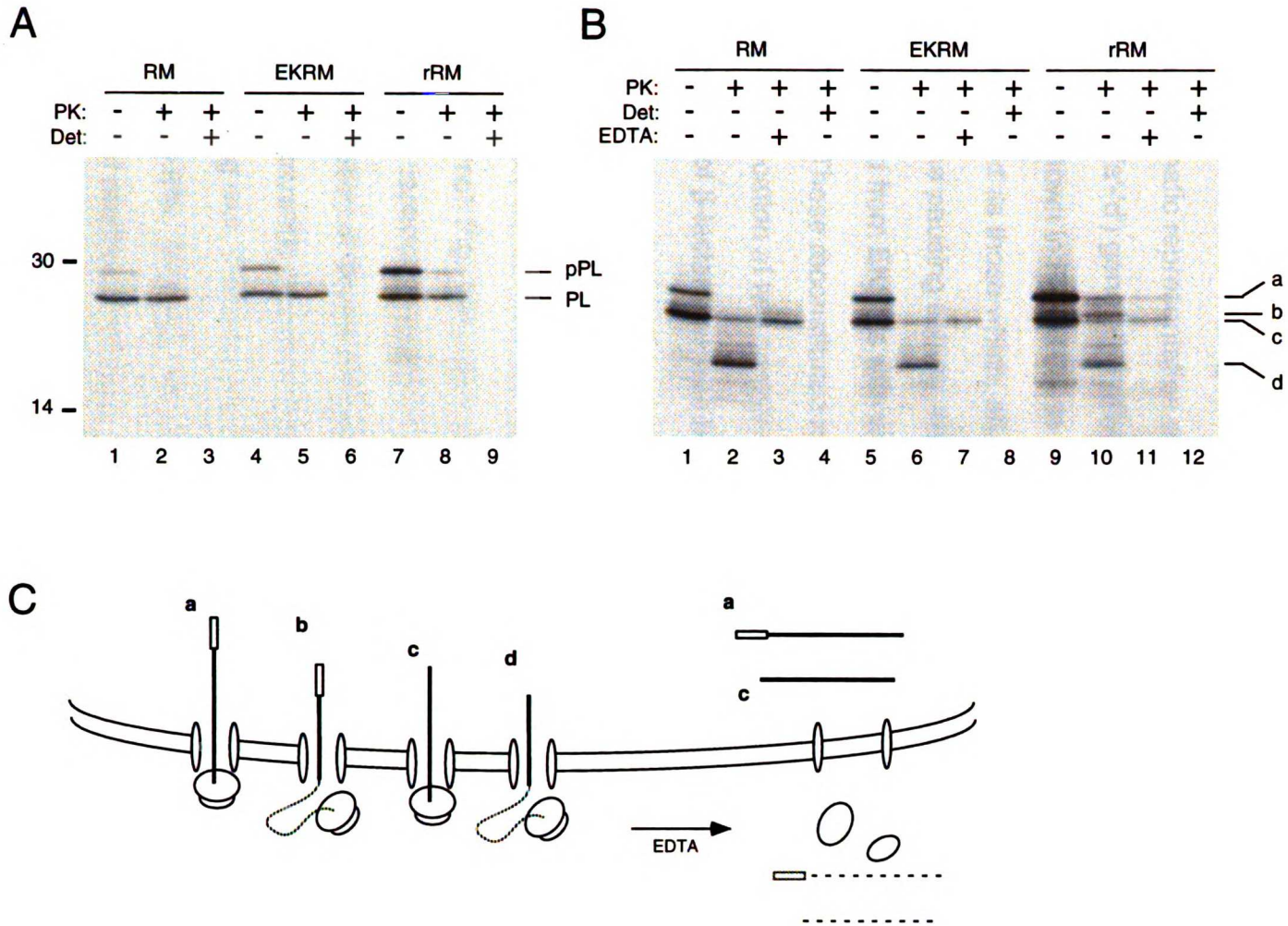
In order to identify any putative regulatory components involved in various aspects of translocational pausing, our first aim was to reconstitute some or all of these events in a system tractable to fractionation. We therefore examined whether pausing would occur in proteoliposomes reconstituted from 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 EKRMs) and extracted with 0.8% Cholate. The solubilized proteins were then re-incorporated into vesicles by the removal of detergent to yield reconstituted microsomal membranes (rRM). Assays of prolactin translocation across each of these membranes demonstrated that the rRMs, like RMs and EKRMs, were active in supporting transport into the lumen of the microsomal

vesicles (Fig. 11A). However, not all of the translocated (and thus protease protected) 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., 1992b).

Analysis of the Prl-pause construct in the same set of membranes showed that translocational pausing was equally well restored (Fig. 11B). In RMs and EKRMs, the majority of the Prl-pause translocation intermediate was accessible to PK, resulting in the digestion to a specific lower molecular weight fragment (species 'd'; see Fig. 11B, C). This indicated that these nascent chain 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 non-cleaved paused nascent chains (labeled species 'd' and 'b', respectively; see Fig. 11C), again reflecting the incomplete action of signal peptidase.

If paused translocation intermediates are treated with EDTA, the ribosome-nascent chain-translocon complex is disrupted, resulting in "restarting" of the pause (Chuck and Lingappa, 1992). In RMs, and to a lesser extent in EKRMs, the nascent chain is found to largely restart translocation into the lumen, and is subsequently fully protected from PK digestion. Restarting was also seen in rRMs, but in this case, the formerly paused nascent chains largely "fall back" into the cytosol, where it is entirely accessible to PK digestion. 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 (RSH and VRL, unpublished results). Thus, rRMs are able to assemble a paused translocation

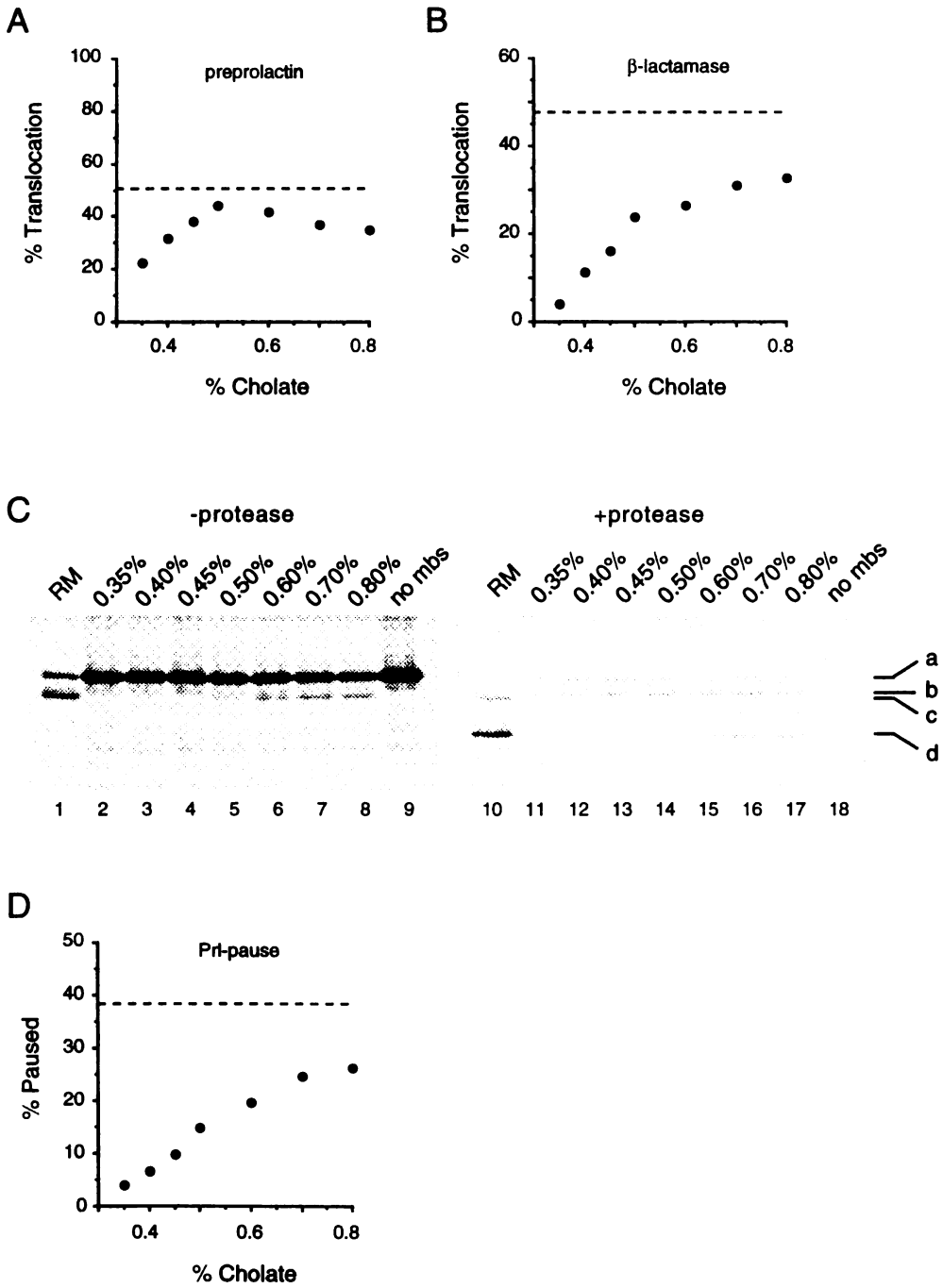
Figure 11 - Translocational pausing in reconstituted membranes.
Rough microsomal membranes (RM), EDTA washed rough microsomal membranes (EKRM), and proteoliposomes reconstituted from a 0.8% cholate extract of EKRMs (rRM) were each used to assay translocation and pausing. (A) Proteolysis assay for translocation 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 minutes 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) The interpretation of the proteolysis data in panel B is schematized, with the species 'a' through 'd' indicated. 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 or cytosol (where they are accessible to PK digestion).


A
RM
EKRM
rRM
PK:
Det:
30
14
pPL
PL
1 2 3 4 5 6 7 8 9
B
RM
EKRM
rRM
PK:
Det:
EDTA:
a
b
c
d
1 2 3 4 5 6 7 8 9 10 11 12
C
a
b
c
d
EDTA

intermediate, which subsequently restarts upon treatment with EDTA. Furthermore, because the size of the protected fragment 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. A schematic representation of these results, showing the origin of the various species ('a'-'d') generated after PK treatment of a paused translocation intermediate is shown in Fig. 11C. In subsequent experiments, paused nascent chains are 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 pausing as well as translocation of two control substrates, prolactin and β-lactamase (Fig. 12). The choice of β-lactamase was based on the previous observation that the translocation of this substrate, in contrast to prolactin, is largely dependent on TRAM (Görlich et al., 1992b; Voigt et al., 1996). We found that the membranes which showed maximal translocation of prolactin (occurring in rRM made from 0.5% cholate extract; Fig. 12A) were different from those that showed maximal translocation of β-lactamase (occurring at 0.8%; Fig. 12B). Thus, although optimal reconstitution of Sec61 complex and SRP-receptor (needed for prolactin translocation) apparently occurs at 0.5%, the combination of these minimum components along with TRAM (required for β-lactamase translocation) are best reconstituted at 0.8%. Similarly, we observed that pausing activity, both as measured by total number of chains paused as well as percent of chains paused, was maximal at 0.8% (Fig. 12C, D). These data suggested that factor(s) in addition to the minimal components required for prolactin translocation might be needed for reconstitution of pausing.

Figure 12 - Characterization of pausing in various reconstituted membranes. Proteins were extracted from EKRMs using the various concentrations of cholate from 0.35% to 0.80% and reconstituted into proteoliposomes. These were then used to assay prolactin translocation (panel A), β-lactamase translocation (panel B), and pausing of Prl-pause by the PK digestion assay (panels C and D). (C) SDS-PAGE analysis of Prl-pause translocation intermediates in each membrane preparation before (left panel) and after (right panel) protease digestion. The positions of species 'a' through 'd' (see Fig. 11C) are indicated. 'RM' and 'no mbs' are reactions that contained control rough microsomal membrane and no membranes, respectively. (D) Quantitation of pausing (from panel C) in each membrane preparation. Percent pausing was determined by summing the amount of translation product represented by species 'b' and 'd' (after protease), and dividing by amount of synthesized translation product (before protease), then multiplying by 100. The dotted line in panels A, B, and D indicate the corresponding value determined for RMs.


A
preprolactin
% Translocation
100
80
60
40
20
0
0.4
0.6
0.8
% Cholate
B
β-lactamase
% Translocation
60
40
20
0
0.4
0.6
0.8
% Cholate
C
-protease
+protease
RM
0.35%
0.40%
0.45%
0.50%
0.60%
0.70%
0.80%
no mbs
a
b
c
d
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18
D
Prl-pause
% Paused
50
40
30
20
10
0
0.4
0.6
0.8
% Cholate

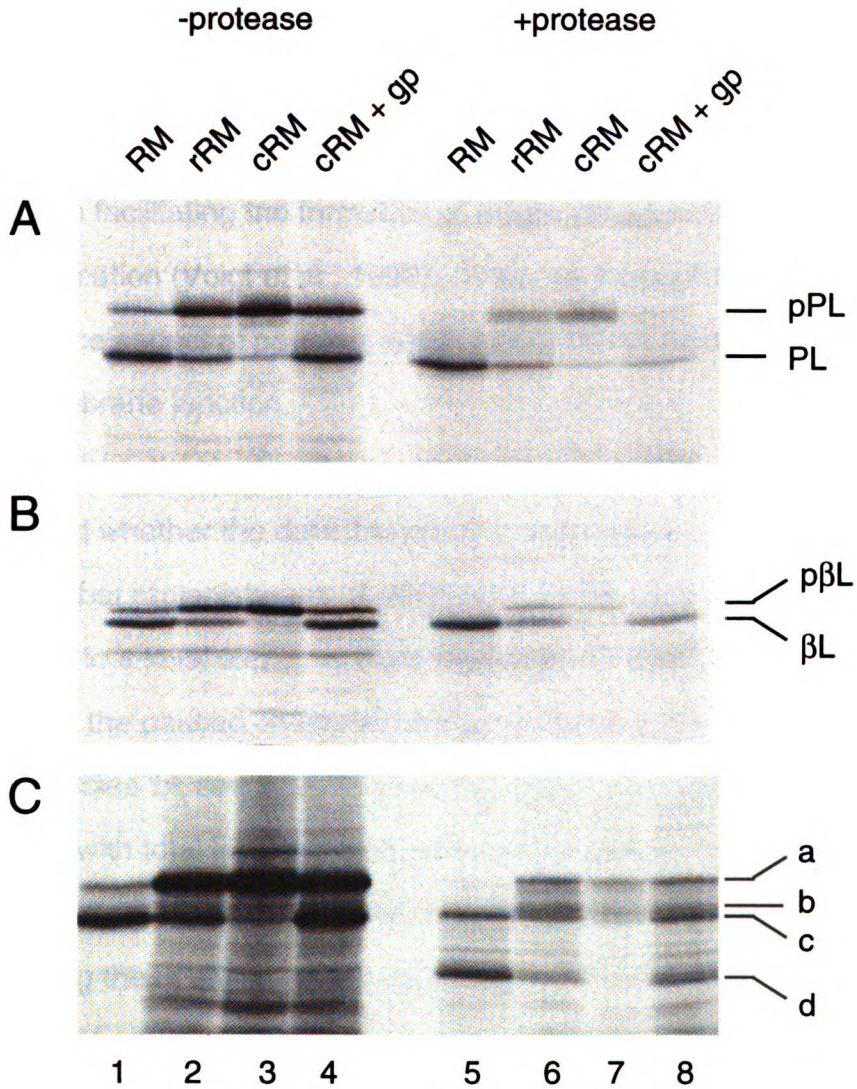
Uncoupling pausing from translocation

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., 1992b). Reconstituted membranes were prepared that either contained the full complement of proteins (rRM), were depleted of Con A-binding glycoproteins (cRM), or depleted but subsequently replenished with the glycoprotein fraction (cRM+gp). Each of these membranes was then assayed for pausing and translocation.

As expected, all of the membrane preparations supported the translocation of prolactin (Fig. 13A). By contrast, the cRMs failed to translocate β-lactamase efficiently, presumably reflecting the requirement for the glycoprotein TRAM (Fig. 13B). 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 which still retain translocation activity (of at least prolactin), indicating that certain aspects of translocational pausing require component(s) in addition to the minimal translocon.

Additionally, we consistently observed that the total number of paused and non-paused nascent chains (i.e., the sum of species 'a'-'d') was significantly less in the cRMs than when glycoproteins were present. Initially however, we focused on the identification of the biochemical basis of the observed diminishment in the number of paused chains. Subsequent experiments addressed the basis of this apparent disparity in translocation and revealed it was not a defect in translocation *per se*, but a surprising lesion in one particular aspect of pausing.

Figure 13 - 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 prolactin and β-lactamase translocation (panels A and B, respectively), and pausing activity of Prl-pause (panel C). The positions of the precursor and processed products of prolactin and β-lactamase are indicated to the right of the gels, as are the positions of species 'a' to 'd' of the Prl-pause analysis.

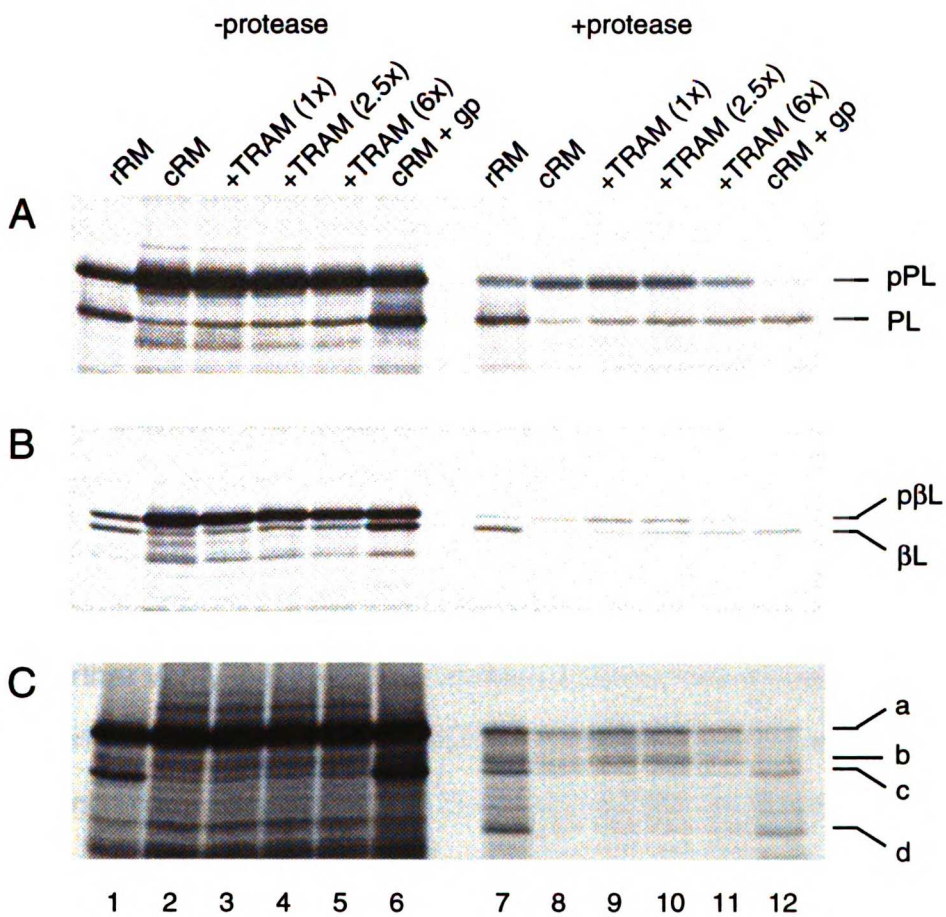

-protease
+protease
RM
rRM
cRM
cRM + gp
RM
rRM
cRM
cRM + gp
A
pPL
PL
B
pβL
βL
C
a
b
c
d
1
2
3
4
5
6
7
8

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 co-fractionate with TRAM. For example, the detergent extraction (Fig. 12) and glycoprotein fractionation properties (Fig. 13) 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. Fig. 14C 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 Fig. 14C). By comparison, replenishment with total glycoproteins restored pausing as well as signal sequence cleavage, resulting in an increase of species 'd' (lane 12). Notwithstanding these differences in signal sequence cleavage, the extent of replenishment of pausing again paralleled the extent of translocation activity of β-lactamase (compare Fig. 14B to 14C). 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.

Figure 14 - 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 Fig. 13. 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 prolactin and β-lactamase translocation (panels A and B, respectively), and pausing activity of Prl-pause (panel C) exactly as in Fig. 13.


-protease
+protease
rRM
cRM
+TRAM (1x)
+TRAM (2.5x)
+TRAM (6x)
cRM + gp
rRM
cRM
+TRAM (1x)
+TRAM (2.5x)
+TRAM (6x)
cRM + gp
A
pPL
PL
B
pβL
βL
C
a
b
c
d
1 2 3 4 5 6 7 8 9 10 11 12

Of the non-glycoproteins 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 prolactin, but not β-lactamase (Fig. 15A). The additional inclusion of TRAM in the proteoliposomes (DST membranes) restored translocation of β-lactamase.

Assays of translocational pausing (and restarting with EDTA) in these membranes demonstrated that DST, but not DS membranes were able to assemble paused nascent chains (Fig. 15B). The size of the PK protected fragment of paused chains (species b), as well as its disappearance with EDTA treatment, further verify 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). Thus, it appears that TRAM is the only component, in addition to the minimal translocation machinery, that is absolutely required for pausing. Although paused nascent chains were no longer present after EDTA treatment (reflecting restarting of the pause), they were not found in the lumen of the microsomes (i.e., species 'a' does not increase corresponding to the decrease of species 'b' after EDTA). Rather, it appears that the paused chains "fall back" to the cytosol upon restarting. This is similar to the

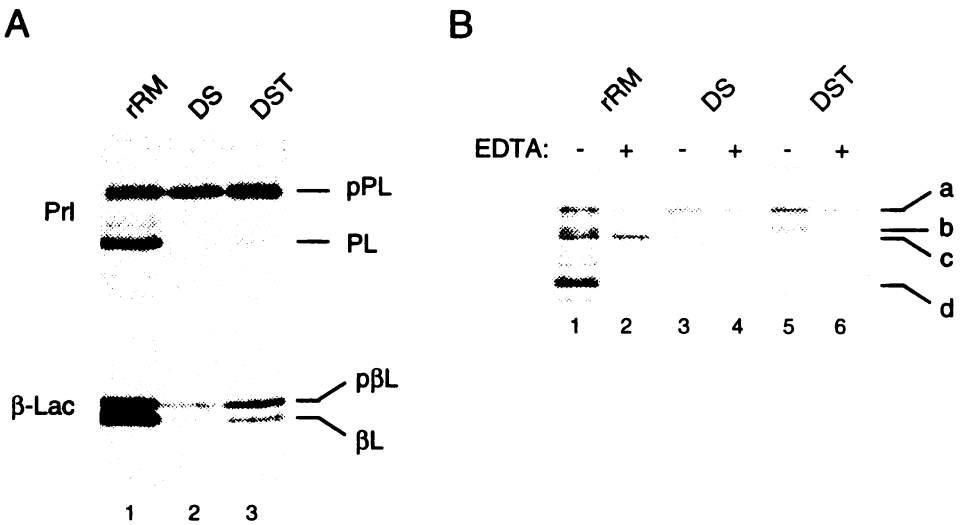


Figure 15 - Translocational pausing in proteoliposomes containing purified components. Purified SRP-receptor and Sec61 complex, without (DS membranes) or with TRAM (DST membranes) were reconstituted with pure phospholipids into proteoliposomes. These were compared to rRMs with respect to prolactin and β-lactamase translocation (panel A), and pausing activity of Prl-pause (panel B). The products remaining after protease digestion of the samples are shown. A portion of each Prl-pause translocation product was treated with EDTA prior to proteolysis (as in Fig. 11B) to assay restarting.

restarting behavior of paused chains seen in rRM (see Fig. 11C), a lesion that can be corrected by including lumenal proteins in the microsomal membranes (unpublished data).

Crosslinking of TRAM to paused nascent chains

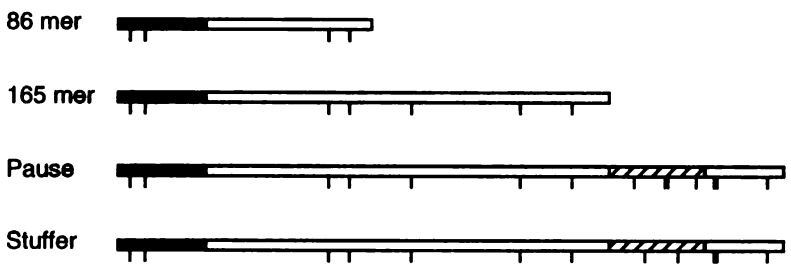
Until these studies, the only functional role for TRAM during secretory protein biogenesis was at an early stage of translocation (Görlich et al., 1992b), when it is required for certain proteins to be inserted properly at the translocon (Voigt et al., 1996). This was supported by crosslinking studies which demonstrated that TRAM was adjacent to the nascent chain early in translocation (Görlich et al., 1992b; High et al., 1993; Mothes et al., 1994). At later points during secretory protein translocation, crosslinks 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 crosslinking, 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 (Fig. 16). 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. Fig. 16A shows a schematic depiction of these points of truncation used in the crosslinking experiment.

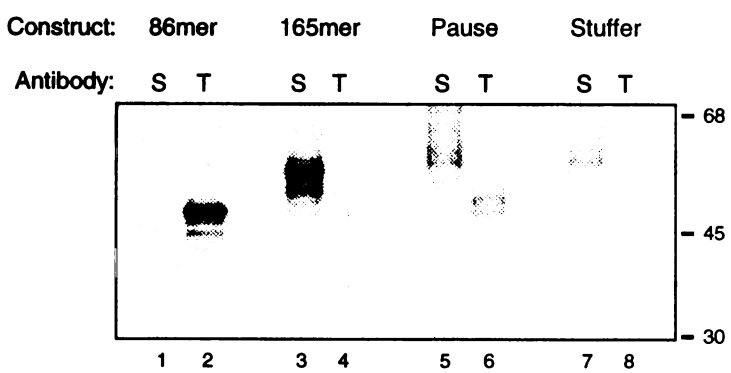
Figure 16 - Crosslinking of paused nascent chains to TRAM.

(A) The pPL-86mer, pPL-165mer, 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 crosslinker. (B) Nascent chain translocation intermediates of pPL-86mer, pPL-165mer, Prl-pause, and Prl-stuffer were prepared with the TDBA-lysyl nascent chain crosslinker. Following crosslinking 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 four fold higher for the 86mer and 165mer than the pause and stuffer substrates. The heterogeneity of migration seen in some of the crosslinks is likely to be due to crosslinks from different positions in the nascent chain.

A


86 mer
165 mer
Pause
Stuffer

B


Construct:
86mer
165mer
Pause
Stuffer
Antibody:
S T S T S T S T
68
45
30
1 2 3 4 5 6 7 8

We found that at point (i), the majority of crosslinks were to TRAM, and not Sec61α (lanes 1, 2). At point (ii), the crosslinks to TRAM had largely disappeared, with concomitant appearance of crosslinks to Sec61α (Fig. 16B, lanes 3, 4). Finally, at point (iii), substantial crosslinks to both Sec61α and TRAM were observed (lanes 5, 6). The crosslinks to TRAM, but not Sec61α, were specific to the pause, since the TRAM crosslinks disappeared (lanes 7, 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 translocon 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 apparant 'loss' in translocation efficiency of Prl-pause appeared to be of approximately the same magnitude as the loss of translocation efficiency of β-lactamase (compare Fig. 13B to 13C). The defect in β-lactamase 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 prolactin signal sequence is not TRAM-dependent (Görlich et al., 1992b), and prolactin translocation did not diminish significantly in these same membranes (Fig. 13A).

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 non-paused chains (see for example, Fig. 13C, lane 6 vs. 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.

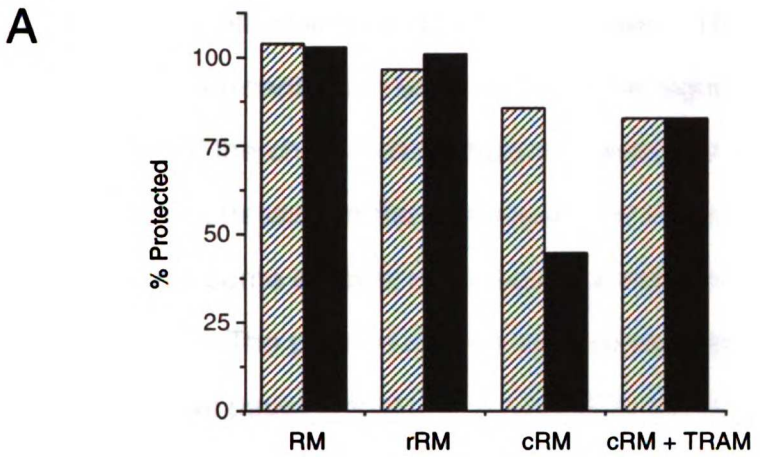
One possible explanation was that in the absence of TRAM, the ribosome-nascent chain complex of a paused substrate might dissociate from the membrane, and thus not be scored as translocated. This seemed plausible, since it was shown that the ribosome-membrane junction is significantly altered during 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 non-paused chains.

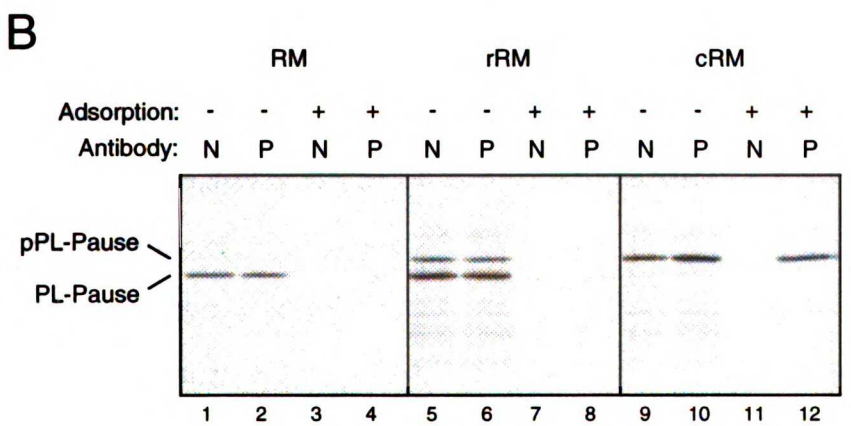
In order to distinguish between these possibilities, we first determined whether the unaccounted chains in cRMs had detached from the membrane, or were still tightly bound to the translocon. Following the assembly of Prl-pause translocation intermediates in various reconstituted membranes, one aliquot 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 (Fig. 17A). For a non-paused translocation intermediate (the 86mer of preprolactin), no discrepancy was observed between the proteolysis and membrane binding data (striped bars). By contrast, analysis of the Prl-pause translocation intermediate (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 (RM, rRM, and cRM+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 which 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 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 Fig. 11), and as expected, is not accessible to antibodies in the cytosol (Fig. 17B, lanes 1-4 and 5-8). However, in cRMs, this domain is found to be inappropriately accessible to cytosolic anti-prolactin antibodies (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.

Figure 17 - Inappropriate exposure of paused nascent chains to the cytosol. (A) Translocation intermediates of pPL-86mer (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' through 'd' (see Fig. 11C). (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 non-specific 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 non-denaturing 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.


A
100
75
50
25
0
% Protected
RM
rRM
cRM
cRM + TRAM


B
RM
rRM
cRM
Adsorption: - - + + - - + + - - + +
Antibody: N P N P N P N P N P N P
pPL-Pause
PL-Pause
1 2 3 4 5 6 7 8 9 10 11 12

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 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 is 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

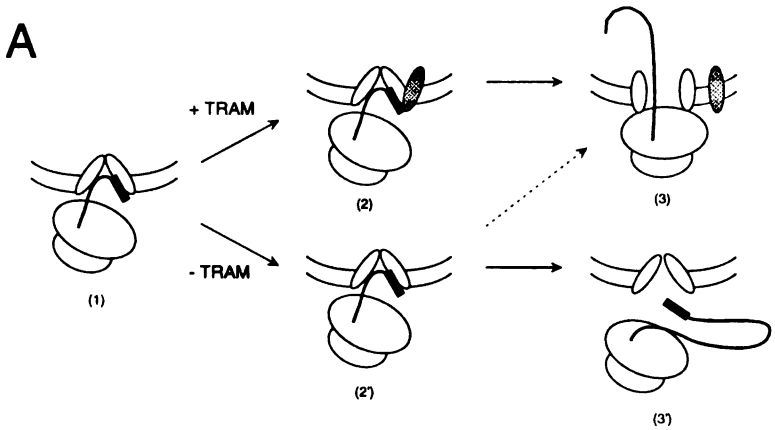
of some, but not other proteins (Görlich et al., 1992b). Subsequently, it was shown that this requirement was dependent on the structure of the signal sequence of the substrate (Voigt et al., 1996). Proteins containing TRAM-dependent signal sequences did not become properly inserted into the translocation site in the absence of TRAM. Crosslinking studies which 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 (Mothes et al., 1994; High et al., 1993).

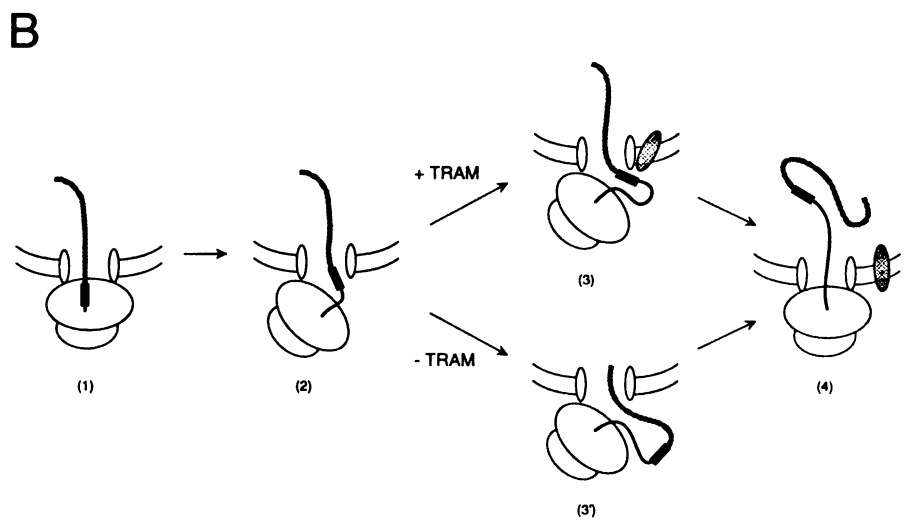
The second proposed role of TRAM was based on experiments demonstrating that TRAM could be crosslinked to a transmembrane segment during multiple discernible steps of its integration into the bilayer (Do et al., 1996). Although a functional requirement in this process has not yet been demonstrated, it was speculated that TRAM may serve to position or temporarily retain a 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 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 18 shows models depicting the proposed roles of TRAM at the signal sequence dependent (panel A) and pause transfer sequence dependent (panel B) phases of translocation. In both scenarios, a topogenic sequence causes the recruitment of TRAM to the site of translocation (diagram 2 in both panels). For the signal sequence dependent role of TRAM, a transition to a tightly docked

Figure 18 - Model of TRAM action during translocation.

Comparison of the suggested roles of TRAM during the early (panel A) and later (panel 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 panel A) and pause transfer sequence (in panel B) are represented by the shaded box in the nascent chain. In Panel 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.


A
+ TRAM
- TRAM
(1)
(2)
(2')
(3)
(3')


B
+ TRAM
- TRAM
(1)
(2)
(3)
(3')
(4)

ribosome-nascent chain-translocon complex is somehow facilitated (diagram 3, panel A). In the absence of TRAM (diagram 2', panel A), this productive insertion is usually not achieved, resulting in the failure of the chain to initiate transfer across the membrane (diagram 3', panel A). 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 (diagram 3 vs. 3', panel B). Instead of failing to be translocated however, the consequence at this later stage of translocation is the excess exposure of the nascent chain. Thus, in both situations, TRAM appears to facilitate 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, that of 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.

More than translocation is regulated by machinery

Historically, the proposal of the signal hypothesis (Blobel and Dobberstein, 1975a, 1975b) and the demonstration of a dedicated machinery for targeting to the ER membrane (Walter et al., 1981), was followed by debate as to whether machinery was involved in the actual translocation across the membrane. It was argued that the post-targeting events of translocation could be accounted for solely by the thermodynamics of protein-lipid interactions in the membrane bilayer (Engelman and Steitz, 1982). This debate was resolved by a series of experiments, first demonstrating the presence of aqueous protein-conducting channels in the ER membrane and culminating in the identification, functional reconstitution, and visualization of this machinery (Simon and Blobel, 1991; Görlich and Rapoport, 1993; Hanein et al., 1996). The question now arises of whether events accessory to protein transport, such as translocational pausing and nascent chain folding, can be accounted for solely by sequences encoded within the substrate.

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.

Perhaps under certain circumstances, TRAM activity is tempered by the effects of other cellular factors, resulting in the cell deliberately prolonging or otherwise altering the events during a translocational pause. Thus, a single nascent chain can be redirected to different fates (such as altered folded conformations or interactions with other polypeptides) by the regulatory activity of the translocon. Such regulation, perhaps acting independently on more than one pause transfer sequence within a given substrate, could conceivably provide the cell with multiple functional products from the same message. Regulation of pausing in this manner may be analogous to RNA splicing, which allows for a combinatorial variety of messages from a single gene.

The possibilities for translocational regulation and dysregulation

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 be regulated largely, if not exclusively by the ER degradation pathway utilizing the cytosolic proteasome (Yeung et al., 1996; Fisher et al., 1997). This pathway of degradation is likely to involve 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.

The most recent findings presented in this study are likely to be only the first glimpses of the many variations on a theme of regulated translocation across the ER membrane. This is because nascency is a unique time, and the translocation channel a unique place, in the life of a polypeptide. In this particular time and space, modifications, folding states, and protein-protein interactions could, in principle, be carried out which might not be possible to do -- or undo -- in the protein's subsequent functional lifetime. The variables available to be shuffled over the course of evolution include not only sequences within the nascent chain, but also alterations in the machinery that allow the chain to be exposed to different environments for variable periods of time. The consequences of such changes in reaction kinetics and compartmental exposure could themselves be dependent on other regulatory events in the cell. The resulting combinatorial possibilities for substantive variations on the theme of protein biogenesis are both enormous and daunting. Once we better understand these interactions, we may be able to manipulate them physiologically and pharmacologically with profound cell biological consequences. The most common human diseases are not simple programmed genetic lesions, but rather are stochastic degenerative or environmentally-induced disorders. It is tempting to speculate that, in many cases, their molecular foundations involve subtle regulatory phenomena.

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, pre-digested for 10 minutes 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.

Plasmid constructions - All manipulations of nucleic acids were done by standard techniques as described by Sambrook et al. (1989). The bovine preprolactin (pPL), β-lactamase, 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 non-paused translocation intermediates. To generate message coding for pPL-86mer and pPL-165mer, the Prl-pause construct was truncated at Pvu2 and EcoR1, respectively, prior to transcription.

Membranes and proteoliposomes - Reconstitutions from crude detergent extracts were performed by minor modifications of previously described procedures (Nicchitta and Blobel, 1990; Görlich et al., 1992b). EKRMs were prepared from crude rough microsomes as described previously (Walter and Blobel, 1983), but resuspended in extraction buffer (350 mM KAc, 50 mM Hepes, 12 mM $MgAc_2$, 15% glycerol (v/v), 5 mM 2-mercaptoethanol) at 1 equivalent per µl (see Walter and Blobel, 1983). 10% w/v detergent (either cholate or deoxyBigCHAP) dissolved in extraction buffer was added to the appropriate final concentrations as described in the figure legends and mixed gently but thoroughly. After a 15-30 min incubation on ice, particles larger than 30 S were sedimented and the soluble proteins, representing the detergent extract, were removed to ice. In some experiments, glycoproteins were depleted by incubation at 4°C, with gentle mixing, for 12 h with 0.2 volumes of packed Con A sepharose. For glycoprotein replenishment, elution from Con A was carried out at room temperature by incubation for 12 h with extraction buffer 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. 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 h 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 5 times the initial concentration in 100 KAc, 50 mM Hepes, pH 7.4, 1.5 mM $MgAc_2$, 0.25 M sucrose, 1 mM DTT. Reconstitutions from purified components (Fig. 15) were performed exactly as described previously (Görlich and Rapoport, 1993).

Cell-free translation and proteolysis - 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 minutes. Following translation, the samples were chilled briefly on ice before further manipulations, which were all performed at 4°C. The proteolysis reactions were initiated by the addition of PK to 0.5 mg/ml, and in some cases, Triton X-100 to 0.5%. Following a 30 minute incubation at 0°C, the samples were adjusted to 5 mM phenylmethylsulfonyl fluoride, incubated 2-5 minutes on ice, and transferred to a 10 fold excess volume of boiling 1% SDS, 0.1 M Tris pH 8.9. The samples were boiled an additional 2-5 minutes before further manipulation. Samples were either analyzed directly by SDS-PAGE and autoradiography, or the translation product first immunoprecipitated with specific antisera.

Membrane flotation studies -Following translation, aliquots of the samples were adjusted to 2 M sucrose, 500 mM KAc, 50 mM Hepes, pH 7.6, 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 minutes 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 minutes 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 µl immobilized protein A (BioRad), and incubated for 60 minutes with overhead mixing. The beads were washed four times with TXSWB and the immune complexes solubilized in 1% SDS, 0.1 M Tris pH 8.9 prior to analysis by SDS-PAGE.

Nascent chain crosslinking - Synthesis of TDBA-lysyl incorporated nascent chains and subsequent UV crosslinking 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, 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 its use.

IV. Prion protein topology and Neurodegenerative Disease

Development of spontaneous neurodegenerative disease in mice expressing a transmembrane form of the prion protein

Abstract

At the endoplasmic reticulum membrane, the prion protein (PrP) can be synthesized in multiple distinct topological forms, one of which is C-trans transmembrane (termed ^{Ctm}PrP). To explore the role of ^{Ctm}PrP in neurodegenerative disease, transgenic mice expressing PrP mutations that either favor or abolish synthesis in this topologic form were examined. Favored expression of ^{Ctm}PrP in mice produced neurodegenerative changes similar to that observed in some genetic prion diseases. Biochemical analyses of brains from these mice detected the presence of ^{Ctm}PrP but not PrP^{Sc}, the PrP isoform associated with transmission of prion diseases. These data identify prion protein biogenesis and topology as a novel point of regulation and specifically demonstrate a role for ^{Ctm}PrP in the pathogenesis of at least some PrP induced neurodegenerative diseases.

Introduction

The prion protein (PrP) is a highly conserved 35 kDa brain glycoprotein that has been demonstrated to be essential in the transmission and pathogenesis of several neurodegenerative diseases such as scrapie, bovine spongiform encephalopathy (BSE), Creutzfeldt-Jakob disease (CJD) and Gerstmann-Straussler-Scheinker (GSS) disease (Prusiner, 1996; Weissmann, 1995). These diseases are unusual in that they may be acquired by either an infectious or inherited mechanism. While the normal function of PrP remains unclear, the pathogenesis of prion diseases requires its expression (Brandner et al., 1996; Bueler et al., 1993; Prusiner et al., 1993), and is often accompanied by the accumulation in the brain of an abnormal isoform of PrP (termed PrP^{Sc}). Considerable evidence from biochemical, immunologic, pathologic, and genetic studies argues persuasively that PrP^{Sc} is the major, if not only component of the transmissible prion particle (reviewed in Prusiner, 1996; Weissmann, 1995). Furthermore, the data suggest that PrP^{Sc} is able to propagate itself in the host by catalyzing the conversion of normal cellular PrP (termed PrP^{C}) to PrP^{Sc}, leading to its accumulation (Kocisko et al., 1994; Prusiner et al., 1990; Scott et al., 1993). Thus, although the exact mechanism of PrP^{C} to PrP^{Sc} conversion remains unanswered, a foundation has been laid for the understanding of prion disease transmission upon which subsequent structural, biochemical, and cell biological studies can be based.

More enigmatic at the present time is our understanding of the biochemical and cell biological events that form the basis for the pathophysiological progression of neurodegeneration in prion diseases. The role of PrP^{Sc} in the pathologic process leading to neuronal death is currently unclear. In some cases, the accumulation of large amounts of PrP^{Sc} within the cell have led to

speculation that disease is a result of inappropriate storage and lack of degradation (Prusiner et al., 1983). However, the observation of significant neurodegeneration in the absence of PrP^{Sc} accumulation in many cases of both natural and experimental instances of prion disease argue against non-specific accumulation as the sole cause of pathology (Tateishi et al., 1992; Hsiao et al., 1994; Hayward et al., 1994; Collinge et al., 1995; Tateishi et al., 1995). Conversely, it has been demonstrated that the time course of PrP^{Sc} deposition in the brains of mice expressing low levels of PrP^{C} does not correlate with the time course of neurodegeneration (Manson et al., 1994; Bueler et al., 1994), raising the possibility that PrP^{Sc} is itself not directly toxic. This is further supported by the demonstration that PrP^{Sc} deposition fails to cause disease in brain tissue not expressing PrP^{C} (Brandner et al., 1996). Thus, while conversion of PrP^{C} to PrP^{Sc} appears to be central to *transmission*, other aspects of PrP expression, folding and trafficking may feature in the pathophysiological mechanisms that ultimately cause *disease*

Studies of PrP translocation at the endoplasmic reticulum (ER) membrane have revealed unusual features in its biogenesis. While most glycoproteins are synthesized in a single orientation with respect to the membrane of the ER, PrP synthesized in cell-free translation systems can be found in more than one topologic form (Hay et al., 1987a; Hay et al., 1987b; Yost et al., 1990; Lopez et al., 1990). One form appears to be fully translocated into the ER lumen, and hence is termed the secretory form (^{sec}PrP). This topology is consistent with much of what is known about PrP^{C}, which is on the cell surface, tethered to the membrane by a glycolipid anchor whose cleavage results in release from cells (Stahl et al., 1987). The remainder of the PrP made at the ER spans the membrane, with regions of the molecule exposed to the cytosol. However, the relationship between these multiple topological forms of PrP and

neurodegenerative disease had not been established nor had transmembrane forms of PrP been detected in the brain.

In this study, we have used transgenic mice expressing various mutations of PrP to examine the role of the topological forms of PrP in neurodegenerative disease. First, we identified two independent mutations which result in favored synthesis of a specific transmembrane form of PrP (termed ^{Ctm}PrP, see below) at the ER. When expressed in transgenic mice, both of these mutants were demonstrated to confer rapid and severe neurodegeneration with features typical of prion disease, and a concordant increase in the level of detectable ^{Ctm}PrP in brain. By contrast, mice expressing mutants of PrP that favor expression of the ^{sec}PrP form did not develop neurodegenerative disease and did not contain ^{Ctm}PrP in their brains. Furthermore, if expressed at sub-physiologic levels, even a mutant favoring ^{Ctm}PrP production did not accumulate detectable ^{Ctm}PrP in brain and did not cause disease. Finally, none of these mutations of PrP resulted in the formation of detectable amounts of protease resistant PrP^{Sc} in the brains of transgenic mice. These findings strongly argue that synthesis and accumulation of ^{Ctm}PrP, even in the absence of PrP^{Sc}, can result in the development of spontaneous neurodegenerative disease.

Results

PrP topology

The events of secretory and membrane protein biogenesis can be reconstituted and studied using cell-free translation systems containing ER-derived microsomal membranes (Blobel and Dobberstein, 1975b; Shields and Blobel, 1978). The events of protein translocation, membrane insertion, and achievement of the correct final topology of a wide variety of proteins have been shown to be faithfully reproduced. Topology of a protein can be assessed by determining whether any regions of the molecule are accessible to proteases added to the outside of the membrane vesicles. Chains that failed to target to the membrane are completely digested, removing them from the analysis. Of the targeted chains, full protection from exogenous protease is taken to indicate complete translocation into the ER lumen. Conversely, digestion of certain domains to yield discrete protease-protected fragments is taken to indicate a membrane-spanning topology, the exact orientation of which is clarified by immunoprecipitation of the protected fragments with epitope-specific antibodies. Additionally, an aliquot of the sample is routinely digested in the presence of low concentrations of non-denaturing detergents to disrupt the integrity of the lipid bilayer. Under these conditions, proteins in all compartments are accessible to protease and are completely digested, ruling out any inherent protease resistance as the basis for protection in the absence of detergent.

It is important to note that this use of proteases as a probe of topology is distinctly different from the use of proteases as probes of protein conformation (e.g. protease-resistance of PrP^{Sc}). Since the topology assay is carried out in the absence of detergent, the protection from protease is due to an intact membrane barrier. Addition of detergent to this assay generates a necessary negative

control. In contrast, the assay of PrP conformation by use of proteinase K typically is carried out in detergent solution, where only particular folded conformations of PrP, and not a membrane barrier, can protect it from digestion (Oesch et al., 1985; also see Chapter V).

Previous analyses of PrP topology have shown two distinct forms of PrP made at the ER (Hay et al., 1987a; Hay et al., 1987b). One form (^{sec}PrP) appears to be fully translocated across the membrane, and is thus entirely protected from protease digestion in the absence of detergents. The other form of PrP is transmembrane in orientation, and yields two discrete fragments upon protease digestion: one fragment is C-terminally derived and glycosylated, while the other is N-terminally derived and unglycosylated. This data had been interpreted previously to indicate that transmembrane PrP chains span the membrane twice, with the N- and C-termini of the molecule in the ER lumen, protected from proteases added to the cytosolic side (Hay et al., 1987a; Lopez et al., 1990). Recent studies (to be described elsewhere) suggest that the N- and C-terminal fragments reflect the existence of two different transmembrane forms of PrP. One form, termed C-trans transmembrane (^{Ctm}PrP) has the C-terminus in the ER lumen with the N-terminus accessible to proteases in the cytosol. The other form, termed N-trans transmembrane (^{Ntm}PrP), has the N-terminus in the ER lumen with the C-terminus accessible to proteases in the cytosol. Both transmembrane forms appear to span the membrane at the same hydrophobic stretch in PrP (roughly residues 110-135, previously termed TM1). For this reason, the proteolytic fragments derived from each transmembrane form share a common domain of PrP from approximately residue 105 to 140 (the residues immediately adjacent to the membrane spanning domain are not digested by PK due to steric hindrance by the membrane itself). Thus, both fragments share the epitope for the 3F4 monoclonal antibody (against residues 105-112), while only

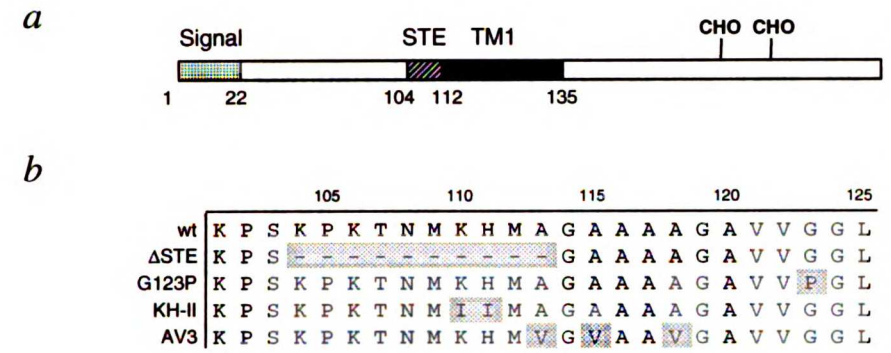
the C-terminal fragment contains the epitope to the 13A5 monoclonal antibody (against residues 138-141). These differences in antibody reactivity, glycosylation, and size allow the ^{Ntm}PrP and ^{Ctm}PrP fragments to be distinguished clearly.

Topology-altering mutations in PrP

Synthesis of both transmembrane forms of PrP is dependent on discrete sequences within the PrP coding region (Hay et al., 1987b; Yost et al., 1990; DeFea et al., 1994). Two adjacent domains within PrP (see Fig. 19a), the hydrophobic, potentially membrane-spanning stretch from amino acids Ala_{113}-Ser_{135} (termed TM1) and the preceding hydrophilic domain [termed STE, and presently narrowed to residues Lys_{104}-Met_{112} (KAD, RSH and VRL, unpublished)], appear to act in concert to generate both transmembrane forms of PrP. Mutations, deletions, or insertions within these domains can alter the relative amounts of each topological form of PrP that is synthesized at the ER (Yost et al., 1990; Lopez et al., 1990). Given these complex and unusual features of PrP biogenesis, it seemed plausible to think that its dysregulation may have dramatic consequences for the physiology of an organism.

In order to explore this hypothesis, we first identified four mutations within STE-TM1 (shown in Fig. 19b) which grossly alter the ratio of the topological forms when assayed by cell-free translation. Two of these mutations (KH-II and ΔSTE) were engineered into SHaPrP, while the other two (AV3 and G123P) were put into MH2MPrP, a mouse-hamster chimera in which residues 94-188 are from hamster PrP (Scott et al., 1993). We found that the species variation between SHaPrP and MH2MPrP (differing at 8 residues) had very little effect on topology (Fig. 19c). However, by comparison to MH2MPrP, MH2MPrP(AV3) showed a dramatic increase (from 8% to 50%) in the relative amount of ^{Ctm}PrP

Fig. 19 Analysis of the topology of mutant PrP molecules at the ER membrane using cell-free translation. *a*, Schematic of topogenic domains in PrP. Topogenic sequences shown are the N-terminal signal sequence (signal), the stop transfer effector sequence (STE), the potential membrane spanning domain (TM1), and glycosylation sites (CHO). Amino acid positions are shown below the diagram. *b*, Mutations used in this study. The amino acid number of hamster PrP is indicated above the figure. Changes are indicated by shading. *c-e*, Topology of wild type and mutant PrP molecules at the ER. *In vitro* synthesized transcript coding for each PrP construct (indicated above the gels) was used to program a rabbit reticulocyte lysate cell-free translation reaction containing ER derived microsomal membranes. Where indicated, a competitive peptide inhibitor of glycosylation (AP) was included in the reaction. Following translation, samples were either left untreated or digested with proteinase K (PK) in the absence or presence of 0.5% Triton X-100 (Det) as indicated below the gel. The positions of unglycosylated (-CHO) and glycosylated (+CHO) PrP species are indicated to the left and molecular weight markers to the right. The positions of the N-terminal and C-terminal fragments generated by PK digestion of the ^{Ntm}PrP and ^{Ctm}PrP forms are indicated at the left of each gel. *f*, Quantitative representation (as described in Methods) of the relative amounts of ^{sec}PrP (black bars), ^{Ctm}PrP (striped bars) and ^{Ntm}PrP (white bars) for each PrP construct analyzed in panels c-e.


a
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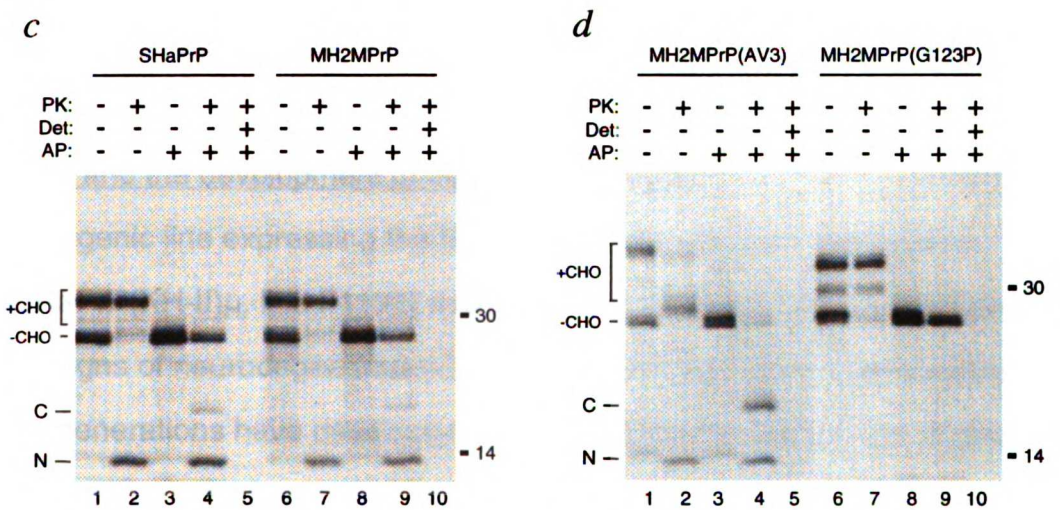
wt K P S K P K T N M K H M A G A A A A G A V V G G L
ΔSTE K P S – – – – – – – – – – G A A A A G A V V G G L
G123P K P S K P K T N M K H M A G A A A A G A V V P G L
KH-II K P S K P K T N M I I M A G A A A A G A V V G G L
AV3 K P S K P K T N M K H M V G V A A V G A V V G G L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MH2MPrP(AV3)
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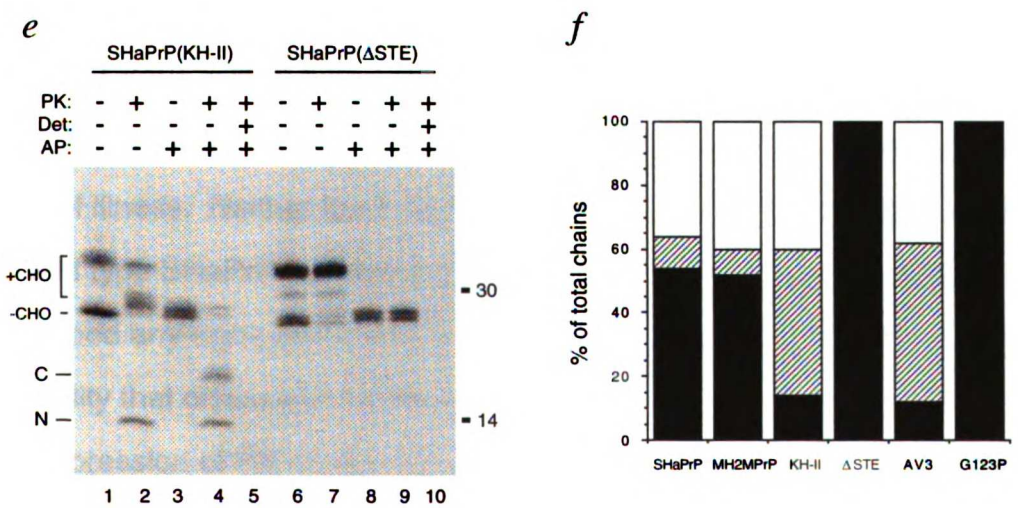
e
SHaPrP(KH-II)
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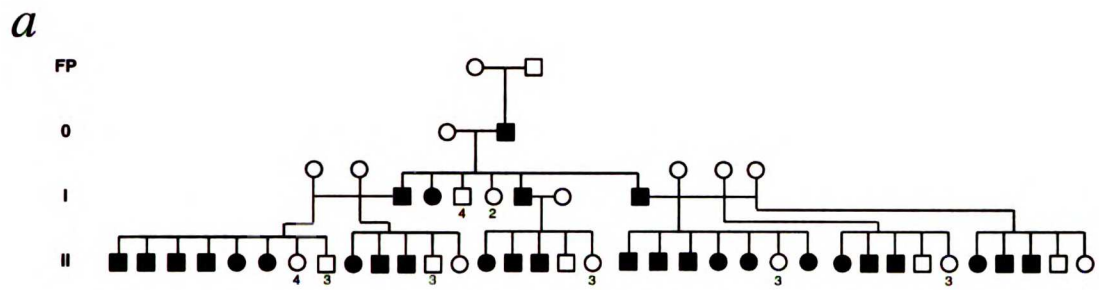
f
% of total chains
100
80
60
40
20
0
SHaPrP
MH2MPrP
KH-II
ΔSTE
AV3
G123P

synthesized, with a concomitant decrease in ^{sec}PrP (Fig. 19d). The amount of ^{Ntm}PrP remained essentially unchanged. Nearly identical results were obtained when SHaPrP was compared to SHaPrP(KH-II) (Fig. 19e). By marked contrast, both MH2MPrP(G123P) and SHaPrP(ΔSTE) were synthesized exclusively in the ^{sec}PrP form (Fig. 19d, e). These results, quantitated and summarized in Fig. 19f, provide the basis with which to examine the effects of aberrant ^{Ctm}PrP synthesis *in vivo*. To do this, PrP transgenes encoding each of these mutations were expressed in mice lacking the PrP gene (FVB/Prnp$^{0/0}$, see Prusiner et al., 1993). These mice were then observed for clinical signs and symptoms, examined for histopathology, and the PrP molecules in their brains were analyzed biochemically for transmembrane topology.

^{Ctm}PrP and the development of neurodegeneration

A transgenic line expressing the SHaPrP(KH-II) transgene [designated TgSHaPrP(KH-II)$_H$, line F1198] was produced, and was observed to develop clear signs of neurodegenerative disease. All 29 transgenic animals spanning three generations have developed clinical symptoms seen in prion disease including ataxia and paresis (Fig. 20a). Analysis of the F2 generation of mice harboring the transgene (n=24) revealed that the average age of onset was 58 days ± 11 days, with the earliest development of symptoms being 41 days (Fig. 20b). By contrast, none of the non-transgenic littermates have exhibited any signs of illness. Neither the FVB/Prnp$^{0/0}$ mice nor FVB/Prnp$^{0/0}$ mice expressing the wild type SHaPrP transgene (designated TgSHaPrP, line A3922) have developed any signs or symptoms of neurologic disease. To rule out the possibility that disease in TgSHaPrP(KH-II)$_H$ mice was simply due to massive overexpression of PrP [which has been shown to cause neurologic disease

Fig. 20 Production and histological characterization of a transgenic mouse line expressing SHaPrP(KH-II). *a*, Genealogy of TgSHaPrP(KH-II)$_H$ transgenic line, generated as described in the Methods. Individuals carrying the transgene are indicated by symbols that are blackened (each of which succumbed to neurodegenerative disease). Open symbols indicate non-transgenic littermates. Males are indicated with a square and females with a circle. Numerals below some symbols indicate multiple individuals represented by that symbol. FP indicates the founding parents, 0, I and II indicate the founder, F1 and F2 generations, respectively. *b*, The F2 transgenic animals (n=24) were observed for signs of neurodegenerative disease, including ataxia and paresis. Plotted on the ordinate axis are the number of asymptomatic animals in the group at any given age (in days). *c*, Assessment of level of expression of TgSHaPrP(KH-II)$_H$ and TgSHaPrP. PrP in varying amounts of brain homogenate (micrograms of total protein is indicated above each lane) from either normal Syrian hamster, TgSHaPrP mouse (wt) or TgSHaPrP(KH-II)$_H$ mouse was detected by immunoblotting with the 13A5 monoclonal antibody. *d*, Haemotoxylin and Eosin stain of immersion fixed brain sections from TgSHaPrP(KH-II)$_H$ mice showing mild to moderate vacuolation. *e*, Analysis of TgSHaPrP(KH-II)$_H$ mice by immunohistochemical staining of immersion fixed brain section with antibodies to glial fibrillary acidic protein, demonstrating reactive astrocytic gliosis.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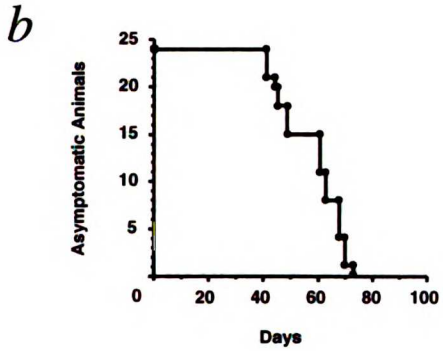
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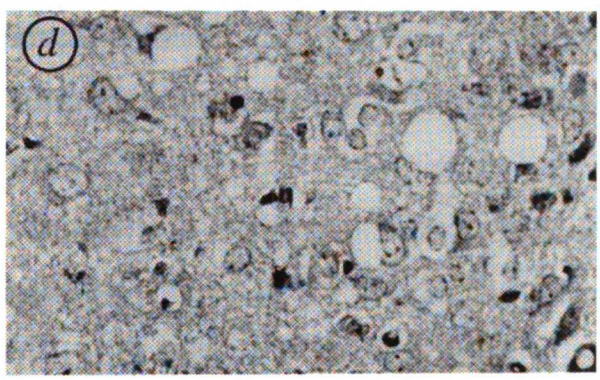
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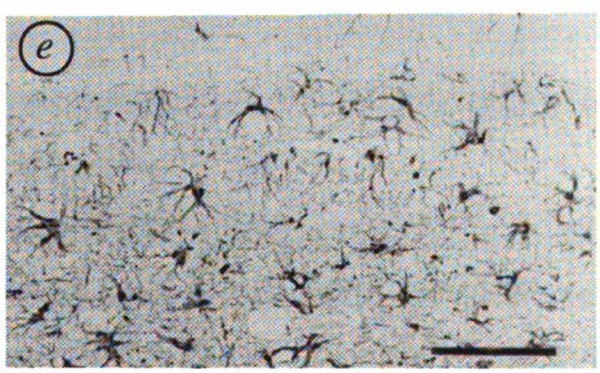

e

(Westaway et al., 1994)] levels of PrP expression were carefully quantitated (Fig. 20c). We found that TgSHaPrP(KH-II)$_H$ expressed PrP at approximately half the level seen in the control mice (TgSHaPrP), indicating that disease development was not simply due to overexpression.

Efforts to generate a stable line expressing MH2MPrP(AV3) at high levels were met with more resistance. Initial attempts to identify founder transgenic animals with high copy numbers of the transgene were unsuccessful. Upon further analysis, we recognized that many animals were showing neurological signs of illness and dying within three or four weeks after birth, prior to screening for the presence of the transgene. To date, every animal identified with a transgene copy number approximately equal to the TgSHaPrP line has succumbed to disease within two months of age. Twelve other founder animals were identified with lower transgene copy numbers and were selected for breeding. Only two have recently allowed formation of breeding lines, which are currently being expanded for further analysis. Half of the remaining founder animals developed neurological illness and died before producing offspring. The other founders did produce offspring, but all the transgenic progeny died before they could be mated. Biochemical analyses of mutant PrP in selected sick animals (see for example, Fig. 21) revealed the expression level to be equal to or lower than in TgSHaPrP, again ruling out a non-specific effect of simple overexpression. These results indicate that, similar to the KH-II mutation, the AV3 mutation causes a neurodegenerative illness of extremely rapid onset upon high level expression in transgenic mice.

Histopathological examination of brains from clinically ill TgSHaPrP(KH-II)$_H$ mice revealed neuropathological changes that were similar to those found in scrapie caused by inoculation of animals with prions. These changes included focal vacuolar degeneration of the grey matter neuropil (most pronounced in the

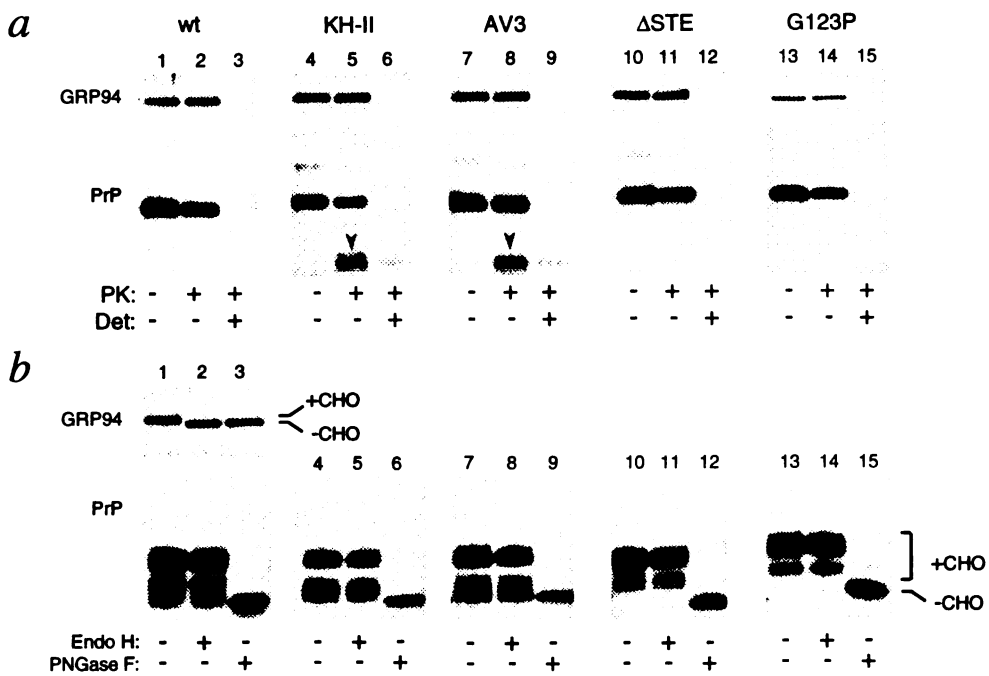
hippocampus and piriform cortex, see Fig. 20d) and an associated astrocytic gliosis (Fig. 20e). Such pathology was seen in each of multiple TgSHaPrP(KH-II)$_H$ mice analyzed, but was not observed in the brains of normal mice, FVB/Prnp$^{0/0}$ mice, or TgSHaPrP mice. Analysis of sick mice carrying the MH2MPrP(AV3) transgene also revealed neurodegeneration with marked astrocytic gliosis (data not shown). However, unlike with TgSHaPrP(KH-II)$_H$ mice, vacuolar degeneration was not consistently observed. Whether this variance in the neuropathology between TgSHaPrP(KH-II)$_H$ and TgMH2MPrP(AV3) mice is due to differences in level of expression, age of examination, the species of PrP gene used (SHaPrP vs. MH2MPrP), or the location or nature of the mutation itself remains to be determined.

Transgenic mice expressing SHaPrP(ΔSTE) and MH2MPrP(G123P) were also constructed, and a high expressing line of each [designated TgSHaPrP(ΔSTE), line F1788, and TgMH2MPrP(G123P), line D13638] was selected for further study. TgSHaPrP(ΔSTE) mice were found to express PrP at ~2.5-3 times that of normal Syrian hamster, a level slightly higher than TgSHaPrP(KH-II)$_H$ mice, but lower than TgSHaPrP mice. TgMH2MPrP(G123P) mice expressed PrP at ~2 times that of normal Syrian hamster, approximately equivalent to the level of TgSHaPrP(KH-II)$_H$. However, in contrast to mice carrying the KH-II or AV3 mutation in PrP, neither TgSHaPrP(ΔSTE) or TgMH2MPrP(G123P) mice showed any signs of illness. Furthermore, even at ages significantly beyond the life-span of the TgSHaPrP(KH-II)$_H$ mice, histological analysis of TgSHaPrP(ΔSTE) and TgMH2MPrP(G123P) mice revealed no abnormal neuropathological changes. Taken together with the above findings, these results are suggestive of the notion that favored synthesis in the ^{Ctm}PrP form as judged by the cell-free translocation assay is indicative of the pathogenicity of the PrP mutation *in vivo*.

The topology of PrP in the brains of these transgenic mice was examined next. For these studies, brains are removed from animals and intact microsomal membranes, containing PrP among other proteins, are prepared. These intact vesicles are then subjected to protease digestion and the accessibility of PrP to protease is assessed by immunoblotting. Generation of a proteolytic fragment encompassing the carboxy-terminus of PrP is taken to indicate that those molecules were in the ^{Ctm}PrP orientation, while full protection from protease indicates that the PrP is in the ^{sec}PrP orientation. Analysis of the proteolytic fragments is simplified by the removal, just prior to SDS-PAGE, of the highly heterogeneous carbohydrate trees with the enzyme PNGase F. This allows us to look specifically for the 18 kD carboxy-terminal fragment specific to the ^{Ctm}PrP form, without the complications of differential electrophoretic migration of variably glycosylated PrP molecules. Again, the ratio of ^{sec}PrP to ^{Ctm}PrP is assessed by comparing the ratio of PrP chains which are protected from protease to PrP chains represented by the 18 kD fragment. To ensure that vesicle integrity was maintained during the proteolysis reaction, the accessibility to protease of GRP94, an ER lumenal protein (Welch et al., 1983), is also evaluated.

Fig. 21a shows the analysis of PrP topology in the microsomal membranes prepared from TgSHaPrP ,TgSHaPrP(KH-II)$_H$, TgMH2MPrP(AV3), TgSHaPrP(ΔSTE) and TgMHM2PrP(G123P) mice. Only the TgSHaPrP(KH-II)$_H$ and TgMH2MPrP(AV3) samples contained PrP molecules spanning the membrane, which after protease digestion in the absence of detergent produced an 18 kD fragment. The PrP chains which were fully protected from protease were in the ^{sec}PrP topology. As expected in each instance, GRP94 was fully protected from protease in the absence of detergent, demonstrating that the protease only had access to the outside of the vesicles. The 18 kD band produced by protease digestion was determined to be a C-terminal fragment of

Fig. 21 Biochemical characterization of mice expressing mutant PrP transgenes. *a,* Intact microsomal membranes isolated from the brain tissue of various transgenic mice (as indicated above each gel) were subjected to digestion with PK in the absence or presence of 0.5% Triton X-100 (det) as described in the Methods. Following completion of the proteolysis reactions, all samples were digested with PNGase F and analyzed by SDS-PAGE and immunoblotting with the 3F4 antibody (lanes 1-3, 7-9, and 13-15) or 13A5 antibody (lanes 4-6 and 10-12). The arrowheads in lanes 5 and 8 point to the C-terminal proteolytic fragment indicative of the ^{Ctm}PrP form. In parallel, the blots were also probed with 9G10 antibody to detect GRP94, an ER lumenal protein. Complete protection of GRP94 in the absence of detergent in each instance indicates that the PK had access to only the outside of the vesicles. *b,* Microsomal membrane proteins isolated from brain tissue of various transgenic mice (as indicated above) were denatured in 1% SDS, digested with either endoglycosidase H (endo H) or PNGase F, and the samples analyzed by immunoblotting with 13A5 or 9G10 antibodies to detect PrP and GRP94, respectively. The positions of glycosylated (+CHO) and de-glycosylated (-CHO) PrP and GRP94 are indicated.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Det: - - + - - + - - + - - + - - +
b
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GRP94
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4 5 6 7 8 9 10 11 12 13 14 15
PrP
+CHO
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Endo H: - + - - + - - + - - + - - + -
PNGase F: - - + - - + - - + - - + - - +

PrP based on its detection with the 13A5 antibody. Consistent with this is its observed slower migration on SDS-PAGE as a heterogeneous set of bands if polysaccharides were not removed from PrP prior to analysis (data not shown). Thus, the PrP molecules found spanning the membrane are in the ^{Ctm}PrP orientation. We were not able to clearly detect any PrP molecules in the ^{Ntm}PrP form in any of the animals examined. Whether specific mutations which increase the generation of this form will show a phenotype in mice, or facilitate its detection in brain remains to be seen.

However, the above results demonstrate that brains from TgSHaPrP(KH-II)$_H$ and TgMH2MPrP(AV3) mice contain ^{Ctm}PrP (comprising ~20-30% of the PrP in the microsomes). Similar results were obtained from multiple TgSHaPrP(KH-II)$_H$ and TgMH2MPrP(AV3) animals, while in no instance was any ^{Ctm}PrP detected in TgSHaPrP, TgSHaPrP(ΔSTE) or TgMH2MPrP(G123P) animals. Taken together, these data indicate that the presence of the ^{Ctm}PrP form of PrP in the brains of transgenic mice correlates well with observations made using the cell-free translocation system, although the absolute amount of ^{Ctm}PrP in brain was consistently less than that observed in cell-free assays (see below). More importantly, the findings suggest that ^{Ctm}PrP is involved in the development of spontaneous neurodegenerative disease.

To determine whether the ^{Ctm}PrP observed in transgenic mice brains had exited the ER, we analyzed the state of maturation of the carbohydrate trees present on PrP. While still in the ER, carbohydrates can be removed efficiently with the enzyme endoglycosidase H (endo H; e.g. Hebert et al., 1995). However, upon transit of proteins to the Golgi apparatus, the sugar trees are trimmed and rendered resistant to endo H, and can only be removed with the enzyme PNGase F. We thus determined whether the PrP molecules in brain microsomes [~20-30% of which are in the ^{Ctm}PrP form for TgSHaPrP(KH-II)$_H$ and

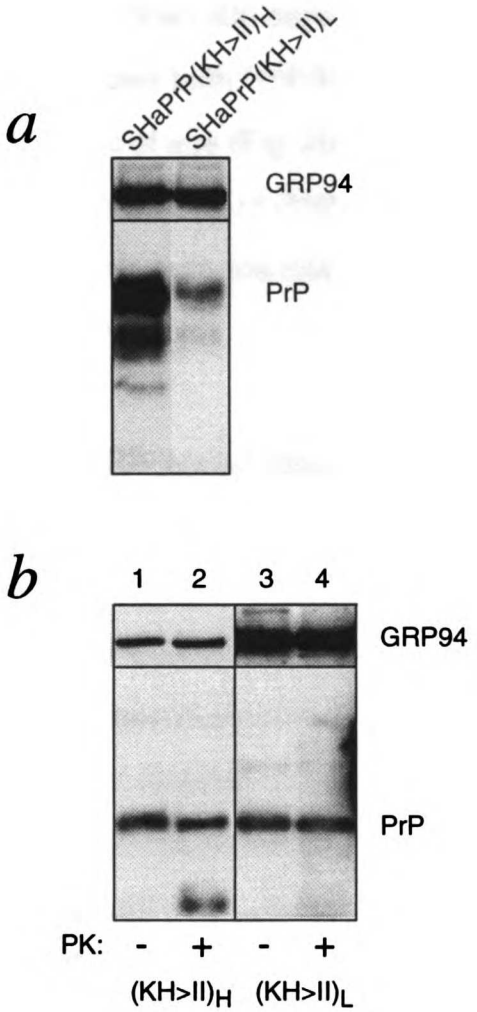
TgMH2MPrP(AV3)] have acquired resistance to endo H digestion. As a control, the resident ER protein GRP94 (Welch et al., 1983), which is not expected to acquire endo H resistance, was also examined. We found that very little (<2%) of the PrP was digested with endo H, while 100% of the GRP94 in the same sample was digested (Fig. 21b). By contrast, both PrP and GRP94 were digested completely with PNGase F. These results indicate that nearly all of the PrP, including the ^{Ctm}PrP form seen in TgSHaPrP(KH-II)$_H$ and TgMH2MPrP(AV3) samples, had exited the ER and resides in a post-ER compartment.

Level of expression modulates ^{Ctm}PrP and disease

The observation that the percent of PrP molecules found in the ^{Ctm}PrP topology *in vivo* is consistently lower than that found *in vitro* (compare Fig. 19 to Fig. 21) raises the possibility that cells normally have mechanisms to prevent the accumulation of this potentially pathogenic form. Thus, the basis for the modest ^{Ctm}PrP accumulation in brain in the KH-II or AV3 mutant may be a combination of overexpression and the severe skew toward ^{Ctm}PrP synthesis , which together exceed the cell's ability to prevent accumulation of ^{Ctm}PrP. If this were the case, then one would predict that lower levels of expression of a ^{Ctm}PrP-favoring mutant should fall below such a threshold, and thus produce only ^{sec}PrP. Such mice would be predicted not to get sick despite the mutation in the PrP gene, owing to the absence of ^{Ctm}PrP.

We explored this idea by first identifying a transgenic line of mice (designated TgSHaPrP(KH-II)$_L$, line E12485) expressing the KH-II mutation at sub-physiologic levels. This line contained approximately one fourth to one half the level of PrP found in normal Syrian hamster, corresponding to levels ~5 fold lower than the TgSHaPrP(KH-II)$_H$ mice (Fig. 22a). Upon biochemical examination of the brain, ^{Ctm}PrP was not detected in TgSHaPrP(KH-II)$_L$ mice, with all of the PrP being in

Fig. 22. Level of transgene expression dictates presence of ^{Ctm}PrP. *a*, Equal amounts of total brain homogenate from TgSHaPrP(KH-II)$_H$ and TgSHaPrP(KH-II)$_L$ mice were analyzed by western blotting for PrP (with the 13A5 mAb) and GRP94. Serial dilutions of the TgSHaPrP(KH-II)$_H$ sample showed it to contain approximately 4-8 fold more than the TgSHaPrP(KH-II)$_L$ sample (data not shown). *b*, The topology of TgSHaPrP(KH-II)$_H$ and TgSHaPrP(KH-II)$_L$ mice was analyzed as in Fig. 21a. The amount of PrP that was visualized was normalized by adjusting the exposure time to the film. Identical results were obtained when the amount of visualized PrP was normalized by adjusting the amount of sample analyzed (data not shown).


a
SHaPrP(KH>II)H
SHaPrP(KH>II)L
GRP94
PrP
b
1 2 3 4
GRP94
PrP
PK: - + - +
(KH>II)H (KH>II)L

the ^{sec}PrP form (Fig. 22b). Thus, by decreasing the level of transgene expression ~5 fold, the percent of ^{Ctm}PrP form is reduced from ~30% to undetectable levels. Corresponding to this lack of ^{Ctm}PrP generation, observation of the animals from the TgSHaPrP(KH-II)$_L$ line have thus far revealed no signs of illness at ages greater than 300 days. This is in sharp contrast to the TgSHaPrP(KH-II)$_H$ line which shows both ^{Ctm}PrP and clear clinical signs of disease at approximately 60 days of age (Fig. 20b). These data lend substantial support to the hypothesis that generation of ^{Ctm}PrP is causative of a neurodegenerative disease in mice, with the role of the mutation being limited to one of favoring synthesis of ^{Ctm}PrP.

Spontaneous disease without PrP^{Sc} accumulation

The data thus far presented suggest that the basis of disease pathogenesis for the KH-II and AV3 mutants of PrP is an increase in ^{Ctm}PrP production. However, given the association of protease resistant PrP^{Sc} and various prion diseases, one might wonder whether the mutations described in this study predispose towards spontaneous conversion to PrP^{Sc}, and that accumulation of this isoform is the cause of the disease observed. Although further studies will be required to determine whether the spontaneous disease caused by the KH-II and AV3 mutations is transmissible, we have analyzed brains of the sick transgenic mice for the presence of protease resistant PrP^{Sc}. As shown in Fig. 23, we were unable to detect PrP^{Sc} in either the TgSHaPrP(KH-II)$_H$ or TgMH2MPrP(AV3) mice. Even overloaded gels probed at maximal sensitivity failed to detect protease resistant PrP molecules. Consistent with these findings, immunohistochemistry of brains sections (following hydrolytic autoclaving) from TgSHaPrP(KH-II)$_H$ and TgMH2MPrP(AV3) failed to detect PrP^{Sc} (data not shown). Whether these low amounts of protease resistant PrP^{Sc} will reflect low

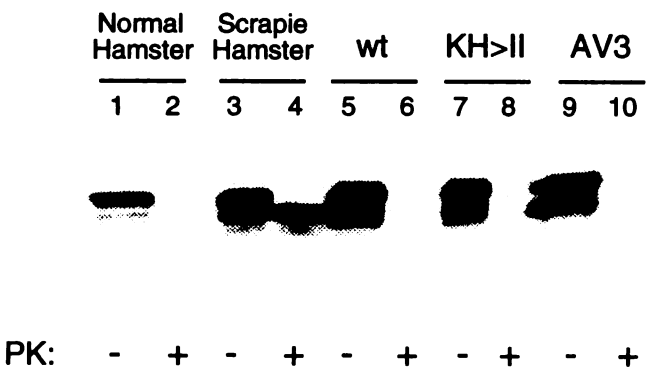


Fig. 23 Analysis of spontaneously sick transgenic mice for PrP^{Sc}. 10% Brain homogenates from normal hamster, a hamster sick with experimental scrapie, TgSHaPrP mouse (wt), TgSHaPrP(KH-II)$_H$ mouse, or TgMH2MPrP(AV3) mouse were prepared in PBS containing 1% NP-40 and 1% dexoycholate. The samples were either left untreated (odd numbered lanes) or digested with 100ug/ml proteinase K (PK) for 60 minutes at 37° (even numbered lanes) as described previously (Telling et al., 1996). Following termination of the PK reaction with PMSF, samples were analyzed by immunoblotting with the 13A5 antibody.

titers of infectious particles remains to be seen. Regardless, these findings further support the notion that it is ^{Ctm}PrP overexpression, and not PrP^{Sc} accumulation, that is the cause of neurodegeneration observed in these transgenic mice.

Discussion

Our studies were motivated by the need to determine the role in prion diseases played by a transmembrane form of PrP that was first identified through the use of cell-free translation-translocation systems (Hay et al., 1987a). The hypothesis that a specific topologic form of PrP, namely ^{Ctm}PrP, is involved in development of neurodegeneration is supported in several ways (summarized in Table 1). First, two independent mutations of PrP (KH-II and AV3), that were initially identified by their ability to markedly favor the synthesis of ^{Ctm}PrP in cell-free systems, are found to cause accelerated spontaneous neurodegeneration and death in transgenic mice. Second, other PrP mutations in the STE-TM1 domain that abolish expression of ^{Ctm}PrP in cell-free systems (ΔSTE and G123P) do not cause spontaneous disease in transgenic mice. Third, biochemical examination of PrP topology in the brains of transgenic mice expressing pathogenic mutations of PrP reveal the presence of ^{Ctm}PrP *in vivo*, while this form is not seen in mice expressing a comparable level of wild type PrP. Fourth, mice expressing the potentially pathogenic KH-II mutation at sub-physiologic levels do not contain ^{Ctm}PrP in brain, and correspondingly fail to show signs of disease. Finally, examination of the brains of sick mice failed to reveal the presence or accumulation of protease resistant PrP^{Sc}. Taken together, these data strongly suggest that expression of PrP in the ^{Ctm}PrP form causes neurodegenerative disease.

An unanswered question is whether the spontaneous disease developed in TgSHaPrP(KH-II)$_H$ and TgMH2MPrP(AV3) mice is transmissible, a property traditionally used to define prion diseases. While demonstration of this feature will require further experiments, several lines of evidence currently indicate that the pathologic processes resulting in ^{Ctm}PrP-associated neurodegeneration are

Table 1 Mutational analysis of PrP *in vitro* and *in vivo*

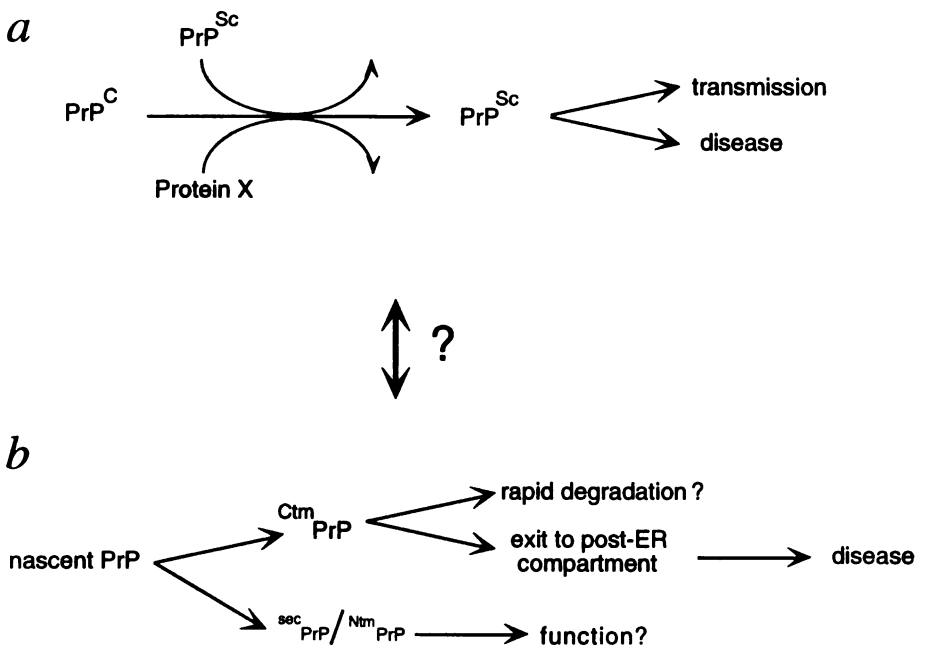
	Ctm^{PrP} cell-free (rel to wt)	Level of expression (rel to SHa)	detection of Ctm^{PrP} in brain	Clinical Symptoms	Neuropath in brain	PrP^{Sc} detection in brain
wild type	=	4x	-	-	-	-
ΔSTE	<	2.5-3x	-	-	-	-
G123P	<	2x	-	-	-	-
$(KH\text{-}II)_H$	>	2x	+	+	+	-
$(KH\text{-}II)_L$	>	0.25-0.5x	-	-	-	-
AV3	>	2-4x	+	+	+	-

directly relevant to prion diseases. First, the neuropathology observed in TgSHaPrP(KH-II)$_H$ and TgMH2MPrP(AV3) is very similar to that observed in several demonstrated prion diseases (Hsiao et al., 1989; Tateishi et al., 1990). Second, the apparent lack of protease resistant PrP^{Sc} in TgSHaPrP(KH-II)$_H$ and TgMH2MPrP(AV3) mice is not unprecedented in prion disease. Rather, it is quite common in several genetic forms of this disease, with certain mutations causing neuropathology despite little detectable PrP^{Sc} and low titers of infectivity (Tateishi et al., 1990; Hsiao et al., 1994; Tateishi and Kitamoto, 1995). Finally, it does not appear that the disease observed in this study is due to non-specific accumulation of misfolded or aggregated PrP molecules. This is supported by the observation of nearly all of the ^{Ctm}PrP being located in a post-ER compartment, a lack of excessive accumulation of PrP (i.e., no plaques or other localized deposition of PrP), and a lack of neuropathology characteristic of a storage type disorder. Together, these data support the hypothesis that expression of ^{Ctm}PrP may be involved in the pathologic process occurring in at least a subset of genetic prion diseases.

At present, the data in this study are suggestive of at least three distinct steps in the pathogenesis of ^{Ctm}PrP-associated neurodegenerative disease (Fig. 24b). The first step is preferential synthesis of ^{Ctm}PrP over other topological forms at the ER membrane. Second, newly synthesized ^{Ctm}PrP, which may normally be degraded rapidly, exits from the ER. Finally, in a post-ER compartment, ^{Ctm}PrP is proposed to serve an as yet unknown function to cause, directly or indirectly, neurodegeneration.

There are three lines of evidence for the first step. First, PrP can be made in more than one topological form in cell-free translation systems (this study and Hay et al., 1987a; Hay et al., 1987b; Yost et al., 1990). Second, the ^{Ctm}PrP form has now been detected *in vivo* (see Fig. 21a). Finally, PrP topology is

Fig. 24 Structural (top) and cell biological (bottom) models of prion disease pathogenesis. In the structural model, PrP^{C} is converted to PrP^{Sc} in a reaction catalyzed by PrP^{Sc} and an unknown protein X. PrP^{Sc} is then able to transmit disease to other animals and/or cause disease. In the cell biological model, nascent PrP is synthesized in either the ^{sec}PrP, ^{Ntm}PrP or ^{Ctm}PrP form. The ^{Ctm}PrP form may subsequently be rapidly degraded in the ER, or in some cases, able to escape degradation to a post-ER compartment. Upon exit from the ER, ^{Ctm}PrP is proposed to cause disease. The arrow between the two boxes indicates the currently unknown relationships linking one set of events with the other. See text for details.


a
PrP^Sc
PrP^C
PrP^Sc
transmission
disease
Protein X
?
b
nascent PrP
Ctm PrP
rapid degradation ?
exit to post-ER compartment
disease
sec PrP / Ntm PrP
function?

dependent on both the amino acid sequence (Yost et al., 1990 and this study) and *trans*-acting factors in the cytosol (Lopez et al., 1990) and in the ER membrane (Hegde *et al.* in preparation). Thus, not only is PrP able to be made in multiple topological forms, but this step appears to be regulated in a complex and novel manner. One prediction of this hypothesis is that, just as mutations in *cis* are able to affect both PrP topology and development of disease, loss or inactivation of specific *trans*-acting factors might also alter PrP topology in favor of ^{Ctm}PrP and potentiate development of neurodegeneration.

Once synthesized at the ER, ^{Ctm}PrP is thought to exit to a post-ER compartment based on the acquisition of resistance to digestion by endo H (Fig. 21b). We postulate that while exit from the ER can occur in some instances, ^{Ctm}PrP normally may be degraded in the ER itself, a fate observed for some posttranslationally regulated proteins (Bonifacino et al., 1990; Wang et al., 1996). This may explain why expression of any given PrP construct at the ER in the cell-free system results in a higher percentage of transmembrane PrP chains than are detectable for that construct in brain at steady state. The observation of a lack of ^{Ctm}PrP accumulation in the TgSHaPrP(KH-II)$_L$ line of mice is supportive of this model. Alternatively, it is possible that differences in the level or efficiencies of one or more *trans*-acting factors involved in PrP synthesis and translocation are responsible. Further work will be required to distinguish between these and other models.

At the present time, we do not know how ^{Ctm}PrP, upon exit from the ER, is able to cause disease. The correlation of two independent PrP mutants that promote ^{Ctm}PrP synthesis in cell-free systems, with development of neurodegenerative disease in transgenic mice, and detection of ^{Ctm}PrP in brain suggest a causative role in disease. Whether inappropriately expressed ^{Ctm}PrP is able to initiate specific signaling events to cause cell death, or accomplishes

this end by another mechanism remains to be determined. Similarly, it is currently unclear how, in the case of transmissible prion disease, accumulation of PrP^{Sc} results in disease. Perhaps PrP^{Sc} accumulation, either directly or indirectly, can serve as a signal for the synthesis of ^{Ctm}PrP to cause neurodegeneration. Future work correlating disease transmission, PrP^{Sc} generation, and specific events in ^{Ctm}PrP biogenesis will allow the above hypotheses to be tested, and the present findings integrated into an overall model of prion pathogenesis.

Experimental Procedures

DNA constructions and antibodies - For cell-free translation of PrP and mutants, each coding region was engineered using standard methods (Sambrook et al., 1989) into an sp64 plasmid vector containing the SP6 promoter followed by the 5' untranslated region of Xenopus globin (Krieg and Melton, 1984). The SHaPrP and MH2MPrP constructs have been described (Scott et al., 1993). For transgenic mice production, PrP coding regions were engineered into the cosSHa.Tet cosmid expression vector (Scott et al., 1992) at the Sal1 site. Use of the 3F4 and 13A5 monoclonal antibodies, and the R073 polyclonal antibody, have been described (Scott et al., 1992; Rogers et al., 1991). GRP94 was detected using the 9G10 monoclonal antibody (Stressgen Biotechnologies).

Cell-free transcription, translation, and translocation assays - Rabbit reticulocyte lysate and microsomal membranes from dog pancreas were prepared as described previously (Merrick, 1983; Walter and Blobel, 1983). Transcription using SP6 polymerase, translation, and proteolysis were as described previously (Hegde and Lingappa, 1996). All translation reactions were carried out at 32°C for 60 minutes, and proteolysis reactions at 0°C for 60 minutes using 0.5 mg/ml proteinase K (PK). Products were immunoprecipitated with either the 3F4 antibody (Fig. 19c and 19d) or R073 antibody (Fig. 19e), analyzed by 10% tricine-SDS-PAGE (Schagger and vo Jagow, 1987), and the proteins visualized by autoradiography. Relative amounts of the topological forms of PrP were quantitated by densitometry of the three products resulting after protease digestion in the absence of detergent: full length, undigested PrP (^{sec}PrP), the 18 kD C-terminal fragment (^{Ctm}PrP), and the 14 kD N-terminal fragment (^{Ntm}PrP). Densitometric values for C- and N-terminal fragments were adjusted (to

compensate for loss of radioactivity due to proteolytic digestion of a portion of the molecule) to obtain quantitative estimates of ^{Ctm}PrP and ^{Ntm}PrP.

Transgenic mice - cosSHa.Tet cosmids containing the appropriate PrP transgenes were digested with NotI, the transgene purified and used for microinjection into the pronuclei of fertilized FVB/Prnp$^{0/0}$ oocytes as previously described (Hsiao et al., 1990). The presence of the transgene in weanling animals was assessed by screening genomic DNA isolated from tail tissue with a probe specific to the 3' untranslated region of the SHaPrP gene contained in the cosSHa.Tet vector. PrP expression was assessed by immunoblotting of brain tissue homogenate with 13A5 monoclonal antibody, comparing to serial dilutions of normal Syrian hamster brain tissue (see Fig. 20c for an example). Levels of expression (relative to normal hamster) of each transgenic line are shown in Table 1.

Histopathological examination of brain - Immersion fixation, sectioning, and histological staining of brain tissues has been described previously (Hsiao et al., 1990). Astrocytes were visualized by immunohistochemical staining for glial fibrillary acidic protein as described previously (Hsiao et al., 1990).

Assessment of PrP topology in brain - Microsomal membranes were isolated from fresh brain tissue following homogenization in approximately 20 volumes of ice cold buffer (0.25 M sucrose, 100 mM KCl, 5 mM $MgAc_2$, 50 mM Hepes, pH 7.5, 0.5 mM PMSF) by successive passage, 5 times each, through 16, 18, 20 and 22 guage needles. Homogenate was centrifuged 10 minutes at 12,000 x g at 4°C. Supernatant was removed and centrifuged either 20 minutes at 100,000 rpm in TLA-100.3 rotor (Beckman) or 60 minutes at 65,000 rpm in 70.1Ti rotor

(Beckman). Supernatant was discarded and pellet resuspended in same buffer as above, but without PMSF, at a concentration of 500 ul per gram of starting tissue. Topology was assessed by digestion of membranes with PK as described above, followed by digestion with PNGase as directed by manufacturer (New England Biolabs). Samples were precipitated with TCA, separated by 10% tricine-SDS-PAGE, transferred to nitrocellulose, and probed with either 3F4 or 13A5 monoclonal antibodies as indicated in the Figure legends.

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V. Topological Dysregulation in Human Disease

A transmembrane form of the Prion protein is implicated in the pathogenesis of Gerstmann-Straussler-Scheinker syndrome

Abstract

A variety of mutations in the gene encoding the prion protein (PrP) have been shown to result in the development of Gerstmann-Straussler-Scheinker syndrome (GSS), a heritable neurodegenerative disorder of humans. However, the mechanism by which such mutations result in disease has been unclear. In this study, one of these mutations, an alanine to valine substitution at position 117, is shown to result in the increased synthesis of a transmembrane form of PrP (termed ^{Ctm}PrP) *in vitro*. Elevated levels of ^{Ctm}PrP were also observed in the brain tissue of GSS patients carrying this mutation. By contrast, PrP^{Sc}, a misfolded form of PrP associated with transmissible prion diseases, was not detected. Together with the recent demonstration that ^{Ctm}PrP overexpression in mice causes neurodegenerative disease, these data demonstrate that at least some heritable cases of GSS are due to ^{Ctm}PrP and provide the first example of dysregulated protein topogenesis as the basis for a human disease.

Introduction

The biogenesis of PrP at the endoplasmic reticulum (ER) membrane has proven to be extraordinary in at least two respects. First, PrP is capable of adopting multiple topological forms at the ER membrane when synthesized in cell-free systems. One of these forms (^{sec}PrP) is fully translocated across the membrane (Hay et al., 1987b) and is consistent with the topology ascribed to PrP^{C}, the normal cellular isoform (Stahl et al., 1987). The other topoloical forms of PrP span the membrane (Hay et al., 1987a), with either the C-terminus or N-terminus located in the lumen of the ER (and thus termed ^{Ctm}PrP and ^{Ntm}PrP, respectively; see Chapter IV). The second remarkable feature of PrP biogenesis is that the ratio of the topological forms relative to each other can be modulated. Both mutations in the PrP chain (Yost et al., 1990 and Chapter IV), or manipulation of *trans*-acting factors in the cytosol (Lopez et al., 1990) or ER membrane (Hegde *et al.*, in preparation) can alter the final topological form achieved by PrP. However, the relevance of these unusual events in PrP biogenesis, or the consequences of its dysregulation, had previously remained unclear.

Recent studies employing transgenic mice expressing mutant PrP genes revealed that alterations in PrP topology *in vivo* can have severe consequences (see Chapter IV). Mutations which favored the synthesis of PrP in the ^{Ctm}PrP form at the ER (termed KH-II and AV3, see Methods) were found to cause a neurodegenerative disease similar to GSS (with clinical ataxia, paresis, astrogliosis, and/or spongiform changes) upon overexpression in mice. Furthermore, the generation of ^{Ctm}PrP (rather than the mutation *per se*) correlated with disease, as mice expressing the KH-II mutation at sub-physiologic levels showed neither ^{Ctm}PrP nor neurodegenerative disease. Although these

results established that ^{Ctm}PrP could cause disease, whether it indeed was the cause of any currently known human disease remained a matter of speculation.

Given that PrP biogenesis is readily manipulated with mutations, we reasoned that there may exist in nature similar mutations which also affect topology and thus cause disease. A GSS causing mutation in PrP (Hsiao et al., 1991; Mastrianni et al., 1995; Tranchanc et al., 1992), an alanine to valine substitution at position 117, was a likely candidate for several reasons. First, this mutation lies in the hydrophobic domain (termed TM1) that has been shown to be crucial to the biogenesis of the transmembrane forms of PrP (Yost et al., 1990; DeFea et al., 1994). Second, the pathological findings in these cases of GSS appear to share some features with mice sick due to ^{Ctm}PrP overexpression (see Chapter IV). Third, the biochemical examination of brain tissue from these cases of GSS has revealed little or no protease resistant PrP (Tateishi et al., 1990; Mastrianni *et al.*, in preparation), raising the possibility that a mechanism other than PrP^{Sc} accumulation was involved.

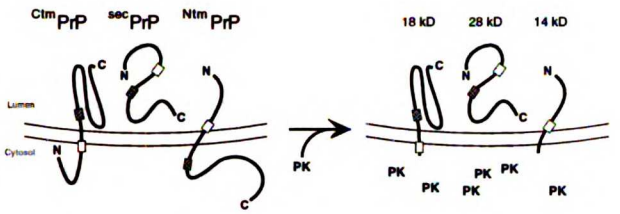
Results and Discussion

To initially explore the mechanism by which the A117V mutation may cause disease, we compared the biogenesis in a cell-free system of this mutant PrP to its wild type counterpart (both of which contained a valine at the polymorphic position 129). The topology achieved by PrP can be assayed using protease added to the cytosolic side of an intact membrane (see Chapter IV). In this manner, cytosolically exposed domains of membrane spanning proteins are digested, while lumenal domains are protected (see Fig. 25a). Analysis of the products of this topology assay for wild type human PrP (HuPrP) reveals three distinct products following protease digestion: i) PrP that is fully protected from protease digestion, indicative of ^{sec}PrP, ii) PrP digested to yield a C-terminal fragment (whose unglycosylated size is ~18 kD), indicative of ^{Ctm}PrP, and iii) PrP digested to yield a 14 kD N-terminal fragment, indicative of ^{Ntm}PrP. These three products are most readily resolved in the absence of glycosylation, allowing direct comparison of their relative amounts (Fig. 25b, lane 4). Upon examination of the GSS mutant PrP molecule [HuPrP(A117V)], we noticed that it consistently contained higher amounts of the transmembrane forms of PrP than HuPrP (compare lanes 4 and 9, Fig. 25b). Quantitative analysis of multiple independent translocation reactions, shown in Fig. 25c, demonstrated clearly that the A117V mutation causes a significant favored synthesis of both ^{Ctm}PrP and ^{Ntm}PrP , with a concordant decrease of the ^{sec}PrP form.

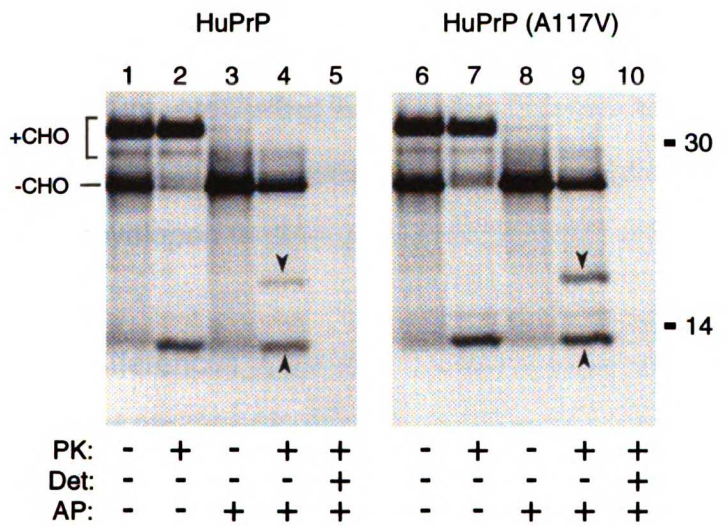
Thus, a mutation of PrP in the STE-TM1 region that is associated with human disease shows a relative preference for synthesis in the transmembrane forms of PrP upon expression in cell-free translation systems. Alterations in membrane association were also reported to occur in cultured cells expressing certain pathogenic human mutations of PrP (Lehmann and Harris, 1995, 1996a, 1996b).

Fig. 25. Analysis of HuPrP and HuPrP(A117V) topology at the ER membrane using cell-free translation. *a.* Schematic of the standard proteolysis assay for PrP topology determination. The three topologic forms of PrP are shown before (left) and after (right) digestion with cytosolically disposed proteinase K (PK). The approximate sizes of the fragments generated from each form are indicated above the diagram. The positions of the epitopes recognized by the 3F4 and 13A5 antibodies are indicated by the white and grey boxes, respectively. *b. In vitro* synthesized transcript coding for HuPrP (lanes 1-5) or HuPrP(A117V) (lanes 6-10) was used to program a rabbit reticulocyte lysate cell-free translation reaction containing ER derived microsomal membranes. Where indicated, a competitive peptide inhibitor of glycosylation (AP) was included in the reaction. Following translation, samples were either left untreated or digested with 500 ug/ml proteinase K (PK) in the absence or presence of 0.5% Triton X-100 (Det) as indicated below the gel. The positions of unglycosylated (-CHO), and glycosylated (+CHO) PrP species are indicated to the left and molecular weight markers to the right. The N-terminal and C-terminal fragments generated by PK digestion of the ^{Ntm}PrP and ^{Ctm}PrP forms are indicated by the upward and downward pointing arrowheads, respectively. *c*, Quantitative analyses of HuPrP and HuPrP(A117V) topology at the ER membrane are shown by the black and striped bars, respectively. Data from multiple experiments performed exactly as above were used to quantitate (as described in Experimental Procedures) the relative amounts of ^{sec}PrP, ^{Ctm}PrP, and ^{Ntm}PrP. Numbers above each bar indicate the mean ± SEM (N=6).

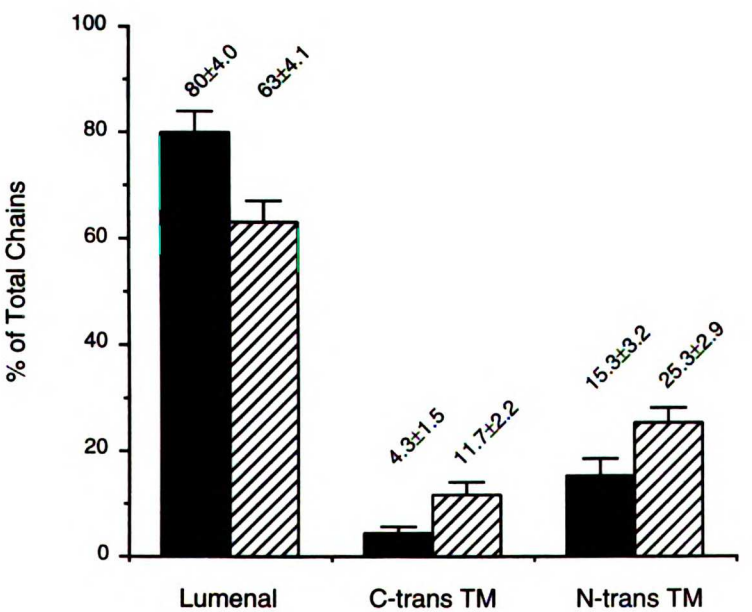
a


Ctm PrP
sec PrP
Ntm PrP
Lumen
Cytosol
N
C
PK
18 kD
28 kD
14 kD

b


HuPrP
HuPrP (A117V)
1 2 3 4 5
6 7 8 9 10
+CHO
-CHO
30
14
PK: - + - + +
Det: - - - - +
AP: - - + + +
- + - + +
- - + + +

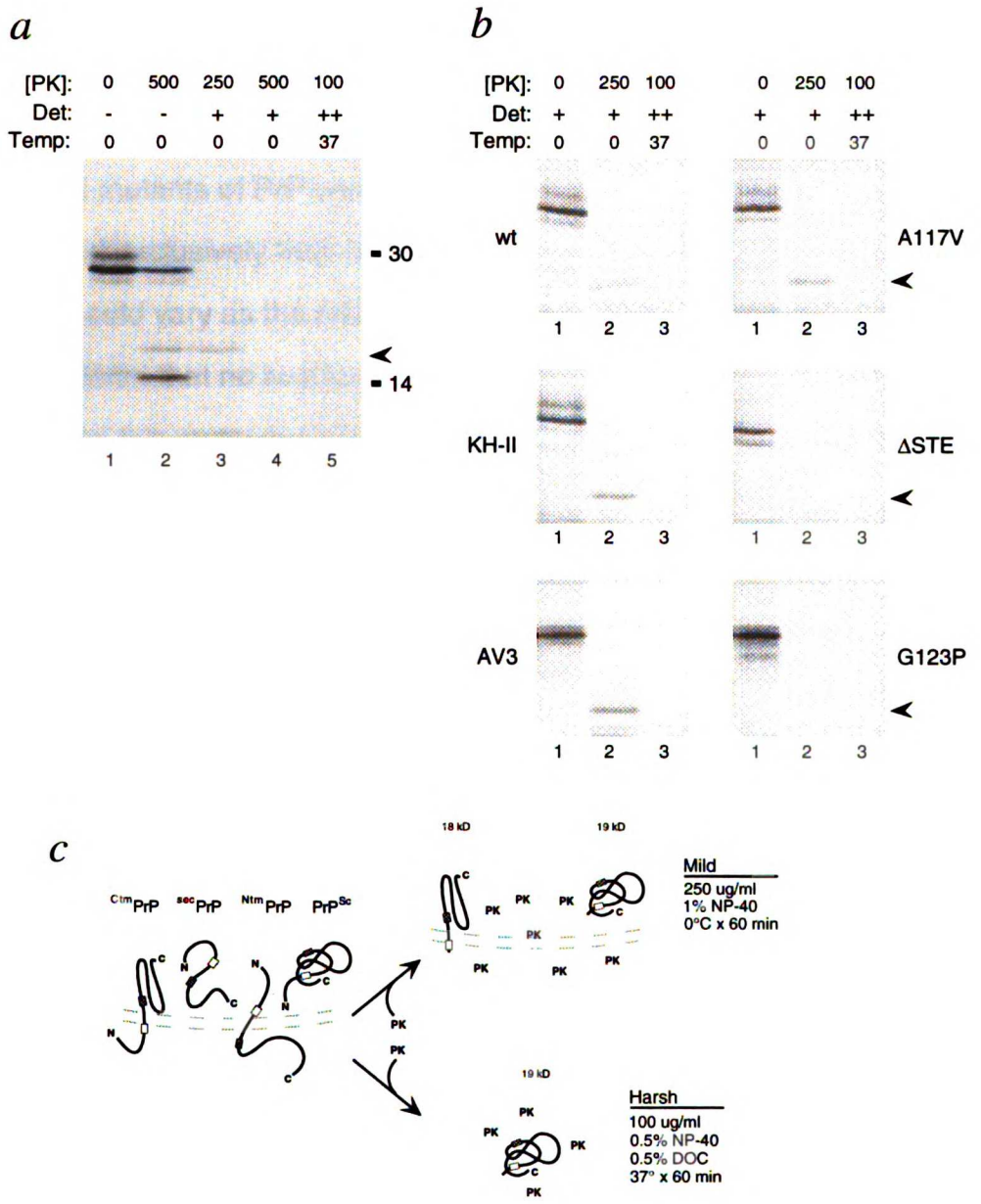
c


% of Total Chains
100
80
60
40
20
0
80±4.0
63±4.1
4.3±1.5
11.7±2.2
15.3±3.2
25.3±2.9
Lumenal
C-trans TM
N-trans TM

These data are consistent with the hypothesis that a transmembrane form of PrP plays a role in GSS. To explore this idea further, we wished to biochemically examine brain tissue from a GSS patient and determine whether *in vivo*, elevated levels of transmembrane forms of PrP could be detected. However, assays for protein topology from human tissue are not feasible due to the lack of availability of sufficient quantities of fresh tissue suitable for subcellular fractionation. The available frozen tissue samples (usually taken at autopsy) do not maintain adequate cellular architecture, precluding the isolation of intact intracellular membranes suitable for topology determination. To overcome these obstacles, an alternate assay was developed to distinguish the different topological forms of PrP.

We sought to exploit differences in the native folded conformation that would be expected of the different topologic forms of PrP. Such differences in folded conformation would likely be maintained in extracts from frozen brain, and would only be lost completely upon denaturation. Conformational differences are often reflected in the accessibility of certain domains of a protein to macromolecular probes such as antibodies or proteases (Ou et al., 1995; Mathieu et al., 1996). An extreme example of this is the differential sensitivity to proteinase K (PK) of PrP^{C} and PrP^{Sc}, with the latter being highly resistant to digestion (Oesch et al., 1985). To determine whether a similar assay based on differential protease resistance could be used to distinguish the different topological forms of PrP, we digested cell-free translation products of PrP with PK under various conditions (Fig. 26a). Non-denaturing detergent(s) were included to permeabilize membranes, and allow the PK access to all of the PrP molecules in the reaction. We found that although harsh digestion at elevated levels of PK (at >500 ug/ml, lane 4) or elevated temperature (37°C, lane 5) could digest completely all topological forms of PrP (but not PrP^{Sc}, data not shown; see Oesch et al., 1985),

Fig. 26. *a.* Characterization of topologic forms of PrP by limited PK digestion. SHaPrP, translated as described in Fig. 25b (with ER derived microsomal membranes and a competitive peptide inhibitor of glycosylation), was subjected to proteolytic digestion with PK for 60 min under the conditions indicated above each lane. A '+' indicates the presence of 1% NP-40, and '++' the presence of 0.5% NP-40, 0.5% deoxycholate during the digestion. Samples were immunoprecipitated with the 3F4 mAb and analyzed by SDS-PAGE and autoradiography. The arrowhead indicates the position of a relatively resistant fragment generated upon PK digestion (lane 3) that comigrates with the C-terminal fragment derived from ^{Ctm}PrP (lane 2). *b.* Various mutants of PrP (see Methods) were translated as in panel a and either left untreated (lane 1), digested for 60 min at 0°C with 250 ug/ml PK in the presence of 1% NP-40 (lane 2), or digested for 60 min at 37°C with 100 ug/ml PK in the presence of 0.5% NP-40, 0.5% deoxycholate (lane 3). Samples were immunoprecipitated with the 13A5 mAb and analyzed by SDS-PAGE and autoradiography. The arrowhead indicates the position of the proteolytic fragment generated upon limited PK digestion of some of the constructs. c. Schematic of limited PK digestion of the various topologic forms of PrP. The three topologic forms of PrP are shown before (left) and after (right) digestion with PK under mild (top) or harsh (bottom) conditions. The approximate sizes of the fragments generated from each form are indicated above the diagram. The positions of the epitopes recognized by the 3F4 and 13A5 antibodies are indicated by the white and grey boxes, respectively. The detergent solubilized membrane is indicated by a dashed line.


a
[PK]: 0 500 250 500 100
Det: - - + + ++
Temp: 0 0 0 0 37
= 30
= 14
1 2 3 4 5
b
[PK]: 0 250 100 0 250 100
Det: + + ++ + + ++
Temp: 0 0 37 0 0 37
wt
A117V
KH-II
ΔSTE
AV3
G123P
1 2 3 1 2 3
c
Ctm PrP
sec PrP
Ntm PrP
PrP Sc
18 kD
19 kD
PK
Mild
250 ug/ml
1% NP-40
0°C x 60 min
Harsh
100 ug/ml
0.5% NP-40
0.5% DOC
37° x 60 min

a subset of PrP was only partially digested under the milder conditions of 250 ug/ml PK and 0°C (lane 3). The fragment generated under these conditions comigrated on SDS-PAGE with the protected C-terminal domain generated in the standard topology assay (compare lanes 2 and 3).

To determine whether this relatively resistant fragment of PrP was derived from a particular topological form of PrP, we examined the digestion of various topological mutants of PrP under the same conditions. If this resistant fragment was derived exclusively from one of the topological forms, then its relative amount should vary as the relative amount of the topological form from which it is derived. Given that no two topological forms share the same pattern of change between the five mutants examined, the results should be unambiguous. We found that under the limited digestion conditions, the generation of the protease resistant fragment correlated exactly with the amount of ^{Ctm}PrP form: mutations which increase the relative amount of PrP in this form (A117V, KH-II, AV3, see Chapter IV) resulted in increased generation of the PK resistant fragment, while mutations which abolished synthesis of this form (ΔSTE, G123P, see Chapter IV) did not yield a PK resistant fragment (Fig. 26b). As expected, all of the topological forms were completely digested under the harsher conditions that PrP^{Sc} is able to survive. Thus, at least for the topological forms synthesized in the cell-free system, we have identified conditions that are able to distinguish ^{sec}PrP and ^{Ntm}PrP from ^{Ctm}PrP. Furthermore, ^{Ctm}PrP can also be distinguished from PrP^{Sc}. The different digestion conditions and properties of this assay are diagrammed in Fig. 26c.

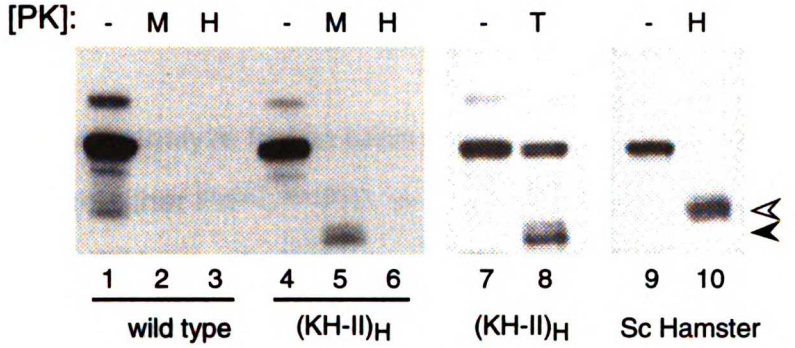
Unlike membrane integrity, the folded conformation of a protein is reliably maintained after freezing and thawing of tissue, and thus the assay described above should allow examination of frozen tissue samples without the need to first fractionate intact membranes. To confirm that we could distinguish ^{Ctm}PrP from

^{sec}PrP from PrP^{Sc} in frozen brain tissue samples, we performed the assay on tissue from transgenic mice in which the distribution of PrP among the topologic forms had previously been established (see Chapter IV). Transgenic mice expressing the wild type SHaPrP (TgSHaPrP, line A3922) or the KH-II mutation ($TgSHaPrP(KH\text{-}II)_H$, line F1198) were analyzed, and compared to products of the standard topology assay done with intact membranes. We further included as a marker the PrP^{Sc} fragment generated from PK digestion of scrapie infected hamster brain homogenate. We found that similar to the results of the standard topology assay (see Chapter IV), the conformational assay generated a protease protected fragment only in the SHaPrP(KH-II) sample (Fig. 27a). Thus, the presence of this fragment appears diagnostic for the presence of ^{Ctm}PrP for three reasons: i) it was only detected in samples previously established to contain ^{Ctm}PrP by the topological assay, ii) it was detected with mild, but not harsh digestion conditions (compare lanes 5 and 6), and iii) it comigrated with the ^{Ctm}PrP fragment from the standard topology assay (compare lanes 5 and 8), but not the PK resistant core of PrP^{Sc} (compare lanes 5 and 10).

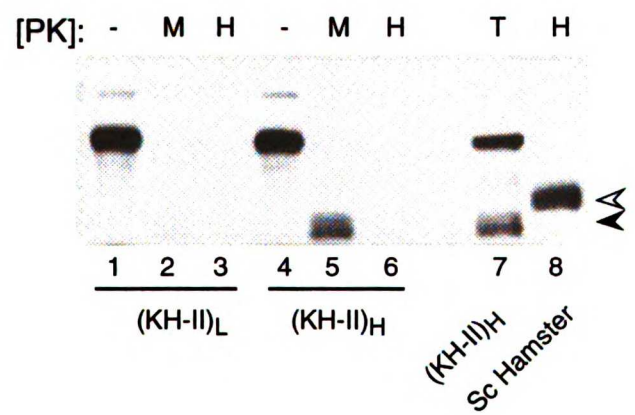
As a final test of the conformational assay, we compared the $TgSHaPrP(KH\text{-}II)_H$ line to a second transgenic line containing the KH-II mutation (termed $TgSHaPrP(KH\text{-}II)_L$, line E12485). By contrast to the $TgSHaPrP(KH\text{-}II)_H$ line, these mice express the mutant PrP molecule at significantly lower (sub-physiologic) levels, do not contain ^{Ctm}PrP in brain (by the standard topology assay, see Chapter IV) and thus do not exhibit illness. Thus, comparison of these two transgenic lines should ascertain whether the conformational assay is truly diagnostic of ^{Ctm}PrP (as opposed to differences in digestion pattern due to the mutation). Perfectly paralleling the results obtained using the standard topology assay, the conformational assay did not generate the proteolytic

Fig. 27. a. Characterization of limited digestion of PrP in homogenate from frozen brain. 10% homogenate from the frozen brain of either a TgSHaPrP mouse (lanes 1-3), TgSHaPrP(KH-II)$_H$ mouse (lanes 4-6), or scrapie infected hamster (9,10) were digested under mild (M) and/or harsh (H) conditions (as defined in Fig. 26c). Additionally, microsomal membranes isolated from the freshly removed brain of a TgSHaPrP(KH-II)$_H$ mouse (see Chapter IV) were analyzed by the standard topology assay (see Fig. 25a). The black and white arrowheads indicate the position of the ^{Ctm}PrP derived fragment from the standard topology assay (lane 8) and the PrP^{Sc} derived fragment (lane 10), respectively. b. Brain tissue from a TgSHaPrP(KH-II)$_L$ mouse and TgSHaPrP(KH-II)$_H$ mouse were analyzed by limited PK digestion exactly as in panel a. Lanes 7 and 8 provide markers for the ^{Ctm}PrP and PrP^{Sc} derived fragments (indicated with the black and white arrowheads, respectively) and are comparable to lanes 8 and 10 of panel a.

a


[PK]: - M H - M H - T - H
1 2 3 4 5 6 7 8 9 10
wild type
(KH-II)H
(KH-II)H
Sc Hamster

b


[PK]: - M H - M H T H
1 2 3 4 5 6 7 8
(KH-II)L
(KH-II)H
(KH-II)H
Sc Hamster

fragment indicative of ^{Ctm}PrP from TgSHaPrP(KH-II)$_L$ brain homogenate (Fig. 27b). Together with the results in Fig. 27a, these results demonstrate that ^{Ctm}PrP can reliably and easily be detected from frozen brain tissue, and clearly distinguished from PrP^{Sc}. Furthermore, these data underscore the point that elevated levels of ^{Ctm}PrP (as judged by either the topology or conformational assays) correlate well with the development of spontaneous neurodegenerative disease.

With the ability to analyze frozen brain tissue for the presence of ^{Ctm}PrP (distinguished from either PrP^{C}, ^{sec}PrP, or PrP^{Sc}) we now were able to ask whether GSS caused by the A117V mutation was due to elevated levels of ^{Ctm}PrP. Frozen brain tissue from a neurologically normal as well as GSS patient, taken at autopsy, were analyzed for the presence of ^{Ctm}PrP and PrP^{Sc} using the appropriate assays (Fig. 28). We found that tissue from the GSS patient, but not normal individual, contained increased levels of ^{Ctm}PrP. By contrast, neither brain contained detectable PrP^{Sc} under conditions where it was readily found in tissue from a sporadic CJD patient. These results were independently confirmed by analysis of tissue from a second patient carrying the same mutation (data not shown). Thus, consistent with observations *in vitro*, the A117V mutation resulted in increased generation of the ^{Ctm}PrP form *in vivo*.

These data strongly indicate that in the case of GSS caused by the A117V mutation, the disease is due to the increased production of ^{Ctm}PrP. This conclusion is consistent with and supported by several observations. First, it has previously been established in mice that increased production of the ^{Ctm}PrP form leads to neurodegeneration and ultimately death. Second, the histopathology of these ill mice due to ^{Ctm}PrP is similar to that observed in GSS. Third, PrP containing the A117V mutation is demonstrated to result in favored synthesis in

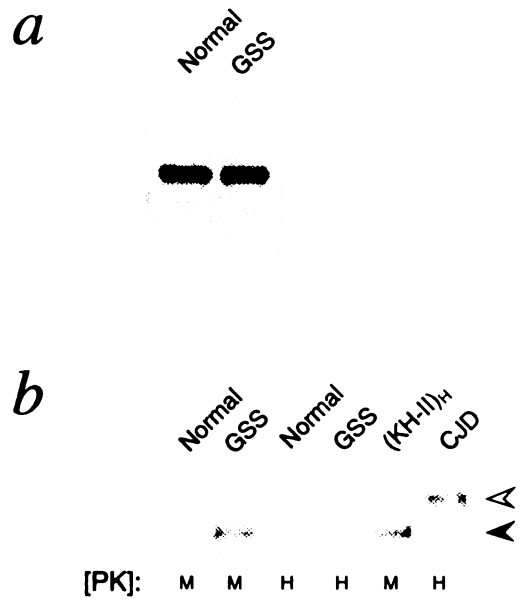


Fig. 28. Analysis of human tissue samples for ^{Ctm}PrP. 5% homogenates were prepared from frozen brain tissue removed at autopsy from a neurologically normal individual and a GSS case containing the A117V mutation. Aliquots were digested with PK under either mild (M) or harsh (H) conditions as described in Fig. 26c, and the samples were normalized for individual variations in starting PrP levels prior to analysis by SDS-PAGE and western blotting with the 3F4 mAb. Panel *a* shows the samples prior to digestion with PK, and panel *b* after the digestions. As a marker for human PrP^{Sc}, homogenate from the brain of a sporadic CJD case was digested under the harsh conditions and an aliquot analyzed in parallel (lane 6, panel b). The position of the ^{Ctm}PrP and PrP^{Sc} derived fragments are indicated with the black and white arrowheads, respectively.

the ^{Ctm}PrP form relative to the wild type protein (Fig. 25). Fourth, the ^{Ctm}PrP form, as judged by the conformation assay developed in this study, is found in a GSS patient carrying the A117V mutation (Fig. 28). And finally, cases of GSS (resulting from the A117V mutation) do not form or accumulate PrP^{Sc} (Tateishi et al., 1990; Mastrianni *et al.*, in preparation).

Given that ^{Ctm}PrP is pathogenic, both in transgenic mouse model systems as well as inherited human disease, it will be of great interest to determine in which other disease processes this molecule plays a role. It is possible that expression of this form of PrP is one of several potential triggers of neuronal death. Since expression of the topological forms of PrP can be modulated by *trans*-acting factors, it is plausible that a variety of diseases, including transmissible prion diseases such as BSE and CJD, share in common the final pathway of cell death caused by ^{Ctm}PrP. The observation that PrP^{Sc} deposition does not cause cell death in neurons lacking PrP is consistent with such a hypothesis (Brandner et al., 1996). With the tools developed in this study to detect ^{Ctm}PrP and distinguish it from PrP^{Sc}, these questions can now be addressed.

Experimental Procedures

cDNA constructions - For cell-free translation of PrP and mutants, each coding region was engineered using standard methods (Sambrook et al., 1989) into an sp64 plasmid vector containing the SP6 promoter followed by the 5' untranslated region of Xenopus globin (Krieg et al., 1984). The PrP constructs used in Fig. 26 have been described and characterized previously (Chapter IV) and are as follows: wild type PrP, A117V mutant, KH-II mutant (residues Lys_{110}, His_{111} both changed to Ile) and ΔSTE mutant (deletion of residues 104-113) are in the SHaPrP backbone; AV3 mutant (residues Ala_{113}, Ala_{115}, Ala_{118} all changed to Val) and G123P mutant are in the MH2MPrP backbone (a mouse-hamster PrP chimera, see Scott et al., 1992).

Transgenic mice - The phenotypes and biochemical analyses for ^{Ctm}PrP and PrP^{Sc} in the transgenic lines [TgSHaPrP, line A3922], [TgSHaPrP(KH-II), line F1198] and [TgSHaPrP(KH-II)L, line E12485] have been described previously (Chapter IV).

Cell free translocation reactions - Rabbit reticulocyte lysate and microsomal membranes from dog pancreas were prepared as described previously (Merrick, 1983; Walter and Blobel, 1983). Transcription using SP6 polymerase, translation, and proteolysis were as described previously (Hegde and Lingappa, 1996). All translation reactions were carried out at 32°C for 60 minutes, and except lanes 1,2,6,7 of Fig. 25b, included a glycosylation acceptor peptide to competitively inhibit glycosylation of translation products. Products were immunoprecipitated with the 3F4 antibody, analyzed by 10% tricine-SDS-PAGE (Schagger and von Jagow, 1987), and the proteins visualized by

autoradiography. For Fig. 25c, relative amounts of the topological forms of PrP were quantitated by densitometry of the three products resulting after protease digestion in the absence of detergent: full length, undigested PrP (^{sec}PrP), the 18 kD C-terminal fragment (^{Ctm}PrP), and the 14 kD N-terminal fragment (^{Ntm}PrP). Densitometric values for C- and N-terminal fragments were adjusted (to compensate for loss of radioactivity due to proteolytic digestion of a portion of the molecule) to obtain quantitative estimates of ^{Ctm}PrP and ^{Ntm}PrP.

Conformational assay for ^{Ctm}PrP - Brain tissue (frozen) was homogenized in PBS (at 5% w/v or 10% w/v) by successive passage through 16, 18 and 20 guage needles. Cell free translation reaction were assayed directly. For ^{Ctm}PrP detection ('mild' proteolysis conditions), aliquots of the sample were adjusted to 1% NP-40, 0.25 mg/ml PK and incubated for 60 min on ice. For PrP^{Sc} detection ('harsh' proteolysis conditions), samples were adjusted to 0.5% NP-40, 0.5% deoxycholate, 0.1 mg/ml PK and incubated for 60 min at 37°C. The proteolysis reactions were terminated by the addition of PMSF to 5 mM, incubating an additional 5 minutes, and transferring the sample to 5 volumes of boiling 1% SDS, 0.1M Tris, pH 8.9. Brain derived samples were then digested with PNGase as directed by the manufacturer, resolved by 10% tricine-SDS-PAGE, transferred to nitrocellulose, and probed with either the 3F4 (Fig. 28) or 13A5 (Fig. 27) monoclonal antibody. In vitro translation products were immunoprecipitated with either 3F4 (Fig. 25, 26a) or 13A5 (Fig. 26b), and analyzed by SDS-PAGE and autoradiography.

VI. Conclusions and Perspectives

The signal hypothesis proposed, among other things, the existence of a protein machinery dedicated to the recognition and transport of secretory and membrane proteins. A corollary of this view is that lesions in the machinery can, in principle, affect the biogenesis of proteins that themselves do not contain any defect. This implication of the signal hypothesis, not predicted by spontaneous insertion models of translocation, was appreciated even before any of the translocation machinery had been identified:

> A second important area for future research concerns signal pathology. The elucidation, at least in its general features of a novel machinery required in proper working order by all living cells has obvious implications for disease. In so far as cellular membranes are a likely first line of impact of environmental insults to the cell, such a complex mechanism is a likely early target within intracellular membranes. A variety of degenerative diseases might be excellent candidates for Signal Pathology. A degenerative rather than a genetic origin for such lesions would be expected since those individuals lacking properly functioning signal machinery would probably be aborted while those who develop lesions in specific organ sites later in life, perhaps as a result of local or general environmental influences, would exhibit organ-specific pathology of degenerative diseases, with longer range delayed onset systemic implications.
>
> (Lingappa, 1979)

This notion, that certain aspects of translocation can be regulated, or misregulated in the pathogenesis of some diseases, was in idea that at the time was difficult to explore in the absence of a mechanistic understanding of the machinery involved. Upon elucidation of the basic mechanism and machinery for simple secretory protein translocation, the stage was finally set to ask whether more complex substrates can be subject to regulation in some aspect of their biogenesis.

The work described in this thesis represents efforts to identify and understand potential examples of translocational regulation. The results of Chapter II and III provide substantial support to the view that protein translocation can be regulated

in trans. Using apolipoprotein B as a model, we were able to identify events occurring during the translocation of complex but not simpler secretory proteins: i) opening of the normally sealed ribosome-membrane junction in response to specific domains (pause transfer sequences) within the nascent translocating chain, ii) subsequent exposure of defined regions of the nascent chain to the cytosol, and iii) alterations in the protein environment (as probed by crosslinking) of the nascent chain. The selective nature of these events (both with respect to the substrate in general and particular sequences within the substrate) suggested that they were directed by the machinery of the translocon, and thus might be regulated.

Exactly this point was demonstrated in Chapter III, where the translocon component TRAM was identified as being required for proper translocational pausing. Remarkably, the data indicate a role for TRAM in controlling the extent of exposure of a paused nascent chain to the cytosol. Thus, the environment(s) seen by a nascent chain during its translocation can be regulated by such translocation accessory factors. This has several implications. First, the function and ultimate fate of an apo B chain is likely to be dependent on these regulatory events during its biogenesis. Exaggerating or eliminating particular aspects of translocational pausing may result in an apo B chain that is folded differently, modified differently, or interacts with different proteins in the cell. Thus, multiple versions of apo B-containing lipoprotein particles (perhaps each of them serving related but subtly different functions) could in principle be produced depending on the extent of translocational pausing. Indeed, it has been observed that lipoprotein particles play roles entirely apart from their lipid carrying function, such as binding to and clearing certain toxins from the blood. Whether these different functions are served by the same, or different (but not easily

distinguishable by the currently employed assays) subsets of lipoprotein particles remains unknown.

A second direct implication is that pausing can be manipulated via the activity of TRAM. Physiologically, this may occur through the binding or modification of TRAM by yet other (perhaps organ specific) proteins. Under certain metabolic conditions, apo B biogenesis could be altered (via modulating TRAM activity) to direct it to a fate suited for the situation at hand. Determining if and how such regulation of pausing occurs will be an important goal for future studies. Assays will need to be developed to detect subtle consequences of translocational pausing. In the case of apo B, its association with lipids, its folded state, and its interaction with other proteins are all candidates for aspects of its biology that could be regulated via pausing.

The development of experimental systems that allow the reconstitution and manipulation of these substrate specific (and organ specific) events will facilitate the elucidation of the physiologic role of translocational pausing. It is likely that apo B biogenesis and lipoprotein secretion can be affected by the pharmacologic modulation of translocational pausing. The identification of at least one component involved in pausing (TRAM) provides a potential target for such manipulations and highlights the feasibility of these ideas. It is entirely possible, if not likely, that there exist diseases, either acquired or hereditary, whose molecular lesion is at the level of translocational pausing. Future studies in which the consequences of altered (either exaggerated or abolished) translocational pausing are examined *in vivo* will shed light on the nature of such diseases. The manipulation of TRAM expression or activity *in vivo* should be a means by which these questions can be addressed.

While the studies on apo B provide evidence that translocation can be regulated, the physiological and pathological consequences of this regulation

have not yet been elucidated. The examination of these specific ideas await future studies; however, the general concept that there are pathophysiologic consequences of dysregulated protein translocation has been explored. The results described in Chapters IV and V demonstrate that achieving the proper protein topology at the ER, in this case of PrP, is crucial to the normal function of an organism. The pathologic consequences of altered topology were highlighted in both mouse model systems, as well as naturally occurring human disease.

The studies exploring the role of PrP biogenesis in disease relied on mutations within PrP to modulate topology. However, analogous to translocational pausing, protein factors are likely to be able to modulate the topology of wild type PrP *in trans* . Evidence for such factors, both in the cytosol (Lopez et al., 1990) and in the ER membrane (Hegde et al., unpublished), have been provided. Together, these observations imply that modulation of trans-acting factors can not only potentially cause disease, but perhaps repair otherwise disease causing lesions in PrP. Thus, a crucial area of future studies will focus on the identification of these translocation accessory factors that can modulate the topology of PrP (and perhaps many other proteins).

Ongoing studies employing solubilization and reconstitution techniques to fractionate ER membrane proteins have indicated that a glycoprotein (provisionally termed TrAF for Translocation Accessory Factor) is required to prevent synthesis of ^{Ctm}PrP, the pathological transmembrane form of PrP. Initial fractionation steps show that TrAF is not TRAM, suggesting that potentially novel proteins currently not implicated in translocation play a role in PrP topology. Once TrAF and related factor(s) are identified, it will be of great interest to determine how they are used to regulate PrP topology during various stages of an organisms life. Given that even wild type PrP can be synthesized in more than topological form, regulated by the action of trans-acting factors, it seems

likely that PrP topology play a role in the currently mysterious normal function of this highly conserved brain protein. Perhaps during certain periods of development or aging, PrP topology is shifted from secretory to C-trans transmembrane to selectively cause subsets of neurons to die in a programmed manner. This shift in topology could be mediated by external cues, acting via signal transduction pathways, that modulate the activity or expression of the TrAFs that govern PrP topology at the ER.

The relationship between the transmissible diseases of prions (such as bovine spongiform encephalopathy - BSE) and the different topological forms of PrP are currently not understood. However, given that the mechanism of neuronal cell death following the replication and accumulation of transmissible prions (PrP^{Sc}) in the brains of affected organisms remains unknown, a link to ^{Ctm}PrP-mediated cell death seems plausible. Perhaps the accumulation of PrP^{Sc} results in a change in the activity of the TrAFs that control PrP topology, resulting in a shift toward synthesis of the ^{Ctm}PrP, which subsequently leads to cell death. Future experiments examining PrP topology and the functional state of the ER membrane (with respect to TrAF activity) during the course of PrP^{Sc} replication and accumulation should allow these and other hypotheses to be tested.

Together, the studies on apo B and PrP biogenesis provide a working framework for understanding the emerging field of translocational regulation. As other complex substrates are examined, it seems likely that the early stages in the life of a wide variety of proteins will prove to be a key point of regulation for the cell. Nascency provides the cell with a unique opportunity to direct the fate of a protein in one of several directions by subtle variations in the pathway of translocation and folding utilized by the substrate. The responsibilities of directing the translocational regulation used by the cell are likely to reside with a diverse family of TrAFs. Indeed, the proposed function of TRAM in

translocational pausing suggests that it represents the first of these TrAFs. The regulated expression of various combinations of TrAFs in an organ specific manner is envisioned to play a role in many of the currently enigmatic aspects of physiology and disease. The directions for future studies are clear.

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