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Mechanisms of prion protein biogenesis

Author
Lopez, Charles D.

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Mechanisms of Prion Protein Biogenesis

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Charles D. Lopez

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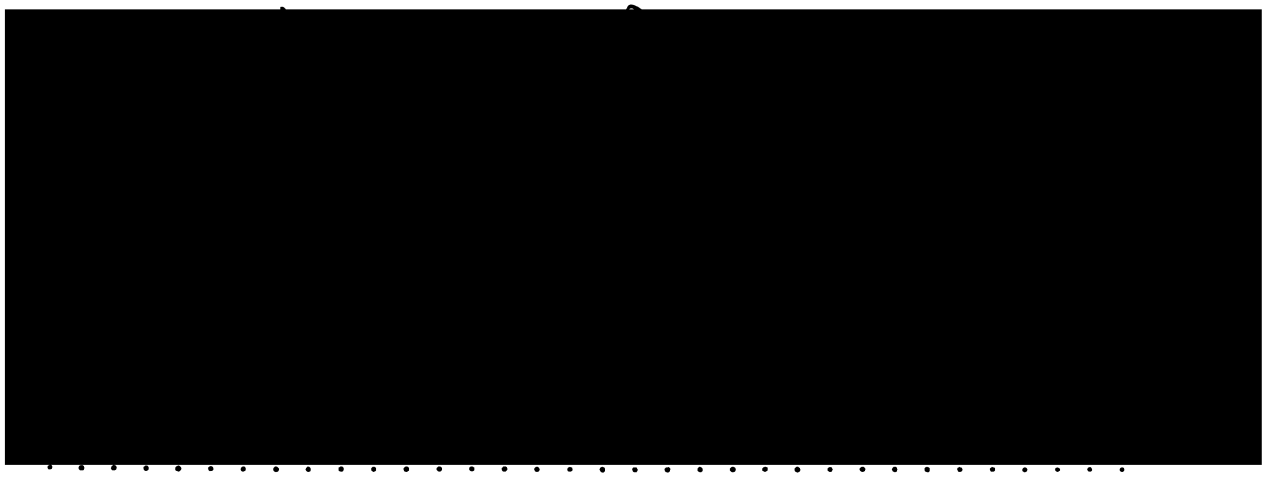

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This thesis is, of course, dedicated to my family and especially to Dad for hanging tough in the ICU when things were going from bad to worse. Without their support this could have not been possible.

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ABSTRACT.

Biosynthetic studies of the prion protein (PrP) have shown that two forms of different topology can be generated from the same pool of nascent chains in cell-free translation systems supplemented with microsomal membranes. A transmembrane form is the predominant product generated in wheat germ (WG) extracts, whereas a completely translocated (secretory) form is the major product synthesized in rabbit reticulocyte lysates (RRL). An unusual topogenic sequence, (R_{74-114}), within PrP is now shown to direct this system-dependent difference.

This topogenic sequence contains a lumenally disposed charged domain that is required for stop transfer at the adjacent hydrophobic domain. This domain, or stop transfer effector (STE), requires a precise spatial relationship with the hydrophobic domain for efficient stop transfer. Codons encompassing STE confer transmembrane topology to a heterologous protein when engineered adjacent to the codons for a normally translocated hydrophobic domain.

The actions of R_{74-114} were independent of on-going translation and could be conferred to heterologous proteins by the engineering of a discrete set of codons. Furthermore, system-dependent topology was conferred by addition of RRL to WG translation products.

These results identify an unexpected functional domain (STE) of stop transfer in the prion protein. Additionally, the data demonstrates that R_{74-114} interacts with one or more cytosolic factors present in RRL. The implications for membrane protein biogenesis are (1) the process of stop transfer may involve proteinaceous receptors in the endoplasmic reticulum that interact with domains NH_2-terminal to

membrane spanning hydrophobic amino acids and, 2) topogenesis can be a regulated, rather than a constitutive, process.

CHAPTER I. INTRODUCTION AND BACKGROUND.

INTRODUCTION.

Eukaryotic cells distinguish themselves from prokaryotic cells by the formation of intracellular organelles via membrane limited compartments. The complexity of eukaryotes is a direct result of such specialized compartmentalization with organelle identity determined by associated proteins. In order to carry out their specific functions, non-cytosolic proteins synthesized by the eukaryotic cell must first target to the correct membrane, orient accurately with respect to the bilayer (e.g. either fully translocated or transmembrane), and sort to the proper compartment. Proteins aberrently localized or with an improper topology can be dysfunctional to a cell/organism.[1] Thus, it is not surprising that sophisticated cellular machinery has developed to ensure high fidelity during the process of topogenesis and sorting.

Protein biogenesis has been traditionally viewed as a constitutive process. That is, a process that once set in motion will not deviate from a predictable outcome. Nevertheless, in certain circumstances, protein biogenesis may be under some form of regulatory control.[2] Regulation at this level could be a potentially powerful form of biological control. For instance, proteins redirected to other than their usual compartment, or refolded into a different conformation, may take on different functions altogether. This notion will be further developed in this thesis.

Protein synthesis (with the exception of some mitochondrial proteins) occurs in the cytosol. Nuclear[3,4], mitochondrial[5,6], chloroplast[7], peroxisomal[8], and secretory[9] proteins have developed different methods for the initial targeting event and subsequent

translocation across their respective membrane bilayers. Discussion of targeting and translocation will remain limited to the events of the secretory pathway.

Fully translocated and integral membrane proteins destined to traverse through the secretory pathway target first to the membrane of the rough endoplasmic reticulum (RER). These proteins acquire their orientation with respect to the membrane in a manner that is both predictable and absolute.[9] This reflects the action of discrete regions within such nascent proteins, termed signal and stop transfer sequences, that initiate and terminate translocation across the RER.[10] The preponderance of evidence to date suggests that these "topogenic sequences" interact with proteinaceous receptors in the cytosol, RER membrane and RER lumen. Engagement of these receptors in a sequential fashion directs the biogenesis of a nascent protein[11]. Examples of the known components of this cellular machinery will be discussed below. Within the last two decades it has become clear that the process of protein biogenesis is quite complex. Yet, as we learn more, the level of complexity will probably increase several-fold as the regulatory aspects are elucidated. Chapters II and III will provide evidence for a novel mechanism of regulated topogenesis in the Prion Protein (PrP).[12,13]

BACKGROUND.

I. Cellular Machinery Involved in Protein Biogenesis of the Secretory Pathway.

A. Cytoplasm.

The presence of a signal sequence capable of recognizing SRP determines whether a newly synthesized protein targets to the secretory pathway. As the signal sequence on the nascent chain emerges from the ribosome, it is recognized by the ribonucleoprotein signal recognition particle (SRP).[14,15,16] This well characterized cytosolic particle consists of six proteins and a 7S RNA. The different functions of SRP appear to be mediated by distinct functional domains.[22] After the signal sequence binds to the 54 kD subunit of SRP, chain elongation pauses.[17,18] The resultant polysome-SRP complex is targeted specifically to the cytoplasmic face of the RER membrane where it binds SRP-receptor (SRPR).[19,20,21] Binding to SRPR releases SRP from the nascent chain allowing the resumption of translation. With secreted proteins, translocation then proceeds into the lumen of the RER, while in the case of transmembrane proteins, integration into the membrane via the action of stop transfer sequences occurs.

There are examples of SRP-SRPR independent targeting of proteins to the RER membrane.[9,23] These exist for only a few specialized proteins and reflect the versatility of evolution in utilizing diverse mechanisms. Most proteins target to the RER via an SRP-SRPR mediated pathway.

Other cytosolic proteins aside from SRP are known to be involved in protein biogenesis. A 70-kilodalton family of heat shock proteins (Hsp 70) contains cytosolic forms (Hsp 72,73) that interact with nascent chains in normal cells.[24] This interaction has been shown to be transient

and adenosine triphosphate (ATP)-dependent. It has been suggested that this is necessary to facilitate the proper folding of the growing peptide chain. Proper conformation of the nascent chain may be necessary to permit binding with the subsequent receptors involved in targeting and translocation. Yeast cytosol has been shown to contain two constitutively expressed 70-kilodalton hsps that increase the rate of translocation across the ER membrane.[25] At least one other organelle appears to utilize similar cytosolic machinery since hsp 70 proteins also interact with precursor polypeptides during post-translational import into mitochodria.[26] Furthermore, hsp 70 functions in the uncoating of clathrin-coated vesicles.[27]

An abundant cytosolic protein involved in the enzymic catalysis of isomerization of the stereochemistry about the imide bond in the sequence [-X-Proline-] is peptidyl-prolyl-cis/trans isomerase (PPI) (reviewed ref. 29). This may be a rate determining step in the folding of some proteins which could have profound effects on how a polypeptide is handled by the translocation machinery.

The concept of cytosolic translocation factors (especially those that can effect the conformation of a peptide chain) is not only also appreciated for molecules destined to organelles outside of the secretory pathway, but for prokaryotic organisms as well (reviewed ref. 28).

B. Rough endoplasmic reticulum membrane.

The actual events involved in chain translocation through the lipid bilayer of the RER are only now beginning to be understood. Two major hypothesis have existed. One postulates the process of peptide chain translocation is driven by the thermodynamics of protein and lipid

interactions such that the free energy cost of moving polar and charged polypeptides through a hydrophobic lipid bilayer is made up by the reduction in free energy achieved by inserting the hydrophobic signal sequence in the bilayer as a helical hairpin.[30,31] The other hypothesis poses that a proteinaceous channel is formed in the membrane by the interaction of receptors with the signal sequence as well as possibly stop transfer sequences.

The preponderance of evidence suggests the latter hypothesis is correct. The demonstration that chain translocation required nucleotide triphosphate hydrolysis independent from that of translation strongly suggested that the spontaneous insertion model was too simplistic.[32] Furthermore, it was shown that translocation of protein domains occurs through an aqueous channel.[33] Proteinaceous import receptors exist in other organelles (e.g. mitochondria[5], chloroplasts[7], nucleus[34]) and therefore by analogy may also exist in the RER. Taken together, these results imply the existence of a channel in the RER lipid bilayer that allows the passage of a hydrophilic protein domain. Conclusive proof will require identification of the channel components and reconstitution of translocation function in vitro. Much work has been recently done along such lines as will be discussed below.

Several proteins have been identified in the RER membrane that are involved in different aspects of translocation and protein processing. Whether they are components of the actual proteinaceous channel involved in chain translocation remains to be seen. Certainly their intimate involvement in these events might suggest at some point during

protein biogenesis they are at least transiently associated with the translocation machinery.

Proteins entering the secretory pathway are synthesized on ribosomes associated with the RER (hence the term "rough"). Ribosome attachment must presumably be an important initial step during the targeting-translocation process. Ribophorins I and II have been proposed to serve as ribosome receptors.[35,36] Recently, a 180 kD integral membrane protein with a large cytoplasmic domain was shown to function as such a receptor by its ability to compete with ribosome binding to intact membranes as well as its functional reconstitution into lipid vesicles.[37] The precise roles of the ribophorins and the 180 kD protein remain unclear. Perhaps binding to the RER membrane and translocation are each mediated by these different receptors.

As mentioned previously, SRPR binds the SRP-nascent chain-ribosome complex on the RER membrane to relieve the translation arrest induced by SRP binding. SRPR is a cytoplasmically oriented two subunit (alpha and beta) RER membrane protein[38,39] with unique features involved in its biogenesis.[40] The cytoplasmic domain of the alpha subunit is thought to interact with SRP to release translation arrest and initiate translocation.[20] This displacement reaction is GTP-dependent[41] and probably occurs via the GTP-binding domain on the alpha subunit.[42]

After the nascent chain is released from SRP via interactions with SRPR, the signal sequence binds another specific receptor in the RER membrane as shown by chemical cross-linking . This two-subunit (35 kD alpha and 22 kD beta integral membrane glycoproteins), signal

sequence receptor (SSR), was found associated to an in vitro-synthesized precursor protein in an SRP-dependent manner.[43,44] Nascent chains at different stages in translocation predominantly crosslinked with alpha SSR.[44] Other data has indicated that alpha SSR may be identical to a 39 kD integral membrane glycoprotein (mp39) that also binds to different portions of a nascent preprotein during the course of translocation.[45] Thus, SSR is very likely an important component in the translocation process and perhaps even part of the channel itself. Recently, similar crosslinking experiments have revealed RER specific membrane proteins that remain associated with the transmembrane segment of a nascent integral membrane protein.[46] This association was no longer observed upon the termination of translation. The crosslinked proteins (Mr = 15, 20, 24, 27, 37-38, 39, and 70 kD) have not been characterized with the exception of the 39 kD species that is thought to be alpha SSR (mp39). These proteins may also be part of the translocation channel.

It was recognized early that many signal sequences were endoproteolytically cleaved during translocation.[47] The enzyme responsible was signal peptidase, a multisubunit glycoprotein complex found in the lumen of the RER.[48,49] Signal peptidase co-exists in stoichiometric amounts relative to ribosomes attached to the RER membrane.[48] It is unclear what role the multiple subunits in the eukaryotic system may play but such intimate association with the translocation process hints that signal peptidase complex may be an important part of the translocation mechanism.

Functional reconstitution is necessary to achieve a true mechanistic understanding of the translocation process. The conditions

for assembly of translocation-competent proteoliposomes have been established by first solubilizing canine pancreas rough microsomes in detergent, preparing an extract by centrifugation, and reconstitution of vesicles by a detergent dialysis procedure.[50] The reconstituted vesicles normally translocated proteins efficiently, however if the detergent extracts were depleted of glycoproteins, they became deficient in translocation (but not targeting) activity. Membrane protein subfractions were prepared by ammonium sulfate precipitation and translocation reconstitution assays were performed. Two fractions were prepared that were incapable of translocation activity individually, however when the fractions were combined to reconstitute vesicles, translocation activity was restored.[51] The components of these membrane fractions are now being identified and presumably contain some (or all) of the translocation channel. One fraction is known to contain SR alpha, SR beta, and ribophorin I (but deficient in signal peptidase and SSR) and is capable of targeting. The other fraction is known to contain SSR and signal peptidase (but deficient in SR alpha, SR beta and ribophorin I). This is direct evidence that the protein components for targeting and translocation are separate.

C. Endoplasmic reticulum lumen.

Folding of newly synthesized proteins in the RER lumen occurs both during and after translocation across the membrane. While basic topology (e.g.- secretory or transmembrane) is determined by signal or stop transfer sequences, final three-dimensional structure is often achieved by other mechanisms.

Proteins can receive a significant number of covalent modifications within the RER lumen. These modifications can be common to many types of proteins as is the case with signal sequence cleavage[48], N-linked glycosylation[52], and disulfide bond formation.[53] Other modifications are reserved for select types of proteins such as procollagens which have their proline and lysine residues hydroxylated or the gamma-carboxylation of glutamate residues in blood clotting and other types of Ca^{2+}-binding proteins.[54]

The various modifications a protein can receive do not necessarily impart a particular final conformation. Rather, modifications may occur as a consequence of protein folding and perhaps act to stabilize or only slightly alter the basic three dimensional structure of the peptide backbone. The process of protein folding in the RER lumen is currently thought to be a facilitated one; that is, one that proceeds with the assistance of one or more other specialized proteins.

One protein that may be involved in such events is protein disulfide isomerase (PDI). This very abundant RER lumen resident homodimer catalyzes thiol:disulfide interchange reactions in protein substrates.[55] It has been shown that the presence of PDI within the interior of pancreas rough microsomes is necessary for efficient co-translational disulfide bond formation during in vitro protein synthesis.[53] Recent evidence has suggested that PDI has multiple roles.[54] For example it was shown that the beta subunit of prolyl-4-hydroxylase (involved in modification of nascent procollagen polypeptides) is identical to PDI. Furthermore, PDI may be a component of the co-translational N-linked glycosylation machinery in

the RER since the glycosylation site binding protein (GSBP) is probably PDI. With such high lumen concentrations and multiple co-translational functions, it is conceivable that PDI may be also utilized during the process of nascent chain translocation. For instance, if proteins being translocated require a conformation that is different from the final native state, PDI might function to ensure that the proper intermediate conformational state is maintained. Thus, for at least some proteins, PDI could be an important component of the translocation and folding machinery.

Another protein that has received great attention in recent years because of its intimate involvement with protein folding in the RER lumen is the immunoglobulin heavy chain binding protein (BiP or GRP 78).[56] Like hsp 72 and 73, BiP is an ATP-binding protein and exhibits 50 percent sequence homology with hsp 70 proteins.[57,58] However, BiP contains a signal sequence and is therefore found in the RER lumen.[59] BiP was originally found to be transiently associated with immunoglobulin heavy chains until light chains were bound, whereupon release of heavy chains occured and the completed immunoglobulin complex exported from the RER.[60] Subsequently, BiP was found to transiently associate with influenza hemaglutinin subunits before final trimerization and has also been shown to bind mutant or malfolded proteins.[61,62] In vitro assays have been developed to demonstrate the specific binding and ATP-dependent release of synthetic peptides to BiP (and its cytosolic counterpart hsp 70).[63] Thus, it has been suggested that BiP functions as part of a system for clearing the RER of malfolded or aggregated proteins.[66] Alternatively, BiP (and hsp 70) binding of

polypeptides could be a common, if transient, event that facilitates normal protein folding. Recently it was shown that BiP associated with proteins that are not inherently defective.[64] This supports the idea that BiP functions as a normal component of the secretory pathway. The importance of conformation in protein biogenesis is underscored by the roles of hsp 70 and BiP (and probably other similar yet to be characterized proteins). Incorrectly folded secretory and integral membrane proteins are usually not transported to their ultimate destinations. Such tightly controlled homeostasis could theoretically provide the cell with a powerful point of regulation, and hence, another level of control over protein function.

Both PDI and BiP are soluble proteins that are retained in the RER. Both share a carboxy terminal sequence (KDEL) that binds a 72 kD intracellular membrane protein which serves as a "salvage receptor" to return these proteins to the RER.[65] How this receptor operates is unknown but it is no doubt extremely important for both PDI and BiP function.

D. Transport beyond the endoplasmic reticulum.

The original topological conformation acquired by a protein during its synthesis does not change during transit through the secretory pathway. Translocation through a lipid bilayer occurs only at the RER membrane. Thus, the mechanisms that determine whether a molecule is to be secretory or transmembrane act early during protein biogenesis. After first passing through the RER, proteins sort through the Golgi stacks (cis, medial and trans)[67], Trans-Golgi Network (TGN)[68] and

beyond to either the plasma membrane (in a constitutive or regulated fashion)[69] or lysosomes.[70]

The fate of newly synthesized proteins is determined in the RER. That is, proteins which are malfolded or assembled improperly are in most cases not released from the RER. As discussed above, in many cases this is probably through interactions with BiP. Proteins retained in the RER must be eventually degraded in order to maintain cellular homeostasis. It has been shown that a proteolytic system in the RER exists which is distinct from lysosomal degradation.[71] This highly selective RER degradative pathway[73] is not only involved in the clearance of incompletely assembled proteins but can also function in the regulation of cellular processes. For example, regulation of T-cell receptor expression during thymic differentiation utilizes the RER degradative pathway in a posttranslational fashion via signalling through CD4 surface molecules.[72] HMG-CoA reductase, an enzyme that catalyzes the rate limiting step in cholesterol biosynthesis also undergoes regulated RER degradation. With high cholesterol levels, HMG-CoA reductase is rapidly degraded in the RER, while with low levels of cholesterol, its stability is enhanced. Interestingly, the majority of this multispanning membrane protein is in the cytoplasm and deletion of various transmembrane regions abolishes rapid degradation.[74] This poses unique questions as to the machinery of the degradative pathway. Apolipoprotein B (apoB) is also degraded in the RER depending on nutrient conditions.[75] Interestingly, the biogenesis of apoB involves nonintegrated transmembrane intermediates.[76] Under conditions that favor intracellular RER degradation, apoB is only partially translocated

across the membrane and appears to exist as a transmembrane protein.[76] Thus, it is not unreasonable to hypothesize that the RER degradative machinery might be intimately associated with the process of translocation and sorting

The traffic of proteins between the different compartments of the secretory pathway is mediated by vesicles which bud from one membrane and fuse with the next membrane in the pathway.[77] This process of budding and fusion is still incompletely understood. However, studies utilizing yeast genetic and biochemical approaches have shed some light on the nature of these transport mechanisms especially at the level of Golgi transport.[78] Several proteins crucial for Golgi vesicle movement have been purified. N-ethyl maleimide-sensitive factor (NSF)[79] is a peripheral membrane protein which is isolated in a soluble form and is involved in membrane fusion after uncoating of the bound transport vesicle. It reversibly binds Golgi membranes only in the presence of a cytosolic fraction termed SNAP (soluble NSF attachment proteins).[80] At least three different SNAPs have been purified using transport activity as an assay. These SNAP proteins can bind NSF and link it to the Golgi localized NSF membrane receptor (NMR). Other evidence has implicated GTP binding proteins, GTP hydrolysis[81,82], and phospholipid transferases, as additional factors that play an important role in directing vesicle flow through the Golgi stacks.[78]

As mentioned previously, transport beyond the TGN is responsible for the actual sorting of vesicles to their different compartments (e.g. - plasma membrane or lysosomes).[68] Additionally,

it appears that the pathways of vesicle transport from the TGN and from receptor-mediated endocytosis share common compartments.[83] The obvious implication is that sorting through the secretory pathway could also utilize receptors to control the precise movement of proteins.The sorting of lysosomal enzymes represents one such receptor mediated transport system that has been partially characterized to date.[70,84] Additionally, the cell is quite capable of even more sophisticated forms of vesicular traffic as seen in polarized epithelial cells and in regulated secretory cells[77]. However the precise molecular mechanisms remain to be worked out.

II. Protein Topogenesis.

It should be clear from the preceding discussion that the mechanisms of protein biogenesis are as complex as they are diverse. Many points of regulation are present in the secretory pathway that the cell can take of advantage of to control a wide array of biological processes with ever increasing fidelity. The concept that the actual translocation event across the RER membrane can also function as a point of regulation has not been fully explored. Yet, this seems like a profound step to regulate since it is the only point where a protein crosses or integrates into a lipid bilayer to acheive its final topological orientation. Presumably, control of topological orientation at this level would involve the interactions of different topogenic sequences and their respective receptors.

The nature of what constitutes a "topogenic sequence" remains a functional definition. For instance, an amino acid stretch suspected of

being a topogenic sequence can be placed into another protein context and the topology of the resultant chimeric molecule can provide insite into the topogenic information encoded within the putative sequence.[85]

Different transmembrane proteins can display quite distinct topologies with respect to the disposition of amino and carboxy termini as well as the number of membrane spanning domains. Aside from the signal and stop-transfer sequences already mentioned, there exist other variants of topogenic sequences that may or may not require proteinaceous machinery.[86] The actions of signal-anchor sequences may occur via different mechanisms than the actions of signal and stop-transfer sequences though the evidence for this remains speculative.[87,88] Certainly for a subset of proteins and translocation events, non-receptor mediated mechanisms of membrane insertion are likely to exist. Again, this reflects the ability of evolution to utilize multiple mechanisms to achieve similar goals. How common a role these mechanisms play in the biogenesis of the majority of proteins in the secretory pathway is not clear but it appears that interaction with receptors represents the usual theme.

A. Signal sequences.

Many proteins that are translocated through the membrane of the RER contain amino terminal signal sequences. These sequences range from 15 to 30 amino acids in length and are often cleaved off of the mature protein by signal peptidase. Sequence analysis of known signal sequences has failed to reveal direct homologies at the primary amino acid level.[89] Nevertheless, three distinct regions seem to exist in most signals. A positively charged amino terminal consisting of one or more

basic amino acids is often followed by a central hydrophobic core of at least six or more amino acids. The carboxy terminal amino acids of signal sequences tend to be polar with small neutral side chains at positions -1 and -3 of the cleavage site. The degeneracy of signal sequences might suggest that their function is a relatively non-specific one based loosely on hydrophobic properties. A more plausible explanation advances that sequence divergence might represent the evolution of a particular signal sequence for it's particular passenger domain to provide optimal translocation. This notion is supported by the finding that even subtle changes in a passenger domain can affect translocation.[90] As previously discussed, signal sequences interact with at least three known receptors of the translocation apparatus-- SRP, SSR, and signal peptidase. Specific interactions despite a lack of primary amino acid sequence homology between signal sequences highlights the ability of the translocation machinery to recognize secondary and tertiary structural characteristics that have not yet been elucidated. Interactions at this level are not unlike receptor-ligand interactions seen in enzymic catalysis. The translocation machinery appears highly conserved. For instance, bacterial and eukaryotic signal sequences can often operate interchangebly in directing protein translocation across membranes as demonstrated in both in vitro and in vivo experiments.[91-94]

B. Stop transfer sequences.

The stable integration of proteins into a membrane bilayer requires a hydrophobic stretch of amino acids which span the bilayer.[97-101] These membrane spanning domains are generally comprised of 20

to 30 uncharged, hydrophobic amino acids thought to span the bilayer as an alpha helix.[102] As with signal sequences, no sequence homologies have been observed, although there appears to be a general trend toward having basic amino acid residues at the carboxy terminal end of the hydrophobic membrane spanning stretch.[103] It has been suggested that direct partitioning of the membrane spanning domain into the bilayer can account for the process of stop tranfer in a non-receptor mediated fashion[86], however simply the presence of a long enough stretch of hydrophobic amino acids cannot reliably predict whether a peptide sequence will or will not span a membrane.[85] Evidence suggests that the process of stop transfer requires more than just hydrophobicity. Some studies have demonstrated that hydrophobic signal sequences are not necessarily functionally interchangeable with hydrophobic stop transfer sequences in halting chain translocation.[85] Additionally, while it is seen that in special circumstances stop transfer sequences can initiate chain translocation, they do not behave as pure signal sequences and cannot translocate themselves through the bilayer as do signal sequences.[95] Stop transfer activity of certain hydrophobic sequences can depend on the translation system used and have led some to conclude that interactions bewteen the ribosome and RER membrane can influence the termination of protein translocation by hydrophobic sequences.[112] Receptors for stop transfer sequences[95,96] have not been as well characterized although recently, as previously discussed, several potential candidate proteins have been identified in the RER membrane via photoaffinity cross-linking experiments.[46]

It is clear that signal and stop transfer sequences are distinct from each other and that hydrophobic amino acid stretches cannot account for the complex functions of stop transfer. Final integration of the hydrophobic sequence into the lipid bilayer must not be confused with the halting of the chain being actively translocated through the membrane. In this light, it might be useful to think of stop transfer as a two-step process. The first step is a receptor-mediated event that terminates the proteinaceous channel mediated translocation of a peptide chain into the RER. The second step then occurs, via dissolution of the channel components and the subsequent thermodynamically favorable spontaneous integration of the lipid soluble core of the stop transfer sequence into the bilayer. The role of the non-hydrophobic amino acid sequences flanking a hydrophobic membrane spanning domain may be to interact with the stop transfer receptor(s).[10,12,104,105]

C. The study of topogenesis.

The advent of cell-free systems was crucial to the advancement of our current models of targetting and translocation. Such systems reconstitute the process of protein translation and, when performed in the presence of microsomal membranes derived from RER, the process of translocation across the lipid bilayer.[47,107] The cytosolic lysates contain ribosomes and soluble factors necessary for protein synthesis and are quite active in translation when programmed with poly-adenylated selected mRNA or cell-free transcription products of cDNA (engineered behind specific bacteriophage RNA polymerase promoters). Different sources of tissue can be used to prepare cell-free systems. Two commonly used sources for cytosolic extacts are derived from wheat

germ embryo (WG) and rabbit reticulocyte lysate (RRL).[108,109] A typical source of translocation competent membranes is from canine pancreas rough microsomes, the equivalent of the lumen of the ER in vivo. Newly synthesized protein is detected by including at least one radiolabelled (e.g. ^{35}S methionine) amino acid and analyzing by SDS-PAGE.

The transcription-linked translation coupled translocation system has permitted the detailed analysis of topogenic sequences. Recombinant DNA technology has allowed for the manipulation of cDNA encoding topogenic sequences. Construction of clones that express fusion proteins combining different topogenic sequences within defined passenger domains has allowed for direct comparisons of topogenic sequence function. Mutagenesis of topogenic sequences themselves has permitted microanalysis of important subdomains.

Sophisticated studies probing the molecular mechanisms of protein translation and translocation have been possible because of the versatility of the cell-free systems. For instance, the uncoupling of translocation from translation[32] was a major conceptual advancement and would have been very difficult to confirm in vivo. Additionally, utilizing different tissue sources for cell-free translation-translocation experiments has led to the discovery of various components of the targeting and translocation machinery. For example, the discovery of SRP and SRPR capitalized on differences between the WG and RRL systems.[9] RRL was discovered to be enriched for SRP when compared to WG, thus creating a complementation assay that ultimately led to the purification of these factors. Differences between the WG and RRL

systems have also been exploited to study functional interactions between newly synthesized polypeptide and cytosolic factors. For example the binding of hsp90 to glucocorticoid receptor near the termination of translation was directly demonstrated in RRL but not WG.[110] In vitro systems programmed with microsomal membranes are capable of synthesizing complex functional proteins. For example, ligand binding beta-adrenergic receptor has been expressed in a time dependent, rough microsomal membrane, ATP, and RRL cytosolic factor (> 30 kD) requiring manner.[111] No doubt that further cell-free characterization of the membrane and cytosolic factors involved in the post-translational processing of this receptor will prove fruitful in advancing our understanding of protein biogenesis.

Functional studies provide assays for which components of the translocation machinery can be characterized and purified. Some might argue that because the in vitro systems are not necessarily a total reconstitution of in vivo events, they are therefore limited in their usefullness. On the contrary, it is precisely this partial reconstitution that makes the cell-free systems powerful. The incomplete translocation events often seen in cell-free systems can provide unique insite onto mechanisms that would otherwise be missed. More important however is that different functions can be complemented with other cellular fractions to achieve a more complete reconstitution of translocation events--including regulatory events. Thus, functional dissection and biochemical purification of the machinery is possible.

The study of the process of stop transfer in cell-free systems has yielded interesting results that suggests the process is more complex then

initially imagined. In the case of the prion protein (PrP), the same molecule can adapt different topological orientations (secretory verses transmembrane) depending on the translation system used.[113,114] This occurs by the actions of a novel topogenic sequence as will be discussed extensively in Chapters II and III.

CHAPTER II. NON-HYDROPHOBIC EXTRACYTOPLASMIC DETERMINANT OF STOP TRANSFER IN THE PRION PROTEIN.

Introduction.

A universal feature of integral transmembrane proteins is a hydrophobic peptide segment that spans the lipid bilayer. These hydrophobic domains are important for terminating the translocation of the polypeptide chain across the endoplasmic reticulum (a process termed stop transfer) and for integrating the protein into the bilayer.[97-101] But a role for extracytoplasmic sequences in stop transfer and transmembrane integration has not previously been shown. The prion protein (PrP) is a brain glycoprotein that exists in two isoforms identical in primary amino acid sequence but differing in either subcellular location or transmembrane orientation.[115] Consistent with this is the observation of two different topological forms in cell-free systems.[113,114] An unusual topogenic sequence in the prion protein seems to direct these alternate topologies (see Chapter III).[13] In the wheat-germ translation system, this sequence directs nascent chains to a transmembrane orientation; by contrast, in the rabbit reticulocyte system, this sequence fails to cause stop transfer of most nascent chains. We have now investigated determinants in this unusual topogenic sequence that directs transmembrane topology, and have demonstrated that (1) a lumenally disposed charged domain is required for stop transfer at the adjacent hydrophobic domain, (2) a precise spatial relationship between these domains is essential for efficient stop transfer, and (3) codons encompassing this hydrophilic extracytoplasmic domain confer transmembrane topology to a heterologous protein when engineered adjacent to the codons for a normally translocated

hydrophobic domain. These results identify an unexpected functional domain for stop transfer in the prion protein and have implications for the mechanism of membrane protein biogenesis.

Results.

We constructed a series of deletions throughout the prion protein (PrP) to demonstrate the significance of the unusual topogenic sequence. Figure 1 illustrates these mutants and compares them with the wild-type (wt) PrP molecule. We expressed these PrP mutants in the wheat-germ (WG) translation system supplemented with dog pancreas rough microsomal membranes. We digested aliquots with proteinase K in the presence or absence of the non-ionic detergent Triton X-100, and subjected them to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein encoded by the mutant PrP ha 32 (Figure 2 lanes 1-4) had the same topology as wt PrP (Figure 3 lanes 5-8), as determined by size, intensity and proportion of the characteristic N-terminal transmembrane fragment generated upon proteolysis of intact membranes (compare Figure 2 lane 3 verses Figure 3 lane 7).[113] Proteolysis in the presence of detergent completely hydrolyzed translation products for PrP ha 32 and all other PrP mutants (Figures 2,3, and 4). These results were expected in view of the large body of evidence indicating that only very discrete regions of a protein are involved in determining topology.[9,10] Indeed, deletion of other regions in the C-terminal end of PrP did not affect the transmembrane topology (data not shown).

The proteins encoded by PrP ha 30 (Figure 2 lanes 5-8) and PrP ha 31 (Figure 4) which have deleted the transmembrane regions have, as

predicted, fully translocated topologies. They are largely glycosylated as well as protected from protease digestion in the absence of detergents. This is consistent with previous studies demonstrating an absolute requirement for the hydrophobic domain in transmembrane integration.[97,98,116]

FIGURE 1. Deletion mutagenesis of PrP ha 1. The 254-amino-acid residues of Syrian hamster PrP are shown at the top. Open box indicates 22-residue signal peptide. Hatched boxes show membrane-spanning regions. Vertical bars indicate possible N-linked glycosylation sites. Arrows indicate positions at which insertions generating PrP ha 40 and PrP ha 45 were made. Black boxes below indicate the regions of PrP ha 1 that were deleted to generate each of the indicated mutants, PrP ha 32, PrP ha 30, PrP ha 31, and PrP ha 28, corresponding to the deletions of codons 39-62, 91-158, 63-158, and 63-87 respectively. METHODS. Plasmids were constructed as follows. Plasmid pSP PrP ha 1: PrP cDNA from Syrian hamster isolate was cloned into pSP64T. Plasmid pSP PrP ha 28: a Kpn1 site was introduced by site-directed mutagenesis at codon 73 of PrP ha 1 construct to generate PrP ha 23. Plasmid was opened at Kpn 1 site, treated sequentially with nuclease Bal31, Klenow fragment of DNA polymerase 1 and T4 DNA ligase. Plamid pSP PrP ha 30: plasmid PrP ha 1 was cut with Nae1 in the presence of ethidium bromide, recut with Hinc2 and closed with T4 DNA ligase. Plasmid pSP PrP ha 32: plasmid PrP ha 1 was cut with Nco1, and a 270-base pair(bp) fragment isolated and religated back into the vector Nco1 site. Plasmid PrP ha 40: 350-bp EcoR1-Bste2 fragment encoding chimpanzee globin residues 1-110, and including a previously inserted sequence encoding an N-linked glycosylation site, was generated from pSPSLSTgG.[85] After treatment with Klenow, Kpn1 8mer linkers were added to the blunt ends and the fragment was religated into the Kpn1 site of plasmid PrP ha 23. Plasmid pSP PrP ha 45: BamH1 10mer linkers were added to the same blunted fragment from plasmid pSPSLSTgG described above treated with Bam methylase. An upstream Nae1 site in a noncoding region of PrP ha 1 was eliminated by digestion wih Nhe1 and SpH1, and treated with Klenow, mung bean nuclease, and T4 ligase. The sole remaining Nae1 site located in the coding region of PrP ha 1 was cut with Nae1, and BamH1 10mer linkers added. The plasmid was then cut with BamH1, and the BamH1-tailed fragment inserted. All constructions involving Bal31 treatment were sequenced. All constructions were confirmed by transciption-linked expression and immunoprecipitation with relevant antisera, and the expected size confirmed by SDS-PAGE.

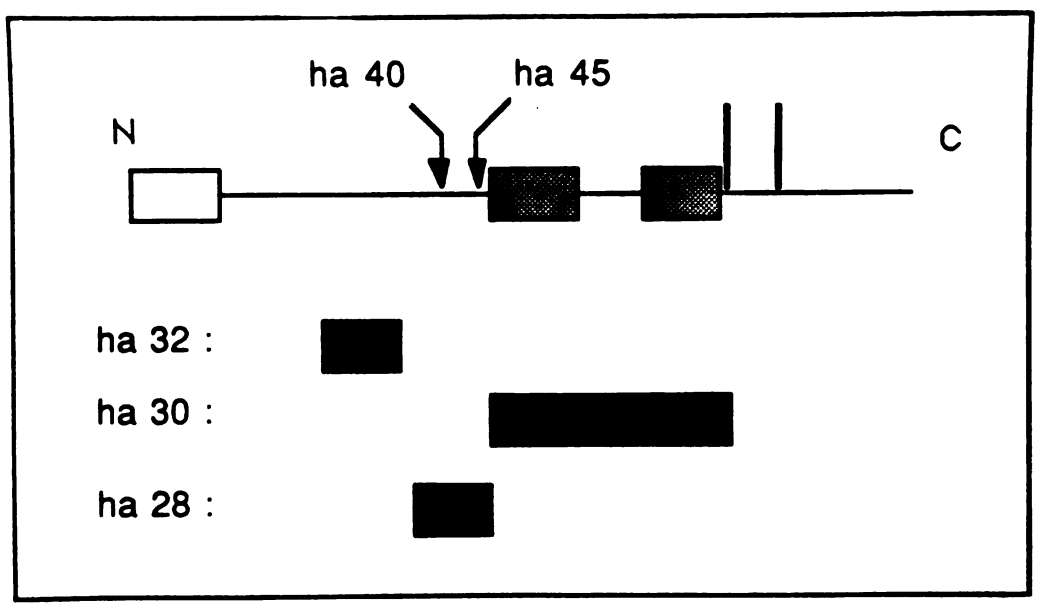
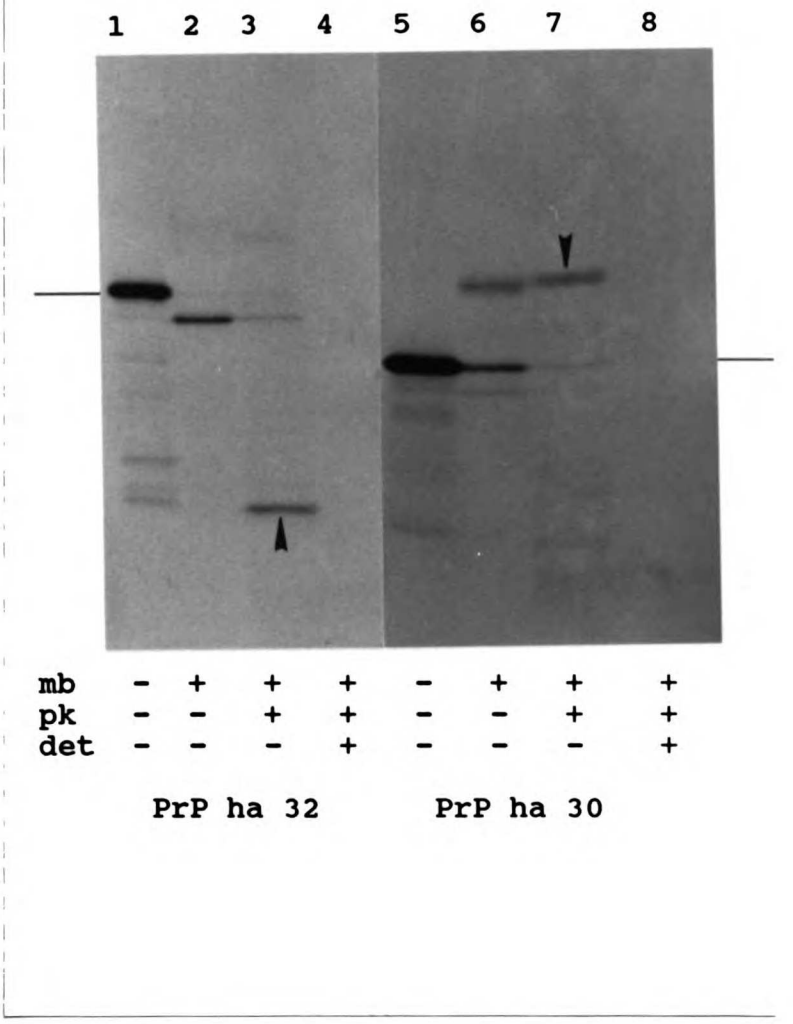

ha 40
ha 45
N
C
ha 32 :
ha 30 :
ha 28 :

FIGURE 2. Cell-free transcription-linked translation-coupled translocation of products encoded in plasmids pSP PrP ha 32 (lanes 1-4) and pSP PrP ha 30 (lanes 5-8) as described in Figure 1 analyzed by SDS-PAGE and flourography of total products as previously described.[12] Transcriptions were performed using SP6 polymerase. Translations used WG extracts with dog pancreas microsomes (2.5 A_{280} units/ml.) where indicated as previously described.[113] Aliquots posttranslationally proteolyzed with or without detergent are indicated below each panel. All proteolysis was performed at 4°C for 1 h with 0.2 mg/ml PK, 10 mM $CaCl_2$, 10 mM Tris-HCl buffer pH 8.0, and with or without 1% Triton X-100. PK digestions were terminated by adding phenylmethylsulphonyl flouride to 1 mM in dimethylsulfoxide and boiling in 1% SDS-0.1 M Tris-HCl pH 8.9 for 5 minutes. These conclusions were confirmed by quantitative densitometry adjusted for methionine content. Abbreviations: Mb, membranes; PK, proteinase K; Det, detergent. Arrows denote the main protected products.


1 2 3 4 5 6 7 8
mb - + + + - + + +
pk - - + + - - + +
det - - - + - - - +
PrP ha 32
PrP ha 30

The gene encoding the mutant PrP ha 28 (Figure 3 lanes 1-4) has a deletion of 24 codons just 5' to the region encoding the first transmembrane region, but leaves the entire transmembrane region intact. Surprisingly, this deletion had a dramatic effect on transmembrane topology. Many chains were glycosylated and fully protected from protease with few chains displaying the characteristic transmembrane topology expected for wt PrP (Figure 3 lanes 5-8). We noted that the fraction of chains glycosylated varied with each mutant.

Experiments in which N-linked glycosylation was abolished using an acceptor tripeptide[117] demonstrated that glycosylation was not a factor in determining transmembrane or secretory topology of either PrP or its deletion mutants (Figures 5 and 6).

The region deleted in PrP ha 28 has strikingly positive charge distribution, containing four lysine and two histidine residues and completely lacking hydrophobic character (see Figure 10). The behavior of this mutant is consistent with the hypothesis that this hydrophilic, extracytoplasmic region (henceforth termed STE for Stop Transfer Effector) promotes the integration of the adjacent hydrophobic sequence into the bilayer.

FIGURE 3. Cell-free transcription-linked translation coupled translocation of products encoded in plasmids pSP PrP ha 28 (lanes 1-4) and pSP PrP ha 1 (lanes 5-6) analyzed by SDS-PAGE and fluoragraphy of total products. Transcriptions, translations, translocations and proteolysis as described in figure legend 2. Arrows denote the main protected products.

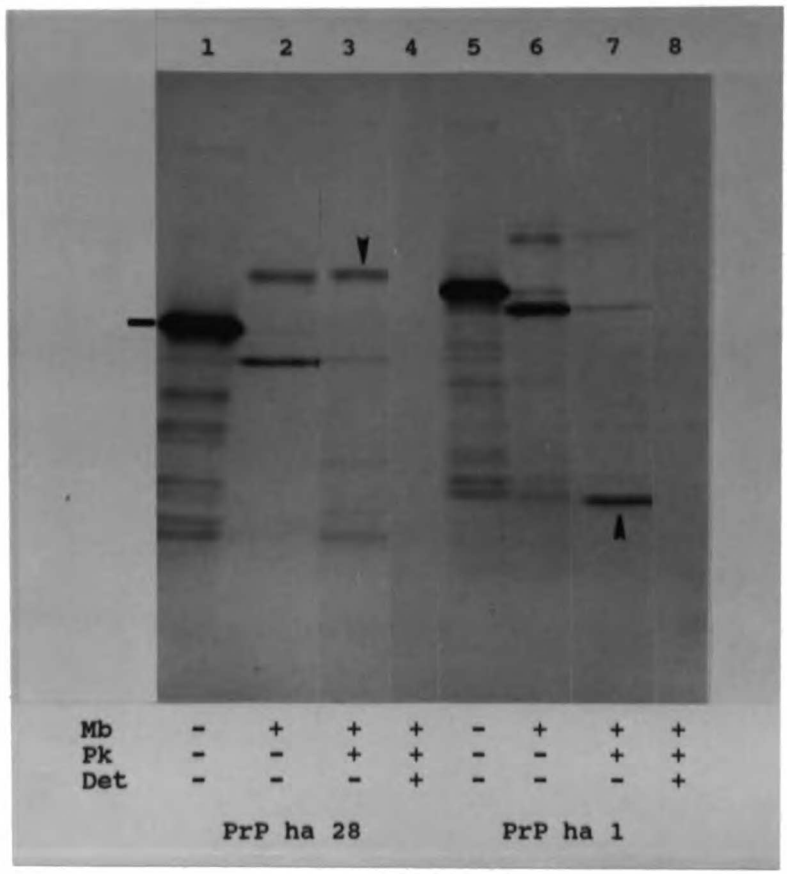

1 2 3 4 5 6 7 8
Mb - + + + - + + +
Pk - - + + - - + +
Det - - - + - - - +
PrP ha 28
PrP ha 1

FIGURE 4. Cell-free transcription-linked translation-coupled translocation of products encoded in plasmid pSP PrP ha 31 analyzed by SDS-PAGE and fluorography of total products. Transciptions, translations, translocations and proteolysis as described in previous figure legends. Arrows denote the main protected products.

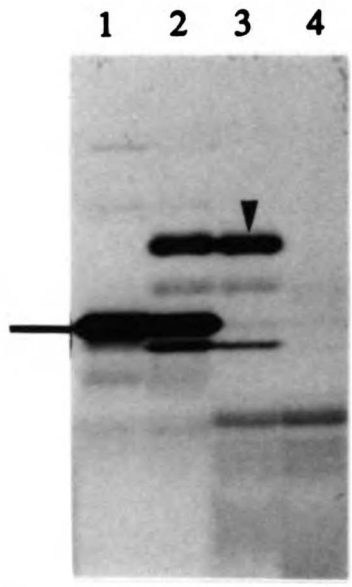

1 2 3 4
Mbs - + + +
PK - - + +
Det - - - +

FIGURE 5. Cell-free transcription-linked translation-coupled translocation of products encoded in plasmids pSP PrP ha 28 (lanes 1-6) and pSP PrP ha 1 (lanes 7-12) with or without acceptor tripeptide (AP) as indicated, analyzed by SDS-PAGE and fluorography of total products. Transcriptions, translations, translocations and proteolysis as described in previous figure legends.

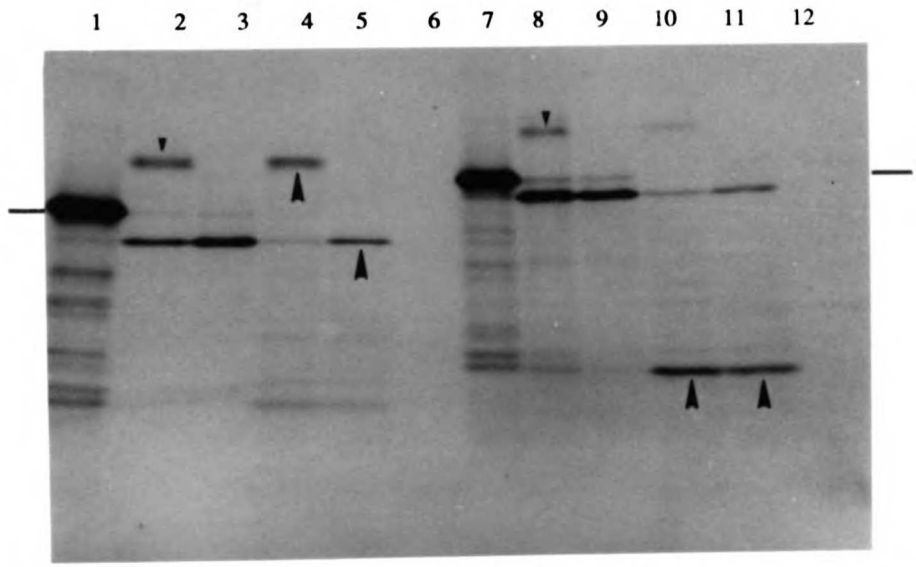
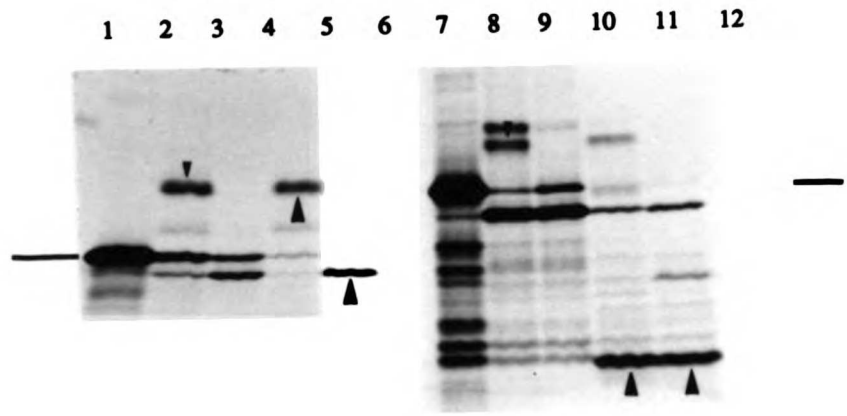

1 2 3 4 5 6 7 8 9 10 11 12
Mbs - + + + + + - + + + + +
AP - - + - + - - - + - + -
PK - - - + + + - - - + + +
Det - - - - - + - - - - - +

FIGURE 6. Cell-free transcription-linked translation-coupled translocation of products encoded in plasmids pSP PrP ha 30 (lanes 1-6) and pSP PrP ha 32 (lanes 7-12) with or without acceptor tripeptide (AP) as indicated, analyzed by SDS-PAGE and fluorography of total products. Transcriptions, translations, translocations and proteolysis as described in previous figure legends.



Mbs	-	+	+	+	+	+	-	+	+	+	+	+
AP	-	-	+	-	+	-	-	-	+	-	+	-
PK	-	-	-	+	+	+	-	-	-	+	+	+
Det	-	-	-	-	-	+	-	-	-	-	-	+

To understand better the spatial relationship of STE to the hydrophobic domain, we inserted codons from the cytosolic protein alpha-globin into the coding region of PrP. Because alpha-globin contains no information for either start or stop transfer[85,94,104], it served as a "spacer" and allowed us to determine the importance of location for the function of STE. Codons 1-110 of the chimpanzee alpha-globin gene, as well as an eight-codon N-linked glycosylation site[118], were inserted either just N-terminal to the sequence encoding STE (PrP ha 40), or between the sequences encoding STE and the hydrophobic transmembrane region (PrP ha 45), as indicated by arrows in Figure 1. The identities of the constructs were further confirmed by immunoprecipitation (Figures 7 and 8).

The fusion protein PrP ha 40 had the same topology as wt PrP ha 1 (compare Figure 9 lanes 1-4 and Figure 3 lanes 5-8). However, with globin inserted between STE and the hydrophobic region, the resulting fusion protein, PrP ha 45, had mainly the topology observed for deletion mutant PrP ha 28 (compare Figure 9 lanes 5-8 and Figure 3 lanes 1-4). Thus, globin residues placed between STE and the hydrophobic transmembrane domain disrupt stop transfer. Apparently the spatial relationship of STE to the hydrophobic domain is critical for transmembrane integration of PrP.

FIGURE 7. Cell-free transcription-linked translation of plasmid pSP PrP ha 40 immunoprecipitated with domain specific antisera as described previously[13] and analyzed by SDS-PAGE and fluorography. Hatch mark indicates full-length product. Lane 1, anti-carboxy terminal PrP antisera (P3); Lane 2, anti-amino terminal PrP antisera (P1); Lane 3, anti-globin antisera; Lane 4, non-immune sera.

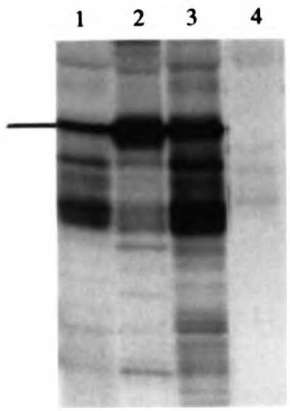

1
2
3
4

FIGURE 8. Cell-free transcription-linked translation of plasmid pSP PrP ha 45 immunoprecipitated with domain specific antisera as described previously[13] and analyzed by SDS-PAGE and fluorography. Hatch mark indicates full-length product. Lane 1, non-immune sera; Lane 2, anti-carboxy terminal PrP antisera (P3); Lane 3, anti-amino terminal PrP antisera (P1); Lane 4, anti-globin antisera.

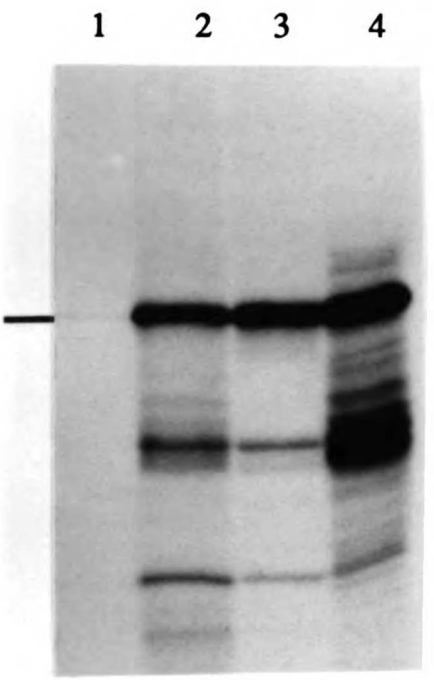
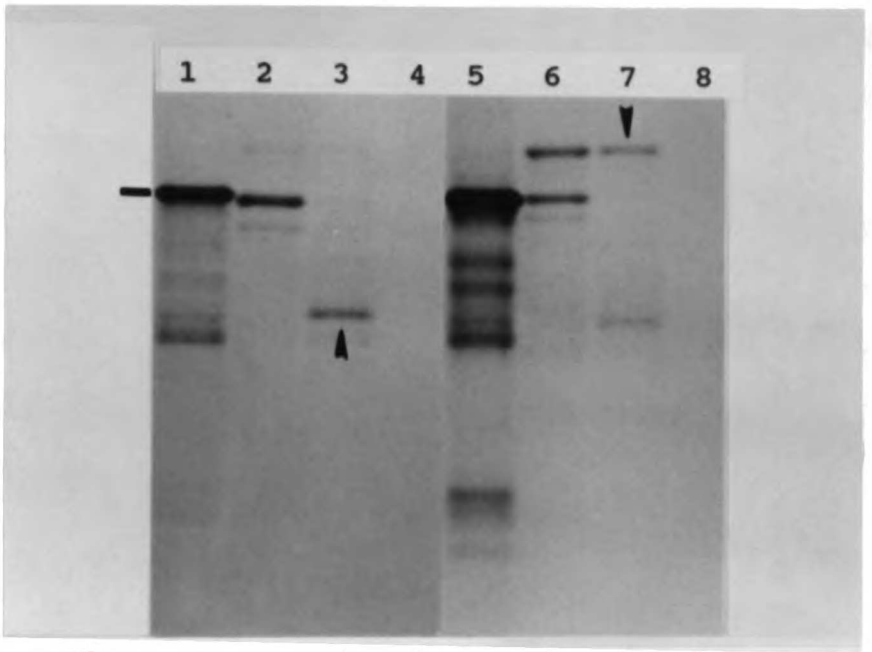

1
2
3
4

FIGURE 9. Cell-free transcription-linked translation-coupled translocation of products encoded in plasmids pSP PrP ha 40 (lanes 1-4) and pSP ha 45 (lanes 5-8) analyzed by SDS-PAGE and fluorography of total products. Transciption, translation and proteolysis are as described in previous figure legends. Arrows denote the main protected products.


1 2 3 4 5 6 7 8
Mb - + + + - + + +
Pk - - + + - - + +
Det - - - + - - - +
PrP ha 40
PrP ha 45

The hydropathy profile of PrP ha 1 and the mutants PrP ha 28, ha 40 and ha 45 using the consensus program of Eisenberg[119] are shown in Figure 10. The amino acid sequences of the pertinent regions of these molecules are also listed. The plot shows that the disruptive effect of the deletion in PrP ha 28 and of the insertion in PrP ha 45, are not a result of alteration in hydrophobicity or introduction of specific sequences. Rather, the observed effects seem to be a consequence of abolishing the functional relationship between STE and the hydrophobic anchor.

Finally, we determined whether STE could induce stop transfer at a normally translocated hydrophobic domain, and if so, whether these observations could be extended to stop transfer in living cells. To do this we took advantage of earlier observations with chimeric proteins[85]. In Xenopus oocytes (XO), translocation initiated by an N-terminal signal sequence cannot be terminated by another signal sequence that follows internally (construct S•G•S•P, so termed to indicate component protein domains see Figure 11 legend). By contrast, a genuine stop transfer sequence in the same context results in transmembrane topology (S•G•ST•P). Thus, we engineered STE adjacent to the internal signal sequence of the former chimera and expressed the new hybrid (termed S•G•STE•S•P) in XO. If STE can confer stop-transfer activity it would be expected to terminate chain translocation at the normally translocated hydrophobic internal signal sequence. Figure 11 shows the topology of S•G•STE•S•P and of the original chimeric proteins as controls. The STE domain conferred transmembrane topology to 50% of the chains at the internal signal sequence (as determined by quantitative densitometry

corrected for methionine distribution) in contrast to the behavior of S.G.S.P chains which were completely translocated (see lanes 8 verses 5). This percentage is similar to that observed for other chimeras in which STE is adjacent to the TM1 hydrophobic sequence from PrP (see chapter III).[13]

FIGURE 10. Hydropathy plots for PrP ha 1, PrP ha 28, PrP ha 40 and PrP ha 45. A. Upper plot shows Eisenberg consensus profile (14-residue window) of Syrian hamster PrP. The single amino-acid code for the hydrophilic region preceding the transmembrane segment is listed below. Lower plot shows the profile for the 230-amino-acid sequence of PrP ha 28 in which the hydrophilic domain has been deleted. Listed above is the amino-acid sequence across the deletion junction as determined by dideoxy-chain-termination sequencing of plasmid DNA. Boxed residues show the full sequence of the transmembrane domain. B. Hydropathy profile of PrP ha 40 and PrP ha 45. Upper panel shows plot of PrP ha 1 with globin inserted in-frame before STE, with the amino-acid sequence of points of globin-PrP fusion shown below. Lower panel shows plot of PrP ha 1 with globin inserted between STE and the hydrophobic domain. Sequence above shows points of globin-PrP fusion.

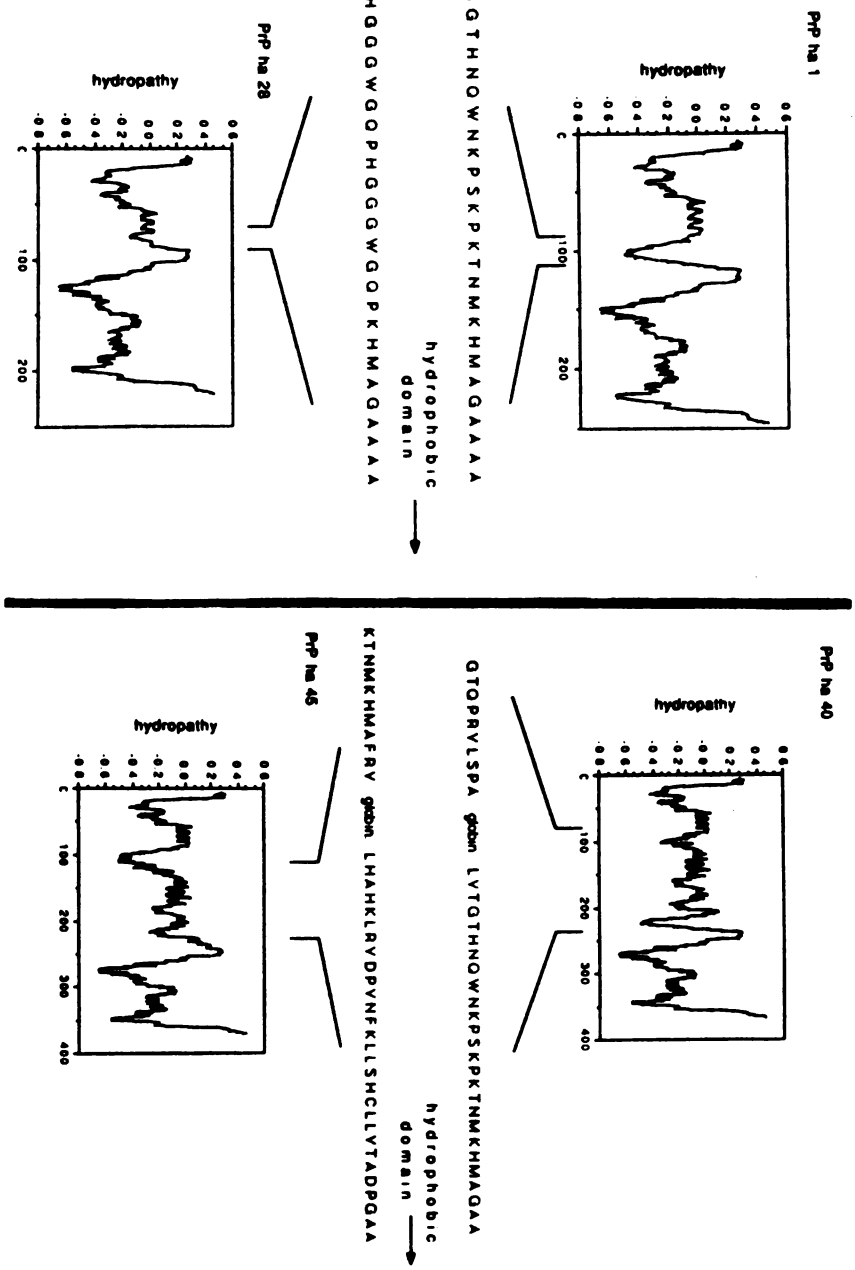
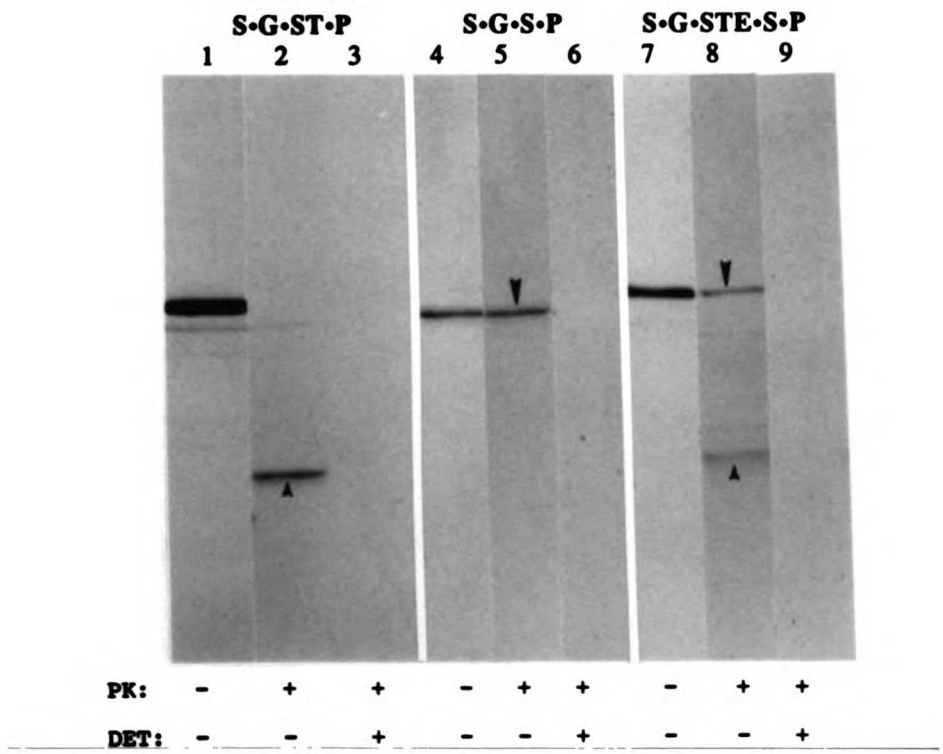

PrP hs 1
hydropathy
QGGGTHNQWNKPSKPKTNMKHMAGAAAA
hydrophobic domain
PrP hs 28
hydropathy
GQPHGGGWGQPHGGGWGQPKHMAGAAAA
PrP hs 40
hydropathy
GTQPRVLSPA globin LVTGTHNQWNKPSKPKTNMKHMAGAA
hydrophobic domain
PrP hs 45
hydropathy
KTNMKHMAFRV globin LHAHKLRVDPVNFKLLSHCLLVTADPGAA

FIGURE 11. Expression of chimeric proteins in Xenopus oocytes. Chimeric proteins were engineered (see ref. 85 and below) and expressed in stage VI Xenopus oocytes by microinjection of RNA transcripts and [^{35}S]-methionine as previously described.[85] Oocytes were labelled for 4 h, homogenized in 0.25 M sucrose, 0.05 M Tris buffer pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, 1mM DTT, and an aliquot of homogenate proteolyzed with proteinase K in the presence or absence of detergent as previously described.[85] Products were then immunoprecipitated with antisera raised against human globin or with non-immune serum (not shown) and analyzed by SDS-PAGE. Arrows pointing downwards denote fully protected secreted products; arrows pointing upwards denote protease-protected fragments from transmembrane chains. Plasmid pSP SGSTESP was engineered as follows. pSP SGSP was cleaved with BstE2, treated with Klenow, and ligated to Xba1 12mer linkers. The resulting intermediate plasmid was cleaved with Xba1, treated calf intestinal phosphatase and religated in the presence of an excess of a fragment encompassing the 90 bp of the PrP encoding region form Nae1 to Nco1 sites generated from intermediates by treatment with Klenow, addition of Xba1 8mer linkers, cleavage with Xba1 and purification by agarose gel electrophoresis.


S•G•ST•P
S•G•S•P
S•G•STE•S•P
1 2 3 4 5 6 7 8 9
PK: - + + - + + - + +
DET: - - + - - + - - +

Discussion.

Because of the requirement for thermodynamic stability of proteins in membranes, it is not surprising that hydrophobic domains are essential for transmembrane integration[97-101,120,121]. But a role for extracytoplasmic sequences in stop transfer, although previously suggested[10,104] has not been demonstrated. The studies presented here indicate such a role for STE in PrP biogenesis.

In view of the growing evidence that the process of translocation across membranes is mediated by receptors[43], and that translocation itself occurs through an aqueous channel[33] it might be expected that stop transfer would display some receptor-mediated features. By analogy to other protein-mediated systems, in some cases the efficiency of stop transfer might depend on regulatory factors in the cytosol or the membrane, or in both. The seemingly "inefficient" stop transfer activity of PrP is independent of ongoing protein synthesis and dependent on cytosolic factors (see chapter III).[13] We suggest here that STE operates as a ligand for such receptor-mediated stop transfer.

Studies on other integral transmembrane proteins are necessary to determine whether these findings are a universal feature of transmembrane protein biogenesis or an unusual feature limited to a small class of proteins including PrP. The corresponding extracytoplasmic regions of other transmembrane proteins are quite divergent in sequence and charge. Also, the hydrophobic domains of various native and engineered transmembrane proteins differ significantly in their overall hydrophobicity as well as in the specific

amino acid residues they contain[96,106,122,123]. It is noteworthy that two positively charged residues are retained immediately preceding the transmembrane region in the deletion mutant PrP ha 28. Thus the observed effect of STE is probably not specifically mediated by charge but rather by more subtle structural features as is likely to be the case for other receptor-ligand interactions involving chain translocation.[22,77]

It is also provocative that the PrP protein from mouse strains with different scrapie incubation times as well as an isoform of human PrP linked to inherited prion disease, Gerstmann-Straussler syndrome, all have unique substitutions of non-charged amino acids within STE.[124-126] Perhaps control over the action of STE is important for the generation of both secreted and membrane-bound forms of PrP observed previously[113,114], for the as yet unknown function of PrP in normal brain, or for the generation of PrP^{Sc} during scrapie.

Chapter III. UNUSUAL TOPOGENIC SEQUENCE DIRECTS PRION PROTEIN BIOGENESIS.

Introduction.

Biosynthetic studies of the prion protein (PrP) have shown that two forms of different topology can be generated from the same pool of nascent chains in cell-free translation systems supplemented with microsomal membranes. A transmembrane form is the predominant product generated in wheat germ (WG) extracts, whereas a completely translocated (secretory) form is the major product synthesized in rabbit reticulocyte lysates (RRL). An unusual topogenic sequence within PrP is now shown to direct this system-dependent difference. The actions of this topogenic sequence were independent of on-going translation and could be conferred to heterologous proteins by the engineering of a discrete set of codons. System-dependent topology conferred by addition of RRL to WG translation products suggests that this sequence interacts with one or more cytosolic factors.

Secretory and transmembrane proteins acquire their orientation with respect to the membrane of the endoplasmic reticulum (ER) in a manner that is usually both predictable and absolute.[9] This reflects the action of discrete regions within such nascent proteins, termed signal and stop-transfer sequences, that initiate and terminate translocation across the ER.[10] Studies in eukaryotic cell-free systems have revealed that at least some topogenic sequence actions are mediated by receptor proteins.[22,25,26,42,43] The subcellular components that function in such systems appear conserved. Thus, mRNA from any tissue or species can be translated in cytosolic extracts of either WG or RRL.[108,109] When these translation systems are supplemented with microsomal

membranes derived from the ER, the newly synthesized protein achieves a topology identical to that observed in the ER in vivo.[127] Topology can be assayed by means of proteases to distinguish domains protected by the lipid bilayer.[85,104]

An exception to this conservation of components occurs during biogenesis of the prion protein (PrP). PrP, a brain glycoprotein, exists in two isoforms: (i) PrP^{C}, which is found in normal brain and is expressed at specific times during developement[128,129] and (ii) PrP^{Sc}, which is a component of the infectious agent causing scrapie (a degenerative neurologic disease of animals that is related to several diseases of humans).[115] The primary amino acid sequences of PrP^{C} and PrP^{Sc} are identical.[115] Both isoforms contain a phosphatidylinositol glycolipid anchor at their COOH-terminals that is cleavable by a phosphatidylinositol specific phospholipase C (PIPLC).[130] However, only PrP^{C} is released from cells when treated with PIPLC.[131] These findings are consistent with a difference between the two isoforms in either subcellular localization or transmembrane orientation.

Results.

PrP synthesized in vitro displays two system-dependent topologies. When a cloned cDNA encoding PrP is expressed by transcription-linked translation in WG[113], a transmembrane form predominates (Figure 12). When the same transcript is translated in RRL[114], the predominant product is a completely translocated (that is secretory) protein (Figure 13). Some feature or process occuring in the cytosolic fraction seems to recognize information within nascent PrP to direct transmembrane or secretory forms.

The characteristic transmembrane form of PrP observed in WG^{113} spans the bilayer twice, such that both the NH_2- and COOH-terminal domains are within the lumen of the ER (Figure 14A). The first membrane-spanning domain synthesized, TM1, is approximately 90 amino acid residues from the NH_2-terminal-cleaved signal sequence. Therefore it seemed plausible that the difference in PrP topology observed in WG versus RRL was a reflection of a difference in the ability of these systems to stop translocation of the chain at TM1. One explanation for this difference might be that the rate of chain elongation (and hence the rate of chain translocation) is unequal in WG versus RRL, thereby influencing the extent of stop transfer at TM1. However, when translations were carried out at higher temperatures in a WG system, no changes in topologies were observed for wt PrP or PrP ha 28 (Figure 15). This suggested, although did not prove, that the rate of translation did not influence PrP topology.

FIGURE 12. Wheat germ cell-free transcription-linked translation-coupled translocation of products encoded in wild-type PrP (Ha PrP; PrP ha 1) immunoprecipitated with PrP NH_2-domain specific antisera (P1) and analyzed by SDS-PAGE and fluorography. Transcription, translation, translocation and proteolysis as described in previous figure legends. Arrows denote major products.

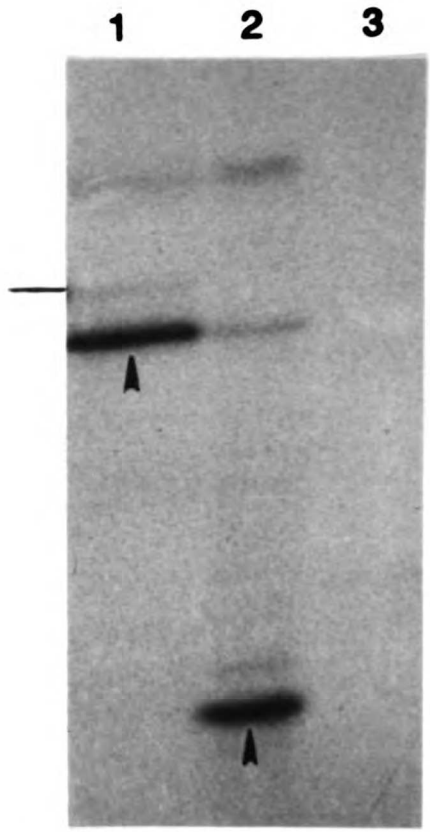

1
2
3
Mb + + +
PK − + +
Det − − +

FIGURE 13. Rabbit reticulocyte lysate cell-free transcription-linked translation-coupled translocation of products encoded in wild-type PrP (Ha PrP; PrP ha 1) immunoprecipitated with PrP NH_2-domain specific antisera (P1) and analyzed by SDS-PAGE and fluorography. Transcription, translation, translocation and proteolysis as described previously.[114] Arrows denote major products.

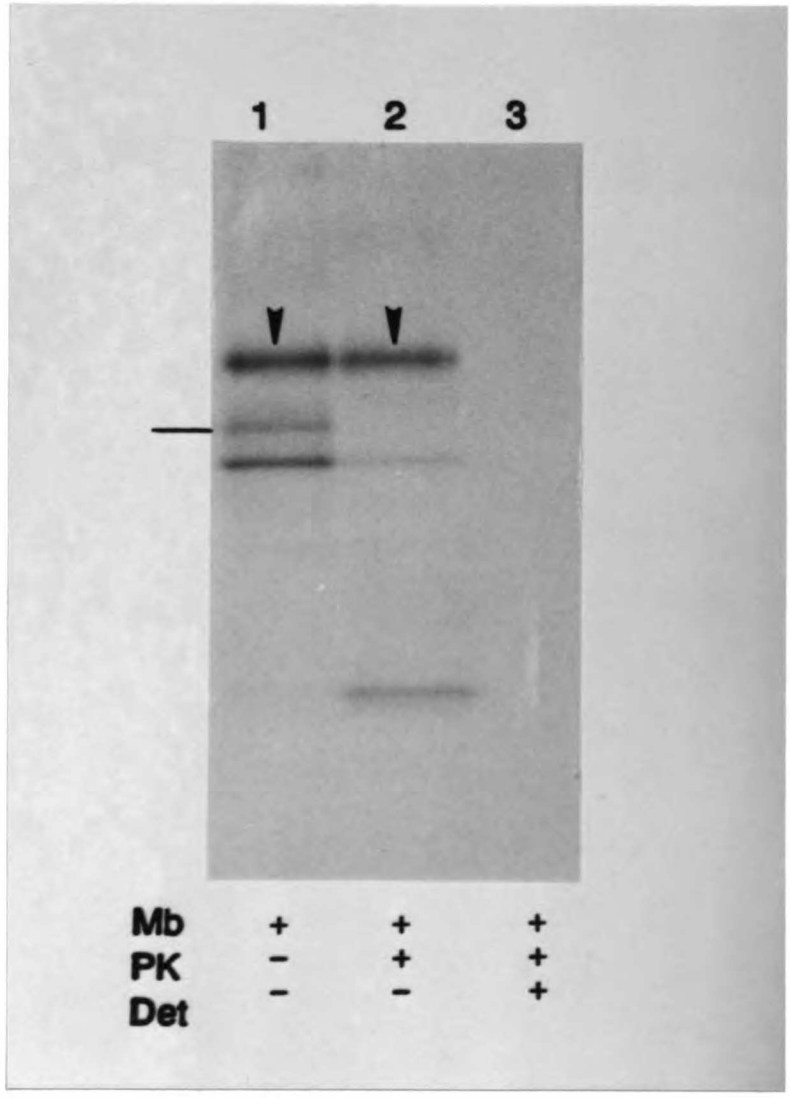

1
2
3
Mb + + +
PK − + +
Det − − +

FIGURE 14. Schematic peptide maps of HaPrP, VSV G, and VSV Gxk. The 232 amino acid residues of mature Syrian hamster PrP are shown at top. Residues -22 to 0 represent the cleaved NH_2-terminal signal sequence. Vertical bars topped by circles indicate potential N-linked glycosylation sites. The open box represents amino acids 74 to 114, with the larger box representing TM1 (24 hydrophobic amino acid residues 90 to 114), which make up the first membrane-spanning region. The smaller box represents the extracytoplasmic hydrophilic domain (STE). The stippled box represents TM2 (an amphipathic helix from amino acids 135 to 160), which makes up the second membrane-spanning region. A schematic diagram of the transmembrane and fully translocated forms is depicted at right.[113,114] The 520-amino acid residues of the mature VSV G protein are depicted with potential N-linked glycosylation sites indicated. The cleaved NH_2-terminal signal sequence is depicted by the striped box. The black box labelled ST represents the native stop transfer sequence from amino acid 474 to 491. The SP6 expression plasmids were constructed as follows. Plasmid pSPHa PrP: as described for PrP ha 1 in Chapter II. Plasmid pSPVSV G: pRSVG was cut with HindIII and BglII, gel-purified and ligated with T4 DNA ligase into vector pSP64T (that had been opened with HindIII and BglII and treated with calf intestinal phosphatase). Plasmid pSPVSV Gxk: a KpnI site and an XbaI site were introduced via site-directed mutagenesis at codons 72 and 114, respectively of pSPHaPrP. The resultant plasmid was opened at the XbaI site, blunted with Klenow, and ligated to a KpnI 8mer with T4 DNA ligase. The plasmid was then cut with KpnI and the 126-bp fragment was gel-purified and religated into pSPVSV G vector opened at codon 346 with KpnI to create an in-frame fusion.

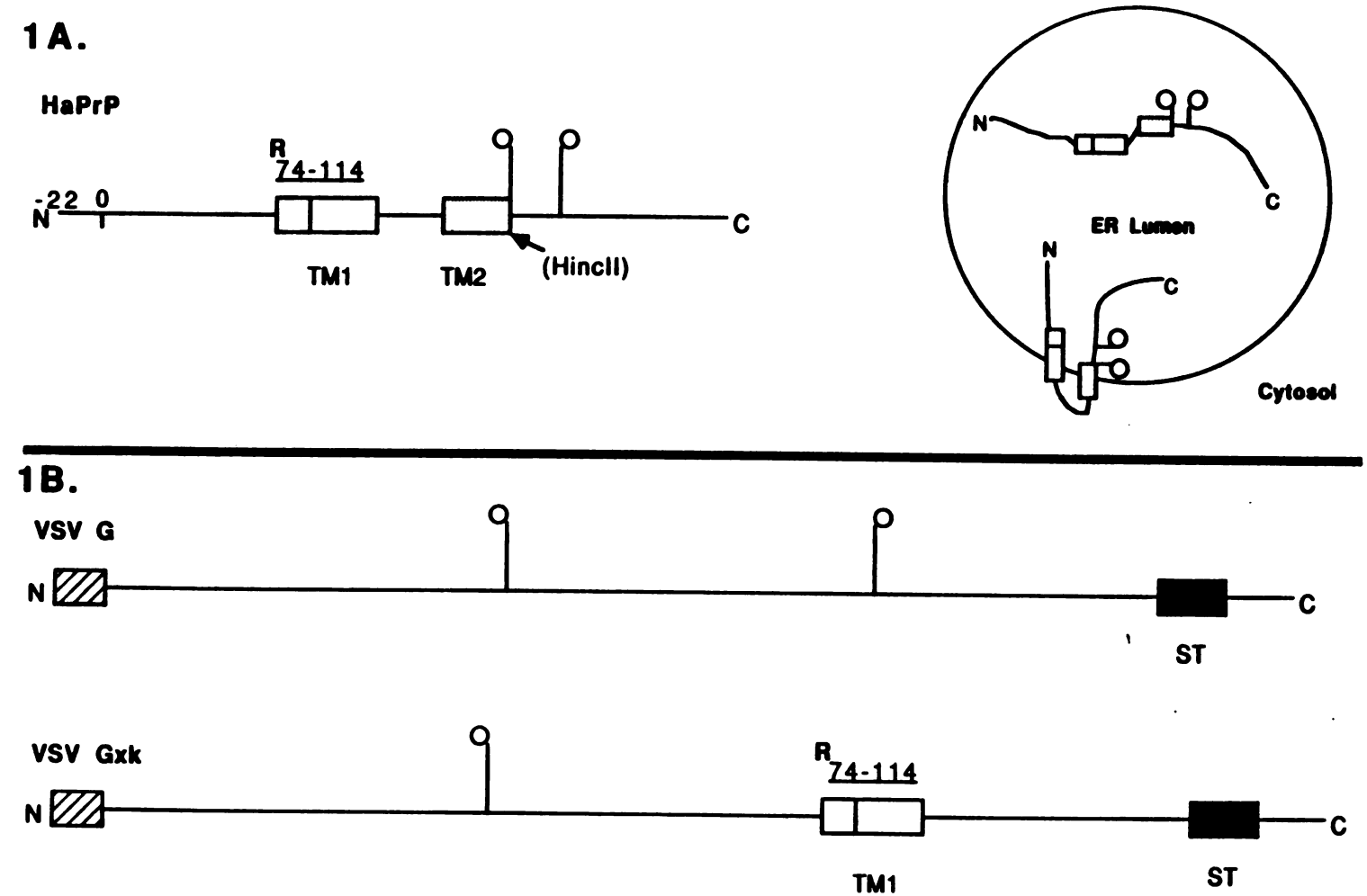
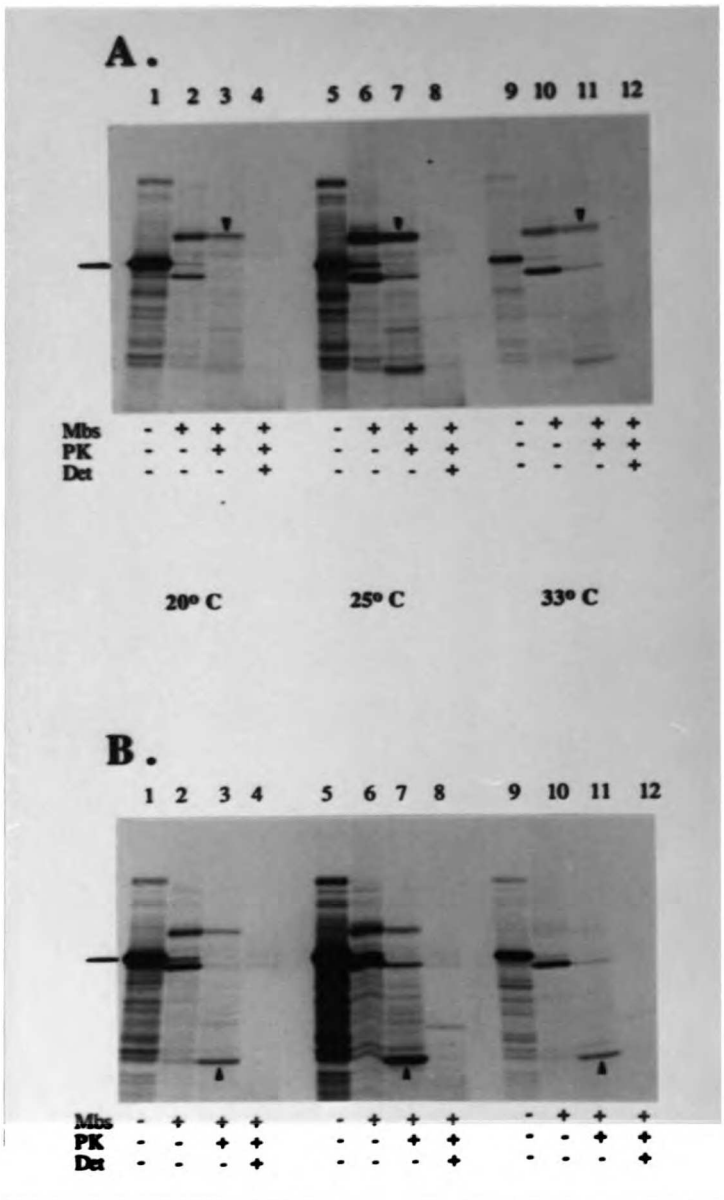

1A.
HaPrP
R
74-114
-22 0
N
C
TM1
TM2
(HincII)
N
C
ER Lumen
N
C
Cytosol
1B.
VSV G
N
C
ST
VSV Gxk
R
74-114
N
C
TM1
ST

FIGURE 15. Wheat germ cell-free transcription-linked translation-coupled translocation of pSP PrP ha 28 (Panel A) and pSPHaPrP (Panel B) at different temperatures as indicated, and analyzed by SDS-PAGE and fluorography of total products. Arrows denote the main protected products as confirmed by quantitative densitometry corrected for methionine distribution. Except for translation-translocation temperatures, the transcriptions, translations, translocations and proteolysis were performed as described in previous figure legends.


A.
1 2 3 4 5 6 7 8 9 10 11 12
Mbs
PK
Det
20° C
25° C
33° C
B.
1 2 3 4 5 6 7 8 9 10 11 12
Mbs
PK
Det

The first step toward analysis of the molecular basis for the alternate fates in PrP biogenesis was to uncouple translocation of the chain from protein synthesis. Normally, translocation occurs only while peptide chains are in the process of being synthesized (co-translationally). By truncation of a cDNA within the coding region, it becomes possible to generate mRNA lacking a termination codon. In this case, the initial engaged ribosome will be unable to release the nascent peptide chain. These nascent chains remain translocation-competent even in the absence of further chain elongation.[32]

PrP cDNA was truncated at a HincII site 74 codons 5' to the termination codon. Transcription-linked translation of this DNA (PrP/HcII) in either WG (Figure 16) or RRL (Figure 17) in the absence of microsomal membranes resulted in a nascent chain-ribosome complex in which PrP had been synthesized from the NH_2-terminal through TM1. The translation product was presented to the membrane either co-translationally (with membranes present during its synthesis) or posttranslationally (with membranes added after completion of synthesis and in the presence of translation inhibitors). As expected for translocation-competent chains, cleavage of the signal sequence occured in the presence of membranes either co- or posttranslationally in both WG and RRL (Figures 16 and 17). Unlike native $PrP^{113,114}$, these truncated products were not glycosylated, since the recipient asparagine residues for N-linked carbohydrates were lost after truncation. Glycosylation has no influence on generation of either form of PrP, as

shown by use of a tripeptide inhibitor of N-linked glycosylation in both WG and RRL (see Chapter II).

The topology of these translocated chains was distinguished by subjecting them to proteolysis with proteinase K. Proteolysis of the transmembrane form generates characteristic protected fragments, one of which is immunoprecipitated by peptide-specific antisera directed to the NH_2-terminal domain of PrP.[113] The NH_2-terminal fragment predominated during proteolysis and immunoprecipitation of the products of both co- and posttranslational translocation of PrP/HcII in WG (Figure 16 lanes 3 and 7). In contrast, full-length PrP/HcII would be protected if stop transfer did not occur. No protected fragments were observed when the membrane bilayer was solubilized with nondenaturing detergent during proteolysis. When PrP synthesized in RRL and translocated either during or after translation was subjected to proteolysis, the predominant protected immunoreactive band was of full size (Figure 17 lanes 3 and 7). Quantitative densitometry, corrected for methionine distribution, confirmed that both co- and posttranslational translocation generated predominantly the transmembrane topology in WG, while the secretory topology was predominant in RRL (Table 1). To ensure translation was completely blocked, a mock control was done in parallel without transcript during the initial 30-minute translation. The sample was then split and one portion was treated with ATA and emetine (Figure 18 lanes 2) and the other with H_2O and compensating salts (Figure 18 lanes 1). The mock samples synthesized protein while the samples receiving inhibitors did not. Previous data had suggested that a precursor product relationship between the transmembrane and

secretory products did not exist.[114] To confirm these findings, PrP/HcII was synthesized in WG, presented posttranslationally to microsomal membranes, and then allowed to incubate for varying amounts of time with the membranes before assaying topology by protease protection (Figure 19). Quantitative densitometry confirmed that there was no precursor product relationship between the transmembrane and secretory forms of PrP/HcII.

Thus, these system-dependent differences appeared independent of chain elongation. Moreover, the topogenic sequence responsible for this behavior was encoded 5' to the HincII site.

FIGURE 16. Posttranslational translocation of PrP/HcII nascent chains synthesized in WG and analyzed by SDS-PAGE and autoradiography, of NH_2-terminal domain specific antisera immunoprecipitates, lanes 5 to 8. Co-translational translocation of PrP/HcII, lanes 1 to 4. Template cDNA was prepared as described in the text. Transcription and immunoprecipitation conditions as previously described.[113] Posttranslational translocation was performed as follows: translation was carried out for 30 minutes, then adjusted to 0.1 mM of aurin tricarboxylic acid (ATA) for 15 minutes followed by addition of emetine to 0.1 mM for 15 minutes. Microsomal membranes (Mb) were then added to 2.5 A_{280}/ml and incubated for 40 minutes. Proteolysis was performed as described in previous figure legends. Hatch mark at left denotes the PrP/HcII precursor. Arrows denote major products. A decrease in the number of chains after proteolysis of posttranslational translocation reactions was reproducibly observed. This phenomenon is observed with other proteins translocated after translation.

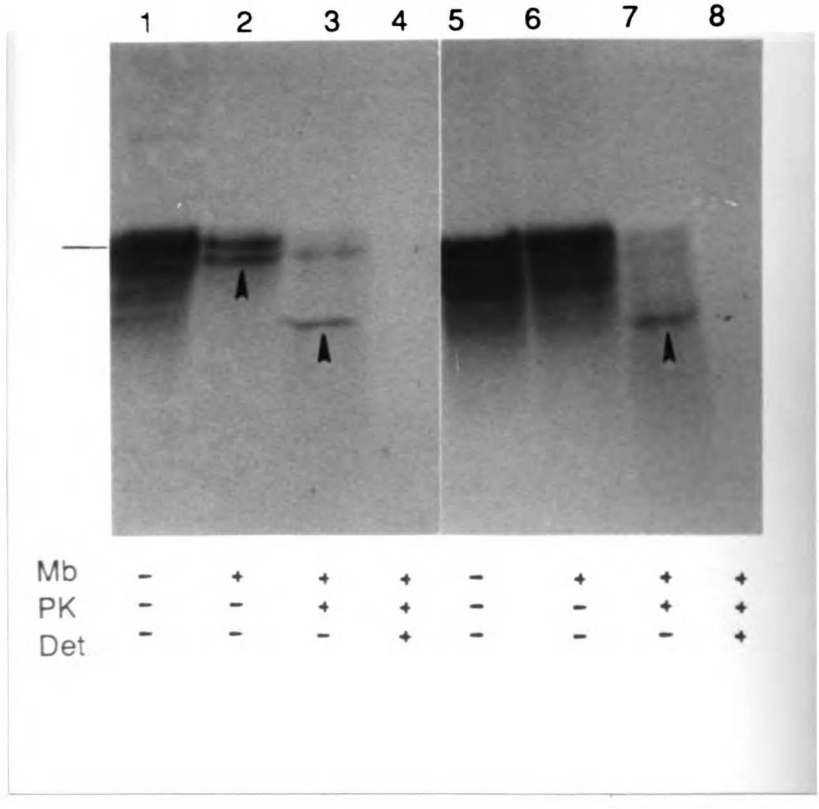
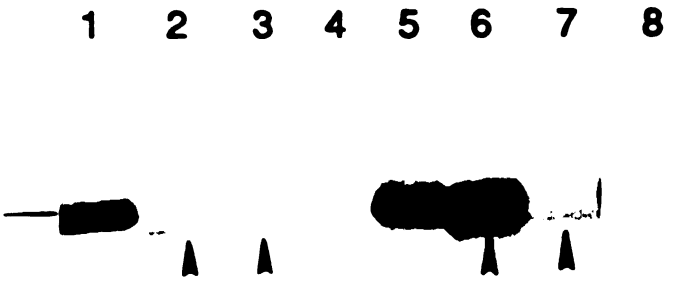

1 2 3 4 5 6 7 8
Mb - + + + - + + +
PK - - + + - - + +
Det - - - + - - - +

FIGURE 17. Posttranslational translocation of PrP/HcII nascent chains synthesized in RRL and analyzed by SDS-PAGE, and autoradiography of NH_2-terminal specific antisera immunoprecipitates (lanes 5 to 8). Simultaneous co-translational translocation of PrP/HcII is also shown (lanes 1 to 4). Transcription, co- and posttranslational translocation, and proteolysis were as described in figure 16, except performed with RRL.[114] Hatch mark at left denotes the PrP/HcII precursor. Arrows denote major products.



Mb	–	+	+	+	–	+	+	+
PK	–	–	+	+	–	–	+	+
Det	–	–	–	+	–	–	–	+

TABLE 1. Scanning laser densitometry (LKB Ultroscan) was performed on autoradiograms (of both total translation products and immunoprecipitates) to quantify relative proportions of protected fragments generated by proteolysis. Data was corrected to account for the differing methionine content of the various fragments.

% Chains Transmembrane at TM1			
		Reticulocyte	Wheat Germ
haPrP		40	90
VSVGxk		33	100
PrP/hcII	(post)	38	84

FIGURE 18. WG and RRL PrP/HcII translation inhibition controls analyzed by SDS-PAGE and autoradiography of total products. Experiment was performed as described in text.

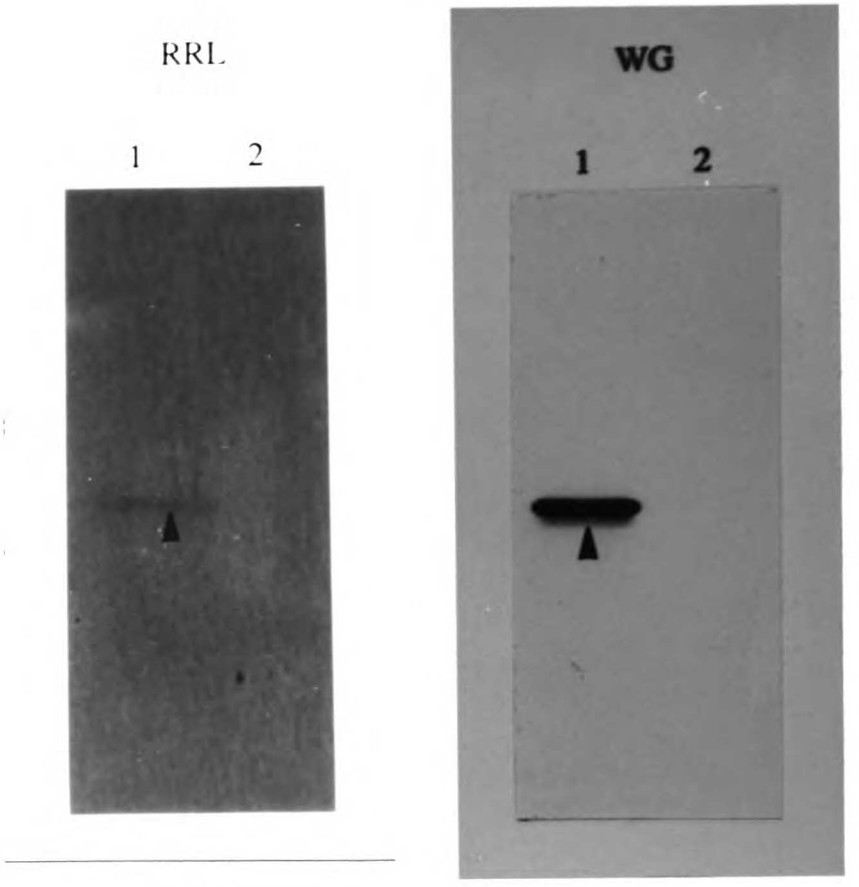

RRL
1
2
WG
1
2

FIGURE 19. Time-course of posttranslational translocation of PrP/HcII nascent chains synthesized in WG and analyzed by SDS-PAGE and autoradiography, of NH_2-terminal domain specific antisera immunoprecipitates (as described in previous figure legends). PrP/HcII was synthesized for 30 minutes, adjusted to 0.1 mM ATA and 0.1 mM emetine as described in previous figure legends, and incubated without (lane 1) or with microsomes for 10 minutes (lanes 2 and 3), 35 minutes (lanes 4 and 5), and 55 minutes (lanes 6 and 7). Proteolysis was performed under conditions previously described with (lane 8) or without detergent (lanes 3, 5 and 7). Hatch mark at left denotes the PrP/HcII precursor. Horizontal arrow in lane 2 denotes processed PrP/HcII. Upward pointing arrows denote major protected products.

1 2 3 4 5 6 7 8

When engineered into the coding region of a heterologous protein, the topogenic sequence should confer the system-dependent behavior observed for PrP onto the resulting chimeras. Figures 20 and 21 respectively show expression in WG and RRL of the chimeric protein encoded in plasmid pSPVSV Gxk in which codons 74 to114 of PrP (henceforth called R74-114) have been engineered into codon 346 of the glycoprotein of vesicular stomatitis virus (VSV G) (Figure 14B). R74-114 includes the codons for TM1, as well as the 16 codons preceding the hydrophobic domain (STE). The identity of the fusion protein was further confirmed by immunoprecipitation (Figure 22). VSV G is a transmembrane protein containing an NH_2-terminal signal sequence. It is largely translocated, but anchored by a well characterized stop transfer sequence near its COOH-terminal.[101] If a topogenic sequence responsible for the difference in PrP topology in WG versus RRL is included within R74-114, expression of VSV Gxk in WG should generate predominantly a transmembrane orientation with TM1 spanning the bilayer. However, in RRL most chains of VSV Gxk should not stop at R74-114, and the predominant topology would be expected to be similar to that of native VSV G.

Translation of VSV Gxk in WG in the presence of membranes resulted in a product of higher molecular weight than that observed in the absence of membranes (Figure 20). This decrease in mobility on SDS-PAGE was due to core glycosylation, which offsets the increased mobility resulting from cleavage of the NH_2-terminal signal sequence.[127] As predicted, proteolysis revealed this product to be

transmembrane at the position of the inserted PrP codons (Figure 20 lane 3). The topology of native VSV G is illustrated in lanes 5 to 8 by a similar experiment. Again, a glycosylated product was observed when synthesized in the presence but not the absence of membranes. Proteolysis resulted in a much smaller size shift of the protected product because of the smaller cytoplasmic domain encoded in native VSV G versus VSV Gxk.

When this experiment was performed in RRL, native VSV G displayed the same orientation as in WG (Figure 21). Likewise, most chains of VSV Gxk synthesized in RRL displayed a topology similar to that of native VSV G. Thus, in both WG and RRL, the chimeric protein VSV Gxk displayed the topology preference at TM1 observed for PrP: TM1 was predominantly transmembrane in WG and translocated in RRL. These conclusions were confirmed by quantitative densitometry. Similar chimeras with R74-114 were generated from two other heterologous proteins, rat lactalbumin[132] and a beta-lactamase-chimpanzee globin fusion protein.[104] In both cases expression of these chimeras resulted in similar system-dependent predominance of transmembrane (in WG) and secretory (in RRL) phenotypes. Expression of VSV Gxk in Xenopus oocytes also resulted in both the transmembrane and completely translocated forms observed in cell-free systems (Figure 23).

Taken together with the demonstrated independence from ongoing protein synthesis, these data argue strongly for the presence of a topogenic sequence within R74-114.

FIGURE 20. WG cell-free, transcription-linked, translation-coupled translocation of products encoded by plasmids pSPVSV Gxk (lanes 1 to 4) and pSPVSV G (lanes 5 to 8) as described in Figure 14B, analyzed by SDS-PAGE and autoradiography of total products. Transcription, translation, and proteolysis were as described in previous figure legends. Schematic representation of VSV Gxk and VSV G topologies are shown at the bottom of the figure. Arrows denote major products. Downward pointing arrow in lane 3 indicates band co-migrating with that of upward pointing arrow in Figure 21 lane 3. Hatch mark at left denotes VSV Gxk precursor.

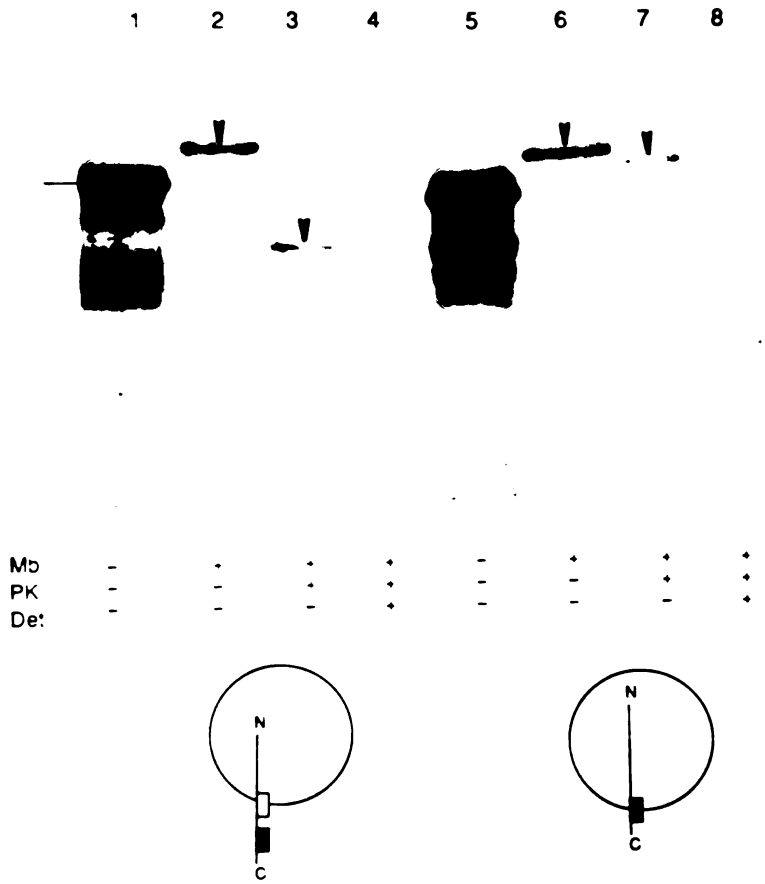

1 2 3 4 5 6 7 8
Mb
PK
Det
N
C
N
C

FIGURE 21. RRL cell-free, transcription-linked, translation-coupled translocation of products encoded by plasmids pSPVSV Gxk (lanes 1 to 4) and pSPVSV G (lanes 5 to 8) as described in Figure 14B, analyzed by SDS-PAGE and autoradiography of total products. Transcription, translation, and proteolysis were as described in previous figure legends. Schematic representation of VSV Gxk and VSV G topologies are shown at the bottom of the figure. Arrows denote major products. Upward pointing arrow in lane 3 indicates band co-migrating with that of downward pointing arrow in Figure 20 lane 3. Hatch mark at left denotes VSV Gxk precursor.

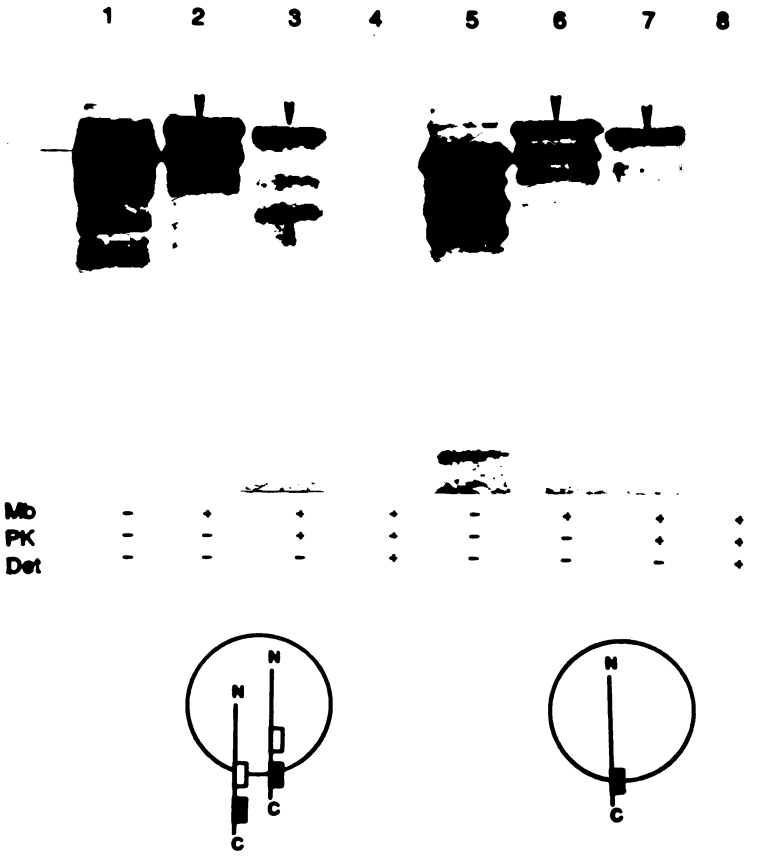
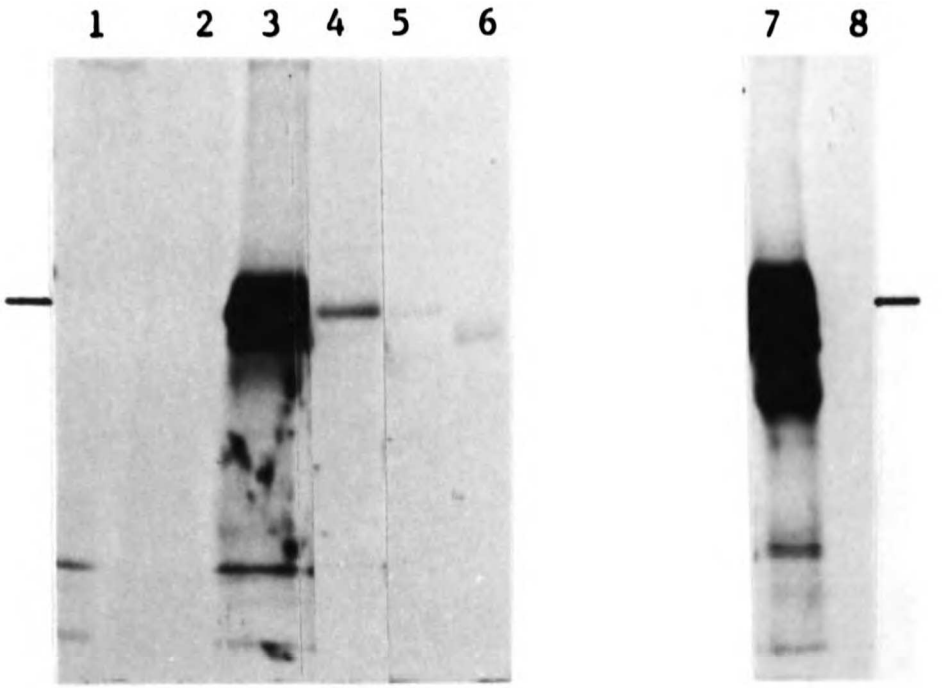

1
2
3
4
5
6
7
8
Mb
PK
Det
N
C
N
C
N
C

FIGURE 22. RRL cell-free transcription-linked translation coupled translocation of pSPVSV Gxk (lanes 1, 2 to 6) and pSPVSV G (lanes 2, 7 and 8) analyzed by SDS-PAGE and fluorography of immunoprecipitates. Lanes 1 and 2, preimmune sera; lanes 3 and 7, polyclonal sera against the Indiana strain of VSV (Lee BioMolecular Research); lanes 4 to 6, and 8, peptide specific antisera against the NH_2-terminal domain of PrP $(P1)^{113}$ which includes a portion of R_{74-114}. Lane 6 treated with PK as described in previous figure legends.


1
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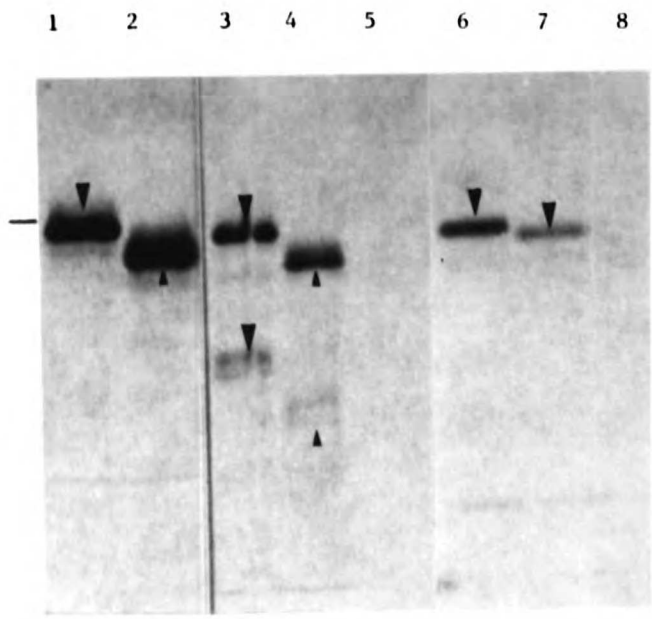
4
5
6
7
8

FIGURE 23. Expression of pSPVSV Gxk (lanes 1 to 5) and pSPVSV G (lanes 6 to 8) in Xenopus oocytes analyzed by SDS-PAGE and fluorography of anti-VSV antisera immunoprecipitates. Transcription, microinjection, labeling, homogenization and proteolysis as described in figure legend 13. Digestion with endoglycosidase H (lanes 2 and 4) as previously described.[113] PK digestions were performed with (lanes 5 and 8) or without detergent (lanes 3, 4 and 7). Arrows denote major products. Downward pointing arrow in lane 3 co-migrates with downward pointing arrow in lane 3 of figure 20.



	1	2	3	4	5	6	7	8
VSVGxk mRNA	+	+	+	+	+	-	-	-
VSVGwt mRNA	-	-	-	-	-	+	+	+
PK	-	-	+	+	+	-	+	+
ENDO H	-	+	-	+	-	-	-	-
Det	-	-	-	-	+	-	-	+

Typically, stop transfer sequences comprise a hydrophobic domain of approximately 20 to 25 amino acid residues preceded by a charged or polar domain of 10 to 15 residues.[9] R74-114 likewise includes a 16-residue charged and polar region preceding a 24-residue hydrophobic domain (TM1) that is similar to other membrane-spanning regions when analyzed for hydropathy by methods of Eisenberg[119] or Kyte and Doolittle.[113] The function of this topogenic sequence is dependent on features of both the polar and hydrophobic domains as described in Chapter II.[12]

Unlike other stop transfer sequences, the topogenic sequence defined here terminates the preponderance of chain translocation across microsomal membranes in WG, but not in RRL. Thus, factors in WG or RRL, independent of protein synthesis, either engage or prevent the action of this unusual topogenic sequence. To establish an assay for these factors, we performed mixing experiments. Increasing amounts of RRL added to PrP/HcII WG translation products resulted in the saturable generation of the secretory form upon addition of membranes (Figure 24). If the RRL was added after the membranes (Figure 27 compare lanes 4 and 5) or if it was first treated at 80°C for 10 minutes (Figure 25 compare lanes 9 and 10), the effect on topology was considerably diminished. In the converse experiment, addition of WG to PrP/HcII RRL translation products, and subsequent incubation with membranes, resulted in no change in topology from that observed for RRL translation alone. Thus, it appeared that a heat-labile factor in RRL is necessary for the efficient generation of the secretory form of PrP.

RRL presents unique problems as a tissue source for the purification of the cytosolic factor(s) because of the large amounts of globin and an intrinsic proteolytic activity present in the crude fraction (Figure 27 lanes 7-10). A preliminary fractionation of RRL will probably be a necessary first step before more refined procedures such as ion-exchange chromatography can be employed. RRL was centrifuged at high speed (100,000 X G for 30 minutes) to pellet ribosomes and associated complexed molecules (Figure 26B lane 3). The resultant post-ribosomal supernatant (PRS) (Figure 26B lane 2) contained the cytosolic factor(s) as determined by the posttranslational translocation mixing assay (Figure 26A). Thus, it appears that the secretory stimulating activity is a soluble factor.

To screen for the cytosolic factor(s) in RRL, the efficiency of the posttranslational translocation assay needed to be improved. Initially, the mixing assay was hampered by the presence of uncharacterized bands when analyzed by proteolysis (Figures 25 and 26). However the assay was considerably improved by 1) using WG that had not undergone more than two freeze-thaw cycles, 2) raising the final concentration of DTT to 2 mM and, 3) increasing the concentration of proteinase K during the protection assay to account for the increase in protein concentration after mixing. Even further improvement of the assay was achieved by either 1) shortening the incubation time between the WG translation products and RRL to less than five minutes at 24° C or, 2) incubating WG translation products and RRL for up to 60 minutes at 4° C (Figure 27).

The globin and background intrinsic proteolytic activity present in both RRL and PRS fractions can be removed by stepwise sequential precipitation with increasing amounts of ammonium sulfate. Globin remained soluble until 60 to 80 percent saturation. The background intrinsic proteolytic activity precipitated at the 40 to 60 percent saturated step. If it can be shown that ammonium sulfate precipitation will not inactivate the factor, this technique will prove useful during purification.

A cytosolic extract prepared from rabbit erythrocyte lysate (REL) was found to be an alternate tissue source for the secretory stimulating factor (Figure 28). Presumably during the process of reticulocyte to erythrocyte maturation the cytosolic factor(s) is not lost or inactivated in appreciable amounts. It remains to be seen if the factor(s) exists in other cell lineages. REL is more readily available and less expensive to prepare than RRL which will prove useful during purification.

FIGURE 24. Plot of representative immunoprecipitation SDS-PAGE densitometry data of a posttranslational translocation assay with PrP/HcII synthesized in WG and mixed with increasing amounts of RRL before addition of membranes (solid squares), with RRL treated for 10 minutes at 80°C (open squares), or RRL added after incubation with membranes (dotted line). The y-axis represents percentage of total translocated chains in the secretory form as defined by protease protection. The x-axis represents percentage of added RRL as final reaction volume.

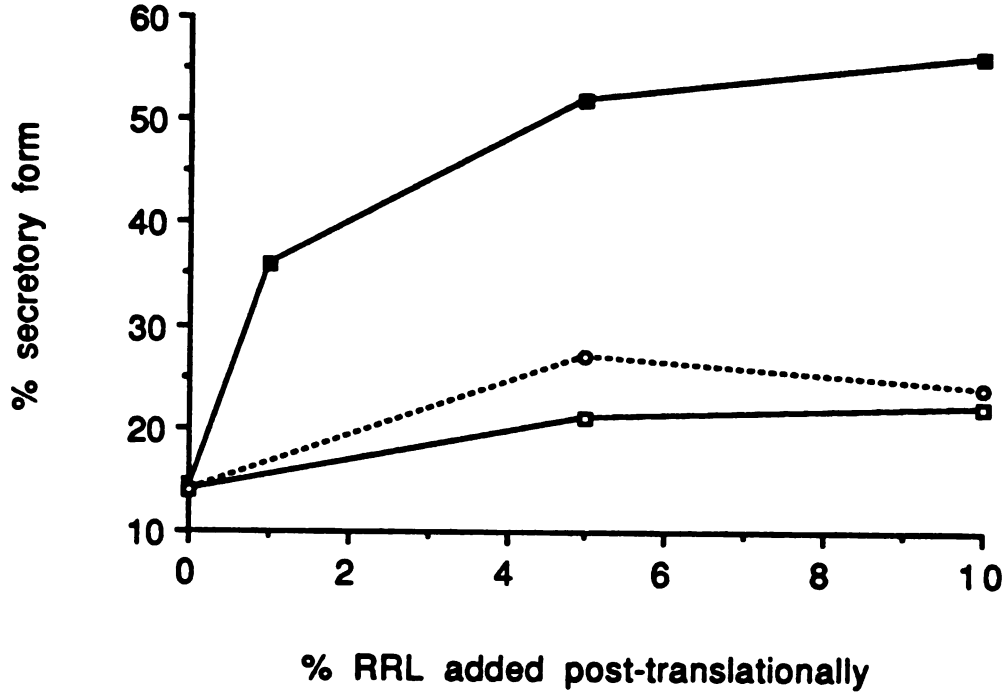
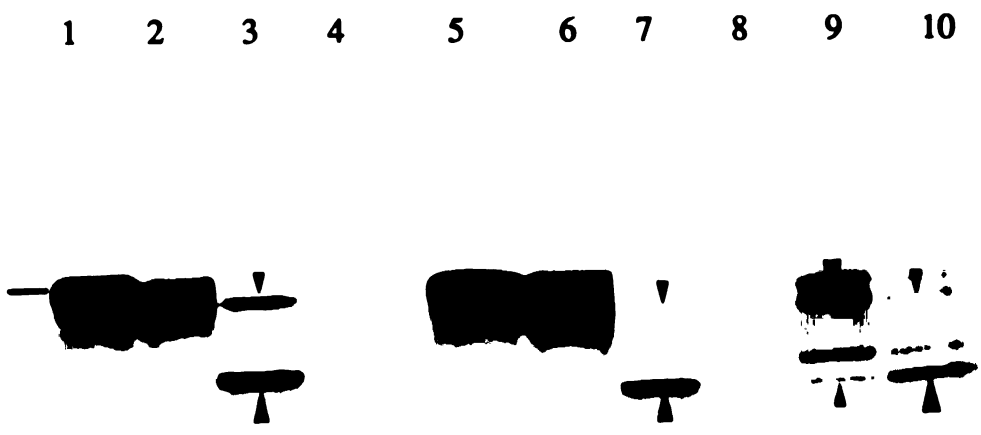

60
50
40
30
20
10
0
2
4
6
8
10
% secretory form
% RRL added post-translationally

FIGURE 25. Posttranslational translocation assay with PrP/HcII synthesized in WG and mixed with RRL before incubation with membranes (lanes 5 to 10) and a simultaneous co-translational translocation assay with PrP/HcII as described in figure 16 (lanes 1 to 6), analyzed by SDS-PAGE and autoradiography. PrP/HcII precursor synthesized in the absence of membranes seen in lanes 1 and 5, while processed PrP/HcII seen in lanes 2 and 6. PrP/HcII translation product incubated at 24°C for 10 minutes with 30 percent RRL (lanes 8 and 9) or 30 percent RRL first treated at 80°C for 10 minutes (lane 10) before posttranslational addition of membranes. Posttranslocational PK digestions were performed as previously described with (lanes 4 and 8) or without detergent (lanes 3, 7, 9, and 10). Downward pointing arrows denote processed, fully translocated PrP/HcII while upward pointing arrows denote NH_2-terminal fragments generated upon proteolysis of chains stopped in the membrane at R74-114. Large arrows denote the major of the two bands indicated in each lane as confirmed by quantitative densitometry corrected for methionine distribution.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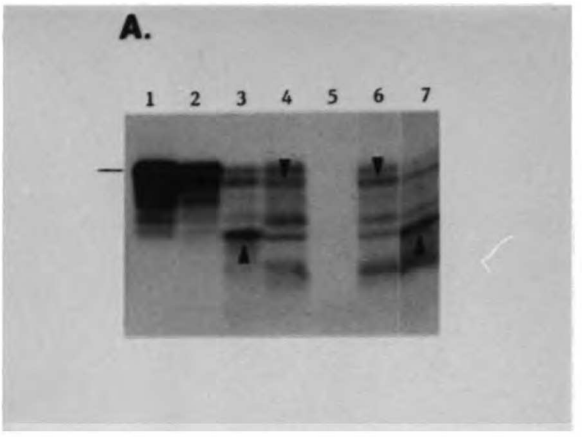
9
10

FIGURE 26. Panel A. Posttranslational translocation assay with PrP/HcII synthesized in WG and mixed with RRL or PRS before incubation with membranes, analyzed by SDS-PAGE and autoradiography. Lane 1 represents PrP/HcII precursor. Lanes 2 to 7 represent PrP/HcII translation products posttranslationally presented to membranes as described in previous figure legend, with no preincubation (lanes 2 and 3), with preincubation with 30 percent RRL (lane 4), with 30 percent PRS (lanes 5 and 6), or with 30 percent PRS first treated at 80°C for 10 minutes (lane 7). PK digestions were performed as described previously with (lane 5) or without detergent (lanes 3, 4, 6 and 7). Downward pointing arrows denote processed, fully translocated PrP/HcII while upward pointing arrows denote NH_2-terminal fragments generated upon proteolysis of chains stopped in the membrane at R_{74-114}. Panel B. Total protein in RRL (lane 1), PRS (lane 2), and ribosomal pellet (lane 3) as analyzed by SDS-PAGE and Coomasie staining. PRS prepared as described in text.


A.
1 2 3 4 5 6 7

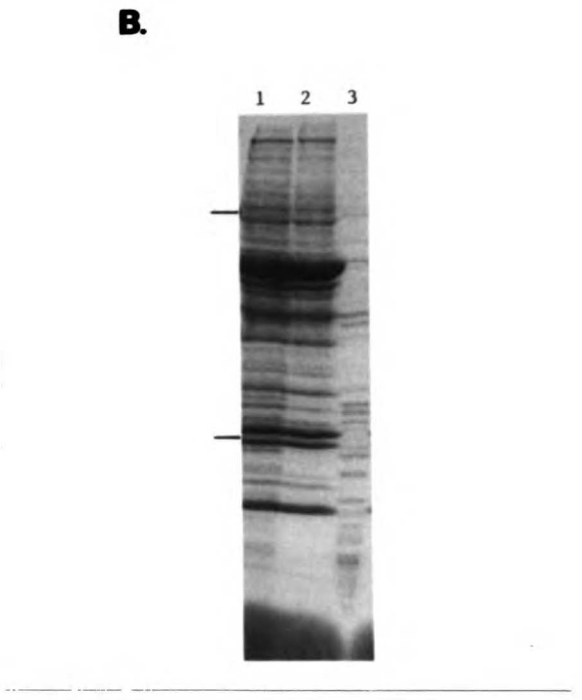
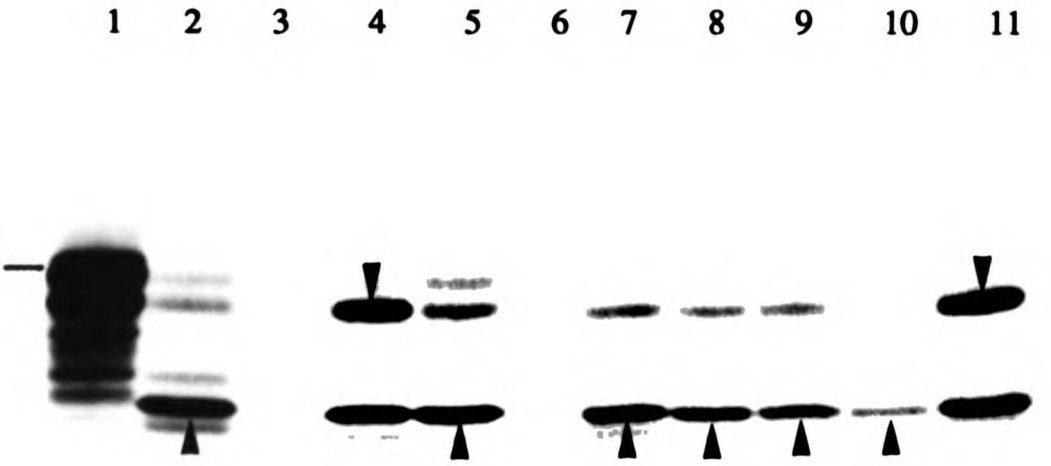

B.
1 2 3

FIGURE 27. Posttranslational translocation assay with PrP/HcII synthesized in WG and mixed with RRL before incubation with membranes (lanes 3, 4, 7 to 11) or after incubation with membranes (lane 5), analyzed by SDS-PAGE and autoradiography. Lanes 1 and 2 respectively represent PrP/HcII precursor alone or presented posttranslationally to membranes without RRL preincubation; lane 6 represents translation product incubated with RRL but without addition of membranes. WG PrP/HcII translation products preincubated with 30 percent RRL at 24°C for 5 minutes (lane 4), 15 minutes (lane 7), 30 minutes (lane 8), 45 minutes (lane 9), and 60 minutes (lane 10) before posttranslational addition of membranes. Lane 11 represents WG PrP/HcII translation products preincubated with 30 percent RRL at 4°C for 60 minutes before posttranslational addition of membranes. Posttranslocational PK digestions performed with (lane 3) or without detergent (lanes 2, 4 to 11) as described previously except the final concentration of PK was at 0.6 mgs/ml. Downward pointing arrows denote processed, fully translocated PrP/HcII while upward pointing arrows denote NH_2-terminal fragments generated upon proteolysis of chains stopped in the membrane at R_{74-114}.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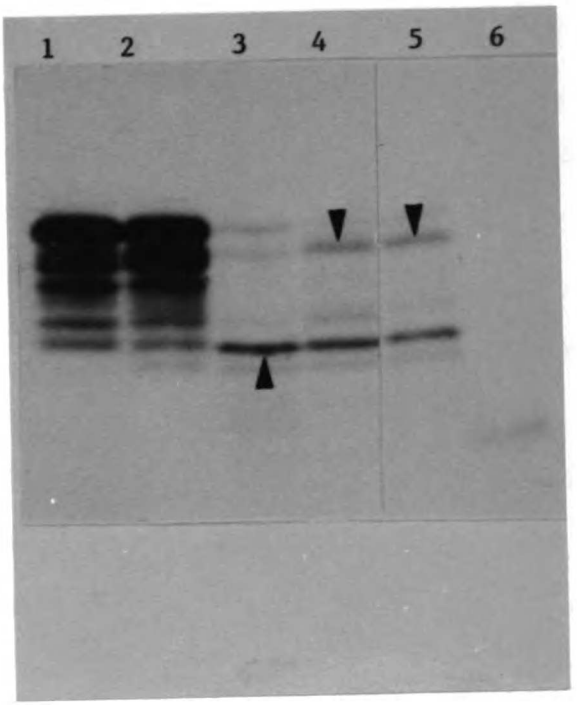
10
11

FIGURE 28. Posttranslational translocation assay with PrP/HcII synthesized in WG and mixed with RRL or REL before incubation with membranes, analyzed by SDS-PAGE and autoradiography. Lane 1 represents PrP/HcII precursor. Lanes 2 to 6 represent PrP/HcII translation products posttranslationally presented to membranes as described in previous figure legend, with no preincubation (lane 2 and 3), with preincubation with 30 percent RRL (lane 4), or with 30 percent REL (lanes 5 and 6). Proteolysis performed as described previously with (lane 6) or without detergent (lanes 3 to 5). Downward pointing arrows denote processed, fully translocated PrP/HcII while upward pointing arrows denote NH_2-terminal fragments generated upon proteolysis of chains stopped in the membrane at $R_{74\text{-}114}$. REL prepared as RRL[13] except rabbits were not injected with phenylhydrazine.


1
2
3
4
5
6

Discussion.

If R74-114 is an unusual form of stop transfer sequence subject to regulation at the level of receptor interactions, perhaps conventional stop transfer sequences also act in a receptor-mediated fashion.[95,96] Alternatively, PrP may be a member of a rare class of proteins that use cofactors to promote or inhibit receptor-mediated stop transfer.

It seems likely that the transmembrane and secretory forms represent different folding states of PrP. Indeed, the expression of this single coding region in two different topologic forms may be a consequence of alternate pathways of protein folding. In this case our findings would suggest (i) a novel solution for the paradox of PrP isoforms (each functionally distinct yet identical in sequence and modifications), (ii) an unusual level of biological regulation with multiple fates and hence multiple functions encoded within a single species of mRNA, and (iii) new variables and caveats to be considered in the prediction of protein structure from primary sequence.

The data presented in Chapter II argues for the presence of a receptor in the membrane of the RER for STE. Engagement of this receptor determines whether the translocation machinery will ultimately integrate an adjacent hydrophobic stretch of amino acids into the lipid bilayer. In light of the mounting evidence for a proteinaceous translocation channel[33,44,51] as well as for a proteinaceous stop transfer receptor[46], the obvious direction to take will be to attempt to characterize and purify the RER membrane associated STE receptor. Whether this mode of stop transfer is unique to PrP or is a general one remains to be seen but at least one other stop transfer sequence has now been shown to contain an STE-like domain.[105]

The data presented in Chapter III provides evidence that the STE-TM1 domain (R_{74-114}) is an unusual topogenic sequence in that a cytosolic factor can control whether it stops in the membrane. This soluble factor present in reticulocyte or erythrocyte lysates prevents the stop transfer machinery in the RER membrane from interacting with STE-TM1. Alternatively, perhaps it engages other receptors that promote complete translocation of nascent peptide. How the cytosolic factor engages STE-TM1 to effect translocation remains a mystery. However, of the cytosolic factors that are known to be involved in protein translocation, many are intimately involved in protein folding.[24-26,28,29] Thus, one could envision the RRL cytosolic factor functioning to alter STE-TM1 conformation such that it is either unable to engage the stop transfer mechanism or can recognize different specialized translocation machinery. Alternatively, the cytosolic factor

could actually modify the nascent chain itself with identical results. Purification of the factor is now possible since the mixing assay has been improved to high efficiency.

The concept that topogenesis can be regulated to confer alternate fates has profound implications for biology; a protein with different topological forms can have different functional phenotypes. The ability to control these fates offers the cell expanded control over its genome in addition to permitting rapid responses to changes in the environment. Examples from other proteins suggest that alternate topological fates play a role in nature. For instance it is known that during the biosynthesis of hepatitis B surface antigen particles (HBsAg), HBsAg is first synthesized as transmembrane monomers that are then posttranslationally assembled into secretory particles.[133,134] While the topology of the transmembrane proteins probably do not change with respect to a membrane, the topology of the generated particle becomes secretory. Perhaps a more dramatic example is that of apolipoprotein B (apoB), which is initially synthesized as a series of non-integrated transmembrane intermediates that chase into the mature secretory form in an energy dependent manner.[135,136] Whether the membrane spanning domains act through an STE-like regulated mechanism is unclear but is being investigated. An interesting example of a potential STE-like domain exists in the stop transfer sequence of the amyloid precursor protein (APP).[137-139] Like PrP, the single hydrophobic membrane spanning amino acid stretch is unusually glycine and alanine rich. Furthermore, the adjacent extracytoplasmic domain is also very positively charged as is STE. APP is known to extract from membranes

under high ionic strength conditions[140] which implies that the hydrophobic transmembrane domain may not necessarily be integrated into the bilayer at all times. Rather, an interaction with proteinaceous receptors in the membrane, perhaps via the extracytoplamsic STE-like domain, may be occuring. This is important because generation of the beta-amyloid peptide in Alzheimers disease is thought to occur via aberrent proteolytic processing.[141] The carboxy terminal cleavage site of the beta-amyloid peptide is normally buried in the lipid bilayer and not cleaved in normal brain. Perhaps regulation at the APP stop transfer sequence (e.g. complete translocation) permits amyloid peptide generation. The recent discovery that a hereditary form of cerebral hemorrhage (involving abnormal deposition of amyloid plaques) is due to a point mutation in the STE-like domain of APP[142], suggests that regulated topogenesis may play a role in this process. It is interesting that point mutations in STE are seen in PrP from mouse strains with different scrapie incubation times as well as in the human prion disease Gerstmann-Straussler syndrome.[124-126]

The normal function of PrP remains unknown. The importance of the regulated topogenesis remains unappreciated at this time although no doubt it will prove crucial to understanding PrP's function. The details of PrP involvement in the pathogenesis of the disease scrapie also remain to be worked out. It is interesting to speculate that the alternate topological fates may play a role in the disease process. It is known that PrP in normal animals is developmentally expressed in early post-natal life. This occurs at a time of neuronal cell death and remodelling in the CNS.[129] Perhaps then, the normal function of PrP is to signal or even

cause neuronal cell death in a programmed fashion. Most interesting is the resemblance of the transmembrane form of PrP to an ion-channel subunit.[143,144] Assembly of a channel in the membrane could permit rapid signalling (for cell death) or even cell death itself. Regulation of the alternate fates of PrP would therefore be equivalent to regulation of cell death. An aberrent state in the cell in which the transmembrane form is inappropriately expressed would result in the massive and uncontrolled cell death seen in the prion induced spongiform encephalapathies. Introduction of a modified form of exogenous PrP (e.g. aggregated or altered conformation) could stimulate loss of control over PrP topogenic regulation and could be the insult leading to an irreversible cascade of events resulting in neuronal cell death. These events themselves would lead to the generation of more modified PrP and hence, continued propagation of the signal for cell death to other neurons. Perhaps cleavage of the PI anchor of PrP may also play an important role in signal transmission.

If scrapie and PrP biogenesis are related, the cytosolic and membrane factors that control PrP topology will prove interesting from a developmental biology perspective and perhaps offer a novel solution to the paradox of PrP isoforms (each functionally distinct yet identical in sequence). Even more importantly, the observation of the phenomena of alternate topology reveals another level of biological regulation with multiple fates, and hence multiple functions, encoded within a single species of mRNA. Control over STE in PrP, and STE-like domains in other proteins, may prove to ultimately occur via the actions of common membrane and cytosolic factors. Likewise, receptor-mediated stop

transfer may also utilize the same cellular machinery. Thus, even though our knowledge of the mechanisms for generating protein topology remains limited, PrP has provided us with a look into the future--and protein biogenesis is proving to be more complex than could have been imagined.

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