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Signal Sequences Control Gating of the Protein Translocation Channel in a Substrate-Specific Manner

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Summary

N-terminal signal sequences mediate targeting of nascent chains to the endoplasmic reticulum and facilitate opening of the protein translocation channel to the passage of substrate. We have assessed each of these steps for a diverse set of mammalian signals. While minimal differences were seen in their targeting function, signal sequences displayed a remarkable degree of variation in initiating nascent chain access to the lumenal environment. Such substrate-specific properties of signals were evolutionarily conserved, functionally matched to their respective mature domains, and important for the proper biogenesis of some proteins. Thus, the sequence variations of signals do not simply represent functional degeneracy, but instead encode critical differences in translocon gating that are coordinated with their respective passengers to facilitate efficient translocation.

Introduction

Segregation of secretory and membrane proteins into the mammalian secretory pathway is mediated by signal sequences that perform two principal functions. The first is to direct targeting of nascent proteins to sites of translocation at the endoplasmic reticulum (ER) membrane (reviewed in Walter and Johnson, 1994; Rapoport et al., 1996; Johnson and van Waes, 1999). As a signal sequence emerges from the ribosome, it is recognized and bound by signal recognition particle (SRP). The ribosome nascent chain-SRP complex is then targeted, via an interaction with the SRP receptor, to a Sec61p-containing translocation channel.

The second, more recently appreciated function of signal sequences is in mediating the opening of the translocation channel to the lumenal environment (Simon and Blobel, 1991; Crowley et al., 1994; Jungnickel and Rapoport, 1995; Hanein et al., 1996). Shortly after targeting, three biochemically discernable reactions, each dependent on a signal sequence, occur in succession: (1) high-salt-resistant binding of the ribosome to the translocon, thought to signify insertion of the nascent chain into the translocation site, (2) formation of a tight seal between the nascent chain exit tunnel in the ribosome and the translocon, and (3) opening of the translocation channel toward the lumen. This series of steps has three important functions. First, by sealing the cytosolic side of the translocon before opening the lumenal side, the permeability barrier of the ER membrane can be maintained during the initial stages of translocation (Crowley et al., 1994). Second, recognition of signals at the translocon may improve fidelity of sorting by effectively "proofreading" a signal sequence before the substrate is allowed to begin translocation (Jungnickel and Rapoport, 1995). And third, completion of these steps results in the formation of a continuous conduit for the nascent chain from the peptidyl transferase center within the ribosome to the ER lumen (Crowley et al., 1993, 1994; Beckmann et al., 1997).

At present, it is not clear whether the same features of a signal sequence that mediate SRP-dependent targeting are also recognized by the translocon. Statistical analyses of signal sequences have revealed that they share little or no sequence homology between substrates (von Heijne, 1985). The only consistently recognizable motif common among signals is a hydrophobic core of at least six amino acids. Aside from this, signal sequences are remarkably diverse even with respect to general features such as length, overall hydrophobicity, and net charge (von Heijne, 1985; Zheng and Gierasch, 1996). This long-standing observation has raised two puzzling questions. First, how can such a degenerate feature be exploited to achieve a high fidelity of sorting? And second, why are signals so complex and diverse, when in principle, protein segregation could be accomplished by a much smaller and specific sequence? Insight into the first question has been steadily growing with the identification and characterization of the protein targeting and translocation machinery that recognizes signal sequences (Bernstein et al., 1989; Jungnickel and Rapoport, 1995; Keenan et al., 1998; Mothes et al., 1998; Batey et al., 2000). In contrast, a satisfactory answer to the second question has been lacking.

Two general possibilities exist for explaining signal sequence diversity. It is plausible that the primary sequence requirements for signal sequence function are sufficiently degenerate that there is little evolutionary pressure to maintain a specific sequence. Thus, as long as certain general features such as hydrophobicity are maintained, polymorphic changes within the signal might be readily accommodated without changes in function. Alternatively, signal sequences may be functionally distinct in ways that are optimized for their substrate. In this view, the sequence diversity would not be due to random variations from a lack of selective pressure, but instead due to the diversity of secretory and membrane proteins for which these signals have been optimized. How might these possibilities be distinguished? If indeed the diversity of signal sequences reflects a substrate specificity, then one might anticipate the following findings. First, substantial differences should be detectable in at least some aspects of signal sequence function for different substrates. Second, the functional features peculiar to a given substrate should be evolutionarily conserved to match the conservation of the mature protein. And third, alterations of the conserved feature, even within the range observed for other normal signal sequences, should result in a substantial

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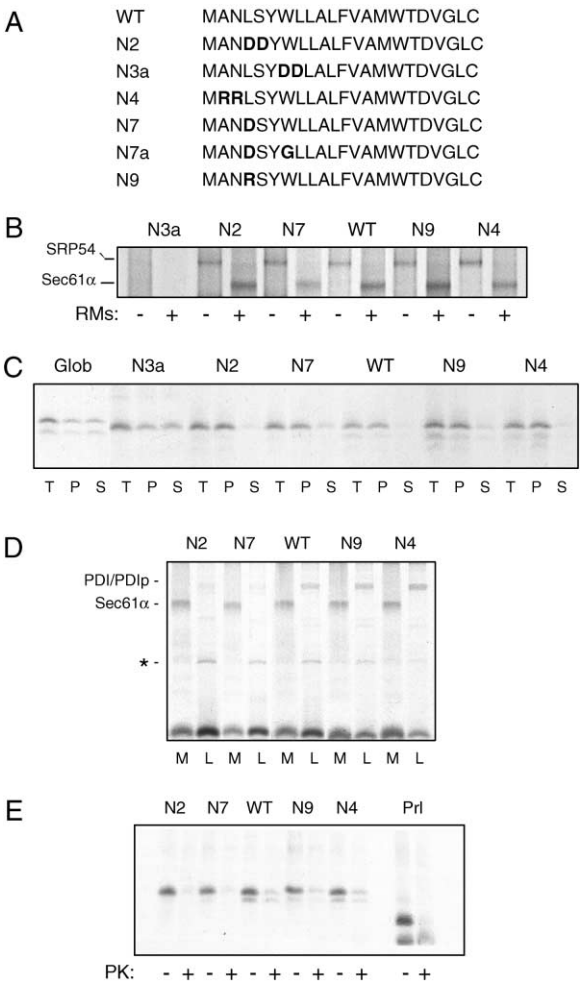

Figure 1. Identification of Signal Sequence Mutants Selectively Defective in Translocon Gating

(A) Sequence of the wild-type and mutant PrP signal sequences, with mutations indicated in bold type.

(B) Nascent chains of 91 residues of various signal mutants were synthesized in the absence or presence of RMs as indicated, and subjected to crosslinking with disuccinimidyl suberate (a homobifunctional amine-reactive crosslinker) as described in Experimental Procedures. The positions of crosslinks to SRP54 and Sec61α (verified by immunoprecipitation; data not shown) are indicated.

(C) 91-mer nascent chains of various signal mutants were synthesized in the presence of RMs, the samples adjusted to high salt (0.5 M KAc), and the RMs isolated by sedimentation. An equivalent amount of the total translated material (T), isolated membrane pellet (P), and supernatant (S) were analyzed.

(D) Translocation intermediates of 112 residues were prepared, isolated after high-salt treatment as in (C), and subjected to crosslinking with disuccinimidyl suberate as in (B). Following crosslinking, samples were fractionated into lumenal (L) and membrane (M) proteins prior to analysis. The positions of crosslinks to ubiquitous and pancreas-specific protein disulfide isomerases (PDI/PDIp) and Sec61α are indicated. The asterisk indicates the position of residual tRNA-associated translation product that was also seen in the absence of crosslinker (data not shown).

(E) Salt-resistant 112-mer translocation intermediates of the PrP signal mutants and the 86-mer of preprolactin were prepared as in (D), and then treated with 1 mM puromycin to release the nascent chains from the ribosomes. Translocation into the lumen was assessed by digestion with proteinase K (PK) as indicated. The lower band observed with the wt, N9, N4, and Prl samples represents signal-cleaved nascent chains. Note that the N7a mutant (identified inadvertently as a mutant of N7) was found to behave essentially identically to N2 by the assays in (B)–(E) (data not shown).

and discernable consequence for the protein's biogenesis. In order to examine these ideas experimentally, sensitive and specific assays of different aspects of signal sequence function are required. In this study, we have taken advantage of recent insights into the role of signal sequences in prion protein (PrP) biogenesis (Kim et al., 2001; Rutkowski et al., 2001) to devise an assay to quantitatively measure differences in the posttargeting functions of signal sequences from a variety of substrates. This has allowed us to explore the question of whether the sequence diversity of signal sequences reflects physiologically relevant functional differences.

Results

Identification of Signal Sequence Mutants that Selectively Affect a Posttargeting Function

To aid in developing a simplified assay for signal sequence functions, we took advantage of a series of point mutants in the signal sequence of the prion protein (PrP) that were generated for another study (Kim et al., 2001). These PrP signal mutants (Figure 1A) were compared by several assays that used short nascent chain intermediates (of less than 112 amino acids) to examine the early stages of their translocation. It is important to note that the only topogenic element in these translocation intermediates is the signal sequence; the potential transmembrane domain (TMD) of PrP (residues 113–135) has not been synthesized yet. In the first assay, nascent chain intermediates of 91 amino acids were compared by crosslinking assays in the presence and absence of rough microsomal membranes (RMs). Figure 1B shows that, with the exception of the N3a mutant, all of the signals crosslinked to SRP54 in the absence but not the presence of RMs. In the presence of RMs, all of the constructs (except N3a) were seen crosslinking to Sec61α (see also Figure 1D).

Next, we assessed whether these translocation intermediates had bound to RMs in a salt-resistant manner. Several previous studies have shown that upon initial transfer to the translocation channel, nascent chains are bound in a salt-sensitive manner (Jungnickel and Rapoport, 1995; Zheng and Nicchitta, 1999). Only after additional chain synthesis (beyond approximately 60 total amino acids in the case of prolactin) is the nascent chain bound in a salt-resistant manner that is dependent on a functional signal sequence. Analysis of 91 amino acid translocation intermediates of the signal mutants revealed that with the exception of N3a, all of them were bound to the membrane in a salt-resistant manner (Figure 1C). N3a was extracted to a substantial degree with high salt, comparable to the negative control Glob-PrP (in which the signal sequence of PrP is replaced with the first 22 amino acids from the cytosolic protein globin). Together with Figure 1B, these data suggest that each of the signal mutants except N3a is functional in directing SRP-mediated targeting, transfer to a

Sec61α-containing translocon, and high-salt-resistant insertion of nascent chains into the translocation site.

The subsequent step in translocation, also dependent on a functional signal sequence, is opening of the channel toward the ER lumen to allow passage of the nascent chain. In the present study, we define this step operationally as *gating* of the translocon because it is the step at which an initially closed channel is opened to the passage of substrate. Thus, our definition of gating is a functional one from the standpoint of substrate translocation, and differs from approaches employing the passage of ions or small molecules to infer the state of the translocon. Although it is likely that the step at which the translocon is open to ions (Crowley et al., 1994) is similar to or the same as the step at which it is open to substrate (Jungnickel and Rapoport, 1995), it is worth emphasizing that the latter is being measured in the experiments that follow.

To assess whether these signal mutants gate the translocon with equal efficiency, we utilized two assays: crosslinking and translocation. Crosslinking to lumenal chaperones was used to determine whether the N terminus of 112 amino acid translocation intermediates of each of these mutants had access to the lumenal environment. Upon comparison of the different signal mutants, we found that translocation intermediates containing the wild-type, N9 and N4 signal sequences each crosslinked efficiently to the lumenal chaperone PDI (and/or the pancreatic homolog PDIp; Volkmer et al., 1997; Figure 1D). By contrast, crosslinking to PDI was markedly reduced for the N2 and N7 signal-containing translocation intermediates. All of these signals, however, crosslinked equally well to Sec61α, showing that they were all docked at the translocation channel. These results demonstrate that the wild-type, N9 and N4 signals have gated the translocon to provide access of the nascent chain to the lumenal environment. By contrast, the reduced crosslinking efficiency to the N2 and N7 signals is consistent with a reduced gating efficiency for these mutants.

To assess gating functionally, we determined whether these same translocation intermediates were competent for translocation into the lumen upon release with puromycin. Consistent with the crosslinking results, we found that the wt, N9, and N4 signals, but not the N2 and N7 signals, were translocated into the lumen upon release with puromycin (as assessed by protection from exogenous protease digestion; Figure 1E). This is exactly what is seen for the positive control, an early translocation intermediate of preprolactin (Figure 1E). These results suggest that at this point in biogenesis, the N2 and N7 signal sequences had not opened the translocation channel to substrate as efficiently as the wild-type, N9 and N4 signals.

Along with the results from Figures 1B–1D, these data allow the discrimination of the various signal mutants into three classes: those that are nonfunctional for targeting (N3a and Glob-PrP), those that target and achieve high-salt-resistant binding but are diminished in their capacity to gate the translocon (N2 and N7), and those that carry out both of these steps efficiently (wt, N9, and N4). In addition to these mutants, previously described mutants of the prolactin (Prl) signal sequence (Jungnickel and Rapoport, 1995; Voigt et al., 1996) provide one additional class of mutants: a signal sequence that is able to target to the translocation channel, but does not achieve high-salt-resistant binding [Prl(Δ13–15)]. An additional two amino acid deletion mutant [Prl(Δ13–17)] diminishes its targeting capability substantially, while the wild-type Prl signal carries out both targeting and gating efficiently (Jungnickel and Rapoport, 1995). Together, these mutants provide a broad range of signal sequences that differ at each of the several well-defined stages of signal sequence function (Table 1).

Table 1. Biochemical Characteristics for Various Native and Mutant Signal Sequences

Signal	Targeting	Salt-resistant binding	Gating
Glob	−	−	−
N3a	−	−	−
Δ13–17*	−/+	−	−
Δ13–15*	+	−/+	−
N7a	+	+	−
N2	+	+	−/+
N7	+	+	−/+
WT	+	+	+
N9	+	+	+
N4	+	+	++
Prl*	+	+	++

Data for constructs indicated with an asterisk are from a previous study (Jungnickel and Rapoport, 1995).

A Simplified Assay for Signal-Mediated Translocon Gating

We next utilized these mutants to characterize a simplified assay for signal sequence function in which the final topology of a reporter protein (Figure 2A) could serve as a sensitive indicator of whether, earlier in biogenesis, the signal sequence had properly targeted, achieved high-salt-resistant binding, and/or gated the translocon. The reporter, PrP(A120L), is a modified version of PrP that lacks an N-terminal signal sequence and contains an alanine-to-leucine substitution within the TMD. This change increases the hydrophobicity of the TMD and, in conjunction with the asymmetric distribution of flanking positively charged residues, should favor membrane integration in a Ctm orientation (type II; N_{cyt}/C_{exo}; Sipos and von Heijne, 1993; Hartmann et al., 1989). However, this TMD is not capable of serving as an efficient targeting signal, either co- or posttranslationally (S.J.K. and R.S.H., unpublished data), making efficient translocation of the protein dependent on introduction of an N-terminal signal sequence.

In this strategy (Figure 2B), chains that fail to target should result in a cytosolic translation product. Similarly, ribosome nascent chains that weakly or nonspecifically bind to the translocon in a salt-sensitive manner should, upon further synthesis, detach from the membrane (Potter and Nicchitta, 2000), also resulting in a cytosolic translation product. By contrast, high-salt-resistant binding ensures efficient translocation of the reporter, with its topology dependent on the gating property of the signal sequence. If signal-mediated gating directs translocation of the N terminus into the lumen, the completed protein should achieve the topology of either

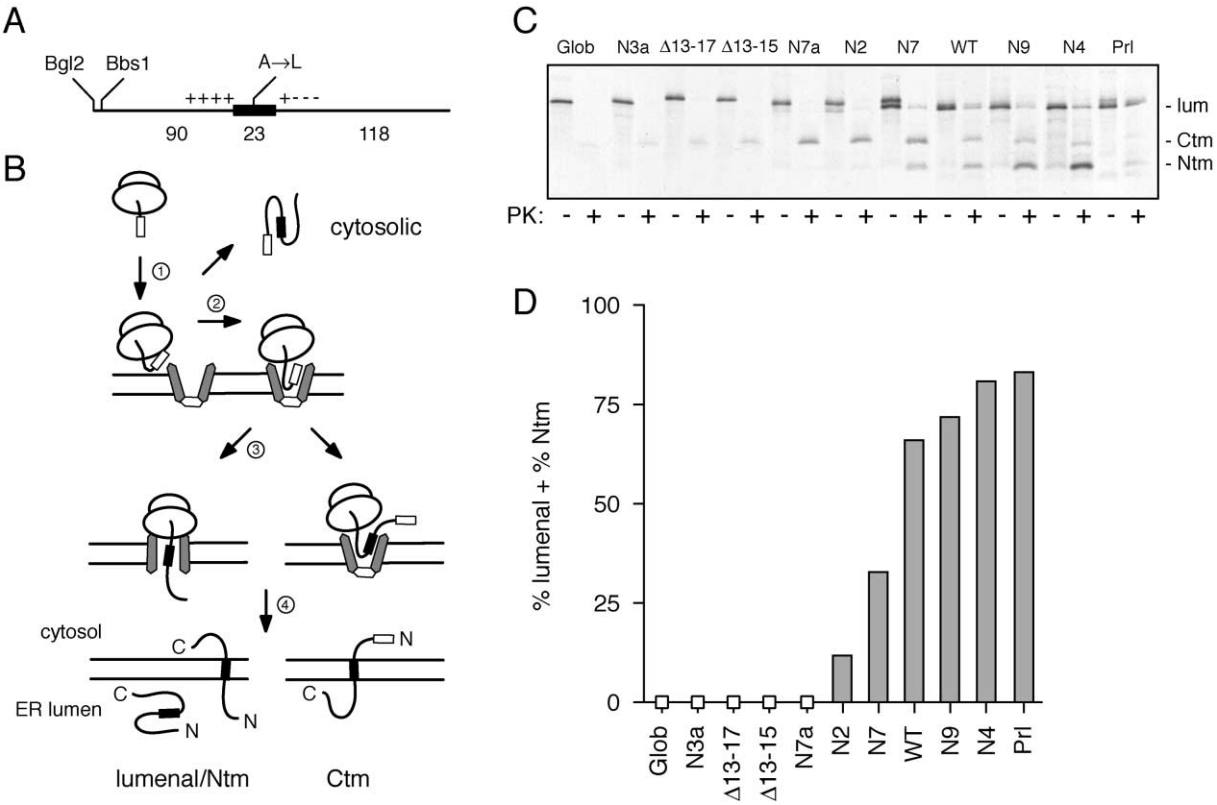

Figure 2. Design and Characterization of an Assay for the Targeting and Gating Functions of a Signal Sequence

(A) Diagram of the PrP(A120L) reporter cassette. Sizes of domains (in amino acids), charged residues flanking the transmembrane domain (TMD), and restriction sites for insertion of signal sequences are indicated.

(B) Design of topologic assay for cotranslational assessment of targeting and gating functions of signal sequences. In this scheme, a cytosolic topology is indicative of a failure of the signal sequence to either target (step 1) and/or promote salt-resistant binding to the translocon (step 2). The Ctm topology is indicative of chains whose signal sequences targeted but did not gate the translocon (step 3) before emergence of the TMD. The Ntm and lumenal topologic forms together indicate chains whose signals targeted and gated the translocon (step 3) prior to emergence of the TMD.

(C) Characterization of targeting/gating assay using defined wild-type and mutant signal sequences. Constructs containing each of the indicated signal sequences fused to the PrP(A120L) reporter were translocated and their resultant topologies were assessed by a protease protection assay. The sizes of the protease-protected fragments indicative of the three topologic forms (lumenal, Ctm, and Ntm) are indicated. Prl indicates the wild-type Prl signal, and Δ13–15 and Δ13–17 the previously characterized deletion mutants (Jungnickel and Rapoport, 1995). The wild-type and mutant PrP signals are from Figure 1A.

(D) Quantitation of data from (C). Plotted on the *y* axis is the sum of nascent chains in the Ntm and lumenal topologic forms (percent of total synthesized chains).

a lumenal or type I membrane protein (Ntm; N_{exo}/C_{cyt}), depending on whether the TMD integrates into the lipid bilayer. However, if the signal is either inefficient or sufficiently slow in gating the translocon, the TMD will emerge from the ribosome while the translocon is still closed to substrate. In this situation, the modified TMD, by virtue of its increased hydrophobicity and flanking charge distribution, has an opportunity to directly interact with the translocon and can become integrated as a type II membrane protein (Ctm; N_{cyt}/C_{exo}).

The validity of this strategy was tested with each of the signal sequence mutants from Table 1 (Figure 2C). Several observations are noteworthy. First, signal mutants that were either defective in targeting (Glob, N3a, and Δ13–17) or achieving high-salt-resistant binding to the translocon (Δ13–15) resulted in the reporter being largely cytosolic. Second, mutants that are able to achieve high-salt-resistant binding but do not gate the translocon efficiently by the time 112 amino acids are synthesized (N7a, N2, and N7; see Figure 1) result in inefficient translocation of the N terminus, with the topology of most chains being Ctm. Third, signal mutants that are able to gate the translocon by 112 amino acids of synthesis (wt, N9, and N4) generate lesser amounts of the Ctm topology, with the majority of chains achieving a combination of the Ntm and lumenal topologies. Fourth, the wild-type Prl signal sequence, which has been extensively studied and demonstrated to gate the translocon efficiently by the time ~70–80 amino acids are synthesized (Crowley et al., 1994; Jungnickel and Rapoport, 1995), was seen to result in a minor proportion of chains in the Ctm topology. Quantitation of the percent of total chains that are synthesized in the Ntm and lumenal topologic forms (indicative of chains that had gated the translocon prior to synthesis of the TMD) revealed a broad range between the signal mutants ranging from 0% for N7a to over 80% for the wild-type Prl signal sequence (Figure 2D).

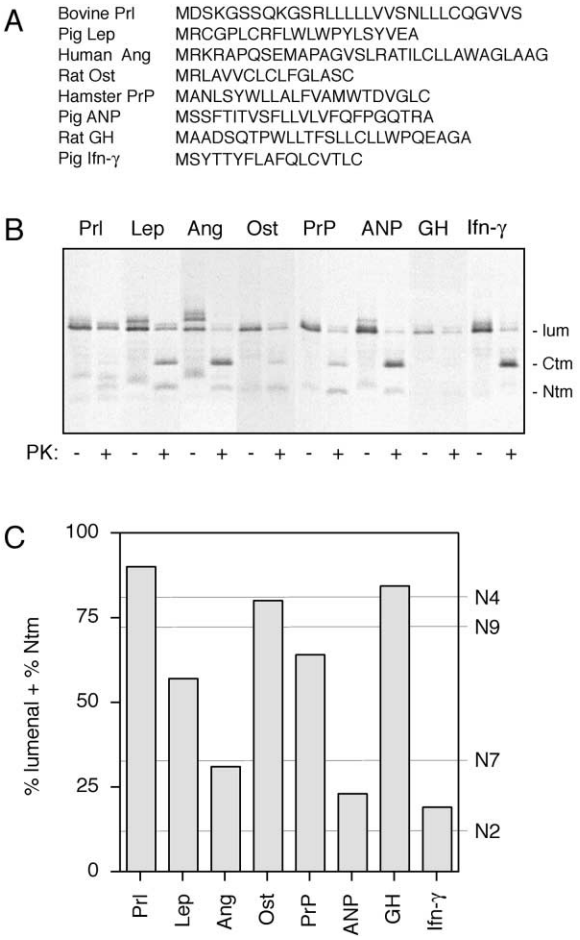

Figure 3. Diversity of Signal Sequence Function at the Translocon Gating Step

Various mammalian signal sequences (A) were assessed by the targeting/gating assay utilizing the PrP(A120L) reporter. The autoradiograph of the topologic analysis and its quantitation for percent gating (i.e., percent of total chains achieving the Ntm and lumenal forms) are shown in (B) and (C), respectively. Shown for reference on the graph are the values for selected PrP point mutants characterized in Figure 2.

Functional Diversity of the Gating Function of Signal Sequences

Having developed and validated a quantitative assay for the targeting and gating functions of signal sequences, we could now ask whether the sequence diversity seen in natural signal sequences also reflects functional differences in either their targeting and/or gating functions. Analysis of eight signals from a variety of mammalian secretory proteins (Figure 3A) by the targeting/gating assay revealed a surprising degree of heterogeneity (Figure 3B). While the overall translocation efficiency of all of the constructs was equivalently high (>90%), the topology achieved by the reporter was dramatically different between the different signals. This suggests that while all of the signals are capable of efficient targeting and high-salt-resistant binding to the translocon, they differ substantially in their gating functions.

Quantitation of these data showed that the percent of Ntm/lumenal forms generated ranged broadly from ~20%–30% (for interferon-γ [Ifn-γ], angiotensinogen [Ang], and atrial naturatic peptide) to over 80% for growth hormone (GH) and Prl signals (Figure 3C). This range is approximately the same as that seen for the various mutant signal sequences analyzed in Figure 2 (with the exception of N7a and the targeting-deficient signals). Together, these data suggest that natural mammalian signal sequences, in the context of a defined reporter protein, differ in their gating properties over a surprisingly broad range.

Evolutionary Conservation of the Gating Function of Signal Sequences

We next wished to address whether the diversity of translocon gating activities observed for different signal sequences is likely to be of physiologic relevance. To gain insight into this question, we first examined whether the substrate-specific differences between these signals were evolutionarily conserved for at least some of the substrates. Such conservation would lend support to the idea that the differences are physiologically important and have been maintained by selection. The second approach (described in a subsequent section) was to directly examine the consequences for the biogenesis of several proteins upon altering the gating property of the signal sequences used to direct their translocation.

To examine the evolutionary conservation of signal sequence function, we used the targeting/gating assay to assess the signals from multiple mammalian species for each of several substrates. As can be seen in Figure 4A, the exact sequences, as well as general features such as length and charge of these signals from the different species, are conserved to varying degrees. However, by the functional assay (Figures 4B and 4C), the gating property of each signal from the different species was remarkably conserved, supportive of the idea that the functional features of a signal are matched to and optimized for the substrate on which it evolved.

If this were the case, the evolutionarily conserved functional diversity of signals may be a consequence of the diversity of the mature domains from which they were taken. Consistent with this idea, we have observed that the degree of sequence conservation of signals was remarkably similar to that found for their respective mature domains, with ~30% of the substrates containing signal sequences that were conserved to the same or greater degree than the mature domain (Figure 4D). Similarly, Williams et al. (2000) have observed that the rate of evolution of signal sequences is significantly slower than expected, and is often correlated with the rate of evolution of their respective mature domain. Although such sequence correlations may be due to various reasons, it raised the possibility that substrate-specific features of a signal are matched with their respective mature domains.

Functional Matching of Signal Sequences with Mature Domains

While the advantages of an effectively gating signal sequence in promoting efficient translocation is apparent, it is less clear why some substrates appear to contain a weakly gating signal. One possibility is that certain

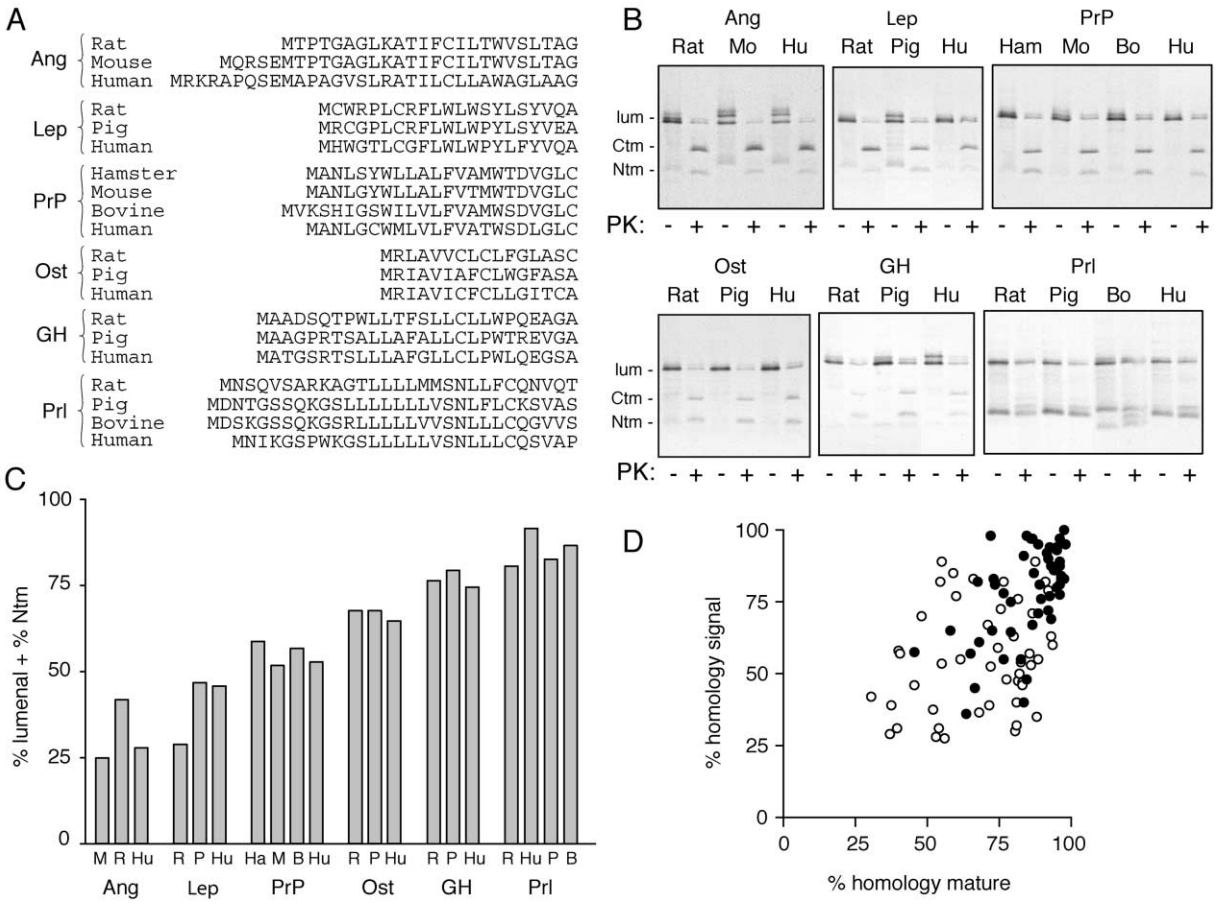

Figure 4. Evolutionary Conservation of Signal Sequence Function

(A–C) The sequences of the signals from multiple mammalian species for each of several proteins are shown (A). These signals were analyzed by the targeting/gating assay (B) and quantitated (C) to assess their relative gating efficiencies.

(D) Plot of the relative homology of the signal (*y* axis) versus mature domain (*x* axis) for 50 independent proteins containing N-terminal signal sequences for ER targeting. For each protein, a comparison was made between the chicken versus human homologs (open circles) and the mouse versus human homologs (closed circles).

signals, in combination with their natural mature domains, are able to attain a translocation-competent conformation (e.g., a "loop") that is not properly achieved in a heterologous context. Thus, a signal and mature domain may be matched to contain specific sequence elements that are sensitive to perturbation. Alternatively, some mature domains may contain structural features that allow it to be effectively translocated by a weakly gating signal sequence, while other mature domains may absolutely require an effectively gating signal for efficient translocation. In this view, the signal and mature domain are matched functionally, and are not necessarily constrained by specific sequences.

To investigate these ideas, we first determined whether, in fact, matching of a mature domain with its appropriate signal sequence influences the initiation of translocation for weakly gating signal sequences. We found that for the Ang and Ifn-γ signal sequences, translocation of the N terminus was substantially improved when the signal sequence was followed by a portion of its own mature domain (62 residues long) instead of the corresponding PrP mature domain of the reporter construct (Figure 5A). By contrast, an effectively gating signal (from Prl) directed efficient translocation of the N terminus regardless of the mature domain following it. Thus, the weakly gating signals from Ang and Ifn-γ are able to initiate translocation of the Ang and Ifn-γ mature domains more efficiently than the PrP mature domain. As discussed above, this might be due to functional matching of a weakly gating signal with an "easy-to-translocate" mature domain, or to matching of specific sequence elements that allow the formation of a particular conformation.

To distinguish between these two possibilities, we determined whether the Ang mature domain could be efficiently translocated by a different weakly gating signal of unrelated sequence. We therefore compared the ability of the weakly gating N7 and the more efficiently gating N9 signals to translocate the Ang, PrP, and Prl mature domains (Figure 5B). The N7 signal was able to translocate the Ang mature domain more efficiently than the Prl or PrP mature domains. By contrast, the N9 signal could translocate the Ang, PrP, and Prl mature domains with comparably high efficiencies. Taken together, these

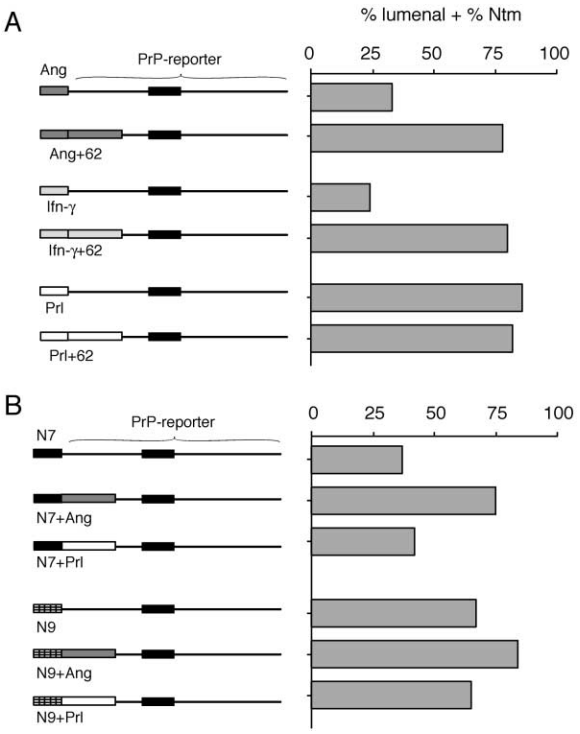

Figure 5. Functional Matching of the Signal Sequence and Mature Domain

(A) The Ang, Ifn-γ, or Prl signals fused to the PrP reporter (from Figure 3) were modified by replacing the first 62 residues of the PrP mature domain with the corresponding residues from the mature domains of Ang, Ifn-γ, and Prl, respectively. The translocation of each of the constructs (diagrammed at left) was analyzed and quantitated to determine the efficiency of N-terminal translocation.

(B) The N7 and N9 signals fused to the PrP reporter (from Figure 2) were modified by replacing the first 62 residues of the PrP mature domain with the corresponding residues from the mature domains of Ang or Prl. The translocation of each of the six constructs (diagrammed at left) was analyzed and quantitated to determine the efficiency of N-terminal translocation.

results suggest that mature domains of proteins differ in the gating requirements of the signals used to translocate them. Mature domains such as Ang are able to be translocated efficiently with a weakly gating signal (see also Figure 6), while other mature domains such as Prl and PrP require strongly gating signals for efficient N-terminal translocation. This interpretation may explain why the signals of some substrates (such as Prl or PrP) are evolutionarily conserved to be strongly gating, while those of other substrates can contain weakly gating signals. Thus, the naturally evolved matching of signals and mature domains appears to be *functional* (from the standpoint of gating), and not simply in sequence.

Consequences of Inappropriate Gating for Protein Biogenesis

We next examined the consequences in vivo for several substrates of altering the gating properties of the signals used to translocate them. Consistent with the in vitro analysis in Figure 5, we found that the Ang mature protein could be efficiently translocated using a variety of signal sequences (Figure 6A). By contrast, the choice of signals substantially influenced the proper biogenesis of PrP, Prl, and GH (Figure 6A), all proteins whose natural signals are evolutionarily conserved to be strongly gating (see Figure 4).

In the case of Prl and GH, less efficient translocation was seen with weakly gating signals than with strongly gating signals, as evidenced by the generation of precursor with the former but not the latter. Indeed, the majority of synthesized GH was precursor with all but the most strongly gating signal sequences (e.g., osteopontin or PrP, each of which showed little or no precursor). In the case of PrP, the choice of signal substantially affected not only the amount of precursor observed but also the efficiency and pattern of glycosylation. The precursor that was generated when Prl was fused to the leptin signal was degraded, as judged by pulse-chase analysis (Figure 6B). Thus, when fused to a weakly gating signal, the percent of synthesized Prl that is eventually secreted is reduced (by ~30%). Qualitatively similar results were seen with other weakly gating signal fusions to either Prl or GH (data not shown; Figure 6D). It therefore appears that for some proteins, changing the signal used to translocate it can have adverse consequences for its proper translocation. Although the signals that were tested varied in a wide variety of ways including length, hydrophobicity, and net charge, at least one key property that influences translocation may be its function in gating the translocon.

To test this idea more directly, we compared the biogenesis of Ang, Prl, PrP, and GH when fused to two signal sequences that differ only in their translocon gating properties. We chose the wild-type PrP signal and the N2 mutant signal because they have been well characterized by in vitro assays and found to be similar in sequence and behave identically in all functional assays except translocon gating (Figures 1 and 2). Expression in cultured mammalian cells of each of these substrates fused to the two different signals and analysis of newly synthesized protein by pulse labeling revealed that Prl, GH, and PrP, but not Ang, were substantially affected by the choice of signal sequences (Figure 6C). Both GH and Prl showed a significant amount of precursor when fused to the N2 but not wild-type PrP signal. By contrast, Ang displayed very similar patterns (with little or no precursor generated) regardless of whether it contained the N2 or wild-type PrP signal. PrP showed not only precursor accumulation, but also a different pattern of glycosylation.

Pulse-chase analysis revealed that with the N2 signal, Prl and GH secretion was reduced by approximately 40% and 70%, respectively, of that observed with the wild-type PrP signal (Figure 6D). By contrast, Ang secretion was essentially identical with both signals (Figure 6D). This is consistent with the observed precursor for Prl and GH containing the N2 signal (Figure 6D), and the observation that in vitro, N2-Ang is translocated with higher efficiency (>50%) than either N2-Prl or N2-GH (<10%; data not shown). These data demonstrate that the N2 mutant signal has a substantial effect on the translocation of some substrates (such as PrP, GH, and Prl, which apparently require and thus naturally contain efficiently gating signals). By contrast, the N2 signal is

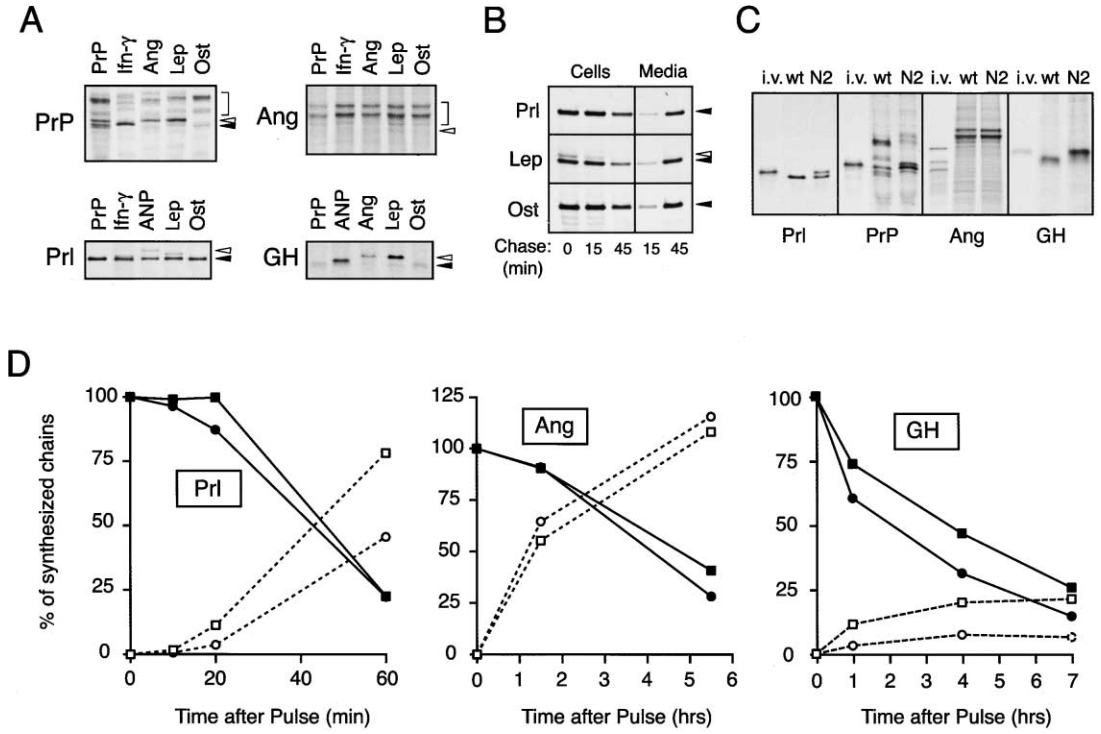

Figure 6. Consequences of Altered Signal-Mediated Gating for Protein Biogenesis

(A) Constructs encoding fusions of various signal sequences (indicated on top) with various mature domains (indicated at left) were transiently expressed in COS-7 cells, pulse labeled with ^{35}S-methionine for 5 min, and the labeled products captured by immunoprecipitation. Substantial amounts of precursor could be detected in several of the PrP, Prl, and GH constructs, but not the Ang constructs. The positions of precursor (open arrowheads), processed (filled arrowheads), and glycosylated (brackets) species are indicated. The various precursor species migrate slightly differently from each other due to differences in their respective signal sequence lengths.

(B) Pulse-chase analysis of Prl containing the Prl, leptin, or osteopontin signals. Following pulse labeling with ^{35}S-methionine for 10 min, cells were incubated with excess unlabeled methionine for the indicated times prior to immunoprecipitation of the labeled Prl from the media and cell lysate, as indicated.

(C) Newly synthesized Prl, PrP, Ang, and GH fused to either the wild-type PrP signal or N2 signal were transiently expressed in COS-7 cells and analyzed by pulse labeling as in (A). A marker for the position of precursor was generated by in vitro translation (i.v.) and also analyzed.

(D) The secretion efficiency of Prl, Ang, and GH containing either the wild-type PrP (squares) or N2 (circles) signals was determined by a pulse-chase analysis similar to (B) above. Plotted are the relative amounts of each protein in the cells (solid symbols) versus media (open symbols) and expressed as a percent of the total synthesized after the initial pulse. For Ang, we consistently observed a higher degree of recovery of the secreted product than the cellular product (and hence values higher than 100% of total synthesized chains are observed at later time points), likely due to more efficient immunoprecipitation of the secreted antigen. Also note that Ang and GH are secreted with substantially slower kinetics than Prl, and that GH is secreted less efficiently than either Prl or Ang.

largely adequate to direct the translocation of a substrate such as Ang that appears not to absolutely require an efficiently gating signal, and naturally contains a signal that is either inefficient or slow in mediating gating. Thus, by altering solely the gating property of a signal sequence, the biogenesis of some, but not other substrates, can be substantially affected.

Discussion

In this study, we have addressed the question of whether the wide sequence diversity of N-terminal signal sequences is accompanied by physiologically relevant differences in their function. To do this, we have developed a simplified assay to quantitatively assess the posttargeting signal sequence function involving translocon gating. Comparisons between a variety of mammalian substrates revealed that the gating function of their signal sequences is remarkably variant. That these differences are physiologically relevant, and not just random variation, was supported by three lines of evidence. First, the substrate-specific features of the gating function of the signal were demonstrated experimentally to be evolutionarily conserved for several proteins. Second, the mature domains of proteins were shown to have different gating requirements, which are precisely matched by their evolutionarily conserved natural signal sequences. And third, altering the gating property of a protein's signal sequence, even within the range observed for naturally occurring signals, can result in a substantial change in the biogenesis of the substrate. Thus, our findings suggest that the highly diverse set of signal sequences observed for secretory and membrane proteins does not simply represent sequence degeneracy secondary to minimal constraints, but instead a

substrate-specific functional diversity that, in many cases, is important to the protein's biogenesis and function.

Targeting and Gating Are Differentially Encoded

This study provides evidence that at least three steps in signal sequence function can be experimentally discriminated, and dissociated with mutations in the signal (Figure 1; Table 1). The ability of the PrP reporter assay to measure a posttargeting, post-salt-resistant signal sequence function in the opening of the translocon to the lumen (operationally defined as gating throughout this study) was critical to identifying differences in function between various signal sequences. This is because the principal point of difference between natural signal sequences was discovered to lie at this step (Figure 3). By contrast, it appears that targeting and transfer (in a salt-resistant manner) to the translocon are steps that are quite similar among various signals. Thus, all signal sequences appear to carry out some functions uniformly while simultaneously displaying distinctive features for other functions. Together, these observations suggest that the different functions of a signal sequence are encoded by different and perhaps partially overlapping features within the sequence.

This idea is consistent with the previous analysis of the Prl(Δ13–15) signal mutant (Jungnickel and Rapoport, 1995), which suggested that perhaps the recognition of the signal by the Sec61 complex was more stringent than the recognition event involving SRP. At present, it is not clear how translocon gating is encoded within a signal. Examination of the sequences of the various signals and mutants analyzed in this study did not reveal an obvious correlation between particular sequence elements and gating function. Both a larger data set as well as systematic mutagenesis studies will be required to address this issue in more depth.

Previous studies of the second signal recognition step by the translocon indicated that, at least for Prl, the Sec61 complex was necessary and sufficient (Jungnickel and Rapoport, 1995). At present, it is not clear whether other signal sequences are also recognized solely by the Sec61 complex, or if additional factors are involved. In support of the latter, it has been shown that TRAM stimulates the translocation of some but not other substrates in a signal sequence-dependent manner (Voigt et al., 1996) and is able to interact with a different region of a signal sequence than Sec61α (High et al., 1993; Mothes et al., 1994). Thus, it is plausible that the regions of the signal contacted by these different proteins are sufficiently different from substrate to substrate to have an effect on translocon gating. In addition, interactions of multiple proteins with various domains of a signal sequence may provide an explanation for how signals that are not discriminated by SRP can be distinguished by the translocon. Future studies analyzing the behavior of various signal sequences in proteoliposomes containing subsets of ER proteins will be required to determine how functionally different signals are discriminated by different components of the translocon.

Substrate-Specific Matching of the Signal Sequence and Mature Domain

In addition to substantial variations in the gating properties of signal sequences, we find that mature domains vary in their gating requirements. Some mature domains, such as those from Prl or PrP, appear to require an efficiently gating signal sequence to facilitate their translocation, while others, such as those from Ang or Ifn-γ, can be translocated with weakly gating signals. Ang can be translocated, not only with its own weakly gating signal sequence, but also with completely unrelated signal sequences with similar or higher gating activity. Similarly, Prl can be translocated equally well with both its natural and unrelated signal sequences, as long as they possess efficient gating activity. These observations provide strong evidence that the gating requirements of a mature domain are *functionally* matched with its signal sequence. Thus, the critical feature of a signal sequence that appears to be required for efficient translocation is its gating property.

Consequences for protein translocation are seen when the gating property of a signal sequence is mismatched with the requirements of the ensuing mature region. Indeed, this principle is, in retrospect, the mechanistic basis behind the PrP-based reporter assay for gating. The data indicate that the PrP mature domain is moderately difficult to translocate. Thus, while an efficiently gating signal sequence such as that from Prl can result in efficient N-terminal translocation (and hence very little generation of the Ctm topologic form), even slightly less efficiently gating signals are readily detected as a decrease in the N-terminally translocated topologic forms. The Prl and GH mature domains also appear to be relatively difficult to translocate, and hence are sensitive (to varying degrees) to having weakly gating signal sequences. This would explain why their natural signal sequences are highly conserved to be efficiently gating. By contrast, the Ang mature domain is relatively easy to translocate and is therefore not nearly as sensitive as PrP, Prl, or GH to having weakly gating signals. Thus, its natural signal sequence is itself relatively weakly gating. Why its weakly gating property should be evolutionarily conserved remains unclear at present.

An Explanation for the Sequence Diversity of Signals

The posttargeting functional differences between signal sequences, their substrate-specific evolutionary conservation, and the observed adverse effects on protein biogenesis of altering this function together provide an explanation for the long-observed sequence diversity of signals. While the sequence requirements for SRP recognition and targeting appear to be quite general, we would propose that the encoding of specific gating information within the same sequence element may add substantially more constraints. Because different substrates appear to require different gating properties, they each would need signal sequences unique to features of the respective mature proteins. Such a hypothesis would reconcile the substantial divergence of signal sequences of different proteins (von Heijne, 1985) with the surprising degree of conservation across species for a given substrate (Williams et al., 2000; Figure 4).

Several previous observations have also suggested that the signal sequences of some proteins are particularly unusual in ways that may be specialized for their respective substrates. For example, it has been observed that some signal sequences, after cleavage, can bind to calmodulin in the cytosol (Martoglio et al., 1997). Other signals are either inefficiently or alternatively cleaved (Li et al., 1996; Kurys et al., 2000; Rehm et al., 2001), perhaps to modulate the length of time the protein is retained at the translocon or within the ER. Alternatively, the prolonged exposure of the nascent chain to the cytosol conferred by an inefficient or slowly gating signal sequence may provide increased opportunity to generate a cytosolic form of certain proteins, or under some conditions, to cotranslationally reroute the substrate to a degradative pathway. Precedence exists for proteins residing in multiple compartments (Dedhar, 1994; Holaska et al., 2001; Johnson et al., 2001) or being cotranslationally tagged for degradation (Sato et al., 1998; Zhou et al., 1998; Turner and Varshavsky, 2000). Whether or not the molecular basis of these observations involves unique functional features of their respective signal sequences will require further study.

Experimental Procedures

Plasmid Constructions

A PrP cassette in the pSP64 vector (Promega) containing unique Bgl2 and Bbs1 sites in place of the signal sequence was generated by PCR mutagenesis. Synthetic oligonucleotides were inserted into these sites to generate fusions of the various wild-type and mutant signal sequences to wild-type PrP. Signal fusions to PrP(A120L) were generated by replacement of the Bsu36I to EcoR1 fragment of PrP with the corresponding domain from PrP(A120L) (Kim et al., 2001). To fuse various mature domains behind a given signal sequence, the signal was first engineered by PCR into pCDNA 3.1 with a unique Pst1 site following the signal. Mature domains, generated by PCR, were ligated to the Pst1/Xba1-digested plasmid (with the 3′ extension of the Pst1 site removed). Sequences for the Ang, PrP, Prl, and GH mature domains were from human, hamster, bovine, and rat, respectively. All signal sequence-mature domain fusions were precise and did not contain any additional codons at the fusion sites. Plasmids were verified by automated sequencing.

In Vitro Translocation Assays

Qualitative and quantitative assessment of PrP topology by translocation and protease protection assays in rabbit reticulocyte lysate (RRL) using canine pancreatic rough microsomal membranes (RMs) were performed exactly as described (Kim et al., 2001). In all of these experiments, an acceptor peptide inhibitor of glycosylation was included to simplify the analysis. The assignment of the N- and C-terminal fragments indicative of the Ntm and Ctm topologic forms has been described (Hegde et al., 1998). Translation intermediates of defined lengths were generated by translation of truncated messages generated from cDNAs digested in the coding region with appropriate restriction enzymes: 91- and 112-mers of PrP and mutants (Kpn1 and NgoM4, respectively) and 86-mer of preprolactin (Pvu2). Translation intermediates were generated in RRL in the presence or absence of RMs as indicated in the figure legends, at 32°C, for 10–30 min. Nascent chains were isolated by sedimentation in a TL100.1 rotor (100,000 rpm for 15 min or 50,000 rpm for 5 min to isolate the RMs) through a 100 μl 0.5 M sucrose cushion in physiologic salt buffer (PSB; 100 mM KAc, 50 mM HEPES [pH 7.4], 5 mM $MgAc_2$). Translation reactions to measure salt-resistant binding were adjusted to 0.5 M KAc and sedimented (50,000 rpm for 4 min) through a 100 μl 0.5 M sucrose cushion in PSB containing 0.5 M KAc. Sedimented nascent chains were resuspended in PSB containing 0.25 M sucrose prior to further manipulations. Crosslinking was with 0.5 mM disuccinimidyl suberate (Pierce) at 23°C for 30 min. In Figure 1A, the reaction was quenched with 0.1 M Tris (pH 8) and analyzed directly. In Figure 1D, samples were adjusted to 0.1 M Tris (pH 8), 1% saponin, 10 mM EDTA and sedimented (100,000 rpm for 15 min) over a 100 μl 0.5 M sucrose cushion in PSB, and the lumenal proteins in the supernatant were precipitated with 15% trichloroacetic acid and washed once in acetone before analysis. The membrane proteins in the pellet were analyzed directly. Treatment with puromycin (Calbiochem) was with 1 mM for 15 min at 25°C (Figure 1E). Proteolysis was with 0.5 mg/ml proteinase K (PK; Merck) on ice for 60 min. Reactions were terminated with 5 mM PMSF and rapidly transferred to ten volumes of boiling 1% SDS, 0.1 M Tris (pH 8).

Cell Culture Studies

COS-7 and NIH3T3 cells were maintained at 37°C in a humidified incubator containing 5% CO_2 and grown in DME-H21 containing 10% fetal bovine serum and antibiotics (GIBCO). Transfection was with Fugene-6 (Roche) as directed by the manufacturer. Tranfected cells were analyzed between 24 and 60 hr after transfection. Detection of products by immunoblotting (Harlow and Lane, 1988) utilized horseradish peroxidase-conjugated secondary antibodies (Amersham) and Supersignal chemiluminescence substrate (Pierce). The following antibodies were each utilized at a dilution of either 1:5000 (immunoblotting) or 1:1000 (immunoprecipitation): rabbit anti-Ang and rabbit anti-GH (ICN), rabbit anti-Prl (USB or ICN), and 3F4 mouse monoclonal anti-PrP (a gift from S.B. Prusiner; Rogers et al., 1991). For pulse labeling, cells were washed once with and preincubated for 5 min in prewarmed (37°C) methionine and serum-free DME-H21 before labeling with 400 μCi/ml of ^{35}S-methionine/cysteine (Translabel; ICN). Chase, if indicated, was performed by replacing the labeling media with low-serum media (OMEM; GIBCO) containing 1 mM methionine. Cells were harvested by rinsing twice with PBS (room temperature) and scraping into 100 μl (per well of a six-well dish) of 1% SDS, 0.1 M Tris (pH 8). Proteins in the media were collected by precipitation with 15% trichloroacetic acid, washed in acetone, and dissolved in 1% SDS, 0.1 M Tris (pH 8). Samples were heated to 100°C, diluted 10-fold with 1% Triton X-100, 50 mM HEPES (pH 7.4), 100 mM NaCl, clarified at 20,000 × g for 10 min, and antigens were immunoprecipitated with the appropriate antibodies (see above) essentially as described (Harlow and Lane, 1988).

Miscellaneous

Sequences of proteins were obtained from searches of the NCBI databases (www.ncbi.nlm.nih.gov). Sites of signal cleavage were either obtained from the annotations of the database sequence, or if not annotated, by alignment with a homologous protein whose signal cleavage site was known. Sequence alignments were performed with the ClustalW algorithm (Thompson et al., 1994) using Macvector software (Kodak). Percent homology, for the purposes of Figure 4D, was defined as percent of identical residues plus 0.5 times the percent of similar residues. SDS-PAGE was either on 12% Tris-Tricine minigels or 10% Tris-Glycine minigels. Unless specifically indicated above, laboratory chemicals were of the highest quality available from either Sigma, ICN, or Mallinckrodt. Enzymes for molecular cloning were from New England Biolabs, except for Pfu polymerase, which was from Stratagene.

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