Signal Sequences Influence Membrane Integration of the Prion Protein†

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ABSTRACT: Biosynthesis of the prion protein at the endoplasmic reticulum generates multiple topological forms. The topology of an individual chain is determined first by the localization of the N terminus and then by potential integration of the transmembrane domain into the lipid bilayer. Here, we provide the first evidence that signal sequences affect the latter of these events by demonstrating that some but not other signal sequences and signal sequence mutations result in significant increases in the fraction of prion protein nascent chains that integrate into the lipid bilayer. Through analysis of the prolactin signal sequence, an especially poor integration effector, we find that the N terminal and hydrophobic regions of the signal sequence affect integration most significantly. Mutations in either region result in a considerable increase in the number of chains that integrate. The effect of the signal sequence cannot be attributed to timing of signal cleavage or the state of the ribosome membrane junction, parameters previously found to affect protein biogenesis. We also present evidence that signal sequences that are poor integration effectors can promote integration under experimental conditions that allow the nascent chain more time to integrate. These findings reveal a previously unappreciated relationship between signal sequences and transmembrane integration.

Signal sequences emerge from the ribosome shortly after translation initiation and are bound by the signal recognition particle (SRP),[1] which targets nascent secretory and membrane proteins to the endoplasmic reticulum (ER) (*1*). After interacting with the SRP receptor, SPR transfers the signal sequence to the translocon, an aqueous channel in the ER membrane. After targeting, the signal sequence is recognized by the core component of the translocon, the Sec61 complex (*2*−*5*). Initially, the signal sequence is oriented with the N terminus closest to the ER lumen. To generate transmembrane (TM) proteins that have their N terminus localized in the cytosol, the signal sequence is reoriented shortly after translocation begins so the N terminus is on the cytosolic side of the translocon (*6*). The targeting and translocation initiation functions of the signal sequence are thought to be independent of the nascent passenger protein. Signal sequences have no strict consensus sequence. They vary in length from 15 to 50 amino acids (aa) but in general have hydrophilic N- and C-terminal domains and a central hydrophobic domain (*7*). Because of these loose structural requirements, signal sequences were long considered interchangeable.

In the past few years, new roles for signal sequences have come to light by studying the effects of their substitution and mutation on secretory protein biosynthesis (*8*−*14*). If signal sequences were interchangeable, then substituting one signal sequence for another would be expected to have little effect. However, examination of the ribosome−membrane junction shortly after translocation initiation reveals that, while some signal sequences close the ribosome−membrane junction early, shielding the nascent chain from the cytosol, other signal sequences close the junction later, allowing longer exposure of the chain to the cytoplasm (*8*). In the one instance studied, determinants in the mature sequence mediate junction closure, later in chain growth. Another step in translocation is opening of the lumenal gate of the translocon to allow passage of the nascent chain into the ER. Kim et al. (*11*) demonstrated that the ability of a signal sequence to open the lumenal gate is matched with the gating requirement of the mature domain. From this, they concluded that signal sequence diversity is functional, not simply random variation.

A recent study revealed that mutation of signal sequences, including that of the well-studied secretory protein, prolactin (Prl), could alter maturation of the attached nascent chain. Specifically, subtle changes in the hydrophobic region of the signal sequence affect the interaction of the nascent chain with the translocon and, as a result, the timing of signal cleavage. These point mutations did not alter the timing of ribosome−membrane junction closure, suggesting another separate, substrate-specific role for signal sequences in regulating biogenesis (*9*).

The prion protein (PrP) is a glycoprotein that has come to prominence because of its involvement in an unusual set of neurodegenerative disorders, termed prion diseases (*15*).

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[1] Abbreviations: aa, amino acid(s); ang, angiotensinogen; ANP, atrial naturatic peptide; βlac, β lactamase; ER, endoplasmic reticulum; GH, growth hormone; IgG, immunoglobulin G; Inf-γ, interferon-γ; Lep, leptin; Ost, osteopontin; PrP, prion protein; Prl, prolactin; PK, proteinase K; SRP, signal recognition particle; TCA, tricarboxcylic acid; TM, transmembrane; TRAM, translocating chain-associated membrane protein.

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Studies of PrP biogenesis have revealed some remarkable features; most notably, it can be synthesized in three different topological forms. Two different steps in biogenesis, the localization of the N terminus and membrane integration, determine the topology of an individual PrP nascent chain (*10*). ^{Ntm}PrP results from lumenal localization of the N terminus and integration of the TM domain. If integration fails to occur, ^{Sec}PrP is generated, which is entirely translocated into the ER lumen. Localization of the N terminus to the cytosol, followed by integration of the TM domain generates ^{Ctm}PrP (see Figure 1A). These forms can be distinguished after proteolysis because they are protected from protease to different extents (see Figure 1B). Several studies have demonstrated that mutation of the PrP signal sequence or substitution of signal sequences from other proteins can alter topologic distribution. Specifically, signal-sequence-mediated regulation of both ribosome−membrane junction closure and translocon gating affect localization of the PrP N terminus and result in increased ^{Ctm}PrP (*8*, *11*). Regulation of the second stage in PrP topology determination, integration, has been considered the task of the TM domain (*10*).

An exception to this paradigm that has not yet been explored is the observation that substitution of the PrP signal sequence with that of Prl (generating the chimera Prl−PrP) alters the topological distribution of PrP, yielding almost entirely ^{Sec}PrP (*8*). In this case, the consequence of swapping the signal sequences includes *both* shifting the localization of the N terminus to the ER lumen and reducing TM domain integration. In this study, we look exclusively at integration of chains that have the N terminus localized to the ER. To do this, we compare the ratio of ^{Ntm}PrP to ^{Sec}PrP generated by several other PrP signal sequence substitutions. We find that other signal sequence substitutions also alter ^{Ntm}PrP integration. To better understand the features of the signal sequence that are important for integration, we examined the effect of a series of mutations on Prl−PrP integration. We also looked at the ability of the mutant signal sequences to mediate integration under different experimental conditions and found that the signal sequence affect on integration is separate from previously described effects of the signal sequence on early events in biogenesis.

EXPERIMENTAL PROCEDURES

Plasmid Construction. Plasmids containing the PrP mature domain with the leptin (Lep), angiotensinogen (ang), osteopontin (Ost), interferon-γ (Inf-γ), and atrial naturatic peptide (ANP) signal sequences were generously provided by D. Mitra and R. Hegde. All Prl−PrP signal mutants and subdomain chimeras were generated by directed mutagenesis of Prl−PrP (*8*). The SN mutant library was generated by directed mutagenesis using primers with degenerate sequences (MHN forward). The +120 constructs were generated by digesting PrP (+120) (*16*) and Prl−PrP or SN−QT PrP with *Bsu36*I and *Pvu*II. The fragment containing the Prl or SN−QT signal sequence was then ligated into the PrP (+120) vector. The Prl−PrP (−GPI) STOP was generated by directed mutagenesis of the Ser 240 to TGA. The 104 aa truncation was generated by *Nae*I digestion. All other truncations were generated by PCR.

In Vitro Translation, Translocation, and Integration Assays. In vitro transcription and translation were performed as described previously (*17*). All translations were carried out at 34 °C for 30 min. Glycosylation was inhibited by 0.2

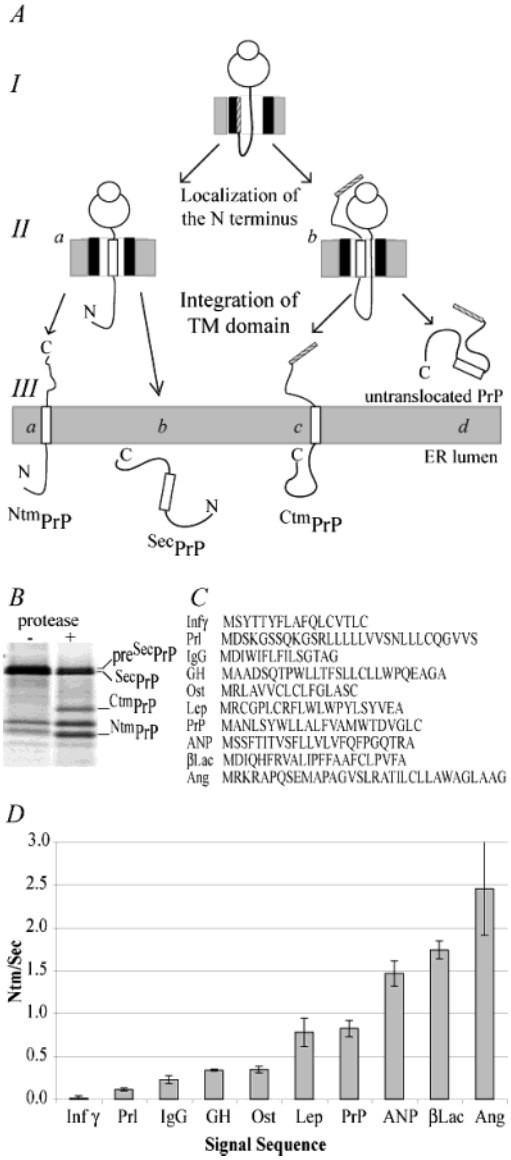


FIGURE 1: Many signal sequences alter ^{Ntm}PrP integration. (A) PrP topology is determined by two different steps during biogenesis. After the signal sequence targets the nascent chain to the translocon (*I*), the N terminus localizes to either the ER lumen (*IIa*) or to the cytosol (*IIb*). Integration of the TM domain is the second step. If the N terminus is localized to the ER lumen and integration occurs, ^{Ntm}PrP is generated (*IIIa*). If integration does not occur the entire protein is translocated into the ER lumen generating ^{Sec}PrP (*IIIb*). ^{Ctm}PrP is generated when the N terminus is localized to the cytosol and the TM domain integrates (*IIIc*). Failure to integrate when the N terminus is in the cytosol results in the entire chain remaining in the cytosol (*IIId*). (B) Different topologic forms of PrP are can be identified after proteolysis based on their size; signal-cleaved and signal-uncleaved (pre) ^{Sec}PrP are completely protected, while ^{Ntm}PrP and ^{Ctm}PrP are protected to different extents. The two lower molecular weight bands in the absence of PK do not contribute to the ^{Ntm}PrP. We conclude this because those bands are not detectable after immunoprecipitation with N-terminal-specific antibodies (data not shown). Constructs with various signal sequences (C) on the PrP mature domain were transcribed and translated in vitro in the presence of microsomal membranes. After isolation of the microsomal membranes, samples were split and incubated in the presence (+) or absence (−) of PK and separated by SDS−PAGE. The fraction of chains synthesized in each topological form was quantitated, and the ratio of ^{Ntm}PrP to ^{Sec}PrP from each reaction is graphed (D).

mM tripeptide competitor (*17*). Where indicated, 1 mM puromycin and 500 mM potassium acetate were added. Microsomal membrane isolation and ammonium sulfate precipitation have been described previously (*9*). Samples were proteolized at 4 °C for 45 min with 0.2−0.4 mg/mL proteinase K (PK). The protease was inactivated by incubation with 10 mM PMSF for 5 min and boiled in 10 volumes of 0.1 M Tris and 1% SDS. Immunoprecipitation of inactivated proteolysis samples was performed in 10 volumes of TXSWB (1% Triton X-100, 100 mM NaCl, 50 mM Tris [pH 8], and 10 mM EDTA). After incubation for 1 h with the RO13 antibody, protein A beads (Bio-Rad) were added and the samples were incubated overnight at 4 °C. Samples were rinsed 3 times in TXSWB prior to SDS−PAGE. Carbonate extraction was performed as described previously (*17*), except that the samples were centrifuged for 40 min at 80 000 rpm, in a TL100.2 rotor (Beckmann). Both pellets and supernatants were then precipitated with 10% tricarboxcylic acid (TCA).

Miscellaneous. A total of 15% tricine and 15% tris-glycine gels were used for SDS−PAGE. Autoradiographs were scanned using an Agfa Arcus II flatbed scanner and quantitated using NIH Image 1.63. Graphs with error bars represent the mean and standard deviation calculated from triplicate reactions.

RESULTS

Signal Sequences Affects Integration of ^{Ntm}PrP. Previous studies revealed that substitution of the PrP signal sequence with that of Prl results in a significant decrease in ^{Ntm}PrP integration. To determine if the ability of the Prl signal sequence to influence ^{Ntm}PrP integration was unique or a general property of a subset of signal sequences, we decided to look at several other signal sequence substitutions. We examined the effect of the immunoglobulin G (IgG), β lactamase (βlac), growth hormone (GH), Lep, ang, Ost, Inf-γ, and ANP signal sequences on integration of the wild-type mature domain of PrP (see Figure 1C for sequences). It was already known that many of these signals alter the levels of ^{Ctm}PrP in the wild-type context (*8*) or in the presence of the A120L mutation (*11*). For the purposes of the present study, we needed a way to discriminate between effects on localization of the N terminus and effects on integration, both of which can alter ^{Ntm}PrP levels. Toward that end, we quantitated the percent of PrP nascent chains generated in each topologic form and then calculated the ratio of percent ^{Ntm}PrP to percent ^{Sec}PrP (Ntm/Sec). This allowed us to distinguished between changes in the localization of the N terminus and integration because the former will increase both ^{Sec}PrP and ^{Ntm}PrP equally, and thus the ratio of the two conformers will remain constant, while a change in the latter will alter this ratio. A small value such as that of Prl−PrP (0.11) indicates that very few chains with the N terminus in the lumen are integrating (see Figure 1D). In contrast, ANP−PrP generates more ^{Ntm}PrP than ^{Sec}PrP and has a larger ratio of 1.46. When the results in Figure 1D are looked at as a whole, it is clear that signal sequences do affect integration of ^{Ntm}PrP in a way that is distinct from any affect that they have on localization of the N terminus. Like the Prl signal sequence, in the presence of the Inf-γ, IgG, GH, and Ost signal sequences, fewer PrP nascent chains, whose N termini are in the ER lumen, integrate. The leptin signal sequence causes similar levels of integration to the PrP signal sequence, while the ANP, βlac, and Ang signal sequences cause increased integration.

N Terminal and Hydrophobic Domains of the Signal Sequence Have the Largest Effect on Integration. To gain a better understanding of the features of the signal sequence that affect ^{Ntm}PrP integration, we chose to study the Prl−PrP construct in more detail. Prl−PrP was selected for several reasons. Very few Prl−PrP chains integrate, and although Inf-γ makes less ^{Ntm}PrP than Prl−PrP, it also makes significantly more ^{Ctm}PrP. The near absence of ^{Ctm}PrP in Prl−PrP means almost all nascent chains are distributed between ^{Ntm}PrP and ^{Sec}PrP, the two populations of most interest in the present study. In addition, Prl signal sequence targeting and translocation initiation has been extensively studied (*2*, *3*, *18*). Mutations in the Prl signal sequence that affect maturation of the Prl mature domain have been identified and are available to study the effects of the Prl signal on PrP biogenesis (*9*).

The first question we wanted to address was what region or regions of the signal sequence are important for mediating the effect on ^{Ntm}PrP integration. We identified the central hydrophobic domain (H domain) and the N- and C-terminal hydrophilic domains of the PrP and Prl signal sequences and then generated constructs with every possible combination of Prl and PrP signal sequence regions (see Figure 2A). Constructs were named based on the region in the N, H, and C domains, with the letters "P" and "L" representing regions from the PrP and Prl signal sequences, respectively (for example, under this nomenclature Prl−PrP is called LLL). We then quantitated the Ntm/Sec value for each construct. In all cases the constructs with the Prl hydrophobic domain integrated less (had lower Ntm/Sec values) than constructs with the PrP hydrophobic domain (compare the upper and lower panels of Figure 2B). In addition, the Prl N and H domains together integrated poorly regardless of the C-terminal domain used. The signal sequence is poorly cleaved from the construct with the Prl N and H domains but not the PrP C domain (LLP), which is surprising because the PrP signal cleavage site is in tact. However, ^{Ntm}PrP with the signal sequence attached can be detected (Figure 2B; see below for the effect of signal cleavage on ^{Ntm}PrP integration). In contrast to the N and H domains, replacement of the PrP C terminal domain with that of Prl (PPL) has a small effect on integration. These results suggest that the Prl N and H domains have the most significant impact on ^{Ntm}PrP integration and are consistent with previous findings on the effect of signal sequence domains on protein biogenesis (*9*).

Characterization of the Effects of Mutations in the N Terminal and Hydrophobic Domains of Prl−PrP on ^{Ntm}PrP Integration. We reasoned that if the N and H domains were responsible for the effect of the Prl signal sequence on ^{Ntm}PrP integration, then mutations in these regions would also be able to alter ^{Ntm}PrP integration. We made several mutations in each region of the Prl signal sequence and quantitated the Ntm/Sec value (see parts A and B of Figure 3). In the N domain, we mutated Lys9, a residue that is highly conserved among Prl signal sequences from different species, to an oppositely charged aa, Asp, and to a hydrophobic aa, Leu. To our surprise, these very different mutations both caused similar increases in integration. We wondered whether changing the Ser and Arg at positions 11 and 12 in the N

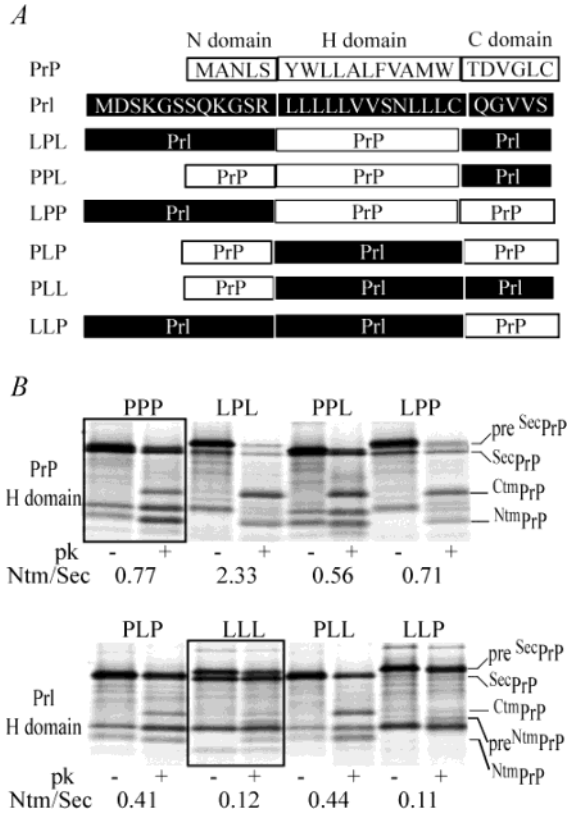


FIGURE 2: Identification of regions of the signal sequence responsible for altering ^{Ntm}PrP integration. (A) N, H, and C domains of the PrP and Prl signal sequences are shown. To assess the affect of each domain on ^{Ntm}PrP integration, we generated chimeras in which the Prl and PrP signal sequence domains were interchanged. Each construct is named according to the sequence in the N, H, and C domains, with P representing a region from PrP and L representing a region from Prl. Each construct was transcribed, translated, and treated as described in Figure 1. (B) Samples were separated by SDS−PAGE on 15% tricene gels, and the Ntm/Sec value for each construct is indicated below the gels. A box indicates the complete PrP (upper panel) and Prl (lower panel) signal sequences. With the exception of LLP, which has a signal sequence that remains uncleaved, all calculations were performed by quanitating only the signal-cleaved material. The PK-resistant band running slightly above ^{Ntm}PrP does not represent an integrated form because it is also present upon analysis of a TM domain deletion construct. Representative data are shown.

domain to two neutral hydrophilic residues (Gln and Thr) or two hydrophobic residues (Leu and Leu) would affect ^{Ntm}PrP integration. Like at position 9, these very different substitutions both resulted in higher Ntm/Sec values. These results support the conclusion that the N domain can affect integration.

Because the H domain of PrP is less hydrophobic than the H domain of Prl (see Figure 2A), we hypothesized that making the Prl H domain less hydrophobic would lead to increased integration of ^{Ntm}PrP. To test this, we changed some of the leucines in the Prl H domain to less hydrophobic aa, Ala or Phe. The construct in which the first five Leu were changed to Ala failed to target to the ER. Substituting Leu 14−16 with Phe or Leu 22−24 with Ala did significantly increase the Ntm/Sec value of Prl−PrP (see Figure 3A). These results suggest that the hydrophobicity of the Prl signal sequence might have a significant impact on ^{Ntm}PrP integration.

An interesting characteristic of the Prl H domain is that the string of hydrophobic residues is interrupted by two neutral hydrophilic aa, Ser and Asn at positions 20 and 21. Mutation of these residues in the context of the Prl mature domain has been shown to affect nascent chain biogenesis by altering the association of the nascent chain with translocon proteins and varying the timing of signal cleavage. In addition, mutation of these residues can affect post-translational modification of the prolactin mature domain with an engineered glycosylation site (*9*). We mutated Ser20 and Asn21 of Prl−PrP to Gln and Thr because this mutation was known to have a dramatic effect on Prl biogenesis. We hypothesized that this mutation would also affect PrP biogenesis and cause increased ^{Ntm}PrP integration. Figure 3B shows that SN−QT PrP has an Ntm/Sec value that is almost 4 times larger than that of Prl−PrP. We also generated SN−TQ, SN−QN, and SN−ST mutants. SN−QN had little effect on integration, while SN−TQ and SN−ST had increased Ntm/Sec values, although not as high as SN−QT (see parts B and C of Figure 3). Like Ser and Asn, Gln and Thr are neutral hydrophilic residues. The ability of SN−QT to significantly affect ^{Ntm}PrP integration suggests that the identity of residues 20 and 21 and not the interruption of the hydrophobic domain influences ^{Ntm}PrP integration.

In addition to the SN−QT mutant, two other mutations whose effects on the Prl mature domain have been well-characterized are SN−MP and SN−NH. On the Prl mature domain, the SN−NH signal sequence behaves similarly to that of the wild type, whereas the SN−QT and SN−MP signal sequences alter biosynthesis (*9*). When we examined the effect of the mutations on PrP biogenesis, we found that, like the wild-type Prl signal sequence, the SN−NH signal sequence generated almost entirely ^{Sec}PrP. In contrast, both SN−MP and SN−QT PrP generated significantly more ^{Ntm}PrP (see shaded regions in Figure 3C). Thus, the results of Prl signal sequence mutations engineered onto a different passenger, PrP, are faithful to their effects on Prl (*9*).

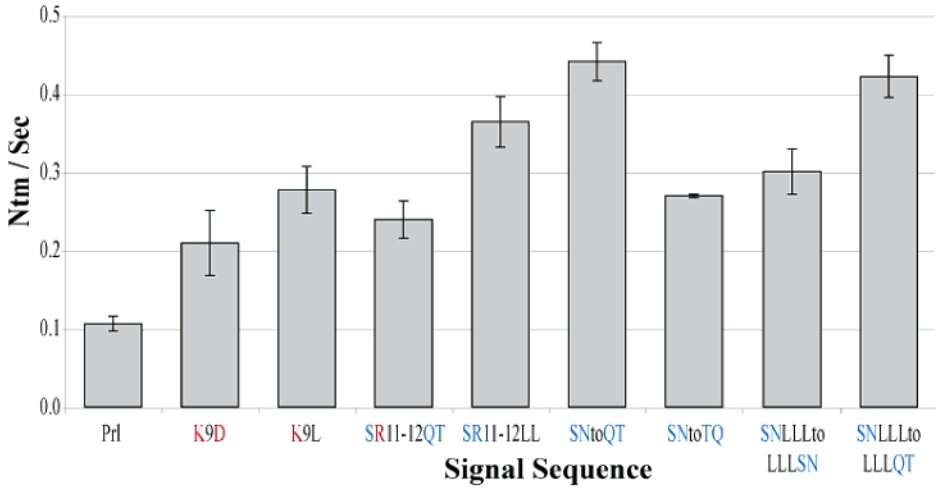
To determine whether changing the location of the Ser and Asn in the Prl H domain altered ^{Ntm}PrP integration, we generated constructs that shifted the Ser and Asn two residues to the left [VVSN(18−21)SNLL] or three positions to the right [SNLLL(20−24)LLLSN]. VVSN(18−21)SNLL altered both the localization of the N terminus (evidenced by a significant increase in ^{Ctm}PrP; data not shown) and integration of the TM domain (Ntm/Sec value of 1.09), while SNLLL-(20−24)LLLSN primarily affected integration. We also generated similar constructs that included QT in place of SN, both of which also had higher Ntm/Sec values than that of Prl−PrP (see parts A and B of Figure 3). The results of the SN-shifted mutants are somewhat surprising because, in contrast to the L(14−16)F and L(22−24)A mutants, these mutations affect the Ntm/Sec value without changing the overall hydrophobicity of the signal sequence.

Mutation of SN(20−21) in the Prl Signal Sequence Affects ^{Ntm}PrP Integration. The initial substitutions that we made at positions 20 and 21 suggested that the identity of the aa at these positions was important for the low ^{Ntm}PrP integration rate of Prl−PrP. Because Ser and Thr, and Asn and Gln are very similar aa, which have very different effects on ^{Ntm}PrP integration, we decided many more mutants were needed to try to determine what aa characteristics are important to mediate the effect of the signal sequence on integration. Toward that end, we generated a library of constructs with random mutation of the SN residues. The presence of charged

A

Signal Sequence		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Prolactin	0.11	M	D	S	K	G	S	S	Q	K	G	S	R	L	L	L	L	L	V	V	S	N	L	L	L	C	Q	G	V	V	S
K9D	0.21									D																					
K9L	0.28									L																					
SR11-12QT	0.24											Q	T																		
SR11-12LL	0.36											L	L																		
L22-24A	0.32																						A	A	A						
L14-16F	0.36														F	F	F														
L13-17A	NA													A	A	A	A	A													
VVSN18-21SNLL	1.09																		S	N	L	L									
VVSN18-21QTLL	0.61																		Q	T	L	L									
SNLLL20-24LLLSN	0.30																				L	L	L	S	N						
SNLLL20-24LLLQT	0.42																				L	L	L	Q	T						

B



C

position 20	position 21	Ntm / Sec
S	D	0.07
H	H	0.09
S	N	0.12
N	Q	0.12
P	K	0.12
P	N	0.13
P	H	0.14
N	H	0.15
Q	N	0.16
P	Q	0.17
L	P	0.19
H	P	0.19
Q	Q	0.21
K	K	0.24
T	Q	0.27
I	N	0.27
P	P	0.27
T	T	0.31
T	L	0.34
P	T	0.35
M	P	0.37
S	T	0.38
L	L	0.44
Q	T	0.44
I	T	0.49
T	I	0.51
L	M	0.55
T	P	0.56
M	L	0.56
Q	L	0.66
K	L	0.81

D

Position 20	N	H	S	P	Q	I	L	T	M	K
n	2	2	3	5	4	2	3	5	2	2
Ntm / Sec	0.13	0.14	0.19	0.20	0.37	0.38	0.39	0.40	0.47	0.53

Position 21	D	H	N	K	Q	P	T	I	M	L
n	1	3	4	2	4	5	5	1	1	5
Ntm / Sec	0.07	0.13	0.17	0.18	0.19	0.32	0.39	0.51	0.55	0.56

= hydrophobic
= hydrophilic, neutral
= hydrophilic, charged

FIGURE 3: Mutations in the Prl signal sequence affect ^{Ntm}PrP integration. (A) Prl signal sequences are shown with the central hydrophobic domain shaded gray. Below are the mutants that were examined including the Ntm/Sec value. (B) Constructs with the specified signal sequences were translated and treated as in Figure 1. Samples were analyzed 3 times. The average Ntm/Sec value and standard deviation are graphed. (C) Library of SN mutants was generated by directed mutagenesis with degenerate primers. Mutants SN−ST, −TQ, −QN, −MP, −NH, and −LL were generated by mutagenesis using specific primers. Samples were analyzed as described above, and the Ntm/Sec value is shown. (D) Average Ntm/Sec value for all SN mutants with specific residues in the 20th (top) and 21st (bottom) positions are shown. The *n* value represents the number of constructs that were used to calculate the average. aa are color-coded according to their properties: hydrophobic residues are black, neutral hydrophilic residues are blue, and charged hydrophilic residues are red.

hydrophilic residues resulted in lower Ntm/Sec values with one exception; no trend is as evident with neutral, hydrophilic, or hydrophobic residues (see Figure 3C).

Because we could draw few conclusions from the raw data, we analyzed the mutant library to try to determine the effect of specific aa at each position. To do this, we averaged the Ntm/Sec values of all mutants with the same aa in the 20th position and separately in the 21st position. For example, we averaged the ratios of QN, QQ, QT, and QL to get a value of 0.37. Figure 3D shows the results of this analysis. The results for some aa vary. For example, Pro in position 20 has a low average Ntm/Sec value, while in position 21, it has a much higher Ntm/Sec value. In contrast, Asn and His have low Ntm/Sec values and Thr has high Ntm/Sec values regardless of location. In general, hydrophobic aa have higher average values and hydrophilic aa have lower average values, but there are several exceptions to this trend. We can conclude that the identity of the aa at position 21 more strongly correlates to hydrophobicity because the average Ntm/Sec values of mutants with hydrophobic aa at position 21 are all quite high.

Signal Sequence Effects on Integration Are Not Mediated by Expected Mechanisms. Because this is the first clear demonstration that the primary structure of the signal sequence can influence integration per se, we wanted to determine whether this affect is mediated by previously described or novel mechanisms. The state of the ribosome−membrane junction has been associated with regulating the localization of the N terminus (*8*). Prl−PrP has a closed ribosome−membrane junction, which means that, shortly

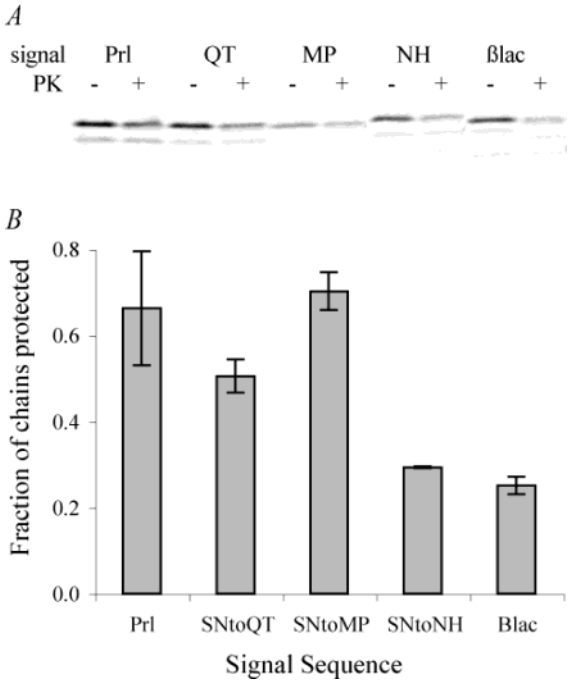


FIGURE 4: Signal sequence mutations have different effects on ribosome−membrane junction closure. (A) Constructs with the indicated signal sequences were digested with *Nae*I. Upon transcription and translation, 104 aa nascent chains were generated that remain associated with the ribosome. The microsomal membranes were isolated, and the samples were incubated in the presence (+) or absence (−) of PK and separated by SDS−PAGE. (B) Intensity of the bands − and + PK was quantitated, and the fraction of chains protected is graphed.

after targeting to the membrane, a tight junction is formed between the ribosome and the translocon. In contrast, the signal sequence from βlac on PrP (βlac PrP) has an open junction (*8*). This was assayed by translating truncated mRNAs that have no stop codon. As a result, the ribosome remains associated with the nascent chain for a relatively long time (*19*). Upon addition of protease, the "closed" ribosome−membrane junction protects Prl−PrP, but βlac PrP is accessible to protease through the "open" ribosome−membrane junction.

The SN−QT mutation on the Prl mature domain affects biogenesis without altering closure of the ribosome−membrane junction (*9*). From extension, we predicted that the mutant signal sequences would have closed ribosome−membrane junctions on PrP. When we tested the protease protection of 104 aa truncations, we found that differences in ribosome−membrane junction closure did *not* correspond to the effect of the mutations on integration (parts A and B of Figure 4). SN−NH PrP, which integrated as poorly as Prl−PrP, had a more open ribosome−membrane junction. However, SN−MP PrP, which integrates relatively well, had a more closed ribosome−membrane junction than that of SN−QT PrP. Thus, there is no clear correlation between the effect of the mutations on integration and closure of the ribosome−membrane junction. The same is true if we look at the signal sequence substitutions. Both βlac−PrP and IgG−PrP have open ribosome−membrane junctions (*8*), but from Figure 1, it is clear that they have opposite effects on ^{Ntm}PrP integration. Therefore, we conclude that the observed differences in ribosome−membrane junction closure had no significant impact on integration.

The signal sequence directs the interactions with the translocon, which in turn, influence the maturation of the nascent chain. In the case of the mutant signal sequences on the Prl mature domain, one consequence of regulated signal−translocon interactions was significantly altered by the timing of the signal cleavage (*9*). In that case, the SN−MP and SN−QT signal sequences began to be cleaved at an early truncation, but the Prl and SN−NH signal sequences were not cleaved until the chains were much longer (*9*). Because it was already clear that the mutations could affect the interaction of the PrP nascent chain with the translocon (resulting in different topological distributions), we expected that, on PrP, the signal sequences would also be cleaved at different times. When we assayed signal cleavage timing, we found, to our surprise, that none of the mutations significantly altered the timing of the signal cleavage (see Figure 5). Although the amount of cleavage varies slightly, all chains begin to be cleaved at the same length. There appears to be subtle differences in the extent of signal cleavage observed at later points, which may contribute to differences in integration, but we found the overall timing of the signal cleavage was similar for Prl−PrP and the mutant signal sequence constructs.

Signal Cleavage Has Little Effect on ^{Ntm}PrP Integration. It seemed possible that the subtle differences in signal cleavage might be responsible for the observed effect on ^{Ntm}PrP integration. To minimize the impact of this difference on our data, we performed almost all calculations using the quantitated data from only the signal-cleaved ^{Sec}PrP and ^{Ntm}PrP material. However, when we included the uncleaved material, we still saw significant differences, for example, between Prl−PrP and SN−QT PrP (data not shown). To test more definitively the effect of signal cleavage on ^{Ntm}PrP integration, we generated two pairs of Prl−PrP and SN−QT constructs. The first mutation we made improved signal cleavage by replacing the first three aa of the PrP mature domain (KKR) with those from Prl (TPV) (see Figure 6A). These constructs are denoted Prl−PrP +3 and SN−QT PrP +3. The second pair had a mutation of the Prl cleavage site from GVVS to WPVP (uncl Prl−PrP and uncl SN−QT PrP). This mutation prevents signal cleavage as shown in Figure 6A. Both making the signal uncleavable and more cleavable may have a small affect on Prl−PrP integration, but neither mutation alters the relationship between Prl−PrP and SN−QT PrP (Figure 6B). Therefore, the observed differences in signal cleavage cannot account for the effect of the signal on integration. It is interesting that the signal has a similar effect whether it remains attached or is cleaved.

Distance between the Signal Sequence and the TM Domain Is Not Significant for Signal Sequence Regulation of ^{Ntm}PrP Integration. It has previously been reported that increasing the distance between the signal sequence and the TM domain can alter the localization of the N terminus (*16*). We wondered whether increasing the distance between the signal sequence and the TM domain might also decrease the effect of the signal sequence on integration. If that were the case, then when the distance is increased, we would expect the Prl signal and the SN−QT signal to generate similar levels of ^{Ntm}PrP. To test this, we put the Prl and SN−QT signal sequences on a construct that has 120 aa of the cytosolic protein globin inserted in the domain between the signal sequence and the TM domain [generating the Prl−PrP

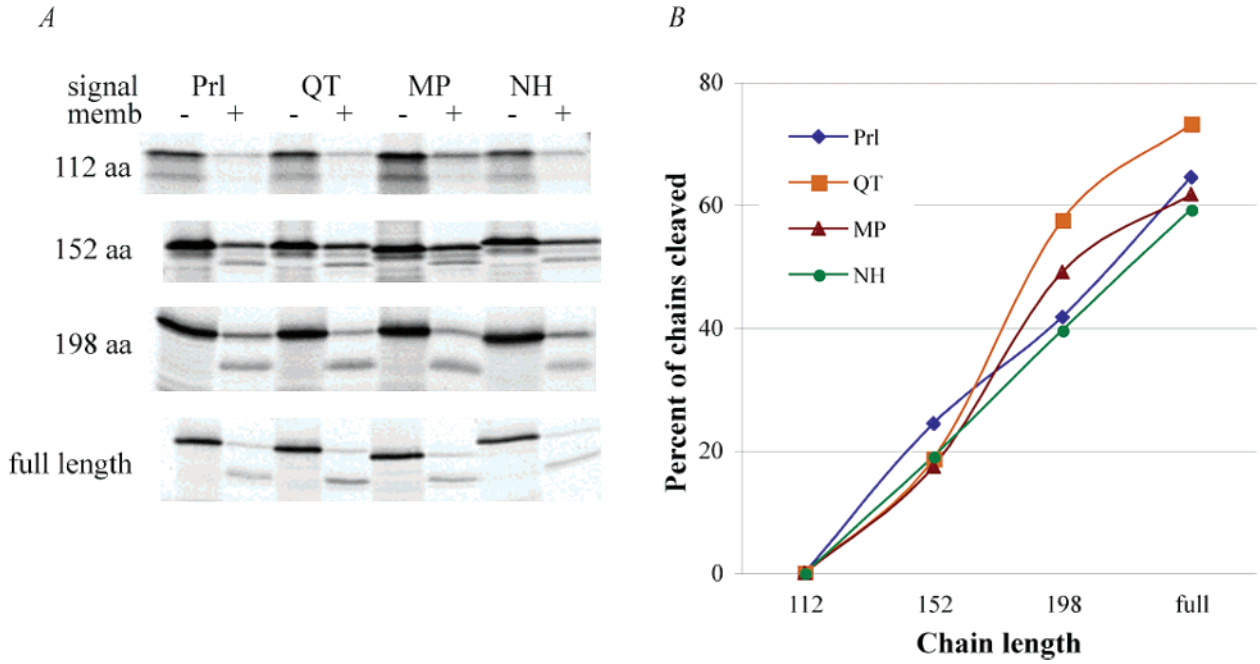


FIGURE 5: Signal sequence mutations do not alter the timing of the signal cleavage. (A) PrP nascent chains with the Prl or mutant signal sequences of the indicated length were translated in the presence (+) or absence (−) of microsomal membranes (memb). Samples were precipitated with 50% ammonium sulfate (− membranes) or pelleted through a sucrose cushion (+ membrane), solubilized, and separated by SDS−PAGE on 15% tricene gels (112 and 152 aa) or 15% tris-glycine gels (198 aa and full length). (B) Intensity of the signal-cleaved and signal-uncleaved bands in the presence of membranes was quantitated, and the percent signal cleavage was calculated.

(+120) and SN−QT PrP (+120) constructs]. This insertion doubles the distance between the signal sequence and the TM domain. To properly identify the +120 ^{Ntm}PrP and ^{Sec}PrP fragments, we immunoprecipitated the samples with an antibody to the PrP N terminal region prior to analysis. We then compared the Ntm/Sec values (see Figure 6C). A few more Prl−PrP chains appear to integrate when the globin spacer is present, but the large discrepancy between the Prl and SN−QT signal sequences is still observed. Although we only examined one pair of insertion constructs, these data suggest that the distance between the signal sequence and the TM domain has little impact on the ability of the signal sequence to affect integration of ^{Ntm}PrP.

SN−QT and SN−MP Mutant Signal Sequences Promote Integration. Because we found no correlation between the affect of the signal sequence on integration and early events in translocation, we decided to look more closely at how the signal sequence affects ^{Ntm}PrP integration. The significantly reduced ability of Prl−PrP to generate ^{Ntm}PrP could be explained by the inability of that signal sequence to adequately promote integration. It seems equally plausible, however, that the Prl signal sequence somehow prevents integration of the TM domain. To try to discriminate between these two possibilities, we decided to extend the time the nascent chain associates with the translocon and assay the effect on ^{Ntm}PrP integration. We hypothesized that if the Prl signal sequence prevents ^{Ntm}PrP integration, then increasing the time the nascent chain resides at the translocon would have little effect on integration. However, if the Prl signal sequence fails to adequately promote integration, then extending the time the nascent chain associates with the translocon may allow an increased fraction of chains to integrate.

To generate experimental conditions where nascent chains had a prolonged time to integrate, we translated truncated mRNAs, allowed the translation to go to completion, released the nascent chains by treatment with puromycin and high salt, and analyzed topology. Truncation greatly increased the amount of time the nascent chain remained at the translocon, and thus the time during which it could potentially integrate. In addition to Prl−PrP, we looked at the affect of truncation on integration of SN−NH, SN−QT, and SN−MP PrP. Figure 7A shows that upon truncation at 262 aa (full length minus the stop codon) all constructs made significantly more ^{Ntm}PrP than identical chains that possess a stop codon. The results were similar for all chain lengths examined (data not shown). These data suggest that, in the full-length context, the Prl signal sequence fails to adequately promote integration. From extension, it seems likely that SN−QT and SN−MP PrP generate more ^{Ntm}PrP because these signal sequences are stronger integration effectors.

Studies of integration have revealed that while some TM domains integrate cotranslationally others remain associated with the translocon until the nascent chain is complete (*20*, *21*). An unexpected implication of the data presented in Figure 7 is that the PrP TM domain remains associated with the translocon until translation is terminated. We conclude this because the Prl−PrP nascent chains truncated at the C terminus generate more ^{Ntm}PrP than identical chains with a stop codon. If PrP TM domains that failed to integrate passed quickly into the ER lumen, we would not expect that leaving full-length chains associated with the translocon would affect integration. However, because more chains integrated upon truncation at the C terminus, we conclude that the TM domain of truncated chains (and by extension of the TM domains of full-length chains) must remain at the translocon until termination to maintain their functional competence for integration. This must be true whether the TM domain integrates or not because the Prl−PrP and SN−NH PrP full-length chains seldom integrate.

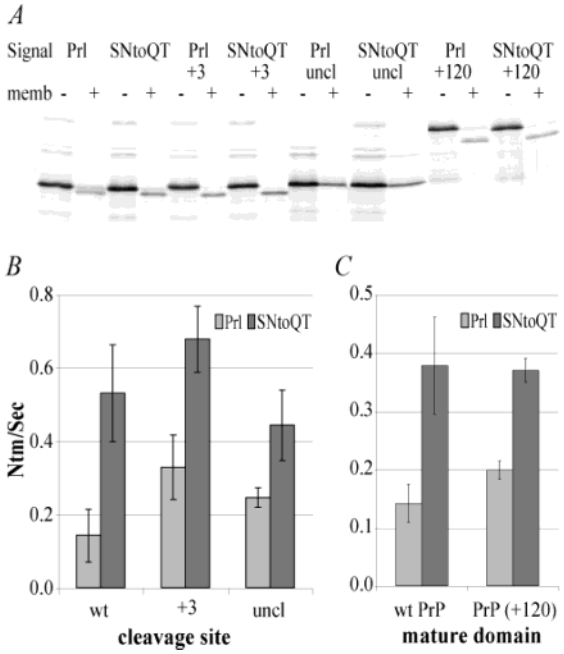


FIGURE 6: Neither signal cleavage nor the distance separating the signal sequence and the TM domain are critical for signal sequence regulation of integration. Three pairs of constructs were made. In the +3 construct, the first three aa of the PrP mature domain (KKR) were changed to the first three aa of the Prl mature domain (TPV). The last four aa of the Prl signal sequence were changed from GVVS to WPVP in the uncl constructs. The +120 constructs have a 120 aa insertion from globin between the signal sequence and the TM domain. (A) Indicated constructs were transcribed in vitro and translated in the presence or absence of microsomal membranes to assess signal cleavage. The samples were then separated by SDS−PAGE on 15% tris-glycine gels. (B) Constructs with the indicated signal sequences and cleavage sites were transcribed and then translated in the presence of microsomes and treated as described in Figure 1. The percent of each topologic form was quantitated, and the average of the Ntm/Sec value of samples assayed 3 times is graphed. (C) Prl and SN−QT PrP constructs with or without the 120 aa insertion were assayed as described above; except after proteolysis, the samples were immunoprecipitated with an antibody to the N terminus of PrP. These samples were then separated by SDS−PAGE on 15% tricene gels. The percent ^{Sec}PrP and ^{Ntm}PrP were quantitated, and the average ratio of the two values from triplicate samples is graphed.

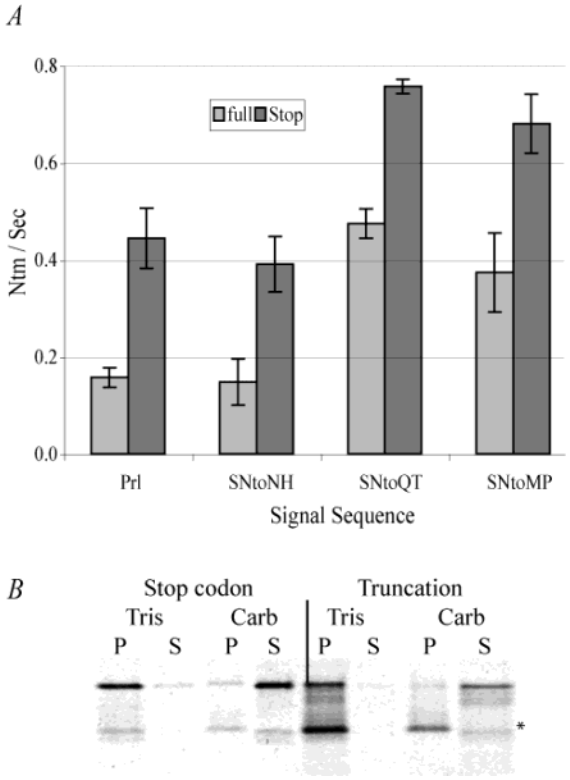


FIGURE 7: SN−QT and SN−MP signal sequences promote integration. (A) PrP nascent chains with the indicated signal sequences were synthesized in the presence of microsomal membranes either as full-length nascent chains, containing the authentic stop codon, or as intermediates truncated at the last aa. After translation, all reactions were treated with puromycin and high salt to release nascent chains still associated with the ribosome. Proteolysis was then used to assess the generation of ^{Ntm}PrP (as described in Figure 1). The average Ntm/Sec value from the full-length and truncated chains is graphed. (B) To assess whether the ^{Ntm}PrP generated in A is integrated, a new Prl−PrP construct was made, which had a stop codon immediately prior to the GPI anchor cleavage site. An identical construct truncated at the GPI cleavage site (no stop codon) was generated by PCR. The samples were translated and released with puromycin and high salt as in A prior to membrane isolation and proteolysis. After addition of the protease inhibitor, microsomal membranes were again isolated. The samples were then split and centrifuged in a Tris-sucrose buffer or in carbonate. Both the supernatants (S) and the resuspended pellets (P) were precipitated with TCA prior to separation by SDS−PAGE. The asterisk indicates ^{Ntm}PrP.

To ensure that ^{Ntm}PrP made upon truncation and release of the Prl−PrP nascent chain was actually integrated into the lipid bilayer, we tested if it could be extracted by sodium carbonate at pH 11.5, a treatment that disrupts protein−protein but not protein−lipid interactions (*22*). Before we could do this, we first had to generate constructs that lacked the GPI anchor at the C terminus, which makes ^{Sec}PrP resistant to extraction. The Prl−PrP (−GPI) STOP construct (with a stop codon at 240) and the Prl−PrP (−GPI) truncation (truncated at codon 240; contains no stop codon) behaved similar to the full-length constructs in all assays (data not shown). Under conditions in which the control secretory protein was extracted but the control membrane protein was retained (data not shown), we observed significant carbonate extraction-resistant ^{Ntm}PrP chains generated by the truncated construct but few chains integrated in the presence of a stop codon (see Figure 7B). This verifies that the ^{Ntm}PrP made by Prl−PrP upon truncation and puromycin release is actually integrated.

DISCUSSION

In this study, we have demonstrated that the signal sequence can affect integration of the PrP TM domain. Previously, the signal sequence was thought to influence just the first step in PrP topology determination, namely, the localization of the N terminus, while only the TM domain and adjacent residues were thought to affect the second step, integration. We found that different signal sequences have different effects on ^{Ntm}PrP integration. In addition, we found that mutation of one of these signal sequences, the ^{Sec}PrP favoring Prl−PrP, actually increased the integration of the TM domain. The mechanism by which the signal sequence has an effect on integration is due neither to the closure of the ribosome−membrane junction nor to an alteration in signal cleavage timing, two parameters previously shown to be important for early functions of the signal sequence. Cleavage of the signal sequence appears to neither promote

nor prevent ^{Ntm}PrP integration. In addition, we found that doubling the distance between the signal sequence and the TM domain has little effect on integration. When we assayed the ability of nascent chains given more time to associate with the translocon to integrate, we found that all constructs generated more ^{Ntm}PrP. These results suggest that some signal sequences intrinsically promote integration better than others.

In addition to being the first example of an N-terminal signal sequence influencing an event as temporally and spatially distant in biosynthesis as integration of the PrP TM domain, this is also the first example of integration of a TM domain being influenced by a region as distant as the signal sequence. It is widely accepted that TM domains influence integration of one another in multispanning membrane proteins (*23*, *24*), but in single-spanning membrane proteins, the only disparate region known to influence integration is a sequence located directly upstream of a TM domain termed the stop transfer effector (*25*, *26*).

PrP has been an important model protein for understanding translocational regulation. It was one of the first proteins found to be synthesized in multiple topological forms (*27*−*30*). More recently, it was used to demonstrate that the signal peptide is not a degenerate sequence, but one that can affect how a nascent protein is synthesized (*8*, *10*). Previously, the presence of two oppositely oriented TM forms prevented the use of PrP as a model for understanding integration. Here, we separate the effect of the signal sequence on N-terminal localization from the regulation of integration. Substitution of two residues in the Prl signal sequence led to an increase in the fraction of chains synthesized as ^{Ntm}PrP. Therefore, we were able to increase integration without affecting the localization of the N terminus. This is significant because the range of the signal sequence effects has previously been limited to the region of the mature domain adjacent to the signal sequence (*9*, *11*). We found, however, that even when the distance between the signal sequence and TM domain was increased we could observe effects of the signal sequence on integration.

Neither of the previously reported mechanisms thought to explain the role of the signal sequence in regulating protein biogenesis that explain the effects described here. In looking at both the ribosome−membrane junction and the timing of the signal cleavage, we found that signal sequences had different effects when connected to PrP than when on the Prl mature domain. This observation is consistent with previous reports that the signal sequence and the mature domain act together to affect biosynthesis (*11*, *31*). The observation that the state of the ribosome−membrane junction does not correlate with signal sequence effects on integration reported here suggests that the protein−protein interactions affecting junction closure, which are critical for localization of the N terminus (*8*), are distinguishable from those controlling integration.

Because the H domain is important for mediating the effects of the signal sequence on integration and the Prl H domain is much more hydrophobic than the PrP H domain, we hypothesized that hydrophobicity of the H domain would be important for mediating the effect of the signal sequence on ^{Ntm}PrP integration. When we looked at the effects of mutations that significantly alter hydrophobicity, we found two examples [mutants L(14−16)F and L(22−24)A] where making the Prl signal sequence less hydrophobic resulted in increased ^{Ntm}PrP integration. Surprisingly, however, we also found mutations that affect ^{Ntm}PrP integration without significantly changing the signal hydrophobicity [SNLLL-(20−24)LLLSN and VVSN(18−21)SNLL]. In addition, the signal sequences shown in Figure 1 that affect ^{Ntm}PrP integration vary widely in hydrophobicity. When taken together, these results suggest that the overall hydrophobicity of the H domain does not mediate the effect of the signal sequence on integration. Analysis of the SN mutant library does suggest, however, that the presence of a hydrophobic aa at position 21 may promote integration.

How the signal sequence influences ^{Ntm}PrP integration is not yet clear. Two observations suggest that a specific consensus sequence for signal sequence integration regulation will not likely emerge. First, we found that, while several signal sequences increase ^{Ntm}PrP integration, other signal sequences have the opposite effect and that there are no obvious similarities between the signal sequences that have comparable effects (Figure 1). In addition, a large variety of mutations both in the N and H domain of the Prl signal sequence were able to improve integration efficiency. If there were a simple consensus sequence, we would have expected mutation at some residues to have no effect.

One practical implication of the surprising observation that increased the length of time in the translocon that alters the fraction of chains integrated is that chemical cross linking of truncated substrates, a technique that has been most revealing for other aspects of ER translocation, cannot be utilized to explore the present phenomenon. This is because, without truncation, it is not possible to sufficiently synchronize the population of nascent chains at a precise point, and yet upon truncation, the difference between the percent of chains integrated as ^{Ntm}PrP in Prl−PrP and SN−QT PrP decreases, undermining the basis for the distinction that such an experimental approach would endeavor to make. Technological advances, such as increased cross-linking efficiency or increased detection sensitivity, may make currently unseen differences in cross linking detectable.

There are several possible mechanisms by which the signal sequence could promote integration. One possibility is that the signal sequence mediates intraprotein interactions that make it easier for the TM domain to partition into the lipid bilayer. This mechanism might be similar to the way TM domains in multispanning membrane proteins facilitate integration of other TM domains (*32*, *33*). Alternatively, the signal sequence could promote integration by preventing nonproductive interactions or conversely by promoting productive interaction of the TM domain with translocon proteins such as the translocating chain-associated membrane protein (TRAM). These explanations are not mutually exclusive and the real mechanism may involve complex effects of the signal sequence through both intra- and interprotein interactions. Dissecting how the signal sequence affects TM domain integration remains an interesting challenge for the future.

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REFERENCES

1. Keenan, R. J., Freymann, D. M., Stroud, R. M., and Walter, P. (2001) The signal recognition particle, *Annu. Rev. Biochem. 70*, 755−775.
2. Jungnickel, B., and Rapoport, T. A. (1995) A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane, *Cell 82*, 261−270.
3. Mothes, W., Jungnickel, B., Brunner, J., and Rapoport, T. A. (1998) Signal sequence recognition in cotranslational translocation by protein components of the endoplasmic reticulum membrane, *J. Cell Biol. 142*, 355−364.
4. Gorlich, D., and Rapoport, T. A. (1993) Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane, *Cell 75*, 615−630.
5. Morgan, D. G., Menetret, J. F., Neuhof, A., Rapoport, T. A., and Akey, C. W. (2002) Structure of the mammalian ribosome-channel complex at 17 Å resolution, *J. Mol. Biol. 324*, 871−886.
6. Goder, V., and Spiess, M. (2003) Molecular mechanism of signal sequence orientation in the endoplasmic reticulum, *EMBO J. 22*, 3645−3653.
7. Martoglio, B., and Dobberstein, B. (1998) Signal sequences: More than just greasy peptides, *Trends Cell Biol. 8*, 410−415.
8. Rutkowski, D. T., Lingappa, V. R., and Hegde, R. S. (2001) Substrate-specific regulation of the ribosome-translocon junction by N-terminal signal sequences, *Proc. Natl. Acad. Sci. U.S.A. 98*, 7823−7828.
9. Rutkowski, D. T., Ott, C. M., Polansky, J. R., and Lingappa, V. R. (2003) Signal sequences initiate the pathway of maturation in the endoplasmic reticulum lumen, *J. Biol. Chem. 278*, 30365−30372.
10. Kim, S. J., Rahbar, R., and Hegde, R. S. (2001) Combinatorial control of prion protein biogenesis by the signal sequence and transmembrane domain, *J. Biol. Chem. 276*, 26132−26140.
11. Kim, S. J., Mitra, D., Salerno, J. R., and Hegde, R. S. (2002) Signal sequences control gating of the protein translocation channel in a substrate-specific manner, *Dev. Cell 2*, 207−217.
12. Li, Y., Bergeron, J. J., Luo, L., Ou, W. J., Thomas, D. Y., and Kang, C. Y. (1996) Effects of inefficient cleavage of the signal sequence of HIV-1 gp 120 on its association with calnexin, folding, and intracellular transport, *Proc. Natl. Acad. Sci. U.S.A. 93*, 9606−9611.
13. Eichler, R., Lenz, O., Strecker, T., Eickmann, M., Klenk, H. D., and Garten, W. (2003) Identification of Lassa virus glycoprotein signal peptide as a trans-acting maturation factor, *EMBO Rep. 4*, 1084−1088.
14. Martoglio, B. (2003) Intramembrane proteolysis and post-targeting functions of signal peptides, *Biochem. Soc. Trans. 31*, 1243−1247.
15. Prusiner, S. B. (1998) Prions, *Proc. Natl. Acad. Sci. U.S.A. 95*, 13363−13383.
16. Kim, S. J., and Hegde, R. S. (2002) Cotranslational partitioning of nascent prion protein into multiple populations at the translocation channel, *Mol. Biol. Cell 13*, 3775−3786.
17. Chuck, S. L., and Lingappa, V. R. (1992) Pause transfer—A topogenic sequence in apolipoprotein-B mediates stopping and restarting of translocation, *Cell 68*, 9−21.
18. Voigt, S., Jungnickel, B., Hartmann, E., and Rapoport, T. A. (1996) Signal sequence-dependent function of the TRAM protein during early phases of protein transport across the endoplasmic reticulum membrane, *J. Cell Biol. 134*, 25−35.
19. Perara, E., Rothman, R. E., and Lingappa, V. R. (1986) Uncoupling translocation from translation—Implications for transport of proteins across membranes, *Science 232*, 348−352.
20. McCormick, P. J., Miao, Y., Shao, Y., Lin, J., and Johnson, A. E. (2003) Cotranslational protein integration into the ER membrane is mediated by the binding of nascent chains to translocon proteins, *Mol. Cell 12*, 329−341.
21. Heinrich, S. U., Mothes, W., Brunner, J., and Rapoport, T. A. (2000) The Sec61p complex mediates the integration of a membrane protein by allowing lipid partitioning of the transmembrane domain, *Cell 102*, 233−244.
22. Fujiki, Y., Rachubinski, R. A., and Lazarow, P. B. (1984) Synthesis of a major integral membrane polypeptide of rat liver peroxisomes on free polysomes, *Proc. Natl. Acad. Sci. U.S.A. 81*, 7127−7131.
23. Heinrich, S. U., and Rapoport, T. A. (2003) Cooperation of transmembrane segments during the integration of a double-spanning protein into the ER membrane, *EMBO J. 22*, 3654−3663.
24. Skach, W. R., and Lingappa, V. R. (1993) Amino-terminal assembly of human P-glycoprotein at the endoplasmic reticulum is directed by cooperative actions of two internal sequences, *J. Biol. Chem. 268*, 23552−23561.
25. Falcone, D., Do, H., Johnson, A. E., and Andrews, D. W. (1999) Negatively charged residues in the IgM stop-transfer effector sequence regulate transmembrane polypeptide integration, *J. Biol. Chem. 274*, 33661−33670.
26. Yost, C. S., Hedgpeth, J., and Lingappa, V. R. (1983) A stop transfer sequence confers predictable transmembrane orientation to a previously secreted protein in cell-free systems, *Cell 34*, 759−766.
27. Hegde, R. S., Mastrianni, J. A., Scott, M. R., DeFea, K. A., Tremblay, P., Torchia, M., DeArmond, S. J., Prusiner, S. B., and Lingappa, V. R. (1998) A transmembrane form of the prion protein in neurodegenerative disease, *Science 279*, 827−834.
28. Hay, B., Barry, R. A., Lieberburg, I., Prusiner, S. B., and Lingappa, V. R. (1987) Biogenesis and transmembrane orientation of the cellular isoform of the scrapie prion protein, *Mol. Cell. Biol. 7*, 914−920.
29. Hay, B., Prusiner, S. B., and Lingappa, V. R. (1987) Evidence for a secretory form of the cellular prion protein, *Biochemistry 26*, 8110−8115.
30. Lopez, C. D., Yost, C. S., Prusiner, S. B., Myers, R. M., and Lingappa, V. R. (1990) Unusual topogenic sequence directs prion protein biogenesis, *Science 248*, 226−229.
31. Andrews, D. W., Perara, E., Lesser, C., and Lingappa, V. R. (1988) Sequences beyond the cleavage site influence signal peptide function, *J. Biol. Chem. 263*, 15791−15798.
32. Ota, K., Sakaguchi, M., Hamasaki, N., and Mihara, K. (2000) Membrane integration of the second transmembrane segment of band 3 requires a closely apposed preceding signal-anchor sequence, *J. Biol. Chem. 275*, 29743−29748.
33. Skach, W. R., Calayag, M. C., and Lingappa, V. R. (1993) Evidence for an alternate model of human P-glycoprotein structure and biogenesis, *J. Biol. Chem. 268*, 6903−6908.

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