Specific Features of the Prion Protein Transmembrane Domain Regulate Nascent Chain Orientation

Carolyn M. Ott, Armin Akhavan, and Vishwanath R. Lingappa

From the Departments of 1Biochemistry and Biophysics and 2Physiology and Medicine, University of California, San Francisco, California 94143 and 3California Pacific Medical Center Research Institute, San Francisco, California 94107

The sequence of a transmembrane (TM) domain and the adjacent regions are important for recognition, orientation, and integration at the translocon during membrane protein biosynthesis. However, the sequences of individual TM domains vary considerably. Although some general effects of electrostatic and hydrophobic interactions have been observed, it is still not clear what features of diverse sequences influence TM domain orientation. Here we utilized the ability of the prion protein (PrP) to be synthesized in multiple topological forms to assay the effects of substitutions and mutations on TM domain orientation. Several of the TM domains we tested appear to contain no inherent information regulating orientation. In contrast, we found that the middle region of the PrP TM domain significantly reduces the ability of the chain to invert its orientation in the translocon. We also observed that the C-terminal region of the PrP TM domain influences orientation, and we characterized the orientation differences between two forms of a physiologically relevant polymorphism in this region. Specifically, we found that the identity of a single amino acid, that at position 129, can significantly alter PrP TM domain orientation. Because position 129 is the location of the disease-associated Met/Val polymorphism, we discuss both how this small change may affect TMD orientation and the larger biological implications of these results.

Nascent membrane proteins are synthesized on cytoplasmic ribosomes and targeted to the endoplasmic reticulum (ER) co-translationally through the interaction of signal or signal-anchor sequences with the signal recognition particle (1). At the ER membrane the ribosome-nascent chain complex associates with the translocon, a proteinaceous pore in the ER lipid bilayer. Signal and signal-anchor sequences are first inserted into the translocon with the N terminus near the ER lumen (2). Inversion of the signal sequence is necessary for proper translocation of secretory proteins and subsequent cleavage of the signal sequence. Signal-anchor sequences, which integrate into the lipid bilayer and become transmembrane (TM) domains, can integrate before or after signal inversion occurs (generating proteins with the majority of the amino acids in the cytoplasm or exoplasm, respectively) (2, 3). Single-spanning membrane proteins without signal-anchors contain internal TM domains, which are recognized within the ribosome exit channel and at the translocon (4). Like signal-anchor sequences, these TM domains can integrate in either orientation. Regulation of signal-anchor and TM domain orientation is essential for proper membrane protein biosynthesis.

Several features of the nascent chain that influence TM domain orientation have been identified. Charged residues adjacent to the TM domain usually orient on the cytoplasmic side of the ER membrane (the positive-inside rule) due to electrostatic interactions (5, 6). In addition, the hydrophobicity of the TM domain is thought to influence orientation (3). It has been observed that more hydrophobic TM domains flip orientations more slowly and presumably less frequently (2). Secondary structure can also influence orientation. Residues that increase flexibility promote TM domain re-orientation, whereas rigid structures are sterically hindered from inverting (8). Although these features can be used to help predict the orientation of transmembrane domains, error rates in prediction algorithms suggest additional features of the translocon and nascent chain influence orientation (9).

Several studies have utilized model signal-anchor proteins (composed of long stretches of leucine residues) to characterize the properties of the nascent chain that influence orientation (2, 8). Although this model system has been very informative, it also has limitations. Because signal-anchor sequences are used for targeting the nascent chain and engaging the translocon in addition to mediating orientation and integration (10–14), it is difficult to be certain that changes in the signal-anchor sequence affect only orientation. An additional limitation is due to the highly regular sequence of the model signal-anchor. An amazing feature of the translocon is the ability to reproducibly process proteins with highly diverse sequences. How is so much variability between TM domain sequences tolerated? What are the features of individual TM domains that influence orientation?

We have employed a different system to try to address these questions. The prion protein (PrP) can be synthesized in mul-
Determinants of PrP Orientation

Multiple topological forms (15–18). During co-translational translocation at the ER, some PrP nascent chains pass completely through the translocon to generate secretory PrP (SecPrP), others integrate into the lipid bilayer with their N terminus in the ER lumen (NtmPrP), and still others integrate in the opposite orientation (CtmPrP; see Fig. 1A). The ability of PrP to be made in multiple topological forms has made it an ideal candidate for studying regulation of membrane protein biosynthesis. Previously it was used to examine the ability of the signal sequence to effect the partitioning of the TM domain into the lipid bilayer (19). Here we use PrP as a model to examine the effect of TM domain substitutions and mutations on orientation.

To generate the different topological variants of PrP, the nascent polypeptide must exist in at least two intermediate states in the translocon (depicted in Fig. 1A). In the first the signal sequence has an Ncyt/Cexo orientation (the N terminus is cytoplasmic, and the C terminus is exoplasmic), the transmembrane domain has the opposite orientation (Nexo/Ccyt), and the connecting sequence is in the lumen of the ER. NtmPrP is generated upon integration of the TM domain in this orientation, whereas SecPrP results if the TM domain does not integrate. The alternate intermediate state is the precursor for CtmPrP and cytoplasmic PrP. In this intermediate state the signal and connecting sequences are in the cytosol, and the TM domain has an Ncyt/Cexo orientation (this is consistent with the observation that CtmPrP contains an uncleaved signal sequence). Movement of the nascent chain between the two intermediate states requires inversion of the transmembrane domain. Presumably all TM domains begin with an Nexo/Ccyt orientation and only re-orient after a sufficient length of polypeptide has been synthesized to span the length of the translocon and connect the C-terminal end of the TM domain to the ribosome. In these studies we use the topological distribution of full-length PrP as a read out for the distribution of the nascent chains in the intermediate stages and the ability of the TM domain to re-orient in the translocon. We utilized substitutions and mutations in different regions of the PrP TM domain to characterize the features that influence orientation.

In addition to providing important information about the role of the TM domain sequence in determining orientation, we were able to gain insights into how the biosynthesis of PrP is regulated, which is especially important for understanding prion pathology. SecPrP in the ER is the precursor of both PrPc and PrPSc, the normal and scrapie-associated forms of PrP which do not contain integrated TM domains but associate with the membrane through a glycolipid anchor (20). NtmPrP has been observed in vivo on the plasma membrane of platelets (21). Generation of CtmPrP has been shown to occur in both infectious and familial prion disease (15, 22). Because we characterized the effect of amino acid substitutions on nascent chain orientation, we were able to detect differences in the topological distribution between two polymorphic forms of wild type PrP. This disease-associated polymorphism is located in the C-terminal region of the PrP TM domain at position 129 (23–25). Our observations provide potential insight into how the polymorphism at 129 may affect prion disease pathogenesis.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Constructs used in vitro transcriptions were derived from the pSP64 hamster PrP (haPrP), mouse PrP (moPrP), and human PrP (huPrP) constructs published previously (14, 15). To generate the TM domain chimeric constructs, the amyloid precursor protein (APP), immunoglobulin M (IgM), vesicular stomatitis virus glycoprotein (VSVG), T-cell specific surface protein (CD28), Cop-coated vesicle membrane protein p24 (p24), interleukin 2 receptor (IL2R), and asialoglycoprotein receptor (ASGP-R) TM domains were PCR-amplified, digested, and ligated into NdeI and NsiI sites outside the PrP TM domain. All other mutants and chimeras were generated by site directed mutagenesis. Transfection vectors were in the pCDNA3.1 vector (Invitrogen).

In Vitro Transcription, Translation, Translocation, and Proteolysis—In vitro transcription and translation were performed as described previously (26). During cell-free translation 35S-labeled Met was incorporated into the nascent chain. Translations were carried out at 34 °C for 30 min. Glycosylation was inhibited by 0.2 mM tripeptide competitor (26). Microsomal membrane isolation has been described previously (10). Samples were proteolized at 4 °C for 45 min with 0.2–0.4 mg/ml proteinase K (pk; Merck). The protease was inactivated by incubation with 10 mM phenylmethylsulfonyl fluoride for 5 min and boiling in 10 volumes of 0.1 M Tris, 1% SDS.

Cell Culture—CHO-K1 cells were cultured in Ham’s F-12 complete medium with 10% fetal bovine serum and antibiotics. Transfections were performed using Lipofectamine Plus (Invitrogen). For each 35-mm well, 3 μg of DNA was incubated with 125 μl of serum-free media and 7.5 μl of Plus reagent for 15 min. In parallel, 5 μl of Lipofectamine was incubated with 125 μl of serum-free media. The two solutions were then mixed and incubated for an additional 15 min at room temperature. This mixture was then added to the well containing 1 ml of fresh serum free media. After 3 h the media was replaced with complete media. Before pulsing with 35S Met, the cells were incubated for 30 min in Ham’s F-12 Cys/Met-free media with 10% dialyzed fetal calf serum and antibiotics. Cells were labeled with 30 μl of 35S EasyTag (PerkinElmer Life Sciences) per well 24 h after transfection for a duration of 4 h.5

Cell Harvesting, Lysis, and Proteolysis—After a 10-min incubation on ice, cells were harvested by scraping and transferred to a 15-ml Falcon tube. They were then pelleted for 5 min at 3000 rpm (2060 × g) and 4 °C in a Beckman GS6R centrifuge. The medium was removed, and hypotonic lysis was carried out in 0.6 ml of 4 °C 10 mM HEPES, pH 7.5. For homogenization, the cells were passed through a 27-gauge needle 10 times. 1/10 volume of 10X physiologic salt buffer (0.5 M HEPES, pH 7.5, 1 M potassium acetate, 50 mM magnesium acetate) was then added.4 For proteolysis, samples were split into three 200-μl aliquots. One sample was treated with both 8 μl of 10 mg/ml pk and 10 μl of 20% Triton X-100, another was treated only with pk, and the third was left untreated. All samples were incubated on ice at 4 °C for 1 h, treated with phenylmethylsulfonyl fluoride, and then transferred to 4 volumes of boiling 1% SDS, 0.1 M Tris, pH 8.0.

5 S. Sagafi and V. Lingappa, unpublished methods.
Determinants of PrP Orientation

Immunoprecipitation and N-Glycosidase F Treatment—400 μl of proteolyzed sample was added to 1.25 ml of 1.5 × Doc TXSWB (75 mM potassium acetate, 75 mM Tris acetate, pH 8.0, 0.75% deoxycholate, 1.5% Triton X-100), 20 μl of packed protein A beads, and 1 μl of 3% F4 or 20 μl of packed protein G beads and 2 μl of 13A5 ascites.4 Samples were mixed gently overnight at 4 °C. The beads were then washed 3 times with 1× Doc TXSWB and 1 time with 10 mM Tris acetate, pH 8.0. The beads were then boiled to evaporate liquid and boiled in 50 μl of 0.5–1% SDS, 0.1 M Tris, pH 8, to release the PrP from the beads.

For N-glycosidase F treatment 50 μl of 2% β-mercaptoethanol, 0.1 M Tris, pH 6.8, was added to each sample, and the tubes were incubated for 15 min at 37 °C then boiled for 2 min. 2.5 μl of 20% Triton X-100 was added, and the samples were mixed by vortexing. The samples were split into two 45–50 μl aliquots; to one, 1 μl of N-glycosidase F was added. The samples were then left at least 6 h at 37 °C.

Calculations and Statistical Analysis—Because 129 PrP has not been detected in cell culture, %CtmPrP in the cell culture experiments was calculated as 100 × (CtmPrP/(CtmPrP + SecPrP)). In all other experiments it was calculated as 100 × (CtmPrP/(CtmPrP + SecPrP + NtmPrP)). In Figs. 4 and 5 some data are shown as normalized %CtmPrP. The normalized value was calculated as the %CtmPrP of the sample divided by the %CtmPrP of wild type Met-129 PrP. Normalization enabled us to compare internally consistent data that varied slightly between experiments.

Mean values and S.D. were obtained from at least three independent experiments and graphed as histogram bars. Student’s t test was used to test for differences between the means of two samples. Statistically significant differences are indicated by the corresponding p value. In Fig. 4C, we claim a difference in percentCtmPrP generated by the Met-129 and Val-129 variants of A117V. This difference is minor and statistically insignificant, possibly because A117V is intrinsically highly Ctm favoring. Nonetheless, this difference is reproducible in all experiments.

RESULTS

The sequence of the hamster PrP TM domain and surrounding regions is shown in Fig. 1B. Although there are charged

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**FIGURE 1.** The PrP TM domain information that influences chain orientation. A, the TM domain emerges from the ribosome it enters the translocon with the N terminus near the ER lumen (or exoplasm) and the C terminus on the cytosolic side. This is the Nexo/Cexy intermediate state. After translation of more of the nascent protein the TM domain can re-orient within the translocon to form the Ncyt/Cexo intermediate. When this inversion takes place the signal sequence moves from the translocon to the cytosol. PrP can be made in four different topological forms at the ER membrane. Shown here are: NtmPrP, which spans the membrane and has its N terminus in the lumen; SecPrP, which is fully translocated across the ER membrane (and subsequently modified with a glycosylphosphatidylinositol (GPI) anchor, shown as a thin white box); CtmPrP, which integrates into the plasma membrane and has its N terminus in the lumen; and 129 PrP. Normalization enabled us to compare internally consistent data that varied slightly between experiments.

**TABLE 1.** Percent of chains made as CtmPrP. In several of the constructs with high levels of CtmPrP (which has an uncleaved signal), both the signal uncleaved (CtmPrP) and signal-cleaved forms can be distinguished in the absence of protease.

**TABLE 2.** Percent of chains made as CtmPrP. In several of the constructs with high levels of CtmPrP (which has an uncleaved signal), both the signal uncleaved (CtmPrP) and signal-cleaved forms can be distinguished in the absence of protease.
residues on either side of the TM domain, one might predict that the TM domain would be more likely to orient Nexo/Ccyt and generate CtmPrP because there are more positively charged residues N-terminal to the TM domain. However, wild type PrP generates less than 10% CtmPrP as shown in Fig. 1C. Surprisingly, substitution of the Lys and His residues at positions 109 and 110 with two Ile causes CtmPrP levels to increase substantially, and leads to neurodegeneration (15). These observations suggest that the positive inside rule is not providing the overriding information to determine PrP TM domain orientation.

We, therefore, proposed that there must be information inherent in the PrP TM domain and possibly other TM domains that influence orientation. To test this hypothesis we substituted the PrP TM domain with the TM domains from proteins, which in their native context integrate in either the Ntm (vesicular stomatitis virus glycoprotein (VSVG), amyloid precursor protein (APP), IgM, CD28, interleukin 2 receptor (IL2R), and p24), or the Ctm (asialoglycoprotein receptor ASGP-R) orientation (sequences shown in Fig. 1B). We intentionally chose sequences that vary in length, hydrophobicity, and presumably structure.

To assess the effect of the substitutions on TM domain orientation, we transcribed and translated the constructs in vitro in the presence of microsomal membranes. The microsomal membranes were then isolated, resuspended, and incubated in the presence or absence of pk. After inactivation of the protease, the different PrP isoforms were separated by SDS-PAGE on Tricine gels. After exposure of the gels to film, the fraction of chains in each topology was quantitated. SecPrP, which translates fully into the microsomal lumen, is protected from the protease, whereas CtmPrP and NtmPrP can be identified by characteristic shifts in molecular weight upon protease addition. We used the increase in CtmPrP as an indicator of shifts in the equilibrium of the two intermediate states. This is valid even without quantitating cytosolic PrP because wild type PrP favors the Nexo/Ccyt intermediate. For some samples the total number of chains appears to decrease upon protease treatment (for example, see – CD28 and + pk in Fig. 1C). It is possible that the missing population is cytosolic PrP that somehow remains associated with the microsomal membranes when they are pelleted but then is completely digested by protease. However, we used only the relative amounts of SecPrP, NtmPrP, and CtmPrP in the + pk lanes for our calculations. Again, this gives us a good barometer for shifts in the integration intermediates because wild type PrP strongly favors the Nexo/Ccyt intermediate.

When we examined the TM domain chimeras we were surprised to find that all constructs generated considerably more CtmPrP than wild type PrP (see Fig. 1C). This was true regardless of the orientation of the TM domain in its native context. Therefore, in the presence of the foreign TM domains the balance between the two states is shifted toward the Nexo/Cexo intermediate. This suggests that the contextual information outside the PrP transmembrane domain favors the Nexo/Cexo intermediate according to the positive inside rule. What then are the features of the PrP TM domain that cause it to favor the Nexo/Cexo intermediate?

It has been speculated that the ability of PrP to be made in multiple topological forms is due in large part to the unique features of the TM domain (18). Unlike conventional TM domains, the PrP TM domain contains many glycines and alamines. The presence of Ala and Val residues in a model signal-anchor sequence has been shown to alter the orientation of the TM region, perhaps by affecting the flexibility of the nascent chain. Because the initial state of all PrP TM domains is Nexo/Ccyt, one might hypothesize that amino acids that increase the flexibility of the chain would promote re-orientation and formation of the Ncyt/Cexo intermediate (and, thus, more CtmPrP), which does not appear to be the case.

To dissect this puzzling situation we decided to substitute smaller regions of the PrP TM domain with the amino acid sequence of the IgM TM domain. We defined three regions within each TM domain, an N-terminal region, a middle region, and a C-terminal region (shown in Fig. 2A). We then generated a series of six new constructs that contain all possible combinations of the different TM regions (Fig. 2A). Constructs were named with the letters representing each domain present. For example, PIP has the middle region from IgM and the PrP N- and C-terminal domains. We then assayed the topological distribution of these constructs and calculated the percent CtmPrP generated. The data from these experiments are represented in Fig. 2, B–D.

In Fig. 2B we compare the effect of the IgM and PrP N-terminal regions on PrP topology. Several disease-associated mutations in the PrP TM domain have been found to significantly increase the generation of CtmPrP (15, 22, 27). These mutations are all in the N-terminal region of the TM domain, suggesting that this region may be important for determining TM domain orientation. However, we found that substituting this region of PrP with IgM had little effect on the topology of an otherwise wild type PrP chain (PPP versus IPP).

The effect of the IgM sequence in the middle region of the PrP TM domain was much more striking (see Fig. 2C). In all cases the presence of the IgM middle region leads to an increase in CtmPrP production. This portion of the TM domain is, therefore, very important for maintenance of the Nexo/Ccyt intermediate. The role of the C-terminal region in TM domain orientation was less obvious. However, we noticed that in the presence of the PrP middle region the IgM C-terminal region significantly reduced CtmPrP (compare PPP and PPI or IPP and IPI, Fig. 2D). The C-terminal region has not been previously implicated in studies of disease causing mutations that alter topology. This result indicates that in the presence of the PrP middle region, the C-terminal region is essential for inversion of the Nexo/Ccyt intermediate.

We next decided to investigate the features of this C-terminal region that are responsible for mediating TM domain re-orientation. We searched through sequence databases and identified two TM domains with features similar to the corresponding region of PrP or IgM; the sixth predicted TM domain from the human growth hormone-inducible transmembrane (GHIT) protein, which has some similarities to the C-terminal region of the PrP TM domain, and the last eight amino acids of the TM domain from the human myelin associated glycoprotein (MAG), which contains several amino acids with similar properties to those found in IgM. We used site-directed mutagenesis to replace these sequences into wild type PrP. In addition we substituted...
the C-terminal region of the PrP TM domain with eight central hydrophobic amino acids and, separately, the last eight amino acids of the asialoglycoprotein receptor because this TM domain orients Ncyt/Cexo in its native context. When we assayed PrP topology we found that all of these chimeras generated levels of CtmPrP well below wild type (see Fig. 3). We were surprised that the growth hormone-inducible transmembrane protein sequence, which has several of the same amino acids as those found in PrP, caused such a significant reduction in the levels of CtmPrP integration. Although these results confirm that a feature of the C-terminal region of the PrP TM domain is important for TM domain inversion, they do not enable us to draw any additional conclusions.

To try to get more information we made another series of constructs in which we substituted parts of the IgM or growth hormone-inducible transmembrane protein sequence for the corresponding sequence in PrP. For example, since growth hormone-inducible transmembrane protein is so similar to PrP, we mutated just the first two or, separately, the last three amino acids. The substitutions and results are shown in Fig. 3. We found that the only constructs that generated wild type levels of CtmPrP were those that have Met at position 129 (see Fig. 3, A and B). Constructs with a Ser in this position have the lowest levels of CtmPrP so we also generated a M129S construct, which had similar low levels of CtmPrP. We were surprised that this single amino acid would have such a dramatic effect on the ability of the nascent chain to convert from the Nexo/Ccyt intermediate to the Ncyt/Cexo intermediate.

It was not lost on us that position 129 is also the site of a Met/Val polymorphism in humans. The identity of the amino acid at position 129 has a significant impact on infectious, sporadic, and familial prion diseases. Although the importance of the polymorphism has been well documented, studies have only revealed slight effects of the substitution on prion stability and conversion (28, 29) and a possible effect on the kinetics of amyloid formation (30). In light of the biological importance of CtmPrP for prion disease, we wanted to compare the levels of CtmPrP generated by the two polymorphic forms of PrP, Met-129 and Val-129. We
Determinants of PrP Orientation

A

|        | hamster | mouse | human |
|--------|---------|-------|-------|
| 129    | Met     | Val   |       |
| pk     | +       | -     |       |
| N1      |         |       |       |
| C1      |         |       |       |
| CtmPrP  |         |       |       |
| CtnPrP  |         |       |       |
| NmPrP   |         |       |       |
| 1117V   |         |       |       |
| 131V    |         |       |       |
| 178N    |         |       |       |

B

![Graph showing % CtmPrP for different PrP constructs](Image)

C

![Graph showing normalized % CtmPrP for different PrP constructs](Image)

FIGURE 4. Methionine generates more CtmPrP than valine at position 129 of PrP. A, PrP from hamster, mouse, and human with either a Met or a Val at position 129 was transcribed and translated in vitro, and the topological forms were assayed as described in Fig. 1. B, the data in A were quantitated. C, human PrP constructs with the indicated polymorphisms and mutations were transcribed and translated as described. Each mutant was analyzed in triplicate parallel to wild type PrP.

made the constructs in the hamster PrP gene, transcribed and translated in vitro, then assayed the levels of CtmPrP. Fig. 4, A and B, demonstrate that hamster Met-129 PrP generates more CtmPrP than Val-129 PrP. This is the first demonstration that

the Met/Val polymorphism affects nascent protein biosynthesis. However, it is possible that the effect of Val/Met polymorphism that we observe is specific to hamster PrP and irrelevant to human disease. This is especially significant because humans are the only organism found to possess a polymorphism at 129 (31). To verify that the difference we observed between Met-129 and Val-129 was also true for PrP from other organisms, we generated mouse and human PrP constructs with either the Met or the Val at position 129. Upon analysis of the topological distribution we found that Met-129 PrP consistently generated more CtmPrP than Val-129 PrP (see Fig. 4, A and B).

Many disease-associated mutations in PrP are consistently found in the presence of either Met or Val at position 129 (25). We wondered whether the reason these mutations were found with a single polymorphic form of the prion disease was due to an effect on CtmPrP biogenesis. To test this hypothesis we generated many mutant constructs with either a Met or a Val at position 129. A previous study had already examined the effect of individual mutations on CtmPrP levels (27), but a 129 polymorphism correlation to CtmPrP synthesis has not been reported. We consistently observe that regardless of the mutation, Met-129 constructs generate more CtmPrP than Val-129 constructs (see Fig. 4C and data not shown). For most mutations, however, we found no convincing effect of the mutations on CtmPrP integration. Fig. 4C shows the levels of CtmPrP generated by the polymorphic forms of wild type human PrP, A117V, G131V (Gerstmann-Straussler syndrome-causing mutations), and D178N (which causes CJD or fatal familial insomnia). A117V, which has already been shown to increase CtmPrP levels both in vitro and in human brain tissue (15), is commonly found allelic to Val at 129. Fig. 4C demonstrates that both polymorphic variants generate more CtmPrP than wild type. The G131V was reported in a Gerstmann-Straussler syndrome patient homozygous for Met at 129 (32). Interestingly, the levels of CtmPrP generated by the two disease causing mutations (A117V Val-129 and G131V Met-129) are very similar. The difference in levels of CtmPrP between wild type and D178N were much less dramatic. D178N Met-129 appears to make more CtmPrP than wild type Met-129 PrP. However, the levels for Val-129 D178N are similar to those seen for Val-129 PrP (see Fig. 4C).

Because the levels of CtmPrP have been shown to be an important part of prion disease pathology (15, 22), we decided it was important to test whether the identity of the amino acid at position 129 affects the level of CtmPrP generated in living cells. The substitutions reported in Fig. 3 indicate that Ser, Val, and Ile at 129 in hamster PrP can all inhibit TM domain inversion. To investigate this further we generated transfection constructs with Met, Val, Leu, Ser, or Gly at position 129 of hamster PrP in the pCDNA3.1 vector. We also included the Tyr-Met to Ser-Ser substitution at positions 128 and 129 shown in Fig. 3. To assess the level of CtmPrP production in living cells we transfected these constructs into CHO-1 cells, labeled the cells with [35S]Met, collected the cells, and homogenized them. We then split the homogenate into three aliquots. One was left untreated, another was treated with proteinase K, and the third was treated with both proteinase K and detergent. PrP was immunoprecipitated from these samples, half of which were
subsequently treated with N-glycosidase F to remove glycosylation. The samples were then separated by SDS-PAGE on Tricine gels. In parallel with the 129 mutants, we also analyzed A120L (a C^TM^PrP favoring mutant) and G123P (a mutant that makes almost no C^TM^PrP). In Fig. 5A C^TM^PrP is visible in the positive control (A120L) as well as Met-129 and Leu-129. Under the mild pk conditions we used here C^TM^PrP is protease-resistant even in the presence of detergent. Fig. 5B, the percent C^TM^PrP from several experiments was quantified and normalized to the value of wild type Met-129 PrP, which enabled us to compare data from different experiments. For all mutants, the value of C^TM^PrP was found to be significantly different from hamster Met-129 (p < 0.01).

DISCUSSION

Here we examined the properties of the nascent chain that influence inversion of the TM domain in the translocon. To this end we utilized PrP as a model system because it can be synthesized in multiple topological forms. C^TM^PrP can only be produced upon inversion and subsequent integration of the PrP TM domain in the translocon. Therefore, we used production of C^TM^PrP as a readout for TM domain re-orientation. We found that properties of the PrP TM domain prevent significant orientation inversions despite the positive charges outside the region, which might be expected to promote such reversals. The middle region of the TM domain is in large part responsible for preventing orientation inversion. We also found that the C-terminal region, specifically residue 129, can alter the ability of the TM domain to invert, both in vitro and in cell culture. This finding is significant because position 129 is the site of a disease-associated Met/Val polymorphism. Below we discuss the implications of these findings for understanding both membrane protein biosynthesis and infectious, sporadic, and familial prion disease.

Determination of Membrane Protein Orientation—An intriguing question in the field of membrane protein biosynthesis is how membrane proteins with such diverse sequences can be consistently oriented and integrated at the translocon. Recent insight has come from studies of the machinery involved in these processes including the crystal structure of the translocon from Methanococcus jannaschii (33). Additional studies using cross-linking and other methods to map the interaction of the nascent chain with the translocon components have provided valuable insight into the mechanisms of integration (34). Additional research has begun to define some of the features of the nascent chain that influence orientation by studying model signal-anchor proteins (2, 8). But it is still not clear what features of diverse nascent chains influence orientation. Here we were able to compare the effect of significant substitutions and subtle mutations on TM domain orientation by utilizing the PrP model system in which all substitutions and mutations affect only the orientation and integration of the TM domain. In this model targeting and translocation initiation are mediated by the signal sequence independent of the TM domain.

When we replaced the PrP TM domain with foreign transmembrane segments we found a significant portion of the nascent chains integrate in the N_cyt/C_Cys orientation regardless of the orientation in their native context. These chimeric proteins all followed the positive-inside rule, indicating that unlike PrP, none of these TM domains contain enough information to overrule the contextual orientation signals outside the TM domain. To identify the features of the PrP TM domain responsible for regulating TM domain orientation we made a series of substitutions. We found that each part of the PrP TM domain makes a contribution to transmembrane topology. The N-terminal region of the PrP TM domain has been shown previously to affect C^TM^PrP biogenesis (15); however, the effects of substituting this region were small in this assay. This could be because the amino acids of the IgM N-terminal region somehow mimic the effects of the PrP sequence.

The middle region of the PrP TM domain is much less hydrophobic than the corresponding regions from other proteins. This region contains many valine and glycine residues, but only one leucine, which is common in the hydrophobic cores of many transmembrane regions. It has been speculated that this difference in part accounts for the ability of PrP to be made in different topological forms (35). The data in Fig. 3 demonstrate that the middle region of the transmembrane domain is essential for PrP versatility. When we replaced this region with the
Determinants of PrP Orientation

corresponding sequence from IgM, a significantly larger fraction of TM domains was able to invert in the translocon and generate CtmPrP. Further studies will be necessary to determine whether these effects are mediated by the flexibility of the nascent chain or specific amino acid interactions.

A role for the C-terminal region of the PrP TM domain was previously unappreciated. We found that the requirement for this region is dependent on the PrP TM middle domain. This can be seen by comparing PPI, which integrates very poorly, to PIIL, which integrates well (compare percent CtmPrP Fig. 2). Perhaps this region of the PrP TM domain was overlooked in previous studies because no disease-associated mutations were identified in this region until 2001 when a patient with the rare prion disease Gerstmann-Straussler-Scheinker was found with the mutation G131V (32). We assayed the topology generated by this PrP mutant and found that it does have increased levels of CtmPrP (Fig. 4).

The observation that the C-terminal region influences orientation only in the presence of the middle domain suggests that specific protein–protein interactions and not passive hydrophobic interactions may mediate PrP TM domain inversion. This is supported by the observation that a single amino acid (at position 129) can completely prevent TM domain inversion. The presence of small hydrophobic amino acids such as Val and Ile was insufficient to promote re-orientation. Only Met and Leu, large hydrophobic amino acids, could promote TM domain inversion.

The mechanism of TM domain inversion is not well understood. One possibility (shown in Fig. 6A) is that the TM domain adopts a secondary structure that reduces the energetic barrier to inversion. The large number of glycine residues in the middle region of the PrP TM domain may make this region more flexible than the other TM domains tested. The amino acid at position 129 may be essential for stabilizing an inversion intermediate. A second (and not mutually exclusive) model is shown in Fig. 6B. The middle and C-terminal regions of the TM domain could interact with Sec61α or some other component of the translocon that promotes TM domain inversion. Further experiments are required to dissect this mechanism.

Implications for Prion Pathobiology—The disease-associated Met/Val polymorphism at position 129 is an unexplained feature of prion biology. The identity of the amino acid at this position has a significant impact on human prion diseases. For example, every human diagnosed with vCJD has been Met homozygotes (23, 39). Yet it is already known that differences in CtmPrP levels affect both the incubation period and time course of prion diseases (22). Perhaps there is an intracellular threshold for CtmPrP. It is possible that cells tolerate low levels of CtmPrP expression (e.g. through ER-associated degradation), but when CtmPrP levels exceed a certain threshold the CtmPrP exits the ER and triggers neurodegeneration. This would be consistent with recent reports that CtmPrP at the plasma membrane inhibits cytokinesis (40). The hypothesis is also supported by the observation that low level expressors of CtmPrP-favoring KH > II Tg mice show no CtmPrP and develop no neurodegeneration, where middle level expressors have low levels of CtmPrP and develop spontaneous disease after an extended period and high level expressors have massive amounts of CtmPrP and develop spontaneous disease rapidly (15).

The threshold model could explain how the subtle difference in CtmPrP production between Met-129 and Val-129 PrP reported here could contribute to the observed difference in vCJD. Upon infection, PrPSc is thought to cause an increase in the amount of CtmPrP made by the cell (22). Perhaps upon infection Met-129 homozygotes generate more CtmPrP than heterozygotes and Val homozygotes. The Met homozygotes could exceed the CtmPrP threshold and develop disease, whereas the CtmPrP levels of heterozygotes and Val homozygotes remain below the

6. C. M. Ott and V. R. Lingappa, unpublished data.

FIGURE 6. Two models for TM domain inversion. A, the middle and C-terminal regions of the PrP TM domain could form a secondary structure within the translocon that makes re-orientation of the TM domain more favorable. Here we have depicted bends in the middle region because it contains several glycine residues. Large hydrophobic amino acids like Met and Leu at position 129 could stabilize the secondary structure (thus, generating more CtmPrP), whereas smaller amino acids like Val and Ser may not support the inversion intermediate as well and, therefore, generate less CtmPrP. B, it is also possible that the middle and C-terminal regions of the PrP TM domain are able to interact with proteins at the translocon that facilitate TM domain inversion. In this case the middle region may establish the initial contacts with the channel component, and the C-terminal region supports or contributes to the interprotein interactions. Small amino acids at position 129 may interact less effectively with channel components and, therefore, result in fewer TM domain inversions.
threshold, e.g. at a level manageable by ER-associated degradation. This hypothesis seems more plausible considering the predicted mean incubation time for vCJD is 11 years (41). Perhaps the incubation time for heterozygotes is far longer, e.g. 30 or 60 years. Interestingly, preclinical vCJD was recently reported in a Met/Val heterozygote who died of a non-neurological disorder, consistent with this hypothesis (7).

Further studies will be necessary to determine both how position 107 influences TM domain inversion and how small changes in the amount of CtmPrP generated affect cell and tissue health. It is clear, however, that examining the diverse nature of TM domain sequences has the potential to reveal interesting and biologically relevant details of membrane protein biosynthesis.

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