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The prion protein (PrP) can adopt multiple membrane topologies, including a fully translocated form (SecPrP), two transmembrane forms (NtmPrP and CtmPrP), and a cytosolic form. It is important to understand the factors that influence production of these species, because two of them, CtmPrP and cytosolic PrP, have been proposed to be key neurotoxic intermediates in certain prion diseases. In this paper, we perform a mutational analysis of PrP synthesized using an in vitro translation system in order to further define sequence elements that influence the formation of CtmPrP. We find that substitution of charged residues in the hydrophobic core of the signal peptide increases synthesis of CtmPrP and also reduces the efficiency of translocation into microsomes. Combining these mutations with substitutions in the transmembrane domain causes the protein to be synthesized exclusively with the CtmPrP topology. Reducing the spacing between the signal peptide and the transmembrane domain also increases CtmPrP. In contrast, topology is not altered by mutations that prevent signal peptide cleavage or by deletion of the C-terminal signal for glycosylphosphatidylinositol anchor addition. Removal of the signal peptide completely blocks translocation. Taken together, our results are consistent with a model in which the signal peptide and transmembrane domain function in distinct ways as determinants of PrP topology. We also present characterization of an antibody that selectively recognizes CtmPrP and cytosolic PrP by virtue of their uncleaved signal peptides. By using this antibody, as well as the distinctive gel mobility of CtmPrP and cytosolic PrP, we show that the amounts of these two forms in cultured cells and rodent brain are not altered by infection with scrapie prions. We conclude that CtmPrP and cytosolic PrP are unlikely to be obligate neurotoxic intermediates in familial or infectiously acquired prion diseases.

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Prion diseases are fatal neurological disorders of humans and animals that appear in sporadic, familial, and infectiously acquired forms. These disorders are caused by conversion of a normal neuronal glycoprotein (PrP<sup>C</sup>)<sup>1</sup> into a conformationally altered isoform (PrP<sub>Sc</sub>) that is infectious in the absence of nucleic acid (1, 2). PrP<sub>Sc</sub>, which is soluble and protease-sensitive, consists of an α-helical, C-terminal domain and an unstructured N-terminal domain. In contrast, PrP<sub>Sc</sub> is rich in β-sheets, aggregated, and protease-resistant. The physiological function of PrP<sup>C</sup> is uncertain but may be related to transport of copper ions or protection from oxidative stress (3).

PrP<sup>C</sup> is unusual because it can adopt multiple membrane topologies. Most PrP<sup>C</sup> molecules are attached to the outer leaflet of the plasma membrane through a C-terminal glycosphosphatidylinositol (GPI) anchor (this topology is designated SecPrP) (4, 5). However, some PrP<sup>C</sup> molecules assume a transmembrane orientation when synthesized in vitro or in cells (6–11). These forms, designated NtmPrP and CtmPrP, span the lipid bilayer once via a highly conserved hydrophobic region in the center of the molecule (amino acids 111–134), with either the N or C terminus, respectively, on the extracytoplasmic side of the membrane. It has been shown that these species are generated in small amounts (<10% of the total PrP) as part of the normal biosynthesis of wild-type PrP in the endoplasmic reticulum (ER). However, mutations within or near the transmembrane domain, including an A117V mutation linked to GSS as well as several “artificial” mutations not seen in human patients, increase the relative proportion of CtmPrP to as much as 20–30% of the total (7, 12).

Recent studies have begun to define the mechanisms responsible for determining PrP topology during the translation process. We discovered that a non-conservative substitution (L9R) within the hydrophobic core of the signal sequence dramatically increased the proportion of CtmPrP (13). Combining this mutation with a triple substitution (3AV) within the transmembrane domain resulted in a molecule that was synthesized exclusively as CtmPrP. These results indicated that the signal sequence as well as the transmembrane domain were major determinants of PrP topology. Work by Hegde and colleagues (9–11) has demonstrated that these two determinants act in mechanistically distinct ways. The signal sequence serves a dual function, first targeting the nascent polypeptide chain to the translocon channel in the ER membrane via binding to the signal recognition particle, and subsequently gating the translocon to allow passage of the N terminus into the ER lumen. In contrast, the transmembrane domain acts primarily to trigger integration of the polypeptide into the lipid bilayer. The combined action of both domains operating during the transloca-

The abbreviations used are: PrP<sup>C</sup>, cellular isoform of PrP; PrP<sub>Sc</sub>, scrapie isoform of PrP; CHO, Chinese hamster ovary; GPI, glycosphosphatidylinositol; PK, protease K; PrP, prion protein; SP, signal peptide; WT, wild type; ER, endoplasmic reticulum; FNase, peptide-N-glycosidase.

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tion process serves to regulate the proportions of the three topological variants of PrP. Regulatory factors associated with the translocon, in addition to sequence determinants within the PrP molecule itself, have also been shown to influence the final topology achieved (14, 15).

Work from our laboratory has identified several novel cell biological features of CtmPrP. First, CtmPrP contains an uncleaved, N-terminal signal peptide (13). This characteristic makes CtmPrP unusual among other type II transmembrane proteins, most of which have internal signal-anchor sequences. Second, CtmPrP has a C-terminal GPI anchor in addition to a transmembrane domain, thus displaying an unusual, dual mode of membrane attachment (13, 16). Finally, by using the L9R/3AV mutant, we found that CtmPrP expressed in cultured cells remains core-glycosylated and is retained completely in the ER (13). This result implies that the protein is recognized as abnormal by the ER quality control machinery that monitors folding of newly synthesized polypeptides.

A great deal of interest in the subject of PrP membrane topology derives from the possibility that topological variants of PrP may play an important pathogenic role in prion diseases. Although PrP<sup>Sc</sup> is widely agreed to be the infectious form of PrP, PrP<sup>Sc</sup> may play an important pathogenic role in prion diseases. In this view, CtmPrP is a key intermediate in the transmembrane topology of the protein.

One candidate for such a neurotoxic intermediate is CtmPrP. Two major pieces of evidence have been used to argue that CtmPrP plays a key pathogenic role. First, transgenic mice have been generated that synthesize PrP molecules carrying the A117V mutation or one of the other CtmPrP-favoring mutations (7, 12). Animals expressing the mutant proteins above a threshold level synthesize CtmPrP in their brains and spontaneously develop a scrapie-like neurological illness, but without PrP<sup>Sc</sup> detectable by Western blotting or infectivity assays. This result implies that certain familial forms of PrP may be due directly to increased levels of CtmPrP. Second, mice have been constructed in which a wild-type hamster PrP transgene serves as a reporter of CtmPrP formation (12). When these animals are inoculated with mouse prions, the amounts of CtmPrP as well as PrP<sup>Sc</sup> in the brain are found to increase during the course of the infection. This result has been interpreted to indicate that PrP<sup>Sc</sup> induces formation of CtmPrP, which is then the proximate cause of neurodegeneration during infectiously acquired prion diseases. In this view, CtmPrP is a key intermediate in both genetic and infectious prion diseases.

Another topological variant of PrP that has been proposed as a neurotoxic intermediate is cytosolic PrP. Expression of an artificial form of PrP lacking a signal sequence, which presumably favors accumulation of PrP in the cytoplasm, has been found to be toxic to cultured cells and transgenic mice (18). However, there is debate about whether PrP is found in the cytoplasm under normal circumstances and, if so, what mechanisms are responsible for delivering it there. Based on the observation that cytosolic PrP accumulates in cells that have been treated with proteasome inhibitors, it has been suggested that some molecules are retrotranslocated into the cytoplasm from the ER lumen as part of normal ER quality control mechanisms (19–21). In contrast, our experiments indicate that cytosolic PrP molecules represent untranslocated chains that have never entered the ER (22). These chains, which are observed primarily under conditions of protein overexpression, contain an uncleaved N-terminal signal peptide and lack a GPI anchor.

A key gap in the experimental evidence supporting roles for CtmPrP and cytosolic PrP in prion-induced neurodegeneration is the lack of data demonstrating that the amounts of these forms increase during the course of a prion infection. In part, this absence of direct methods for detecting CtmPrP and cytosolic PrP in infected cells and tissues. In this paper, we present characterization of an antibody that reacts with both CtmPrP and cytosolic PrP by virtue of their uncleaved signal peptides and our use of this antibody to assay CtmPrP and cytosolic PrP in infected samples. In addition, we carry out a mutational analysis of several sequence determinants in PrP to better understand the factors that influence the topology of the protein.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Synthetic oligonucleotides encoding point mutations in the PrP signal sequence (Fig. 1A) were used to amplify a portion of the PrP DNA sequence by PCR. DNA fragments carrying the mutation were digested with HindIII and PshAI and cloned into a pcDNA3 plasmid (Invitrogen) containing the WT mouse PrP sequence from which the HindIII-PshAI fragment had been removed. Plasmids encoding PrP molecules with a FLAG epitope (DYKDDDDK) inserted at position 2223, and with altered numbers of octapeptide repeats (PG0, Δ51–90, PG1, Δ51–82, PG2, Δ67–90, PG14, +9 repeats) have been described previously (5, 13, 23). Other PrP mutants were constructed by PCR (16). All PrP coding regions carried an epitope tag for monoclonal antibody 3F4, created by changing residues 108 and 111 to methionine.

**In Vitro Translation and PK Protection—**mRNAs encoding WT and mutant PrP molecules were transcribed using the mMessage mMachine kit (Ambion, Austin, TX) and were translated in rabbit reticulocyte lysate (Promega, Madison, WI) containing [35S]methionine as directed by the manufacturer, except that the final lysate concentration was 50%. Translation reactions were supplemented with microsomal membranes from mouse BWS174.3 cells (24) or from canine pancreas (Promega). After translation, 5-µl aliquots of lysate were incubated for 60 min at 4 °C in a final volume of 50 µl with or without 100 µg/ml PK (Roche Applied Science) in the presence or absence of 0.5% Triton X-100. PK was inactivated with phenylmethylsulfonyl fluoride for 5 min, and 12-µl aliquots were added to gel sample buffer containing phenylmethylsulfonyl fluoride for analysis by SDS-PAGE. In some cases, PrP was immunoprecipitated from translation reactions (as described below) prior to SDS-PAGE. For enzymatic deglycosylation, PrP was eluted from protein A-Sepharose beads with 1% SDS, 50 mM Tris-HCl (pH 7.5) and was then diluted 10-fold with 50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100 containing 0.33 units/ml N-glycosidase F (New England Biolabs). After incubation at 37 °C for 1 h, proteins were precipitated with methanol and analyzed by SDS-PAGE. Radioactive bands on gels were quantitated using a PhosphorImager SI (Amer sham Biosciences).

**Scrapie Infection of N2a Cells**—Highly scrapie-susceptible sub-clones of N2a cells were prepared as described (25). Briefly, N2a cells from the ATCC (CCL131) were first sub-cloned by limiting dilution. Each sub-clone was then tested for scrapie susceptibility by incubation for 3 days with an extract of N2a cells that had been infected previously with the Chandler strain of scrapie (26). Cells were then washed and analyzed for PrP<sup>27–30</sup> by cell blotting or by Western blotting after PK digestion. The susceptible sub-clone used for the experiment shown in Fig. 7 was designated N2a.3. It was used in the infected state, as well as in the uninfected state as a matched control.

**Transfection, Metabolic Labeling, and Immunoprecipitation**—CHO and N2a cells were transiently transfected with PrP-expressing plasmids using LipofectAMINE or LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s directions. Twenty-four hours after transfection, cells were labeled for 6 h in methionine- and cysteine-free medium containing 100–200 µCi/ml of [35S]methionine/cysteine (Amersham Biosciences). Cultures were then lysed in 0.5% SDS, 50 mM Tris-HCl (pH 7.5), heated at 95 °C for 5 min, and diluted with 10 volumes of RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.5)). Diluted lysates were incubated with anti-PrP antiserum for 1 h at 4 °C and then with 20 µl of protein A-Sepharose beads for 30 min at 4 °C. Beads were washed...
Scrapie Infection of Mice and Hamsters—Tg(WT)PrP<sup>−/−</sup> mice (E1 line) expressing wild-type PrP carrying a 3F4 epitope have been described previously (27). Tg(L9R/3AV)LtPrP<sup>−/−</sup> mice (B line) express mouse PrP carrying an L9R/3AV mutation and a 3F4 tag, a construct we have expressed previously in cultured cells (13). Full characterization of these mice will be provided elsewhere.2 Scrapie inocula included infected brains that had been passaged once in Tg(WT) mice to introduce the 3F4 epitope. To prepare inocula, infected brains were homogenized (10%, w/v) in phosphate-buffered saline using sterile, disposable tissue grinders. After clearing by centrifugation at 900 × g for 5 min, the homogenates were diluted to a final concentration of 1 or 2.5% in PBS, and 25 μl was injected intracerebrally into the right parietal lobe of 4–6-week-old recipient mice or hamsters using a 25-gauge needle.

Western Blots of Brain Homogenates—Brain lysates were prepared in 0.5% SDS, 50 mM Tris-HCl (pH 7.5). Samples were heated at 95 °C for 5 min and diluted 10-fold with 50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100 containing 0.33 units/ml N-glycosidase F. After incubation at 37 °C for 2 h, proteins were precipitated with methanol, separated by SDS-PAGE, and subjected to Western blotting with 3F4 antibody.

Antibodies—An antibody (anti-SP) that selectively recognizes forms of murine PrP containing an uncleaved signal peptide was generated by immunizing rabbits with a synthetic peptide (TMWTDVGLCKKRPK; 28). An antibody (anti-SP) that recognizes only the C-terminal GPI anchor (29) and polyclonal antibody 8H4 (29) that spans the signal peptide cleavage site at positions 112, 114, and 117 were also used.

Results

Mutations in the Hydrophobic Core of the Signal Peptide Increase the Proportion of C<sub>TM</sub>PrP and Reduce Translocation Efficiency—For these experiments, we translated PrP mRNA in the presence of microsomes from murine thymoma cells, which are efficient at adding the C-terminal GPI anchor (16). Translation reactions were subsequently subjected to PK digestion to reveal protease-protected products corresponding to Sec<sub>PrP</sub> and C<sub>TM</sub>PrP. N<sub>IM</sub>PrP was not quantitated in these experiments, because negligible amounts of this form are produced in the presence of thymoma microsomes (16). We showed previously that substitution of arginine for leucine at position 9 (L9R) in the hydrophobic core (h-region) of the signal peptide had a dramatic effect on PrP membrane topology, with ~50% of the translocated protein assuming the C<sub>TM</sub>PrP orientation, compared with ~10% for WT PrP (13). We then tested the effects of other amino acid substitutions at this site. The results are shown in Fig. 1 and summarized in Table I (lines 1–12). Substitution of either positively charged residues (Arg and Lys) or a negatively charged residue (Asp) for leucine at position 9 increased the proportion of C<sub>TM</sub>PrP, with two non-polar residues (Pro and Gly) having very little effect. All of the substitutions also significantly reduced the efficiency of translocation (the total percentage of PK-protected chains) from ~25% for WT PrP to 5–15% for the mutants (data not shown). To examine the effect of substitutions at another residue in the h-region of the signal peptide, we analyzed V13R and V13D. Both of these mutations completely abolished translocation (Fig. 1).

Previous work demonstrated that certain mutations in the transmembrane segment also increased the amount of C<sub>TM</sub>PrP. One mutation that has been studied extensively is the triple substitution designated 3AV (substitution of valine for alanine at positions 112, 114, and 117) (7, 13). When this mutation was combined with mutations in the signal sequence, an additive increase in the percentage of C<sub>TM</sub>PrP was observed (Fig. 1; Table I, lines 1–12). PrP molecules carrying the 3AV mutation along with substitution of a charged amino acid (Arg, Lys, and Asp) at position 9 were synthesized almost exclusively as C<sub>TM</sub>PrP.

Lack of Signal Peptide Cleavage Does Not Cause Production of C<sub>TM</sub>PrP—We have shown previously (13) that C<sub>TM</sub>PrP has an uncleaved signal peptide. However, it remained unknown whether the lack of signal peptide cleavage was a cause or a consequence of C<sub>TM</sub>PrP formation. To address this question, we used two approaches to prevent cleavage by signal peptidase, and we assayed their effect on synthesis of C<sub>TM</sub>PrP. The −1 and −3 positions in the c-region of signal peptides have

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2 R. S. Stewart and D. A. Harris, manuscript in preparation.

A. Signal Sequence Transmembrane Domain

![Signal Sequence and Transmembrane Domain](image)

B. WT L9R 3AV L9K L9D L9G L9R 3AV L9G L9P V13R V13D

![Amino Acid Substitutions](image)
been shown to have the greatest influence on cleavage (30); small polar residues are strongly preferred. We therefore substituted large, hydrophobic residues at these positions to block removal of the signal peptide completely abolished translocation with the N-terminal signal sequence. We assayed PrP constructs in which the N-terminal sequence had been deleted. We observed that removal of the signal peptide completely abolished translocation of WT PrP, as evidenced by the failure to detect any protected fragment after PK digestion of translation reactions (Fig. 2C, lane 14). The same result was observed after introduction of the 3AV mutation, which normally increases CtmPrP formation and might therefore be expected to enhance the ability of the transmembrane segment to function as a signal-anchor sequence (Fig. 2C, lane 17). Thus, the transmembrane segment cannot function independently to target PrP to the translocon to produce CtmPrP but requires cooperation with the N-terminal signal sequence.

**Residues on the C-terminal Side of the Signal Peptide Cleavage Site Influence PrP Topology**—To demonstrate that CtmPrP has an uncleaved signal, we had constructed previously a PrP molecule with a FLAG epitope inserted at the signal peptide site (DYKDDDDK) changes the downstream amino acid context of the cleavage site from basic (KKRPKKPGG) to acidic. We found that the presence of the FLAG epitope increases the proportion of CtmPrP compared with untagged controls. This effect is relatively modest when microsomes from mouse thymoma cells are used (not shown) but more dramatic when

| Construct | CtmPrP \% |
|-----------|-----------|
| 1. WT     | 12.4      |
| 2. L9R    | 30.1      |
| 3. L9R/3AV| 87.6      |
| 4. L9K    | 62.6      |
| 5. L9K/3AV| 87.7      |
| 6. L9D    | 43.0      |
| 7. L9D/3AV| 77.5      |
| 8. L9G    | 22.3      |
| 9. L9G/3AV| 67.8      |
| 10. L9P   | 11.0      |
| 11. V13R  | =         |
| 12. V13D  | =         |

Note: Neither CtmPrP nor SecPrP were present at detectable levels.

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**Fig. 2. Lack of signal peptide cleavage does not cause production of CtmPrP, and deletion of the signal peptide prevents translocation.** A, amino acid sequence of the N terminus of PrP. Substitutions made at positions 20 and 22 to block signal peptide cleavage are indicated below the corresponding wild-type residues. The upward arrow indicates the site of signal peptide cleavage. B and C, PrP mRNAs encoding the indicated constructs were translated in reticulocyte lysate supplemented with murine thymoma microsomes. Reactions were then incubated with (+ PK lanes) or without (− PK lanes) protease K in the presence (+ Det. lanes) or absence (− Det. lanes) of Triton X-100. Products were then analyzed by SDS-PAGE and autoradiography. The arrowheads indicate the positions of the protected fragments corresponding to SecPrP. The CtmPrP fragments are slightly larger for G20W and C22Y PrP (open arrowheads, lanes 5 and 8) than for WT PrP (filled arrowhead, lane 2), confirming that the mutations prevent cleavage of the signal peptide. The filled arrow (lane 11) indicates the position of the CtmPrP protected fragment. The open arrow (lane 13) indicates the position of unprocessed PrP that has not undergone signal peptide cleavage, GPI anchor addition, or glycosylation.

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The table quantifies the CtmPrP produced by the mutants used in this study.

PrP mRNA was translated in vitro in the presence of either murine thymoma microsomes (constructs 1–18 and 23–28) or canine pancreatic microsomes (constructs 19–22 and 29–32). The CtmPrP and SecPrP fragments produced after PK digestion were quantitated by PhosphorImager analysis of SDS-PAGE gels. The percentage of CtmPrP was expressed as CtmPrP/(CtmPrP + SecPrP) × 100. SecPrP was not included in this calculation because it is present in negligible amounts in translations performed with thymoma microsomes (16). Each value represents the mean of 2–8 replicates.
canine pancreatic microsomes are used (Fig. 3B; Table I, lines 19–22). In this system, the percentage of CtmPrP is doubled by introduction of the FLAG sequence into either WT or 3AV PrP. We have shown previously that, for all PrP constructs, pancreatic microsomes produce a higher CtmPrP/SecPrP ratio than thymoma microsomes (16), possibly due to differences between these preparations in their content of translation accessory factors (14, 15). Insertion of the FLAG epitope does not alter the position of signal peptide cleavage, based on immunoreactivity with the monoclonal antibody M1, which is specific for the FLAG sequence containing a free N terminus (13).

Changing the Spacing between the Signal Peptide and the Transmembrane Domain Affects Membrane Topology—It has been proposed that the two topogenic determinants in PrP (the N-terminal signal peptide and the transmembrane domain) interact with other during the translocation process (9, 11). To test this idea, we determined whether altering the spacing between these determinants affected PrP membrane topology. The spacing was altered by deleting or inserting octapeptide repeat units (P(H/Q)GG(G/S/T)WGQ), five copies of which are normally found in the N-terminal region of PrP (Fig. 4A). The spacing was altered by deleting or inserting octapeptide repeat units (P(H/Q)GG(G/S/T)WGQ), five copies of which are normally found in the N-terminal region of PrP (Fig. 4A). We also observed that replacement of the GPI addition signal with an unrelated sequence (the KDEL ER retention signal) had no effect on membrane topology (data not shown).

An Antibody Directed against the PrP Signal Peptide Specifically Recognizes CtmPrP and Untranslocated PrP—Because CtmPrP, unlike NtmPrP and SecPrP, has an uncleaved signal peptide, we reasoned that an antibody raised against the signal peptide would be a useful tool for specifically assaying CtmPrP in cells and tissues. Because of the hydrophobicity of the signal sequence made synthesis of synthetic peptides encompassing this region problematic, we chose as an immunogen a synthetic peptide that spanned the signal peptide cleavage site (Fig. 6A). This peptide included several positively charged residues on the C-terminal side of the cleavage site that facilitated synthesis of the peptide and improved antigenicity. This peptide was coupled to keyhole limpet hemocyanin via both amino and sulfhydryl groups and was used to raise a polyclonal antiserum (denoted anti-SP) in rabbits.

Initial characterization of the antiserum was performed using PrP synthesized by in vitro translation (Fig. 6B). When WT PrP was immunoprecipitated with an antibody (P45–66) that detects all forms of PrP, two bands were seen (Fig. 6B, lane 1): a 32-kDa species representing core-glycosylated CtmPrP chains, and a 27-kDa species representing unglycosylated, untranslocated chains that have not been processed at either their N or C termini (i.e. they retain both their signal and GPI addition peptides). The anti-SP antibody reacted only with the latter...
cells expressing WT or L9R/3AV PrP were labeled for 6 h with [35S]methionine. PrP was then immunoprecipitated from cell lysates using and the peptide immunogen (lanes 3 and lanes 2). PrP,CtmPrP, and unprocessed PrP are indicated. 

Doubly and singly glycosylated forms of mature PrP, unglycosylated and analyzed by SDS-PAGE and autoradiography. The positions of form migrated at 33 kDa rather than 32 kDa because of with P45 – heads, filled arrows, and open arrows, respectively. CmPrP is not clearly visible for the WT constructs on this autoradiographic exposure, but it is detectable above background levels by PhosphorImager analysis (Table I, lines 29–30).

Fig. 5. Deletion of the GPI addition signal does not affect membrane topology. PrP mRNAs encoding the indicated constructs were translated in reticulocyte lysate supplemented with canine pancreatic microsomes. Reactions were then incubated with (+ PK lanes) or without (– PK lanes) proteinase K in the presence (+ Det. lanes) or absence (– Det. lanes) of Triton X-100. Products were immunoprecipitated with 3F4 antibody and deglycosylated with PNGase F prior to analysis by SDS-PAGE and autoradiography. The positions corresponding to \(^{35}S\)PrP, CtmPrP, and CmPrP are indicated by the filled arrowheads, filled arrows, and open arrows, respectively. CmPrP is not clearly visible for the WT constructs on this autoradiographic exposure, but it is detectable above background levels by PhosphorImager analysis (Table I, lines 29–30).

Fig. 6. Characterization of anti-SP antibody. A, sequence of the synthetic peptide used to raise anti-SP antibody. The arrow indicates the position of the signal peptide cleavage site. B, mRNAs encoding WT or L9R/3AV PrP were translated in reticulocyte lysate supplemented with murine thymoma microsomes. PrP was then immunoprecipitated from translation reactions with P45–66 antibody (lanes 1 and 4), anti-SP antibody (lanes 2 and 5), or anti-SP antibody pre-incubated with the peptide immunogen (lanes 3 and 6). C, transiently transfected CHO cells expressing WT or L9R/3AV PrP were labeled for 6 h with \(^{35}S\)methionine. PrP was then immunoprecipitated from cell lysates using either 3F4 antibody (lanes 1 and 2) or anti-SP antibody (lanes 3 and 4) and analyzed by SDS-PAGE and autoradiography. The positions of doubly and singly glycosylated forms of mature PrP, unglycosylated PrP, CmPrP, and unprocessed PrP are indicated.

form (Fig. 6B, lane 2), and this reactivity could be abolished by pre-incubation of the antibody with the peptide immunogen (Fig. 6B, lane 3). When L9R/3AV PrP was immunoprecipitated with P45–66, both core-glycosylated as well as unglycosylated, untranslocated forms were also observed, but the glycosylated form migrated at 33 kDa rather than 32 kDa because of the presence of an uncleaved signal peptide characteristic of CmPrP (Fig. 6B, lane 4) (13). Anti-SP recognized both the 33- and 27-kDa species (Fig. 6B, lane 5), and again reactivity could be blocked by pre-incubation with the peptide immunogen (lane 6). These results demonstrate that the anti-SP serum recognizes PrP translation products with a retained N-terminal signal peptide and does not react with molecules whose signal peptide has been cleaved.

We then tested the ability of the anti-SP serum to recognize PrP synthesized in transiently transfected CHO cells (Fig. 6C). Cells were metabolically labeled with \(^{35}S\)methionine, and PrP was immunoprecipitated from lysates using either anti-SP antibody or 3F4 antibody (which recognizes all forms of PrP). Cells expressing WT PrP produced two mature, glycosylated species recognized by 3F4 antibody: a doubly glycosylated form at 38 kDa and a singly glycosylated form at 32 kDa (Fig. 6C, lane 1). In addition, small amounts of unglycosylated PrP at 25–27 kDa were present. In contrast, cells expressing L9R/3AV PrP produced a 33-kDa glycosylated form that we have shown previously (13) represents an endoglycosidase H-sensitive form of CmPrP that has an uncleaved signal peptide (Fig. 6C, lane 2). Anti-SP antiserum immunoprecipitated the 33-kDa CmPrP species from cells expressing L9R/3AV PrP (Fig. 6C, lane 4), and trace amounts of the same band were detected in cells expressing WT PrP (lane 3). However, the doubly and singly glycosylated forms of WT PrP were not recognized by this antibody (Fig. 6C, lane 3), demonstrating its selectivity for signal peptide-bearing forms synthesized in cells. We noted that anti-SP also recognized an unglycosylated form of PrP (27 kDa), which we have shown represents untranslocated, unprocessed molecules that accumulate in the cytoplasm at high expression levels (22). These molecules are analogous to the untranslocated species observed after in vitro translation (Fig. 6B). Taken together, the results shown in Fig. 6, B and C, demonstrate that anti-SP antibody is capable of selectively recognizing PrP molecules that contain an uncleaved signal peptide, even in the presence of an excess of N-terminally processed forms. These signal peptide-bearing molecules, which are synthesized both in vitro and in cultured cells, are composed of CmPrP as well as untranslocated forms that remain on the cytoplasmic side of the ER membrane (13, 22).

The Amounts of CmPrP and Untranslocated PrP Are Not Altered by Scrapie Infection of Cultured Cells and Brain— CmPrP has been proposed to be a neurotoxic intermediate whose levels are increased during infectiously acquired as well as familial prion diseases (7, 12). To test this hypothesis, we used the anti-SP antibody to assay the amount of CmPrP in scrapie-infected N2a cells. Infected and uninfected N2a cells were labeled to steady state with \(^{35}S\)methionine, and then PrP was immunoprecipitated with either anti-SP antibody or with an antibody (8H4) that recognizes all forms of PrP. Because the level of endogenous mouse PrP in N2a cells is relatively low, some cultures were transiently transfected to express high levels of WT PrP, with the idea that this might enhance the ability to detect small amounts of CmPrP. In addition, some cells were transfected with constructs encoding PrP mutants (A116V, 3AV, and L9R/3AV) that would serve as positive controls for synthesis of CmPrP. The transfection efficiency in these experiments, measured using a GFP-encoding plasmid, was sufficiently high (–30%) that we could be confident a substantial number of scrapie-infected cells in the culture expressed transfected PrP. The N2a cells used for these experiments represented a sub-clone (N2a.3) which had isolated that was highly susceptible to scrapie infection (see “Experimental Procedures”), so that the infected and uninfected versions could be directly compared without further cloning (25).
Fig. 7. The amounts of CtePrP and untranslocated PrP are not altered by scrapie infection of N2a cells. A, uninfected N2a.3 cells (lanes 1–5) and scrapie-infected N2a.3 cells (lanes 6–10) were untransfected (lanes 1 and 6) or were transiently transfected to express WT (lanes 2 and 7), A116V (lanes 3 and 8), 3AV (lanes 4 and 9), or L9R/3AV PrP (lanes 5 and 10). Cells were labeled for 6 h with [35S]methionine and then lysed. PrP was immunoprecipitated from cell lysates using either anti-SP antibody (upper panels) or 8H4 antibody (lower panels) and analyzed by SDS-PAGE and autoradiography. The filled and open arrowheads indicate the positions of CtePrP and untranslocated PrP, respectively. B, lysates of uninfected N2a.3 cells (lanes 1 and 2) and scrapie-infected N2a.3 cells (lanes 3 and 4) were subjected to digestion with endoglycosidase H (not shown), was present in increased amounts in cells expressing A116V, 3AV, and L9R/3AV PrP (upper panels, lanes 5–8) and 3AV PrP (lanes 5 and 10). Cells were labeled for 6 h with [35S]methionine and then lysed. PrP was immunoprecipitated from cell lysates using either anti-SP antibody (upper panels) or 8H4 antibody (lower panels) and analyzed by SDS-PAGE and autoradiography. The filled and open arrowheads indicate the positions of CtePrP and untranslocated PrP, respectively. B, lysates of uninfected N2a.3 cells (lanes 1 and 2) and scrapie-infected N2a.3 cells (lanes 3 and 4) were subjected to digestion with endoglycosidase H (not shown), was present in increased amounts in cells expressing A116V, 3AV, and L9R/3AV PrP (upper panels, lanes 5–8), confirming its identity as CtePrP (13). The unglycosylated 27-kDa band corresponds to untranslocated PrP, which we have shown to be present in increased amounts in transiently transfected cells (22). Importantly, the amounts of both the 33- and 27-kDa species did not differ between infected and uninfected cells. We conclude from these results that scrapie infection of N2a cells does not detectably increase the amount of either CtePrP or untranslocated PrP. Western blotting confirmed that the scrapie-infected cells produced protease-resistant PrP30, the protease-resistant fragment of PrPSc.

Fig. 7A shows that there was no difference between infected and uninfected cells in the amounts of signal peptide-containing PrP they produced. Although untransfected cells synthesized low levels of PrP that required long autoradiographic exposures to visualize (data not shown), cells transfected with WT or mutant PrP plasmids expressed considerably higher levels of 8H4-reactive protein (lower panels). In cells transfected with the WT PrP plasmid, anti-SP antibody immunoprecipitated two bands of 27 and 33 kDa (upper panels, lanes 2 and 7). The presence of an uncleaved signal peptide (27 kDa) was confirmed by visualizing the bands after prolonged autoradiographic exposures to visualize (data not shown). We also used SDS-PAGE to detect the small (~2 kDa) difference in size between PrP molecules with and without a signal peptide. Proteins were enzymatically deglycosylated prior to SDS-PAGE to eliminate size differences due to differential glycosylation. When the gels were run long enough, we found we could reliably detect the difference in migration between PrP molecules containing a cleaved signal peptide (25 kDa) and an uncleaved signal peptide (27 kDa), as demonstrated by visualization of two bands in brain samples from Tg(L9R/3AV) mice (Fig. 8, lane 5). These mice, which spontaneously develop a severe neurodegenerative illness, synthesize CtePrP as well as signal peptide-cleaved forms. The identity of the 27-kDa form as a signal peptide-bearing form was confirmed by immunoprecipitating it with anti-SP antibody from [35S]methionine-labeled neurons cultured from Tg(L9R/3AV) mice (not shown). Importantly, we did not detect any of the 27-kDa band in brain samples from uninfected mice or from mice and hamsters infected with several different scrapie inocula (Fig. 8, lanes 1–4). Because samples were deglycosylated prior to SDS-PAGE, this analysis would not distinguish CtePrP from untranslocated PrP. We conclude from these data that scrapie infection of mice and hamsters does not increase the amount of either CtePrP or untranslocated PrP in the brain to detectable levels.

DISCUSSION

In this work, we have analyzed several structural features of the PrP molecule that influence its membrane topology. In addition, we have developed an antibody that selectively recognizes CtePrP and cytosolic PrP, topological variants of PrP implicated in prion-related neurodegeneration. We have used this antibody, as well as the distinctive gel mobility of CtePrP and cytosolic PrP, to measure the amounts of these forms in scrapie-infected cells and brain. Our results have implications for the mechanisms of protein translocation in the ER and for the role of transmembrane and cytosolic PrP in neurodegenerative disease.

Topological Determinants in PrP—Initial work on PrP membrane topology demonstrated that mutations within and adjacent to the hydrophobic transmembrane domain influenced the

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relative amount of CtmPrP synthesized (7, 12). Some mutations (e.g. A116V, K109H/H110I, and 3AV) increased the proportion of CtmPrP, whereas other mutations (e.g. G122P) decreased it. Subsequently, we found that a non-conservative substitution (L9R) within the hydrophobic core of the N-terminal signal sequence dramatically increased the amount of CtmPrP (13).

Combining this mutation with the 3AV mutation resulted in a molecule (L9R/3AV) that was synthesized exclusively as CtmPrP both in vitro and in cultured cells. We also found that disease-associated mutations that lie outside the transmembrane region did not alter the membrane topology of PrP (16). Taken together, these results indicated that there were two major topogenic determinants in the PrP molecule: the N-terminal signal peptide and the hydrophobic transmembrane domain. Another potential topological determinant, the C-terminal, GPI addition signal, does not appear to play a significant role. Although the GPI signal can mediate post-translational translocation of PrP molecules lacking an N-terminal signal sequence (8), deletion or substitution of this segment has no effect on the membrane topology of the full-length protein (this paper and see Ref. 11).

To explore further the role of the signal peptide in determining PrP topology, we introduced several additional substitutions in the hydrophobic core (h-region) of the signal peptide. We found that introduction of either positively or negatively charged residues at position 9 significantly increased the proportion of CtmPrP. These substitutions also decreased the overall efficiency of translocation. Introduction of either a positively or negatively charged residue at position 13 completely abolished translocation. These results are consistent with the suggestion (9) that the PrP signal sequence serves two distinct functions: 1) targeting the polypeptide chain to the translocon; and 2) determining the location of the N terminus with respect to the membrane (luminal or cytoplasmic). Introduction of charged residues in the h-region affects both of these functions, reducing the targeting activity of the signal sequence, as well as its ability to promote translocation of the N terminus to the luminal side of the membrane. Previous work has shown that mutations in the n-region of the signal peptide (the polar, N-terminal segment) can also influence the two functions of the signal sequence (9, 31).

Several other features of PrP topogenesis are also addressed by our results. First, we found that mutations in the c-region that block signal peptide cleavage do not alter the topology of PrP. Thus, the presence of an uncleaved signal peptide, which we have found is a characteristic of CtmPrP, does not by itself favor formation of CtmPrP. Rather, failure of signal peptide cleavage is likely to be a consequence of the fact that, in CtmPrP, the N terminus of the protein remains in the cytoplasm and therefore does not come into contact with signal peptidase in the ER lumen.

We have also observed that deletion of the signal peptide prevents translocation, resulting in cytosolic molecules that are fully accessible to added protease. This result, which confirms other published reports (8, 10), indicates that the hydrophobic, transmembrane segment cannot by itself target the polypeptide chain to the translocon. Thus, in contrast to the case for other type II membrane-spanning proteins (whose N termini are cytoplasmic), the transmembrane domain of PrP is not capable of functioning as a signal-anchor sequence. These considerations are consistent with the proposal (9, 11) that the primary function of the PrP transmembrane domain is to determine whether polypeptide chains that have already been targeted to the translocon by the N-terminal signal sequence will integrate into the lipid bilayer. In the model of Hegde and colleagues (11), the signal sequence and transmembrane domain thus mediate two sequential events within the translocon channel that determine the partitioning of PrP chains among the possible topological variants. Consistent with such a model, decreasing the distance between the signal sequence and transmembrane domain increases synthesis of CtmPrP (this paper and see Ref. 11).

We find that introduction of a FLAG epitope just C-terminal to the signal peptide cleavage site increases the proportion of CtmPrP synthesized. This result indicates that, although the signal sequence and transmembrane domain are the primary determinants of PrP topology, other parts of the protein can also have an influence. This observation is reminiscent of experiments in which the translocation of chimeric proteins containing the same signal sequence but different mature domains was measured (31). These experiments indicated that signal sequences and mature domains cooperate in luminal gating of the translocon channel, a step that is correlated with synthesis of NtmPrP and SecPrP. In this light, introduction of the FLAG epitope would decrease the efficiency of luminal gating and thus increase the relative amount of CtmPrP.

An Immunological Assay for CtmPrP and Cytosolic PrP—Previously, the only method available for direct measurement of CtmPrP has been the protease-protection assay using microsomal membranes, which relies upon the generation of a protected fragment representing the luminal domain of CtmPrP (7–9, 11, 16). However, this assay can be cumbersome to perform and is problematic when applied to tissue samples because of the difficulty of preparing purified, ER-derived, microsomal membranes. The assay is also not applicable to scrapie-infected samples because of the intrinsic protease resistance of PrPSC. Another published method (7), which involves PK digestion of detergent extracts under “mild” conditions, has also been claimed to detect CtmPrP, but there is no direct evidence that the PK-resistant fragment produced in this assay represents authentic CtmPrP. Detection of cytosolic PrP has relied upon immunofluorescence staining and the use of proteasome inhibitors (19–21).

The presence of an uncleaved signal peptide on both CtmPrP (13) and cytosolic PrP (22) provides a simple and direct method to detect these forms using an antibody directed against the signal sequence. We have prepared an antiserum against a synthetic peptide that spans the signal peptide cleavage site, and we have shown that this antiserum specifically immunoprecipitates CtmPrP synthesized in vitro and in cells, even in the presence of a large excess of signal peptide-cleaved forms (SecPrP and NtmPrP). The anti-SP antiserum also detects untranslocated forms of PrP that have not been processed at their N or C termini and that we have shown (22) remain on the

Fig. 8. The amount of signal peptide-bearing PrP is not altered in scrapie-infected brain. Brain homogenates were prepared from a Syrian hamster infected with 263K scrapie (lane 1), a Tg(WT) mouse infected with 3F4-tagged RML scrapie (lane 2), a Tg(WT) mouse infected with RML scrapie (lane 3), an uninfected Tg(WT) mouse (lane 4), and an uninfected Tg(L9R/3AV) mouse (lane 5). Infected animals were terminally ill at the time of sacrifice. Homogenates were treated with PNGase F, and proteins were then subjected to Western blotting with 3F4 antibody. The filled and open arrowheads indicate the positions of signal peptide-bearing PrP and signal peptide-cleaved PrP, respectively.
cytoplasmic side of the ER membrane. Thus, this antiserum is a useful reagent for specifically detecting untranslocated, cytosolic PrP as well as \(^{125}\text{I}\)PrP.

Effect of Scrapie Infection on the Amounts of \(^{125}\text{I}\)PrP and Cytosolic PrP—It has been proposed that \(^{125}\text{I}\)PrP is a key pathogenic intermediate in both familial and infectiously acquired prion diseases (7, 12). In this scheme, certain pathogenic mutations in PrP are thought to increase directly the synthesis of \(^{125}\text{I}\)PrP, whereas in cases of infectious origin PrP\textsuperscript{Sc} is hypothesized to indirectly cause accumulation of \(^{125}\text{I}\)PrP. Although it is clear that \(^{125}\text{I}\)PrP-favoring mutations cause a neurodegenerative phenotype when expressed in transgenic mice, there is a paucity of evidence that the amount of \(^{125}\text{I}\)PrP actually increases during the course of a natural prion infection in either humans or animals. The only published experiment that supports this conclusion involved the use of a PrP reporter construct to monitor \(^{125}\text{I}\)PrP levels in scrapie-infected mice (12). However, this assay did not directly quantitate \(^{125}\text{I}\)PrP, and in addition, the maximal increase in \(^{125}\text{I}\)PrP observed was only 3-fold, much smaller than the increase in PrP\textsuperscript{Sc}. Cytosolic PrP has also been proposed as a neurointermediate, but the only experiments to support this claim derive from expression of artificial forms of PrP that lack an N-terminal signal sequence (18).

We report here that scrapie infection of N2a cells and mouse brain does not alter the amount of signal peptide-bearing PrP, based on reactivity with anti-SP antibody, or on SDS-PAGE to detect the slightly larger molecular weight of this form. These results indicate that scrapie infection does not affect the levels of either \(^{125}\text{I}\)PrP or untranslocated (presumably cytosolic) PrP, both of which have an uncleaved signal peptide. Of course, it is possible that these forms were present below the level of detectability of our assay methods. Arguing against this possibility is the fact that we can easily visualize signal peptide bearing forms of PrP in uninfected and scrapie-infected N2a cells that have been transfected to express WT molecules or molecules carrying \(^{125}\text{I}\)PrP-favoring mutations. We estimate that \(^{125}\text{I}\)PrP represents \(0.5\% \text{–} 1\%\) of total PrP in cells expressing the WT protein. In addition, we can detect \(^{125}\text{I}\)PrP in brain extracts from Tg(L9R/3AV) mice that spontaneously develop a severe neurodegenerative illness (Fig. 8). If the amount of \(^{125}\text{I}\)PrP had been elevated in the brains of scrapie-infected mice to levels similar to those observed in the brains of Tg(L9R/3AV) mice, it is thus likely we would have been able to detect this change.

A growing body of evidence indicates that, although PrP\textsuperscript{Sc} is the infectious form of PrP, one or more alternate forms of the protein are responsible for the neurodegeneration observed in prion disorders (17). The results presented here, in conjunction with our previous work showing that levels of \(^{125}\text{I}\)PrP and cytosolic PrP are not altered by disease-associated PrP mutations (16, 22), suggest that these two forms are unlikely to be obligate neurotoxic intermediates in familial or infectiously acquired prion diseases. It will be important now to identify other neurotoxic forms of PrP (32) and to develop specific and sensitive assays to detect these species during the course of prion diseases.

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