Most Pathogenic Mutations Do Not Alter the Membrane Topology of the Prion Protein

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The prion protein (PrP), a glycolipid-anchored membrane glycoprotein, contains a conserved hydrophobic sequence that can span the lipid bilayer in either direction, resulting in two transmembrane forms designated NtransPrP and CtransPrP. Previous studies have shown that the proportion of CtransPrP is increased by mutations in the membrane-spanning segment, and it has been hypothesized that CtransPrP represents a key intermediate in the pathway of prion-induced neurodegeneration. To further test this idea, we have surveyed a number of mutations associated with familial prion diseases to determine whether they alter the proportions of NtransPrP and CtransPrP produced in vitro, in transfected cells, and in transgenic mice. For the in vitro experiments, PrP mRNA was translated in the presence of murine thymoma microsomes which, in contrast to the canine pancreatic microsomes used in previous studies, are capable of efficient glycolipidation. We confirmed that mutations within or near the transmembrane domain enhance the formation of CtransPrP, and we demonstrate for the first time that this species contains a C-terminal glycolipid anchor, thus exhibiting an unusual, dual mode of membrane attachment. However, we find that pathogenic mutations in other regions of the molecule have no effect on the amounts of CtransPrP and NtransPrP, arguing against the proposition that transmembrane PrP plays an obligate role in the pathogenesis of prion diseases.

Prion diseases are neurodegenerative disorders characterized by spongiform destruction of brain tissue and the presence of cerebral amyloid plaques (1, 2). These disorders include kuru and Creutzfeldt-Jakob disease in humans, “mad cow disease” in cattle, and scrapie in sheep. Prion diseases can have an infectious or genetic origin, or can arise spontaneously. The infectious agent is hypothesized to be PrPSc, a conformationally altered isoform of a normal cell-surface glycoprotein of unknown function called PrPSc (3). PrPSc from dietary or other infectious sources is thought to act as a catalyst or template to convert endogenous PrPSc into more PrPSc, which then accumulates, eventually causing disease. Familial forms of prion disease result from germline mutations in the PrP gene on chromosome 20, which are believed to favor conversion of the protein to the PrPSc form (4). Sporadic cases may be due to rare, spontaneous conversion of wild-type PrPSc to PrPSc. No covalent modifications that distinguish PrPSc from PrPSc have been detected (5), but the conformations of the two isoforms are dramatically different, with PrPSc having a much higher content of β-sheet (6, 7). There are several biochemical properties that distinguish PrPSc from PrPSc, the most prominent being protease resistance. After treatment with proteinase K (PK), PrPSc is cleaved near amino acid 90 to yield a protease-resistant core fragment known as PrP 27–30, while under the same conditions PrPSc is completely degraded (8). PrPSc is found in the brain in most cases of infectious, familial, and sporadic prion disease (3). However, in some inherited prion diseases, for example cases of Gerstmann-Sträussler syndrome due to an A117V mutation in PrP, PrPSc has not been detected and brain material does not appear to be infectious when injected into rodents (9–11). These exceptions raise the interesting possibility that PrPSc, although it is the infectious form of the protein, may not be the proximate cause of neurodegeneration in at least some forms of prion disease.

Recently, it has been proposed that an alternate form of PrP that is distinct from PrPSc may play an important role in prion pathogenesis. This form, designated CtermPrP, has an unusual transmembrane topology (12). Most molecules of PrPC do not span the lipid bilayer and are attached to the cell surface exclusively by a glycosyl phosphatidylinositol (GPI) anchor appended to the C terminus of the polypeptide chain (13, 14). In contrast, CtermPrP is thought to span the membrane once, with its C terminus on the exofacial surface and a highly conserved, hydrophobic region in the center of the molecule (amino acids 111–134) serving as a transmembrane anchor. Another form of PrP, NtermPrP, has also been described with the same transmembrane segment, but the reverse orientation (N terminus on the exofacial surface) (12). It has been proposed that the relative proportions of these three topological variants is influenced by as yet unidentified accessory proteins that interact with the translocation apparatus in the endoplasmic reticulum (ER) (15, 16).

Recent studies have brought the potential biological relevance of transmembrane forms of PrP into sharper focus. These species were originally observed only after translation of PrP mRNA in vitro on rabbit reticulocyte or wheat germ ribosomes.

Chinese hamster ovary; WT, wild-type; PAGE, polyacrylamide gel electrophoresis.
in the presence of canine pancreatic microsomes (17–21). In more recent investigations, however, CtmPrP has been identified in brain membranes from transgenic mice that express PrP molecules carrying mutations within or near the transmembrane domain; in vitro translation experiments had indicated that these mutations increased the relative proportion of CtmPrP (12, 22). Transgenic mice expressing such CtmPrP favoring mutations at high levels develop a spontaneous neurodegenerative illness that bears some similarities to scrapie, but without the presence of PrPSc (12, 22). There is also indirect evidence that CtmPrP accumulates in mice expressing wild-type PrP during the course of scrapie infection (22). Based on these results, it has been hypothesized that CtmPrP represents a common intermediate in the pathogenesis of both infectious and genetic prion diseases (22). In this view, CtmPrP is the ultimate cause of neurodegeneration, and PrPSc acts indirectly by increasing the amount of CtmPrP.

We have previously carried out extensive studies of the properties of mutant PrP molecules expressed in cultured cells and transgenic mice (23–27). The mutations analyzed in those investigations lie outside of the conserved hydrophobic region that serves as a transmembrane anchor in CtmPrP and NtmPrP. To test the hypothesis that transmembrane PrP is part of a general pathway for prion-related neurodegeneration, we undertook here to determine whether these mutations induce the formation of NtmPrP and CtmPrP in vitro, in cultured cells, and in transgenic mice. We confirm that mutations in the central, hydrophobic region enhance formation of transmembrane PrP, and we demonstrate for the first time that CtmPrP contains a hydrophobic region that serves as a transmembrane anchor in CtmPrP and NtmPrP.

**Experimental Procedures**

**Antiserum**—P45–66 antibody, raised against a synthetic peptide encompassing amino acids 45–66 of mouse PrP, has been previously (14). Monoclonal antibody 3F4, which recognizes an epitope from hamster and human PrP encompassing residues 109–112 (28), was a gift of Richard Krasnow (Institute for Basic Research, New York). R20 antibody, raised against a synthetic peptide comprising residues 218–232 of mouse PrP (29), was a gift of Byron Caughey (Rocky Mountain Laboratories, Hamilton, MT). Anti-calnexin antibody was from Stressgen (Vancouver, British Columbia, Canada).

**PrP Plasmids and mRNA Synthesis**—All mouse PrP cDNAs were cloned into the vector pcDNA3 (Invitrogen), and carried an epitope tag (amino acids, and penicillin/streptomycin. CHO cells were grown in α-minimal essential medium supplemented with 7.5% fetal calf serum and penicillin/streptomycin. Transfections were performed with LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Cells were harvested 24 h after transfection by brief trypsinization or by mechanical detachment, rinsed twice with PBS, and resuspended in 250 μl of 0.25 M sucrose, 10 mM HEPES (pH 7.4), 1 μg/ml pepstatin, and 1 μg/ml leupeptin. After 5 min on ice, cells were lysed by 10 passages through silastic tubing (0.3 mm, inner diameter) connecting two syringes with 27-gauge needles (34). A post-nuclear supernatant was prepared by centrifugation at 5,000 × g for 2 min. PK protection assays were performed by incubating post-nuclear supernatants in 50 mM Tris-HCl (pH 7.5), 250 μg/ml PK, and in some cases 0.5% Triton X-100. After 60 min at 22 °C, digestion was terminated by addition of 5 mM phenylmethylsulfonyl fluoride. Samples were deglycosylated with peptide-N-glycosidase F prior to analysis by Western blotting.

**Brain Membranes**—Fresh brain tissue (0.5 g wet weight) was homogenized using a Dounce apparatus in 5 ml of Buffer B (0.25 mM sucrose, 10 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM MgCl2, 1 μg/ml pepstatin A, 1 μg/ml leupeptin), followed by passage (five times each) through 16-, 18-, 21-, 23-, and 27-gauge needles. The homogenate was centrifuged first at 12,000 × g for 10 min in a microcentrifuge, and then at 541,000 × g for 20 min in a Beckman TL100.3 rotor, and the microsomal pellet was resuspended in 250 μl of Buffer B. PK protection assays were carried out as described above for transfected cells, except that PR was used at 50 μg/ml.

**RESULTS**

**Microsomes from Canine Pancreas and Murine Thymoma Cells Different in Their Efficiency of GPI Anchoring—PrP is attached to the cell surface via a C-terminal GPI anchor (13, 14). In carrying out studies of the membrane topology of PrP after in vitro translation, it would thus be desirable to utilize microsomal membranes that are capable of attaching the GPI anchor to newly synthesized polypeptides. Previous investigations...**

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have employed microsomes derived from canine pancreas, which do not effectively carry out GPI anchor addition (35). We therefore tested microsomes derived from BW5147.3 murine thymoma cells, which have been reported to be much more efficient in GPI anchoring (31, 36).

We translated mouse PrP mRNA in vitro using rabbit reticulocyte lysate in the presence of pancreatic or thymoma microsomes, and then incubated Triton X-114 lysates of the membranes with or without PIPLC, a bacterial phospholipase that cleaves the GPI anchor. By partitioning the lysate into detergent and aqueous phases, we could then score the amount of PrP that had been rendered hydrophilic by removal of the anchor. As shown in Fig. 1, all translation reactions produced two groups of products of 32 and 25 kDa, representing, respectively, core-glycosylated and unglycosylated forms of PrP. The latter correspond to molecules that had not been translocated into the lumen of the microsomes, since they are susceptible to digestion with PK (see below). Less than 10% of the PrP chains translated in the presence of canine pancreatic microsomes shifted into the aqueous phase after PIPLC treatment, indicating that very few molecules carried a GPI anchor (lanes 1–4). In contrast, about 50–60% of the glycosylated protein produced in the presence of thymoma microsomes shifted into the aqueous phase, indicating relatively efficient GPI anchoring (lanes 5–8). Untranslocated PrP chains, although they lack a GPI anchor, are retained in the detergent phase, presumably because they contain both N- and C-terminal signal peptides that are hydrophobic. Of note, PrP molecules shifted into the aqueous phase migrated with a slightly lower mobility than those that remained in the detergent phase (compare lanes 7 and 8), a phenomenon that is typical for polypeptides that have lost their GPI anchor (37). We believe that the actual efficiency of anchor addition by thymoma microsomes may be even higher than 60%, and that the persistence of some glycosylated PrP in the detergent phase after PIPLC treatment is likely to reflect inefficient PIPLC digestion and phase partitioning. In support of this idea, when samples were denatured in SDS prior to PIPLC treatment, to improve accessibility of proteins to the phospholipase, essentially all of the glycosylated PrP was converted to the more slowly migrating band (not shown). In contrast, PIPLC treatment after SDS denaturation did not alter the gel mobility of PrP synthesized with pancreatic microsomes.

Transmembrane Forms of PrP Are Produced in Both Microsomal Preparations, and Their Proportion Is Increased by Mutations in the Hydrophobic Region—To detect transmembrane forms of PrP, translation products were subjected to digestion by PK, which cleaves off portions of the polypeptide chain residing on the external side of the microsomal membrane and leaves transmembrane and luminal domains intact. Samples were then immunoprecipitated with anti-PrP monoclonal antibody 3F4, and whose lumenal and transmembrane domains have therefore been protected from digestion, and which therefore have been fully translocated into the microsome lumen; for consistency with an earlier report (12), we refer to this form as SecPrP. The two smaller fragments are derived from transmembrane species whose cytoplasmic domains have been digested by the protease, and whose luminal and transmembrane domains have been protected by the microsome. No PrP is detected after PK treatment in the presence of a detergent that disrupts the microsomal membrane (lane 3), confirming that the 19- and 15-kDa fragments do not represent intrinsically protease-resistant portions of the molecule. As will be shown below, the 19-kDa fragment derives from C\textsuperscript{term}PrP, a species whose C terminus resides in the ER lumen, and the 15-kDa fragment from N\textsuperscript{term}PrP, a species whose N terminus is luminal.

Consistent with previous studies (12, 22), we find that two mutations in the central hydrophobic region of the PrP mole-
TABLE I
Proportions of three topological forms of PrP produced by in vitro translation

|          | Sec  | Ctm  | Ntm  |
|----------|------|------|------|
| **Pancreas** |      |      |      |
| WT (n = 7) | 42.3 | 13.7 | 44.0 |
| (11.2)     | (4.8) | (13.9) |      |
| 3AV (n = 7) | 17.7 | 35.0 | 47.3 |
| (9.9)   | (5.9) | (12.9) |      |
| A116V (n = 4) | 22.6 | 25.0 | 52.4 |
| (1.3) | (3.1) | (4.2) |      |
| **Thymoma** |      |      |      |
| WT (n = 6) | 79.6 | 8.8  | 11.6 |
| (12.3) | (6.3) | (6.9) |      |
| 3AV (n = 6) | 61.7 | 25.4 | 12.6 |
| (8.6) | (5.4) | (6.7) |      |
| A116V (n = 2) | 72.5 | 11.7 | 15.9 |
| (6.9) | (4.2) | (2.7) |      |

Fig. 3. Epitope mapping of NtmPrP and CtmPrP. A, messenger RNA encoding WT or 3AV PrP was translated in rabbit reticulocyte lysate supplemented with microsomes from murine thymoma cells. Aliquots were then incubated with (lanes 1, 3–5, and 7–10) or without (lanes 2 and 6) PK in the presence (lanes 1 and 10) or absence (lanes 2–9) of Triton X-100 (Det). PrP was then immunoprecipitated with the indicated antibody, enzymatically deglycosylated, and analyzed by SDS-PAGE and autoradiography. The positions of protease-protected products derived from SecPrP, CtmPrP, and NtmPrP are indicated by arrowheads to the right of the gel. B, schematic of the postulated structures of full-length SecPrP, and the protease-protected fragments of NtmPrP and CtmPrP. The molecular sizes given in parentheses correspond to the polypeptides without oligosaccharides. The epitopes recognized by each antibody are shown. The black segment represents the transmembrane domain. The positions of the C terminus of NtmPrP and the N terminus of CtmPrP are approximate.

In a second experiment, we carried out epitope mapping to define the topology of NtmPrP and CtmPrP (Fig. 3). Translations of both wild-type and 3AV PrP were carried out in the presence of thymoma microsomes, and protease-protected products were immunoprecipitated with three different antibodies: P45–66, which recognizes the octapeptide repeats in the N terminus of PrP (14); 3F4, which reacts with an epitope encompassing residues 108–111 (28) (this epitope, which is not normally present in mouse PrP, was introduced into all of our PrP constructs by changing residues 108 and 111 to methionine); and R20, which recognizes residues 218–231 (29). We found that the SecPrP fragment was recognized by 3F4 and P45–66 but not by R20, indicating that the N terminus of NtmPrP through residue 111 is protected by the microsome. In contrast, the CtmPrP fragment was recognized by 3F4 and R20 but not by P45–66, indicating that the C terminus of CtmPrP starting from residue 108 is protected. As expected, SecPrP, which represents the full-length form of the protein, immunoprecipitates with all three antibodies. These mapping results, in conjunction with the observed sizes of the protected fragments, indicate that NtmPrP and CtmPrP span the membrane once, but in opposite directions, via the central hydrophobic segment (Fig. 3B). Similar results were obtained when pancreatic rather than thymoma microsomes were used.
artificial mutation in the central region (K109I/H110I, referred to as KH-II) that has been reported to increase the amount of transmembrane PrP (12, 22). We found that the proportion of CtmPrP was not increased over wild-type levels by any of the mutations outside of the central, hydrophobic domain (Fig. 5, A and B). As expected, the 3AV, A116V, and KH-II mutations resulted in significantly increased levels of CtmPrP.

Since the amount of CtmPrP associated with wild-type PrP in thymoma microsomes is small (∼10%) and close to the limits of detectability, we carried out similar experiments on mutant PrPs translated with canine microsomes, which generate larger amounts of CtmPrP. In this system as well, the amount of CtmPrP was not significantly altered by pathogenic mutations outside of the central region (Fig. 5B).

When samples were subjected to immunoprecipitation so that NtmPrP could be visualized, the amount of this species produced in the presence of either thymoma or canine microsomes was not changed by any of the mutations (data not shown).

Little CtmPrP Can Be Detected in Transfected Cells—To test whether transmembrane PrP can be detected in cultured cells, we carried out protease protection experiments on membranes in post-nuclear supernatants derived from transiently transfected BHK cells. As shown in Fig. 6, CtmPrP was not detectable in cells expressing wild-type PrP, although small amounts of a 19-kDa band that is likely to represent the deglycosylated, protease-protected fragment of CtmPrP were present in cells expressing 3AV or A116V PrPs. The identity of this band was confirmed by its reactivity with 3F4 and R20, but not with P45–66 (data not shown). Cells expressing PrP molecules with mutations outside of the hydrophobic region did not produce detectable amounts of CtmPrP, indicating that either these mutations do not increase the amount of transmembrane PrP, or if they do, their effect is less pronounced than that of 3AV and A116V. No NtmPrP was detected in any of the transfected cells.

The integrity of the membrane vesicles in these experiments was confirmed by the production of a 70-kDa, protease-protected fragment of calnexin, which represents the transmembrane and lumenal domains of the protein (40). Results similar to those shown in Fig. 6 were also obtained in transiently transfected CHO cells (data not shown). We conclude from these experiments that amount of transmembrane PrP produced in cultured cells is considerably lower than after in vitro translation, with CtmPrP representing <2% of the total PrP even for transmembrane-favoring mutations.

CtmPrP Is Not Detectable in the Brains of Transgenic Mice Expressing a Mutant PrP—To determine whether transmembrane forms of PrP could be detected in brain tissue, we also carried out protease protection experiments on microsomal membranes prepared from Tg(WT) transgenic mice that express wild-type PrP, or from Tg(PG14) mice that express a mutant PrP carrying a nine-octapeptide insertion. Tg(PG14) mice spontaneously develop a neurological illness characterized by ataxia, granule cell apoptosis, synaptic PrP deposition, and accumulation of a weakly protease-resistant form of PrP (26, 27). As was the case in cultured cells, neither CtmPrP nor NtmPrP were observable in the brains of mice expressing either wild-type PrP or PrP carrying the nine-octapeptide insertion (Fig. 7). Again, the integrity of the microsomes was confirmed by the presence of the 70-kDa protease-protected fragment of calnexin. Thus, in contrast to mutations in the hydrophobic region, which have been shown to produce detectable amounts of CtmPrP in the brains of transgenic mice (12, 22), a pathogenic mutation in the octapeptide repeat region does not increase the amount of transmembrane PrP above the level of detectability.

3AV PrP, but Not PG14 PrP, Can Be Released from the Cell
mutated with murine thymoma microsomes. Samples were then incubated with (PK lanes) or without (Det lanes) proteinase K in the presence (Det lanes) or absence (PK lanes) of Triton X-100. Samples were then analyzed by SDS-PAGE and autoradiography without immunoprecipitation of PrP. The SecPrP band is indicated by an open arrowhead, and the CtmPrP band by the filled arrowhead. On the film exposures shown here, the CtmPrP fragment is visible only for KH-II, 3AV, and A116V PrP; however, small amounts of this fragment can be detected above background levels using the PhosphorImager (see panel A). We show that PrP chains translated in vitro or expressed in cultured cells can adopt one of three possible topologies (Fig. 9). Molecules with the SecPrP orientation have been fully translocated into the ER lumen and conjugated to a GPI anchor that serves as their sole means of attachment to the lipid bilayer. This is the topology that is presumably displayed by the majority of molecules present on the surface of cells. In contrast, NtmPrP and CtmPrP span the lipid bilayer once via the central hydrophobic region (residues 111–134), with either the N terminus or C terminus, respectively, in the ER lumen. CtmPrP also acquires a C-terminal GPI anchor, and thus displays an unusual, dual mode of membrane attachment. We find that several mutations within or near the central, hydrophobic region increase the amount of CtmPrP, but pathogenic mutations outside of this region have no effect. The results presented here confirm and significantly extend previous reports on the membrane topology of PrP (12, 22).

All previous studies of PrP membrane topology by in vitro translation utilized canine pancreatic microsomes (12–17, 22), which we show here do not efficiently attach GPI anchors to newly synthesized polypeptide chains. GPI anchor addition occurs in the ER soon after completion of the polypeptide chain, and involves a transamidation reaction in which a hydrophobic, C-terminal sequence is cleaved and replaced by the pre-formed anchor structure (41). Presumably, the transamidase or other enzymes involved in GPI synthesis or addition are relatively inactive in microsomes derived from canine pancreas, or else endogenous phospholipases are more active. In our experiments, we have employed microsomes prepared from BW5147.3 mouse thymoma cells, which attach GPI anchors to the majority of PrP chains. Using this system, we find that both NtmPrP and CtmPrP become GPI-anchored. The demonstration of a GPI anchor on CtmPrP is a novel finding and

Surface by PIPLC—Since CtmPrP is attached to the lipid bilayer by both a transmembrane and a GPI anchor, it would not be released from the membrane after PIPLC cleavage of the anchor. To see if the increased amount of CtmPrP associated with the 3AV mutation caused a reduction in the proportion of the mutant protein that could be released from the cell surface by PIPLC, we labeled transfected CHO cells with a membrane-impermeant biotinylation reagent (Fig. 8). We found that >90% of the surface biotinylated 3AV PrP was released by the phospholipase (lanes 3 and 4), a proportion similar to that for wild-type PrP (lanes 1 and 2). Thus, the 3AV mutation has no observable effect on the PIPLC releasability of surface PrP. In contrast, virtually no biotinylated PrP carrying the PG14 mutation could be released by PIPLC (lanes 5 and 6), even though this mutation does not affect the amount of CtmPrP (Fig. 5). Similar results were obtained with transfected BHK cells (data not shown).

DISCUSSION

We show that PrP chains translated in vitro or expressed in cultured cells can adopt one of three possible topologies (Fig. 9). Molecules with the SecPrP orientation have been fully translocated into the ER lumen and conjugated to a GPI anchor that serves as their sole means of attachment to the lipid bilayer. This is the topology that is presumably displayed by the majority of molecules present on the surface of cells. In contrast, NtmPrP and CtmPrP span the lipid bilayer once via the central hydrophobic region (residues 111–134), with either the N terminus or C terminus, respectively, in the ER lumen. CtmPrP also acquires a C-terminal GPI anchor, and thus displays an unusual, dual mode of membrane attachment. We find that several mutations within or near the central, hydrophobic region increase the amount of CtmPrP, but pathogenic mutations outside of this region have no effect. The results presented here confirm and significantly extend previous reports on the membrane topology of PrP (12, 22).

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FIG. 5. Mutations outside of the hydrophobic domain do not alter the amount of transmembrane PrP. A, messenger RNA encoding WT or mutant PrP was translated in reticulocyte lysate supplemented with murine thymoma microsomes. Samples 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. Samples were then analyzed by SDS-PAGE and autoradiography without immunoprecipitation of PrP. The SecPrP band is indicated by an open arrowhead, and the CtmPrP band by the filled arrowhead. On the film exposures shown here, the CtmPrP fragment is visible only for KH-II, 3AV, and A116V PrP; however, small amounts of this fragment can be detected above background levels using the PhosphorImager (see panel A). We show that PrP chains translated in vitro or expressed in cultured cells can adopt one of three possible topologies (Fig. 9). Molecules with the SecPrP orientation have been fully translocated into the ER lumen and conjugated to a GPI anchor that serves as their sole means of attachment to the lipid bilayer. This is the topology that is presumably displayed by the majority of molecules present on the surface of cells. In contrast, NtmPrP and CtmPrP span the lipid bilayer once via the central hydrophobic region (residues 111–134), with either the N terminus or C terminus, respectively, in the ER lumen. CtmPrP also acquires a C-terminal GPI anchor, and thus displays an unusual, dual mode of membrane attachment. We find that several mutations within or near the central, hydrophobic region increase the amount of CtmPrP, but pathogenic mutations outside of this region have no effect. The results presented here confirm and significantly extend previous reports on the membrane topology of PrP (12, 22).

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B). The NtmPrP product is not detectable because its migration is obscured by globin. The human homologues of the mutations are associated with the following familial prion diseases: Creutzfeldt-Jakob disease (PG11, PG14, V179I, E199K, V209I), Gerstmann-Straussler syndrome (A116V, F197S), and fatal familial insomnia (D177N). B, translations were carried out with either thymoma or pancreatic microsomes. Samples were then analyzed by SDS-PAGE and autoradiography without immunoprecipitation of PrP. The SecPrP band is indicated by an open arrowhead, and the CtmPrP band by the filled arrowhead. On the film exposures shown here, the CtmPrP fragment is visible only for KH-II, 3AV, and A116V PrP; however, small amounts of this fragment can be detected above background levels using the PhosphorImager (see panel A). We show that PrP chains translated in vitro or expressed in cultured cells can adopt one of three possible topologies (Fig. 9). Molecules with the SecPrP orientation have been fully translocated into the ER lumen and conjugated to a GPI anchor that serves as their sole means of attachment to the lipid bilayer. This is the topology that is presumably displayed by the majority of molecules present on the surface of cells. In contrast, NtmPrP and CtmPrP span the lipid bilayer once via the central hydrophobic region (residues 111–134), with either the N terminus or C terminus, respectively, in the ER lumen. CtmPrP also acquires a C-terminal GPI anchor, and thus displays an unusual, dual mode of membrane attachment. We find that several mutations within or near the central, hydrophobic region increase the amount of CtmPrP, but pathogenic mutations outside of this region have no effect. The results presented here confirm and significantly extend previous reports on the membrane topology of PrP (12, 22).

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indicates that this species is attached to the membrane by both a bilayer-spanning segment as well as a C-terminal GPI structure that is covalently linked to the outer leaflet of the bilayer. The existence of such a dual mechanism of membrane attachment is unusual, although several other proteins have been proposed to adopt such a topology (42–44).

We and others have previously observed that PrP molecules carrying pathogenic mutations are resistant to release from the cell surface by PIPLC (14, 23, 45–47). The presence of a dual membrane anchor on the mutant proteins does not explain this phenomenon, since most mutations do not increase the amount of CtmPrP. In fact, PrP molecules carrying the 3AV mutation, which enhances formation of CtmPrP, are fully releasable by PIPLC, suggesting that SecPrP is the major species on the cell surface. Presumably, this is because the amount of CtmPrP that is present in cells is small in comparison to the amount of SecPrP, or because CtmPrP does not reach the cell surface. Our previous studies indicate that the most likely explanation for the PIPLC resistance of mutant PrPs is that the GPI anchors of these molecules are physically inaccessible to the phospholipase, possibly as a result of the conformational change that accompanies conversion to the PrPSc state (45).

It had been previously reported that several mutations in or around the central, hydrophobic region increase the amount of CtmPrP produced in translation reactions in vitro or in brain tissue (12, 22), but the effect of mutations outside of this area had not been examined. The previously studied mutations included one (A117V) whose human homologue (A116V) is associated with Gerstmann-Straussler syndrome (38, 39), and three (N107I, K109I/H110I, and 3AV) that are artificial and have not had been examined. The previously studied mutations included one (A117V) whose human homologue (A116V) is associated with Gerstmann-Straussler syndrome (38, 39), and three (N107I, K109I/H110I, and 3AV) that are artificial and have not been described in humans. The data reported here confirm that these mutations increase the amount of CtmPrP. A plausible explanation for this effect is that the amino acid changes alter the hydrophobicity, α-helical structure, or some other physical property of the central region in such a way as to make it a more suitable transmembrane segment. Two other artificial mutations in the central region of the molecule have been found to have the opposite effect, eliminating formation of NtmPrP and CtmPrP and causing exclusive production of SecPrP (12, 22). One of these (G122P) is likely to disrupt the formation of a transmembrane α-helix, and the other (A103–111) eliminates a segment of hydrophilic amino acids (designated “stop transfer effector”) that strongly influences the topology of the adjacent
transmembrane segment (19, 20). Taken together, the available data indicate that the central region of the PrP molecule acts as a crucial determinant of membrane topology, presumably in conjunction with the N-terminal signal sequence, but that other sections of the molecule, including those where many pathogenic mutations lie, are topologically inert. Exactly how the topology-determining domains of PrP function at the level of the ER translocon, and how the final topology of the protein is regulated will require further investigation (15, 16).

Our observation that most pathogenic mutations do not affect CtmPrP production has important implications for the role of this transmembrane species in the pathogenesis of prion diseases. Hegde et al. (22) have hypothesized that the generation of CtmPrP represents a final common pathway for neurodegeneration in both transmissible and familial forms of prion disease. This idea is based upon their observations that transgenic mice expressing CtmPrP-favoring mutations at high levels develop a spontaneous neurodegenerative illness in the absence of PrPSc, and that the amount of CtmPrP increases during the course of scrapie infection of mice expressing wild-type PrP. However, our observation that seven different pathogenic mutations fail to alter the amount of CtmPrP makes it unlikely that this species is an obligatory intermediate in all cases of prion-associated neurodegeneration. It has been speculated that some mutations like E200K indirectly enhance formation of CtmPrP by first causing accumulation of PrPSc (22). If this were the case, it might be argued that these mutations would not have an observable effect on CtmPrP levels in our experiments if PrPSc were not being produced. However, five of the pathogenic PrPs (PG11, PG14, D177N, F197S, and E199K) do in fact assume a protease-resistant form that resembles PrPSc when expressed in transfected CHO and BHK cells (2, 23), while three of mutants (A116V, V179I, and V209I) are protease-sensitive and display other properties characteristic of PrPC. This comparison makes it clear that there is no correlation between the efficiency with which a mutation promotes conversion to a PrPSc-like state in cell culture and its potency in inducing CtmPrP. A similar pattern is seen in vivo, since the human homologues of some of the mutations we have examined are associated with infectious PrPSc in the brains of patients while others are not (4). These results argue against the proposition that PrPSc causes disease by enhancing formation of CtmPrP. In cases where neither PrPSc nor CtmPrP are present, it seems likely that another form of PrP is the primary pathogenic entity.

Whether CtmPrP is an irrelevant byproduct of PrP biogenesis, or whether it plays some more restricted role in the disease process remains to be determined. One possibility is that CtmPrP is responsible for a subset of inherited prion diseases due to mutations within or near the transmembrane segment. Alternatively, when CtmPrP is present, it may accelerate or enhance neurodegeneration triggered by PrPSc. To evaluate these and other hypotheses, it will be crucial to understand more about the cell biology of transmembrane forms of PrP. We have observed that the amount of CtmPrP produced in transfected cells is considerably less than after in vitro translation. This may reflect differences in the efficiency of transmembrane PrP synthesis between the in vitro and in vivo situations, or, alternatively, it may be due to more rapid degradation of transmembrane PrP in cells. It is also possible that neuronal cells are more efficient at synthesizing CtmPrP than the BHK and CHO cells we have used here. It will be important now to analyze how transmembrane forms of PrP are generated during translation, where they are localized at the subcellular level, and how they are metabolized, to shed further light on the role of these unique species in the biology of prion diseases.

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Note Added in Proof—We find that the P101L mutation, whose human homologue is associated with Gerstmann-Sträussler syndrome, does not alter the amount of CtmPrP produced by in vitro translation performed as in Fig. 5A.

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