Distinct PrP Properties Suggest the Molecular Basis of Strain Variation in Transmissible Mink Encephalopathy

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The molecular basis of strain variation in scrapie diseases is unknown. The only identified component of the agent is the posttranslationally modified host prion protein (PrP^{Sc}). The biochemical and physical properties of PrP from two strains of transmissible mink encephalopathy (TME), called hyper (HY) and drowsy (DY), were compared to investigate if PrP heterogeneity could account for strain diversity. The degradation rate of PrP^{TME} digested with proteinase K was found to be strain specific and correlated with inactivation of the TME titer. Edman protein sequencing revealed that the major N-terminal end of HY PrP^{TME} commenced at least 10 amino acid residues prior to that of DY PrP^{TME} after digestion with proteinase K. Analysis of the brain distribution of PrP^{TME} exhibited a strain-specific pattern and localization of PrP^{TME} to the perikarya of specific neuron populations. Our findings are consistent with HY and DY PrP^{TME} having distinct protein conformations and/or strain-specific ligand interactions that influence PrP^{TME} properties. We propose that PrP^{TME} conformation could play a role in targeting TME strains to different neuron populations in which strain-specific formation occurs. These data are consistent with the idea that PrP^{TME} protein structure determines the molecular basis of strain variation.

Transmissible mink encephalopathy (TME) is a rare neurodegenerative disease of ranch-raised mink that is caused by ingestion of an unidentified scrapie-like contaminant (25, 52). Related diseases include scrapie in sheep and goats, bovine spongiform encephalopathy, chronic wasting disease in captive mule deer and elk, and Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia, and kuru in humans. All of the transmissible spongiform encephalopathies are characterized by long incubation periods followed by a progressive course of neurological disease and death. A hallmark feature of these diseases is the accumulation, primarily in the brain, of the host-encoded prion protein that undergoes unknown posttranslational modification(s) to generate the disease-specific isoform (PrP^{Sc}) (8, 10, 33, 88).

Several studies have demonstrated that PrP^{Sc} is a likely component of the scrapie agent (18, 19, 28, 86, 88, 90). Attempts to demonstrate a scrapie-specific nucleic acid (1, 2, 39, 42, 70, 78, 81, 82, 94) or the requirement of a nucleic acid for agent replication (3, 9, 68) have been unsuccessful. The posttranslational events involved in the conversion of the normal prion protein (PrP^C) to PrP^{Sc} are poorly understood (16, 29). Although distinct biochemical and structural properties are evident, no differences in chemical modifications between PrP^C and PrP^{Sc} have been found by using mass spectroscopy (98). PrP^C is a glycoprotein that is anchored to the plasma membrane via a covalent glycosphosphatidylinositol (GPI) linkage (97), is hydrolyzed by proteases (82), and has a secondary structure that may consist mainly of α helix (84). PrP^{Sc} is also glycosylated (20, 71, 80), is partially resistant to protease digestion (18, 86), forms fibrils or rods in vitro (7, 15, 40, 77, 88, 91), and contains a high β -sheet content (31, 46, 84, 93). PrP^{Sc} is found extracellularly in the form of brain amyloid and plaques (10, 33, 66), while PrP^C is localized to the plasma

membrane of neurons (22, 34). PrP^{Sc} also is found in diffuse deposits located in the neuropil (22, 34), juxtaposed to neuronal perikarya (58, 65), and on or within nerve processes (58, 65, 67).

The nature of the scrapie agent is a true quandary. How can an infectious agent apparently lacking a nucleic acid genome exhibit strain variation? More than 15 strains of mouse scrapie have been identified on the basis of differences in incubation period and brain histopathology (36, 44). These studies suggest that there exist fundamental differences between scrapie strains in the ability to replicate in distinct brain regions or neuronal populations. Several reports have demonstrated that the PrP genotype, which is likely homologous to the scrapie incubation gene (26), can control the length of the incubation period (23, 27, 38), susceptibility to disease (24, 37, 89), and induction of disease in cases of familial Creutzfeldt-Jakob disease (48, 83), Gerstmann-Sträussler-Scheinker syndrome (41, 48, 56), and fatal familial insomnia (47, 76, 96). However, the PrP genotype cannot explain how two or more scrapie strains can be expressed in the same genetic background. Therefore, these agents must encode strain properties that are independent of the host genotype.

In earlier reports, we identified two strains of TME in Syrian golden hamsters, called hyper (HY) and drowsy (DY). HY TME causes a disease characterized by hyperexcitability, head and body tremors, incoordination, and ataxia. DY TME is characterized by a progressive lethargy (13). The incubation period of HY TME is 65 days, compared with 165 days for DY TME. HY TME-infected brain contains a 100-fold-higher concentration of TME infectivity than DY TME-infected brain at the onset of clinical disease (13). Additionally, DY TME is pathogenic in mink whereas HY TME lost its pathogenicity after the third hamster passage (13). Examination of HY and DY PrP^{TME} revealed biochemical and structural diversity (12). Detergent extraction and ultracentrifugation of brain homogenates demonstrated that HY PrP^{TME} was insoluble whereas both DY PrP^{TME} and PrP^C remained in the supernatant.

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Although similar amounts of PrP^{TME} were present in HY and DY TME-infected brains, DY PrP^{TME} was readily degraded by proteases, in contrast to HY PrP^{TME}, which was resistant to degradation. Proteolytic treatment also resulted in a 1- to 2-kDa difference in migration on polyacrylamide gels between the strain-specific PrP^{TME}s. Antigenic mapping revealed that this difference was located at the N-terminal end (12). In the present study, we demonstrate that HY PrP^{TME} and DY PrP^{TME} have distinct protein conformations that may serve as the molecular basis for TME strain variation.

MATERIALS AND METHODS

Source of TME agent and bioassay. Brain tissue from an outbreak of TME in Stetsonville, Wis., was collected as described previously (73) and serially passaged in weanling, outbred (LVK/LAK) male Syrian golden hamsters purchased from Harlan Sprague Dawley (Indianapolis, Ind.). The HY and DY TME strains were biologically cloned by endpoint dilution from the fourth hamster TME passage as described previously (13). Detergent-extracted brain microsome samples were serially diluted 10-fold, and 50 μ l of a 10^{-1} to 10^{-9} dilution was intracerebrally inoculated into the right cerebral cortex of four Syrian hamsters. TME titers were calculated by the method of Kärber (85) after observation for 400 days.

Purification and N-terminal sequencing of PrP^{TME}. Brain PrP^{TME} was isolated as described previously for PrP^{Sc} (12), with modifications (17). The P4 fraction isolated from HY TME brains was digested with 25 μ g of proteinase K (PK) per ml for 4 h at 37°C; for DY TME, PK was added at 2.5 μ g/ml for 1 h. Twenty to 40 μ g of PK-digested HY or DY PrP^{TME} was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon-P membrane in 0.1 M 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11.0) containing 10% sequencing-grade methanol (Sigma, St. Louis, Mo.). PrP^{TME} was identified by Coomassie brilliant blue staining, and the two higher-molecular-weight PrP^{TME} bands were cut from the membrane for N-terminal sequencing. Edman protein sequencing of HY PrP^{TME} and DY PrP^{TME} was performed four times.

Protein sequencing was conducted with an automated model 477A liquid-phase sequencer with on-line model 120A phosphothiohydantoin (PTH) analyzer and model 610A data analysis system (Applied Biosystems, Foster City, Calif.). Data expressed as picomoles of PTH-derived amino acid are quantitated by comparison with a 1-pmol PTH-derived amino acid calibration standard. These data are corrected for background and lag.

Isolation of unique sequence from a mixture of peptides with related ragged N termini was conducted by procedures described by Wadsworth et al. (100). Briefly, a portion (~20 to 30% or ~2 to 20 pmol) of sample protein electroblotted to a polyvinylidene difluoride (PVDF) membrane was subjected to N-terminal sequencing. The resulting peptide map was the basis for a final N-terminal sequencing run in which orthophthalaldehyde (Fluoropa [OPA]) was used to chemically isolate a peptide with proline at the N terminus. Special OPA and PROPVDF cycles were used in series at the cycle(s) containing the selected proline(s); OPA covalently blocks all nonproline N termini, and special PROPVDF cycles reduced carryover caused by inefficient Edman chemistry of proline residues. OPA was obtained from Pierce. All other reagents were sequencing grade (Applied Biosystems, Foster City, Calif.).

Immunohistochemistry and immunoblotting. Brain tissue from clinically ill or age-matched Syrian hamsters was immunostained for PrP^{TME} as described previously (64, 65), with

modifications. Formalin-fixed, paraffin-embedded hamster brains were cut to a thickness of 7 μ m and attached to Proband Plus glass slides (Fisher Scientific, Pittsburgh, Pa.). After deparaffinization, some tissue sections were incubated with PK (25 to 50 μ g/ml) for 1 h at 37°C, and the digestion was terminated by washing in 2 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline. Endogenous peroxidases were blocked by soaking tissue sections in 0.3% H₂O₂ in methanol for 20 min. Tissue sections were submerged in 1 to 3 mM hydrochloric acid and autoclaved for 10 min. After washing, sections were blocked in 5% nonfat dry milk in Tris-buffered saline for 20 min at 37°C and incubated with monoclonal antibody 3F4 ascites fluid (a generous gift of Richard Kascsak, Institute for Basic Research in Developmental Disabilities, Staten Island, N.Y.) (61) diluted 1:2,000 in RPMI 1640 medium containing 10% fetal calf serum and 0.05% Tween 20 for 1 h at 37°C. Washed sections were incubated with goat anti-mouse immunoglobulin G (1:250; Bio-Rad Laboratories, Hercules, Calif.) conjugated with horseradish peroxidase for 1 h at room temperature. Development was performed in 50 mM sodium acetate (pH 5.0) containing 0.8 mg of 3-amino-9-ethylcarbazole per ml and 0.03% H₂O₂. Tissue sections were counterstained with Mayer's hematoxylin.

SDS-PAGE and Western blotting (immunoblotting) for PrP were performed as described previously (12), using monoclonal antibody 3F4 ascites fluid diluted 1:40,000. Quantitation of immunopositive PrP signals was performed with an LKB Ultrosan XL densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden) and Pharmacia Gel Scan XL software.

Brain microsome preparation and analysis. Brain microsomes were prepared from clinically ill hamsters as described previously (12, 51). Microsomes were resuspended at a protein concentration of 1 mg/ml in 25 mM Tris-HCl (pH 7.4)-150 mM NaCl-1 mM EDTA containing 2% *N*-lauroylsarcosine. After removal of an aliquot at time zero, PK was added to a concentration of 100 μ g/ml and microsomes were incubated at 37°C with constant stirring. An aliquot was removed at 1, 12, 24, and 48 h, at which time phenylmethylsulfonyl fluoride was added to 5 mM. After sample collection at the 24-h time point, an additional 25 μ g of PK per ml was added. Following ultracentrifugation in a TLA 100.2 rotor at 225,000 $\times$ g for 1 h, samples were resuspended in 0.1 ml of H₂O. Thirteen microliters was analyzed by SDS-PAGE and Western blotting for PrP^{TME} as described above. To determine the TME activity of these PK-digested brain microsome samples, a 10^{-1} to 10^{-9} dilution (undiluted inoculum contained 13 μ l of the microsome preparation in a 50- μ l volume for each of four hamster recipients) of the microsome preparation was intracerebrally inoculated as described above.

RESULTS

Strain-specific degradation of PrP^{TME} correlates with inactivation of TME. To evaluate potential differences in strain variation, brain microsomes enriched for PrP^{TME} from HY and DY TME-infected hamsters were digested at 37°C with 100 μ g of PK per ml in the presence of 2% *N*-lauroylsarcosine. An aliquot was removed at 1, 12, 24, and 48 h. After 24 h, an additional 25 μ g of PK per ml was added. Brain microsomes were centrifuged at 225,000 $\times$ g, and pellet fractions were analyzed by immunoblotting. At the onset of clinical disease, HY and DY TME-infected hamsters had similar levels of brain PrP^{TME} in the detergent-extracted pellet fractions prior to PK digestion (Fig. 1B; only HY TME is illustrated, but the results were similar for DY TME). PrP^C was found in the supernatant after centrifugation and therefore was not detected (12, 79).

FIG. 1. (A) Brain microsomes isolated from HY and DY TME-infected hamsters were untreated (–) or digested with PK (+) at 100 μ g/ml for 1, 12, 24, and 48 h. Following ultracentrifugation in buffer containing 2% *N*-lauroylsarcosine, the pelleted material was analyzed by SDS-PAGE (15% gel) and immunoblotting using 3F4 ascites fluid (1:40,000). (B) Undigested brain microsomes extracts, as prepared for panel A, from HY TME-infected brains were serially diluted and analyzed by SDS-PAGE and immunoblotting. A relative value of 1.0 is equivalent to the amount of undigested brain microsomes illustrated in panel A. The PrP^{TME} signal was weak but present at a 0.004 dilution. A similar result was obtained for DY TME-infected brain microsome extracts (data not shown). (C) PrP^{TME} digested with PK was purified from HY (H) and DY (D) TME-infected hamster brains. Two micrograms of protein per sample was resolved on a 15% polyacrylamide gel and stained with silver. Molecular mass markers are indicated on the right.

Figure 1A illustrates that PrP^{TME} from HY TME-infected brain microsomes digested with PK was truncated but only partially degraded after 48 h. DY PrP^{TME} was partially degraded at 1 h and completely hydrolyzed by 12 h. Densitometric scanning of immunoblots containing serial dilutions of undigested brain microsomes in Fig. 1A was used to generate a standard curve for using linear regression analysis in order to determine the relative amounts of PrP^{TME} in microsomes digested with PK. Approximately 22% of HY PrP^{TME} remained after 48 h of proteolytic digestion, and 19% of DY PrP^{TME} remained after 1 h (Table 1). Serial dilution of undigested brain microsomes combined with PrP^{TME} immunoblotting revealed that both the HY and DY PrP^{TME} signals were lost between dilutions of 0.004 and 0.0008 (Fig. 1B). Therefore, PK digestion of DY TME-infected brain microsomes for 12 h resulted in at least a 500-fold loss, since no immunopositive PrP^{TME} signal was observed by Western blotting (Fig. 1A). These experiments were repeated three times, with similar results obtained in each trial.

To determine the correlation of TME titer to the relative amount of PrP^{TME} in brain microsome fractions, amounts of

sample identical to those analyzed in Fig. 1A were bioassayed by endpoint dilution in Syrian golden hamsters. Table 1 illustrates that the degradation of PrP^{TME} closely correlated with the inactivation of TME infectivity in a strain-specific manner. There existed almost a 10^2 50% lethal dose (LD₅₀)/g of brain difference between HY and DY undigested brain microsomes (i.e., $10^{8.1}$ versus $10^{6.3}$). Previously, we determined that the brain titer of TME in DY-infected hamsters was 100-fold less than in HY-infected animals at the onset of clinical symptoms, yet we did not detect a 100-fold-lower concentration of brain protein or PrP^{TME} (13). There was a decrease in both PrP^{TME} signal and HY TME titer after 1, 12, and 24 h of PK digestion; after 48 h, there were a $10^{1.3}$ LD₅₀ inactivation of HY TME infectivity (Table 1) and an approximately 80% reduction in the HY PrP^{TME} signal as stated above. A 1-h PK digestion of DY TME-infected brain microsomes resulted in $<10^1$ LD₅₀/g of brain loss of TME titer, and approximately 80% of the DY PrP^{TME} was degraded. Complete loss of the DY PrP^{TME} signal after 12 h of PK digestion resulted in greater than a 10^3 LD₅₀/g of brain loss of TME titer, a reduction from $10^{6.3}$ to $10^{2.8}$ LD₅₀/g of tissue (Table 1). Therefore, a >500 -fold reduction in DY PrP^{TME} levels resulted in a comparable inactivation of DY TME titer. Longer PK treatment resulted in additional loss of DY TME titer, which, like the PrP^{TME} titer, fell below detectable levels (Fig. 1A and Table 1). These data are consistent with previous studies that suggest that PrP^{TME} is an essential component of the TME agent.

Purification and protein sequencing of PrP^{TME}. Purification procedures that use differential ultracentrifugation and PK digestion of detergent-extracted brain fractions yield a preparation consisting predominantly of PrP^{Sc} (54, 88). PK digestion removes at most 67 amino acid residues from the N-terminal end of PrP^{Sc} (55, 90). Previously, when attempting to purify PrP^{TME}, we reported a difference in the sedimentation properties between HY and DY PrP^{TME} that prevented purification of DY PrP^{TME} (12). In the present study, we used a modification of this procedure (17) in order to obtain similar yields of PrP^{TME} from the two TME strains (data not shown). PK digestion of these fractions resulted in the formation of

TABLE 1. TME brain titers of brain microsomes digested with PK

Time (h) of sampling of brain microsomes + PK ^a	HY		DY	
	TME titer ^b (log ₁₀ LD ₅₀ /g of brain)	PrP signal ^c	TME titer (log ₁₀ LD ₅₀ /g of brain)	PrP signal
0	8.1	1.00	6.3	1.00
1	7.8	0.54	6.1	0.19
12	6.8	0.42	2.8	<0.004
24	7.1	0.34	≤1.0	<0.004
48	6.8	0.22	≤1.0	<0.004

^a Brain microsomes were treated as described for Fig. 1A. An aliquot equivalent to that analyzed by immunoblotting was serially diluted and intracerebrally inoculated into Syrian golden hamsters to determine the TME titer.

^b Calculated by the method of Kärber (85).

^c Determined either by linear regression analysis in which dilutions of undigested PrP^{TME} on immunoblots were scanned by densitometry to calculate the amount remaining after digestion or as described for Fig. 1B.

TABLE 2. Amino-terminal sequencing of DY PrP^{TME}

PrP	Amino acid sequence				
	90	95	100	105	110
Hamster PrP ^a	G-Q-G-G-G	-T-H-N-Q-W	-N-K-P-S-K	-P-K-T-N-M	-K-H-M-A-G
	▲	▲	▲		
DY PrP ^{TME}					
No OPA added ^b	G-G-G	-T-H-N-Q-N	-N-K-P-S-K	-P-K-T-N-M	-S-H-M-V-G
			K-P-S-K	-P-K-X-X-M	-X-H-M-A-G
OPA at cycle 5 ^c				-P-K-T-N-M	-K-H-M-A-G
			-P-S-K	-P-K-T-X-M	
OPA at cycles 11 and 14 ^d			-P-S-K	-P-K-T-N-M	-K-H-M-A-G

^a Illustrated is the amino acid sequence of hamster PrP beginning at residue 90. This sequence was reported in several earlier studies (82). The triangles below the indicated amino acids mark the N termini found for DY PrP^{TME} in this study.

^b The major amino acid sequence is illustrated on the top line, while additional signals are shown directly below the major sequence. The experimentally determined amino acid sequences of DY PrP^{TME} were aligned with the known hamster PrP sequence. An X indicates that the amino acid residue could not be assigned. The underlined amino acid residues correspond to cycles 5, 11, and 14.

^c OPA was added at the beginning of the indicated cycle, and the first residue illustrated was the proline at that cycle number. The initial yield of proline at cycle 5 was 2.8 pmol.

^d The initial yield of proline at cycle 11 was 6.8 pmol.

three PrP^{TME} polypeptides of between 19 and 31 kDa (Fig. 1C). The truncated DY PrP^{TME} polypeptides migrated 1 to 2 kDa faster than similar polypeptides of HY PrP^{TME} on SDS-PAGE. The shift in migration was most obvious upon comparison of the lowest-molecular-weight bands. Proteolytic digestion of native proteins with the identical primary amino acid sequence has been used to demonstrate differences in protein conformation (72, 74). The two higher-molecular-weight PrP^{TME} bands were glycosylated whereas the lower one was not (12, 51), but the extent of N-linked glycosylation did not account for differences in gel migration (12).

N-terminal sequencing was performed to evaluate whether the 1- to 2-kDa difference in gel migration was due to cleavage of PK at distinct N-terminal ends of HY and DY PrP^{TME}s. Protein sequencing by Edman degradation revealed that PrP^{TME} from both TME strains had multiple N termini that were likely generated by the use of PK during protein purification. Although at least three N termini were obtained for DY PrP^{TME}, the predominant N-terminal amino acid residue corresponded to Gly-92 of hamster PrP (Table 2). To confirm that Gly-92 was the major N-terminal end, we took advantage of the fact that there were proline residues at positions 102 and 105 of the hamster PrP sequence (82) which corresponds to cycles 11 and 14 of the Edman degradation if, in fact, the N terminus was Gly-92. Therefore, special OPA cycles were used at the beginning of both cycles 11 and 14 in order to block PTH hydrolysis of all non-proline-containing N termini. In this procedure, N-terminal prolines do not chemically interact with OPA, and therefore, Edman degradation proceeds as usual (100). The initial yield of proline at cycle 11 was 6.8 pmol, and the amino acid sequence for the next 20 cycles aligned exactly with the hamster PrP sequence from positions 103 to 123 (82), confirming that Gly-92 was the major N-terminal end of DY PrP^{TME} (Table 2; some data are not shown).

In the absence of OPA, two major peptides and at least three minor peptides were observed. Both a histidine and a proline residue were detected in cycle 5 as significant PTH-derived amino acid signals (Table 2). In the hamster PrP sequence, amino acid residue 97 is a histidine (82). Since the recovery of histidine is low in automated Edman degradation (95), we hypothesized that the proline was part of a less abundant polypeptide that did not have an N terminus at Gly-92. Table 2 illustrates that treatment with OPA at the beginning of cycle 5 resulted in an initial yield of 2.8 pmol for proline at this cycle, and even at this low yield, two minor

sequences were clearly identified. In the more abundant peptide, the proline at cycle 5 corresponded to Pro-105 of the hamster PrP sequence, while the minor sequence aligned with Pro-102. These data indicate that digestion of DY PrP^{TME} with PK results in one major polypeptide product with an N terminus at Gly-92 and two less abundant products with N termini at Lys-101 and Gln-98 of hamster PrP (Table 2). Numerous additional cleavage sites were evident at low abundance; these have not been mapped.

Four attempts to determine the N-terminal sequence of HY PrP^{TME} digested with PK produced equivocal results due to heterogeneity. HY and DY PrP^{TME} amino acid sequencing maps were constructed on the basis of reproducible, strong amino acid signals from the first 12 cycles of Edman degradation. A comparison of these maps that was based on the exclusive presence of each residue in a particular cycle was used. For example, HY PrP^{TME} had a major proline signal in cycle 4 which decreased in cycle 5, whereas in DY PrP^{TME}, no proline signal was detected in cycle 4 whereas a significant proline signal was observed in cycle 5. Our interpretation was that the HY and DY PrP^{TME} sequences differed in proline content in both cycles 4 and 5. From this type of analysis, we determined that the two sequences were different in at least 8 of the first 12 cycles (data not shown). This finding provided strong evidence that HY and DY PrP^{TME}s have different N termini, although a low abundance of similar N-terminal ends cannot be completely ruled out. The major sequence from the first three cycles of HY PrP^{TME} revealed a Gly-Gln-Pro sequence, but thereafter the major signals could not be discriminated in the complex peptide sequencing map. Gly-Gln-Pro is present in the hamster PrP sequence only in the octapeptide repeat region, where it initiates at positions 58, 66, 74, and 82. The HY PrP^{TME} N termini were similar to the reported N termini of hamster PrP^{Sc} digested with PK, which were also located in the octapeptide repeat region (55, 80, 90, 98). These observations indicate that the faster gel migration of DY PrP^{TME} than of HY PrP^{TME} after PK digestion is likely due to additional truncation at the N-terminal end. Differences in proteolytic degradation between two PrP^{TME}s with the same primary structure is an indication of distinct secondary or tertiary protein conformations or ligand interactions (72, 74).

Brain mapping of HY and DY PrP^{TME}. To determine if the strain-specific conformational properties of PrP^{TME} could arise as a result of TME replication in different brain regions or neuronal populations, the distributions of HY and DY

FIG. 2. Light micrographs of coronal brain sections from uninfected (C), HY TME-infected (A and D), and DY TME-infected (B and E) hamsters immunostained for PrP^{TME}. The distribution of immunostaining was examined in the medial geniculate nucleus (G), hippocampus (H), occipital cortex (C), and corpus callosum (cc) in panels A to C (the bar represents 400 μ m). In panel A, the asterisk is located in the molecular layer of the dentate gyrus where there is an absence of PrP^{TME}, while the bracket illustrates a band of immunoreactivity in the middle layers of the cerebral cortex. The cerebellar cortex (D and E; the bar represents 40 μ m) consists of the molecular (m), Purkinje (P), and granular (g) cell layers and the white matter tracts (w). In the HY TME-infected cerebellum, PrP^{TME} plaques were found in the molecular layer (arrows) or adjacent to Purkinje cell perikarya (arrowheads). In the DY TME-infected cerebellum, perivacuolar PrP^{TME} plaques were found in the white matter (arrowheads), and diffuse immunostaining parallel to the axons of the granular neurons was observed in the molecular layer (arrow). PrP^{TME} staining is represented by the red reaction product, and the tissue was counterstained with Mayer's hematoxylin.

PrP^{TME}s were determined by immunohistochemistry. To this end, we used a hydrolytic autoclaving technique of paraffin-embedded brain sections (64, 65). With this procedure, PrP^{TME} immunostaining in control brains was absent or, in a few experiments, a slight background reaction was observed that was easily distinguishable from immunostaining in TME-infected brains. In the indicated examples, pretreatment of paraffin-embedded tissue sections with PK reduced the background and improved detection of PrP^{TME}. Immunopositive PrP^{TME} signals appeared diffuse or in plaques and were found separately or within the same area, depending upon the strain of TME and brain region examined. PrP^{TME} plaques were observed in the neuropil and occasionally were found within perikarya. We examined at least four hamster brains from each group, and although there was slight variation in the intensity

of the PrP^{TME} signal between animals, the location of immunostaining was consistent in each group.

Figures 2A and B illustrate the distribution of HY and DY PrP^{TME}s in the medial geniculate nucleus, hippocampus, corpus callosum, and occipital cortex. A comparison of the PrP^{TME} brain distribution in this region revealed a strain-specific pattern that is summarized in Table 3. The medial geniculate nucleus stained heavily for PrP^{TME} in HY TME-infected brains, but staining was absent in DY TME-infected brains. The dentate gyrus portion of the hippocampus in both HY and DY TME-infected hamster brains was positive for PrP^{TME}, but there was a lack of staining in the molecular layer in HY TME-infected animals (asterisk in Fig. 2A). The distribution of immunostaining in the occipital cortex was also very different among the TME strains. In HY TME-infected

TABLE 3. Brain distribution of PrP^{TME} in HY and DY TME-infected hamsters

Brain region	HY TME		DY TME	
	Diffuse ^a	Plaque	Diffuse	Plaque
Hippocampus				
Dentate gyrus				
Granule layer	0	0	0	0
Polymorphic layer	++	++	+++	0
Molecular layer	0	0	++	0
Corpus callosum	0	+	0	+++
Occipital cortex ^b	++	++	+++	0
Medial geniculate nucleus	++++	0	0	0
Cerebellum				
Granule layer	++	+	+++	+
Purkinje layer	0	+	0	0
Molecular layer	0	++	++	+
White matter	0	++	0	++++
Deep nuclei	++++	++	0	+

^a A scoring system was used to map the brain distribution of PrP^{TME}. The following symbols were used to describe the observed frequency and intensity of PrP^{TME} immunostaining: 0, none; +, rare; ++, mild; +++, moderate; +++++, heavy.

^b HY PrP^{TME} staining was located primarily in the middle cortical layers, while DY PrP^{TME} staining was distributed in all cortical layers.

brains, PrP^{TME} was localized to the middle layers of the cortex, where both diffuse staining and plaques were observed (bracket in Fig. 2A). In DY TME-infected brain, there was a diffuse staining in all cortical layers but plaques were absent. Figure 2C illustrates the absence of PrP^{TME} immunostaining in an uninfected hamster brain from this same region.

Figures 2D and E demonstrate the PrP^{TME} distribution in the cerebellar cortex among the two TME strains. Both strains had mild to moderate staining in the granular layer, but DY TME-infected brains had intense plaque staining in white matter tracts that often surrounded vacuolar lesions (arrow-

heads in Fig. 2E; Table 3). In addition, HY TME-infected brains had PrP^{TME} plaque staining in the molecular layer (arrows in Fig. 2D) and adjacent to the perikarya of Purkinje neurons (arrowheads in Fig. 2D). In DY TME-infected brains, there was a diffuse staining in the molecular layer that appeared to be localized in the axons of granule neurons proximal to bifurcation (arrow in Fig. 2E). HY and DY PrP^{TME} immunostaining was widespread throughout the brain, and we are unable to correlate the PrP^{TME} distribution with clinical signs.

Little is understood concerning the cell types involved in the synthesis of PrP^{TME} in the brain. In HY TME-infected hamster brains, we observed intracytoplasmic PrP^{TME} deposits in neurons in several nuclei of the medulla and pons. The hypoglossal nucleus had a mild, diffuse and plaque PrP^{TME} immunostaining pattern in the neuropil (arrowheads in Fig. 3A and B), but more than 50% of the neurons contained PrP^{TME} plaque-like deposits in the perikaryon (arrows in Fig. 3A). These deposits were usually perinuclear and occasionally were associated with small intracytoplasmic holes. In some cases, the holes, or vacuoles, could occupy most of the perikaryon and were filled with PrP^{TME} (arrow in Fig. 3B). Additional PrP^{TME} laden neurons had shrunken cytoplasm reminiscent of degenerating neurons. No PrP^{TME} immunostaining was observed in the hypoglossal nucleus from DY TME-infected brains (Fig. 3C).

DISCUSSION

The molecular basis of strain variation is central to understanding the mechanism of TME agent replication. Genetic variation in all living organisms is determined by mutation of DNA or RNA. Although the existence of a scrapie-specific nucleic acid cannot be unequivocally disproven at this time, several lines of evidence suggest that the scrapie agent is devoid of a nucleic acid genome and that alteration of the host PrP protein is sufficient to induce neurodegeneration. In this

FIG. 3. PrP^{TME} immunostaining of the hypoglossal nucleus of HY (A and B) and DY (C) TME-infected hamster brain as performed for Fig. 2 except that tissue was treated with PK (25 µg/ml) for 30 min prior to hydrolytic autoclaving. In HY TME-infected brains, PrP^{TME} plaques were found both within the cytoplasm of neurons (arrows) and in the neuropil (arrowheads). No PrP^{TME} immunostaining was detected in the hypoglossal nucleus from DY TME-infected animals. Bars: A and C, 40 µm; B, 20 µm.

report, we provide strong evidence that PrP^{TME}s from the HY and DY strains of TME have distinct protein conformations and propose that these structural features could mediate the biological properties of TME strains. It is possible that multiple PrP^{TME} conformations can be stabilized by interactions with a strain-specific ligand.

In this report, we demonstrate that differences in the rate of proteolytic degradation of HY and DY PrP^{TME}s correlated with the biological inactivation of each strain. Previously, a correlation between hydrolysis of PrP^{Sc} and inactivation of scrapie titer was found (75). However, DY PrP^{TME} has a much greater susceptibility to proteolytic degradation than either PrP^{Sc} or HY PrP^{TME}. Inactivation of HY TME brain titer closely paralleled degradation of PrP^{TME}. After 48 h, there were reductions of 78 and 95%, respectively. In contrast, DY PrP^{TME} was completely hydrolyzed by 12 h of PK digestion (i.e., at least a 500- to 1,000-fold reduction), and DY TME titers had decreased by 10³ LD₅₀ and by >10⁵ LD₅₀ within 24 h. These findings support previous reports that suggest PrP^{TME} is a required component of the TME agent (87). The faster rate of proteolytic degradation of DY PrP^{TME} in vitro could explain its slower accumulation in the brain (11) if it exhibits a higher rate of turnover in vivo.

Proteolytic cleavage of HY and DY PrP^{TME} resulted in distinct N-terminus maps, which is consistent with the two proteins having different conformations. DY PrP^{TME} had N termini beginning at Gly-92, Gln-98, and Lys-101 of hamster PrP. HY PrP^{TME} had a major N terminus at Gln-Gly-Pro, which is located in the octapeptide repeat region (82) that extends from Pro-52 to Gln-91. The HY, but not DY, PrP^{TME} N termini were located in a region similar to that previously reported for hamster PrP^{Sc} (55, 80, 82, 90, 98). Classically, proteases have been used to map ligand-induced conformational changes of proteins (72, 74). In one study (72), ligand binding reduced susceptibility of RNase A to subtilisin cleavage at a bond distant from the ligand binding site. Although we do not have direct evidence of a PrP^{TME}-ligand interaction that can form multiple stable conformations, our findings suggest that differences in the secondary or tertiary PrP^{TME} conformation between the two strains result in distinct PK cleavage sites and rates of degradation. This interpretation is consistent with our earlier findings that HY and DY PrP^{TME}s have different sedimentation properties (12). It is also feasible that ligand binding to PrP^{TME} can sterically hinder the accessibility of PK to potential cleavage sites in a strain-specific manner. Potential ligands include nucleic acids (1, 78, 94, 101) and glycosaminoglycans (98), since both have been detected in highly infectious PrP^{Sc} preparations. Furthermore, specific glycosaminoglycan analogs inhibit the conversion of PrP^C to PrP^{Sc} in neuroblastoma cell lines (28, 30) and were found to result in a concomitant loss of scrapie titer when cell lysates were bioassayed in mice (28).

We predict that the strain differences in PrP^{TME} conformation are due to the extent of β -sheet content. Amyloid proteins found in neurodegenerative diseases can be distinguished from their amyloid precursor proteins by the formation of amyloid plaques, a low α -helix and high β -sheet content (31, 46, 84, 93), and a propensity to form insoluble aggregates when isolated in vitro (54, 79). An investigation of the molecular basis of amyloid formation found that insoluble synthetic β -amyloid peptides had extensive β structure whereas soluble peptides lacked β structure (50). Previously, we reported differences in sedimentation properties between HY and DY PrP^{TME}s (12). Under conditions in which HY PrP^{TME} was found in the pellet fraction, DY PrP^{TME} and PrP^C were in the supernatant. In this study, we modified the centrifugation conditions and were able

to sediment DY PrP^{TME}, while PrP^C remained in the supernatant fraction. We postulate that the ability of PrP^{TME} to form aggregates is due to a higher β -sheet content than in PrP^C, analogous to findings with synthetic β -amyloid peptides (50). Since DY PrP^{TME} sediments more slowly than HY PrP^{TME}, it likely contains less β -sheet content but more than PrP^C. Differences in the ability of HY and DY PrP^{TME}s to aggregate could affect the sedimentation properties of PrP^{TME} as well as its rate of proteolytic digestion and sites of PK cleavage.

Posttranslational modifications also can influence protein conformation and may account for the strain-specific PrP^{TME} properties. N-linked glycosylation (20, 71, 80) and the addition of a C-terminal GPI anchor (97, 99) are the major covalent posttranslational modifications of PrP. Although no differences in the composition of oligosaccharide structures linked to asparagine have been found between PrP^C and PrP^{Sc}, there exists great diversity in these complex structures (43, 51). In the analysis of the GPI anchor from PrP^C and PrP^{Sc}, no differences in the glycan core and in the attachment of sialic acid moieties were found (4, 6, 97). Similarly, the chemical structures of the GPI anchor appeared similar between PrP^{Sc} from mouse strains Sc237 and 139H, indicating that this structure is not likely responsible for strain properties (5). A comparison of proteolytic fragments derived from PrP^C and PrP^{Sc} by mass spectroscopy also failed to find any differences (98). These findings do not exclude the possibility that HY and DY PrP^{TME} exhibit heterogeneity in GPI anchors or N-linked glycosylation that could explain differences in susceptibility to PK degradation or in protein conformation, but they do make it less likely.

Analysis of the brain distribution of HY and DY PrP^{TME}s revealed a strain-specific pattern. We propose that TME tropism may be determined by PrP^{TME} conformation. Structural PrP^{TME} variants may target TME strains to different sites in the central nervous system. Replication of TME strains in separate neuronal populations could facilitate the maintenance of distinct PrP^{TME} conformations through cell-specific ligand interactions, by posttranslational modifications, or by a PrP^{TME}-directed protein-folding mechanism (21, 32, 45, 49, 57, 92). At the level of immunostaining, we could not detect any structural differences between HY and DY PrP^{TME} deposits. Strain-specific brain distribution of PrP^{Sc} in mouse scrapie has been demonstrated in other studies (35, 53). The existence of biochemical and structural diversity of PrP^{Sc} between strains of scrapie in mice with the same PrP genotype has not been adequately resolved (14, 59, 60).

Although the scrapie agent travels at the rate of slow axonal transport (62, 63), it has been difficult to localize PrP^{Sc} within neurons in vivo. In this study, we demonstrate the presence of PrP^{TME} in neuronal perikarya, indicating that neurons are one likely site of PrP^{TME} formation. In HY TME-infected brains, neurons of the hypoglossal nucleus contained intracytoplasmic PrP^{TME} deposits. Some of these deposits occupied the majority of the cytoplasm, while others were found in what appears to be degenerating neurons or neurons adjacent to spongiform changes. These findings strongly suggest that accumulation of PrP^{TME} can be neurotoxic in vivo. Perineuronal PrP^{TME} plaques were observed in several brain regions. Localization of PrP^{TME} to nerve processes was illustrated by a radial distribution pattern parallel to the axons of cerebellar granule neurons as they transverse the molecular layer. Other studies also have described PrP^{Sc} staining juxtaposed to neuronal perikarya and neurites (58, 65) and associated with nerve processes (58, 67) or axons (67, 69). Our results suggest that conversion of PrP^C to PrP^{TME} in neurons does occur in vivo.

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