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Prion Propagation in Mice Expressing Human and Chimeric PrP Transgenes Implicates the Interaction of Cellular PrP with Another Protein

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Summary

Transgenic (Tg) mice expressing human (Hu) and chimeric prion protein (PrP) genes were inoculated with brain extracts from humans with inherited or sporadic prion disease to investigate the mechanism by which PrP^C is transformed into PrP^{Sc}. Although Tg(HuPrP) mice expressed high levels of $HuPrP^C$, they were resistant to human prions. They became susceptible to human prions upon ablation of the mouse (Mo) PrP gene. In contrast, mice expressing low levels of the chimeric transgene were susceptible to human prions and registered only a modest decrease in incubation times upon MoPrP gene disruption. These and other findings argue that a species-specific macromolecule, provisionally designated protein X, participates in prion formation. While the results demonstrate that PrP^{Sc} binds to PrP^C in a region delimited by codons 96 to 167, they also suggest that PrP^C binds protein X through residues near the C-terminus. Protein X might function as a molecular chaperone in the formation of PrP^{Sc}.

Introduction

For three decades, the transmission of human prion diseases was studied largely with apes and monkeys, in which >90% of cases are thought to be transmissible (Brown et al., 1994; Gajdusek et al., 1966). Inoculations of mice, rats, and hamsters produced variable results (Manuelidis et al., 1978; Tateishi and Kitamoto, 1995; Tateishi et al., 1983). In our experience, only ~ 10% of intracerebrally inoculated mice developed central nervous system (CNS) dysfunction, with incubation times of >500 days (Prusiner, 1987; Telling et al., 1994). Since previous investigations had shown that the "species barrier" between mice and Syrian hamsters for the transmission of prions can be abrogated by expression of a Syrian hamster (SHa) prion protein (PrP) transgene in mice (Scott et al., 1989), transgenic (Tg) mice expressing human (Hu) PrP were constructed. These Tg(HuPrP) mice expressed levels of the human cellular prion protein, denoted $HuPrP^C$, that were 4- to 8-fold higher than those of endogenous mouse (Mo) PrP^C, yet upon inoculation with human prions they failed to develop CNS dysfunction more frequently than nontransgenic controls (Telling et al., 1994).

Because of the resistance of Tg(HuPrP) mice to human prions, we constructed mice expressing a chimeric Hu/MoPrP transgene, designated MHu2M. Earlier studies had shown that chimeric SHa/MoPrP transgenes supported transmission of either mouse or Syrian hamster prions (Scott et al., 1992, 1993). The Tg(MHu2M) mice expressing the chimeric transgene at a level slightly below that of endogenous $MoPrP^C$ were found to be highly susceptible to human prions, suggesting that Tg(HuPrP) mice have considerable difficulty converting $HuPrP^C$ into the scrapie isoform, designated PrP^{Sc} (Telling et al., 1994). Although MoPrP and HuPrP differ at 28 residues, only nine or perhaps fewer amino acids in the region between codons 96 and 167 feature in the species barrier in the transmission of human prions into mice, as demonstrated by the susceptibility of Tg(MHu2M) mice to human prions.

To explore why human prions transmit disease to Tg(MHu2M) mice expressing chimeric PrP but not to Tg(HuPrP) mice, we crossed Tg(HuPrP)FVB mice with those in which the MoPrP gene had been ablated, designated $Prnp^{0/0}$ (Büeler et al., 1992). The resulting Tg(HuPrP) $Prnp^{0/0}$ mice were found to be susceptible to human prions, whereas Tg(MHu2M)$Prnp^{0/0}$ mice were rendered only slightly more susceptible. These observations indicate that $MoPrP^C$ inhibited the conversion of $HuPrP^C$ into PrP^{Sc} and that inhibition was abolished once $MoPrP^C$ was removed by gene ablation.

The foregoing results suggest that two separate domains of $HuPrP^C$ participate in the formation of PrP^{Sc}: the central domain delimited by codons 96 to 167 defined by the human sequence in chimeric MHu2M PrP^C that binds to PrP^{Sc} and an additional domain through which $HuPrP^C$ binds to a macromolecule other than PrP^{Sc}. We assume that this macromolecule is a protein and have provisionally designated it "protein X." From our chimeric transgene studies, the second domain of PrP^C must be at the N- or C-terminus, i.e., outside the central region of PrP. Like the binding of PrP^C to PrP^{Sc}, which is most efficient when the two isoforms have the same sequence (Prusiner et al., 1990), the binding of PrP^C to protein X seems to exhibit the highest affinity when these two proteins are from the same species. Although the level of $MoPrP^C$ could be reduced to as low as 5%–10% of $HuPrP^C$ in the brains of the Tg(HuPrP) mice, it prevented the conversion of $HuPrP^C$ into PrP^{Sc}. These findings suggest that $MoPrP^C$ binds to mouse protein X with a considerably higher affinity than does $HuPrP^C$, which provides an explanation for why $MoPrP^C$ inhibits the transmission of human prions in Tg(HuPrP) mice.

Since truncation of the N-terminus of recombinant PrP expressed in cultured cells still permitted the formation of PrP^{Sc}-like molecules (Rogers et al., 1993), it seems likely

that the site at which PrP^C binds to another protein is at the C-terminal end of PrP^C. Mature HuPrP differs from MoPrP at only five positions at the C-terminus lying between residues 215 and 230, some of which are likely to form the protein X–binding site for PrP^C.

The proposed model for prion propagation involving protein X is supported by studies on an inherited form of prion disease modeled in mice. Spontaneous CNS disease was found in uninoculated mice expressing the P102L point mutation of Gerstmann–Sträussler–Scheinker (GSS) disease when this substitution was introduced into MoPrP (Hsiao et al., 1994). As reported here, the P102L mutation expressed in chimeric MHu2M PrP but not in HuPrP produced CNS dysfunction in transgenic mice. These findings argue that, like the transmissible disorder, inherited prion disease requires protein X for the conversion of mutant PrP^C into a pathologic isoform.

Our results argue that the C-terminus of PrP^C binds to protein X, while the central domain binds to PrP^{Sc}. Mismatches in amino acids between the two isoforms at residues 102 and 129, which lie within the central domain, resulted in delayed onset of CNS dysfunction, whereas a mismatch outside this domain at position 200 did not.

Results

Transgenic Mice with Human and Chimeric PrP Genes

FVB mice expressing human, chimeric Hu/Mo, and mutant PrP genes were constructed using the cos.SHaTet cosmid expression vector (Scott et al., 1992). Table 1 shows the designation of the mouse line, the expressed PrP^C molecules, and the approximate level of transgene expression. Also indicated are those mouse lines that were crossed with $Prnp^{0/0}$ mice in which the MoPrP gene had been disrupted (Büeler et al., 1992).

Since the human prion inocula are brain homogenates or purified prion rods from a variety of patients who died of prion disease, the designations for the patients as well as clinical phenotypes are listed in Table 2.

$MoPrP^C$ Inhibits Propagation of Human Prions in Tg(HuPrP) Mice

When Tg(HuPrP)152/FVB mice and nontransgenic littermates were inoculated with human prions from sporadic or iatrogenic Creutzfeldt–Jakob disease (CJD) as well as inherited prion disease cases, ~10% of each group of mice developed CNS dysfunction (Telling et al., 1994). Some of the ill mice produced $MoPrP^{Sc}$ and others produced $HuPrP^{Sc}$, based on Western immunoblots developed with polyclonal anti-PrP antiserum that reacts with both HuPrP and MoPrP and anti-PrP monoclonal antibodies (MAbs) that react with HuPrP but not MoPrP. Those mice that produced $HuPrP^{Sc}$ demonstrated that $HuPrP^{Sc}$ could be formed in mouse cells, but the process was too infrequent for further study.

After the Tg(HuPrP)152/FVB mice were crossed onto the $Prnp^{0/0}$ background, they became susceptible to human prions (Table 3). When Tg(HuPrP)152/FVB mice were inoculated with human prions from a case of sporadic CJD, referred to as RG, only one transgenic mouse out of a group of ten developed neurologic symptoms at >720 days; nontransgenic littermates responded similarly, with one animal out of a group of ten inoculated mice developing neurologic symptoms at >700 days. Similar rates of transmission were observed when Tg(HuPrP)152/FVB and nontransgenic mice were inoculated with a preparation highly enriched for PrP^{Sc} prepared from the brain of RG (Table 3, top). By serial dilution and dot immunoblotting of brain homogenates normalized for protein concentration, we estimated the level of $HuPrP^C$ in the brains of the Tg(HuPrP)152/FVB mice to be approximately 4- to 8-fold higher than $HuPrP^C$ in human brain using the anti-PrP 3F4 MAb (Table 1) (Kascsak et al., 1987).

Since earlier studies had shown that heterologous PrP^C inhibited the conversion of PrP^C homologous to inoculated PrP^{Sc}, as manifested by prolongation of the incubation time (Büeler et al., 1993; Prusiner et al., 1990, 1993), we removed $MoPrP^C$ by producing Tg(HuPrP)152/$Prnp^{0/0}$ mice. When Tg(HuPrP)152/$Prnp^{0/0}$ mice were inoculated with human prions, they developed signs of neurologic

Table 1. Characteristics of Transgenic Mouse Lines

Mouse Line Designation	Expressed PrP^c Molecules	PrP Transgene Expression (Fold)[a]	Sequence[b]
Tg(HuPrP) mice			
Tg(HuPrP)152/FVB	Hu, Mo	~4–8	V129
Tg(HuPrP)152/$Prnp^{0/0}$	Hu	~4–8	V129
Tg(HuPrP)152/$Prnp^{+/0}$	Hu, Mo[c]	~4–8	V129
Tg(HuPrP)440/$Prnp^{0/0}$	Hu	~2	M129
Tg(MHu2M) mice			
Tg(MHu2M)5378/FVB	MHu2M, Mo	~1	M128
Tg(MHu2M)5378/$Prnp^{0/0}$	MHu2M	~1	M128
Tg(MHu2M-P101L)69/$Prnp^{0/0}$	MHu2M-L[d]	~2	M128, L101

[a] Level of PrP transgene expression in brain was measured by serial dilution of the samples, followed by dot immunoblotting. Each sample was compared to PrP^C in human brain.

[b] Amino acid residues at codon 129 (codon 128 in MoPrP) or codon 101.

[c] Tg(HuPrP)$Prnp^{+/0}$ mice are hemizygous for disruption of the MoPrP gene and express ~50% less $MoPrP^C$ than wild-type mice (Büeler et al., 1992).

[d] L indicates substitution of L for P at codon 101 in chimeric MHu2MPrP.

Table 2. Characteristics of Human Prion Inocula

Human Inocula	Prion Disease	Genotype of PrP[a]
Sporadic and infectious CJD prions containing wild-type PrP^{Sc}		
RG, EC, MA, RO	Sporadic CJD	M/M 129
RC	Sporadic CJD	ND
364	Iatrogenic CJD	M/M 129
GSS and familial CJD prions containing mutant PrP^{Sc}		
JJ	GSS	P102L, V/V 129
LJ1, CA	Familial CJD	E200K, M/M 129
FH	Familial CJD	E200K, M/V 129

[a] Patients with sporadic or iatrogenic CJD had wild-type PrP open reading frames. The PrP alleles encode either M or V at position as noted. Mutations in the PrP gene are denoted by the wild-type amino acid followed by the codon number and the mutant residue. ND, not determined.

dysfunction with incubation times of ~260 days (Table 3, middle).

Tg(HuPrP)152/$Prnp^{+/0}$ mice, which are hemizygous for disruption of the MoPrP gene, express about 50% of the $MoPrP^{C}$ found in control mice (Büeler et al., 1992). Like Tg(HuPrP)152/FVB mice, these mice are resistant to human prions (Table 3, bottom).

MoPrP Gene Ablation in Mice Expressing Chimeric PrP

Crossing the transgene array from the already susceptible Tg(MHu2M)FVB mice onto the $Prnp^{0/0}$ background resulted in only a modest decrease (~20%) in CJD incubation times (Table 4, top and middle). Using the 3F4 MAb, we estimated the level of MHu2M PrP^{C} in the brains of the Tg(MHu2M)FVB mice to be slightly less than $HuPrP^{C}$ in human brain.

Any comparison between the incubation times of Tg(HuPrP)152/$Prnp^{0/0}$ and Tg(MHu2M)$Prnp^{0/0}$ mice must take into account the different levels of transgene expression. Generally, the level of transgene expression is inversely related to the length of the incubation time (Prusiner et al., 1990). Although the incubation times are similar for Tg(HuPrP)152/$Prnp^{0/0}$ and Tg(MHu2M)$Prnp^{0/0}$ mice inoculated with human prions (Table 3, middle and Table 4, middle), the Tg(HuPrP)152/$Prnp^{0/0}$ mice express 5- to 10-fold more of the transgene product than Tg(MHu2M) $Prnp^{0/0}$ mice. This suggests that the chimeric transgene or some modified version may be superior to HuPrP in terms of generating mice with the shortest incubation times for bioassay of human prions.

Transmission of Chimeric Prions

Species-specific amino acid variations in PrP are known to contribute significantly to the species barrier (Pattison, 1965; Prusiner et al., 1990; Scott et al., 1989). Primary passage of human prions from a sporadic CJD case, referred to as EC, in Tg(MHu2M)FVB mice with an incubation time of 218 days (Table 4, top) demonstrated that the central region of MHu2M PrP^{C} conferred susceptibility. To determine whether sequences outside this region contributed to the efficiency of transmission, brains from ill mice were collected, and homogenates were inoculated into mice from the same transgenic line. Passage of these chimeric prions in Tg(MHu2M)FVB mice gave incubation times similar to those seen with human prions on the primary passage (Table 5, top). This finding shows that Tg(MHu2M)FVB mice are permissive for human prions. Passage of chimeric prions in Tg(MHu2M)$Prnp^{0/0}$ mice resulted in a shortening of the incubation time by ~20%, presumably owing to the elimination of $MoPrP^{C}$.

Table 3. Transmission of Human Prions to Tg(HuPrP)$Prnp^{0/0}$ Mice

Recipient Mouse Line	Inoculum[a]	Incubation Time in Mean Days ± SEM(n/n_0)	
Tg(HuPrP)FVB mice			
Tg(HuPrP)152/FVB	sCJD (RG)	721	(1/10)[b]
Nontransgenic 152/FVB	sCJD (RG)	701	(1/10)[b]
Tg(HuPrP)152/FVB	sCJD (RG, purified rods)	677	(1/10)[b]
Nontransgenic 152/FVB	sCJD (RG, purified rods)	643 ± 42	(3/10)[b]
Tg(HuPrP)$Prnp^{0/0}$ mice			
Tg(HuPrP)152/$Prnp^{0/0}$	sCJD (RG)	263 ± 2	(6/6)
Tg(HuPrP)152/$Prnp^{0/0}$	sCJD (EC)	254 ± 6	(9/9)
Tg(HuPrP)152/$Prnp^{0/0}$	iCJD (364)	262 ± 8	(6/6)
Tg(HuPrP)440/$Prnp^{0/0}$	iCJD (364)	164 ± 2	(7/7)
Tg(HuPrP)$Prnp^{+/0}$ mice			
Tg(HuPrP)152/$Prnp^{+/0}$	sCJD (RG)	>370	(0/2)
Tg(HuPrP)152/$Prnp^{+/0}$	iCJD (364)	>400	(0/4)

[a] All samples were 10% (w/v) brain homogenates, unless otherwise noted, that were diluted 1:10 prior to inoculation. sCJD is sporadic CJD, and iCJD is iatrogenic CJD. The initials of patients referring to inocula in Table 2 are given in parentheses.
[b] Extended observations of transmissions previously reported (Telling et al., 1994).

Table 4. Transmission of Human Prions to Tg(MHu2M PrP) Mice

Inoculum[a]	Incubation Time in Mean Days ± SEM (n/n_0)
Tg(MHu2M)FVB mice inoculated with sporadic or infectious CJD	
sCJD (RG)	238 ± 3 (8/8)[b]
sCJD (EC)	218 ± 5 (7/7)[b]
iCJD (364)	232 ± 3 (9/9)[b]
iCJD (364)[c]	231 ± 6 (9/9)
sCJD (MA)	222 ± 1 (4/4)
Tg(MHu2M)Prnp$^{0/0}$ mice inoculated with sporadic or infectious CJD	
sCJD (RC)	207 ± 4 (8/10)
sCJD (RG)	191 ± 3 (10/10)
iCJD (364)	192 ± 6 (8/8)
iCJD (364)[c]	211 ± 5 (8/9)
sCJD (MA)	180 ± 5 (8/8)
sCJD (RO)	217 ± 2 (9/9)
Tg(MHu2M)Prnp$^{0/0}$ mice inoculated with inherited GSS or CJD	
GSS (JJ,P102L)	>310 (0/10)
fCJD (LJ1,E200K)	170 ± 2 (10/10)
fCJD (CA,E200K)	180 ± 9 (9/9)
fCJD (FH,E200K)	>290 (0/7)

[a] All samples were 10% (w/v) brain homogenates, unless otherwise noted, that were diluted 1:10 prior to inoculation. sCJD is sporadic CJD, and iCJD is iatrogenic CJD. GSS is Gerstmann–Sträussler–Scheinker disease with the codon 102 mutation, and fCJD is familial CJD with the codon 200 mutation. The initials of patients referring to inocula in Table 2 are given in parentheses. If the PrP gene of the patient carried a mutation, then the mutation is noted after the initials of the patient.
[b] Transmissions previously reported (Telling et al., 1994).
[c] This is a second inoculum prepared from a different brain region of iatrogenic CJD patient 364.

Table 5. Serial Transmission of Chimeric Hu/Mo Prions in Tg(MHu2M) Mice

Recipient Mouse Line	Inoculum[a]	Incubation Times in Mean Days ± SEM (n/n_0)	
		Illness	Death
Chimeric prions produced in Tg(MHu2M) mice inoculated with CJD prions			
Tg(MHu2M)5378/FVB	MHu2M(sCJD)[b]	220 ± 3 (7/7)[c]	226 ± 1 (5)
Nontransgenic 5378/FVB	MHu2M(sCJD)[b]	>420 (0/5)	
Tg(MHu2M)5378/FVB	MHu2M(sCJD)[d]	226 ± 3 (9/9)	228 ± 1 (6)
Nontransgenic 5378/FVB	MHu2M(sCJD)[d]	>420 (0/5)	
Tg(MHu2M)5378/Prnp$^{0/0}$	MHu2M(sCJD)[b]	189 ± 4 (8/8)	192 ± 1 (4)
Tg(MHu2M)5378/Prnp$^{0/0}$	MHu2M(sCJD)[d]	183 ± 5 (7/7)	190 ± 3 (4)
Mouse prions produced in Tg(MHu2M) or nontransgenic mice inoculated with RML prions			
Tg(MHu2M)5378/FVB	Mo(RML)	178 ± 3 (19/19)	203 ± 2 (14)[e]
Nontransgenic 5378/FVB	Mo(RML)	127 ± 2 (18/18)	156 ± 2 (5)
Tg(MHu2M)5378/FVB	MHu2M(RML)[f]	185 ± 1 (7/7)	211 ± 1 (3)
Tg(MHu2M)5378/FVB	MHu2M(RML)[g]	189 ± 2 (7/7)	211 ± 9 (3)
Nontransgenic 5378/FVB	MHu2M(RML)[g]	134 ± 3 (5/5)	ND
Tg(MHu2M)5378/Prnp$^{0/0}$	Mo(RML)	>420 (0/7)	
Tg(MHu2M)5378/Prnp$^{0/0}$	MHu2M(RML)[f]	>380 (0/10)	
Tg(MHu2M)5378/Prnp$^{0/0}$	MHu2M(RML)[g]	>380 (0/10)	

[a] Notation in parentheses indicates inoculum used in initial passage in Tg(MHu2M) mice.
[b] Mice were inoculated with chimeric prions generated in the brain of a Tg(MHu2M)5378/FVB mouse that had been inoculated with a brain homogenate prepared from patient EC who died of sporadic CJD.
[c] Number of mice developing CNS illness divided by the number inoculated is given in parentheses.
[d] Mice were inoculated with chimeric prions generated in the brain of a second Tg(MHu2M)5378/FVB mouse that had been inoculated with a brain homogenate prepared from patient EC who died of sporadic CJD.
[e] Data from Telling et al. (1994).
[f] Mice were inoculated with mouse prions generated in the brain of a Tg(MHu2M)5378/FVB mouse that had been inoculated with RML mouse prions.
[g] Mice were inoculated with mouse prions generated in the brain of a second Tg(MHu2M)5378/FVB mouse that had been inoculated with RML mouse prions.
ND, not determined.

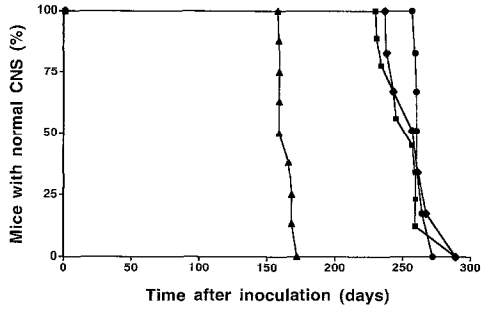

Figure 1. Amino Acid Mismatches at the Codon 129 Polymorphism Prolong the Incubation Times of Tg(HuPrP)Prnp$^{0/0}$ Mice

Tg(HuPrP-M129)440/Prnp$^{0/0}$ mice were inoculated intracerebrally with a case of iatrogenic CJD (364A, triangles). Tg(HuPrP-V129)152/Prnp$^{0/0}$ mice were inoculated with iatrogenic CJD (364A, diamonds) and sporadic CJD (EC, squares; RG, circles). All the CJD cases were from individuals homozygous for M/M at codon 129.

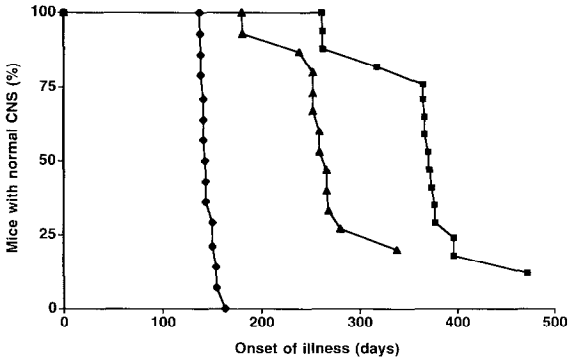

Figure 2. Spontaneous and Transmissible Neurodegeneration in Tg(MHu2M-P102L)69/Prnp$^{0/0}$ Mice

Uninoculated Tg(MHu2M-P102L)69/Prnp$^{0/0}$ mice were observed for the spontaneous development of neurologic disease (squares). At ~70 days of age, Tg(MHu2M-P102L)69/Prnp$^{0/0}$ mice were inoculated intracerebrally with brain homogenate prepared from either a patient with GSS who carried the P102L mutation (diamonds) or another patient who died of sporadic CJD (triangles).

Specificity of Chimeric Prions and Transgenes

While nontransgenic mice expressing only MoPrP^{C} are resistant to chimeric prions, Tg(MHu2M)Prnp$^{0/0}$ mice appear to be unaffected by mouse prions. At the time of writing, both groups of mice remain well at >420 days after inoculation (Table 5). These observations, along with the serial passaging experiments, provide strong support for the hypothesis that homology between PrP^{C} and PrP^{Sc} in the region bounded by residues 96 and 167 facilitates prion propagation.

Although Tg(MHu2M)FVB mice are permissive for Mo(RML) prions, the incubation time of ~178 days was protracted compared with that of 127 days for nontransgenic littermates (Table 5, middle). Two homogenates derived from Tg(MHu2M)FVB mice inoculated with Mo(RML) prions were passaged in the same line of transgenic mice, nontransgenic littermates and Tg(MHu2M) Prnp$^{0/0}$ mice. The incubation time in Tg(MHu2M)FVB mice did not change, while the incubation time in the nontransgenic mice shortened to that registered for primary passage of Mo(RML) prions in these mice (Table 5, middle). This behavior, and the fact that MoPrP^{Sc} is made in response to inoculation with mouse prions (Telling et al., 1994), argues that Tg(MHu2M)FVB mice propagate mouse prions from endogenous MoPrP^{C} and not from MHu2M PrP^{C}. Conversely, Tg(MHu2M)Prnp$^{0/0}$ mice were resistant to mouse prions; they have remained well for >340 days after inoculation (Table 5, middle).

Residue 129 Mismatches between PrP^{Sc} in the Inoculum and Transgene-Encoded PrP^{C}

In Caucasians (Palmer et al., 1991), but not Asians (Tateishi and Kitamoto, 1993), homozygosity for M or V at codon 129 has been reported to predispose people to development of sporadic CJD. The Tg(HuPrP)152 mice express HuPrP with V at codon 129, while another line, Tg(HuPrP)440, synthesizes HuPrP with M at 129. When Tg(HuPrP)152/Prnp$^{0/0}$ and Tg(HuPrP)440/Prnp$^{0/0}$ mice were inoculated with prions from iatrogenic and sporadic CJD cases, the shortest incubation times occurred when the amino acid residues at position 129 were the same in PrP^{C} and inoculated PrP^{Sc} (Figure 1). Tg(HuPrP)440/Prnp$^{0/0}$ mice inoculated with a case of iatrogenic CJD from a patient with an M/M codon 129 haplotype, referred to as case 364, exhibited a mean incubation time of 164 days, while the same inoculum produced disease in Tg(HuPrP)152/Prnp$^{0/0}$ mice with a mean incubation time of 253 days. Two cases of sporadic CJD derived from patients with the M/M codon 129 haplotype (EC and RG) produced disease in Tg(HuPrP)152/Prnp$^{0/0}$ mice with mean incubation times of 254 and 263 days, respectively (Table 3, middle).

Tg(MHu2M-P101L) Mice Expressing the GSS Mutation

In our initial studies designed to produce a model of GSS, we created lines of mice carrying the P102L point mutation in both the MoPrP and HuPrP genes. The Tg(MoPrP-P101L)87 and Tg(MoPrP-P101L)174 mice expressing the mutant PrP^{C} at high levels developed disease spontaneously between 50 and 300 days of age (Hsiao et al., 1994). In contrast, a line designated Tg(HuPrP-P102L)FVB was observed for >700 days and, unlike the Tg(MoPrP-P101L) mice, did not develop spontaneous neurologic disease.

The successful transmission of human prions to Tg(MHu2M)FVB mice prompted us to produceTg(MHu2M-P101L)Prnp$^{0/0}$ mice. Unlike the Tg(HuPrP-P102L) mice, these Tg(MHu2M-P101L) mice spontaneously developed neurologic disease (Figure 2). By 480 days, ~90% of Tg(MHu2M-P101L) mice developed CNS dysfunction. An intense, reactive astrocytic gliosis was found in the gray matter of all mice expressing the MHu2M-P101L transgene at the time they exhibited CNS dysfunction (Figures 3A–3C). Modest spongiform degeneration and PrP immunoreactivity were found in the white matter of all mice examined. Besides the Tg(HuPrP-P102L)FVB mice, additional controls include Tg(HuPrP)FVB, Tg(MHu2M)FVB, and Tg(MHu2M)Prnp$^{0/0}$ mice, none of which has developed CNS degeneration spontaneously. Whether Tg(HuPrP-

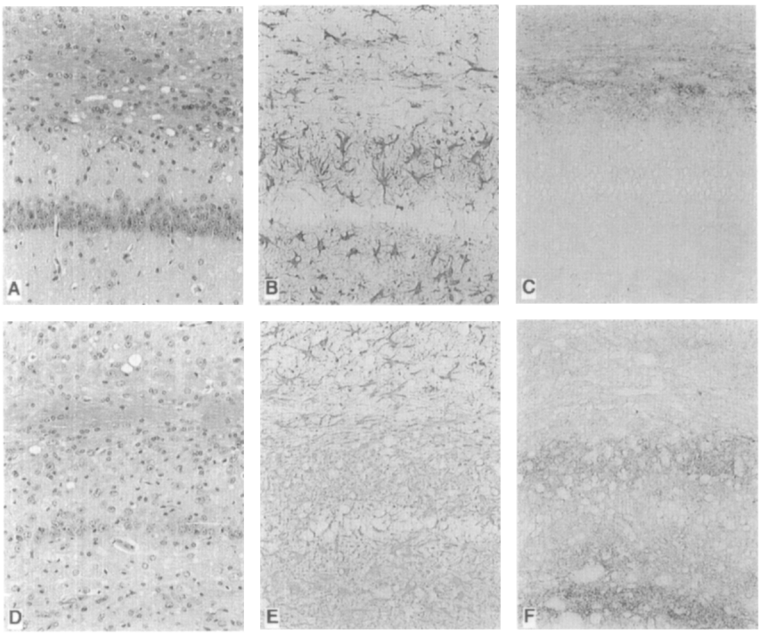

Figure 3. Spongiform Degeneration, Reactive Astrocytic Gliosis, and PrP Immunostaining in Mice Expressing the Chimeric Mutant Transgene Designated MHu2M PrP-P101L

Mice were sacrificed when they showed signs of CNS dysfunction.

(A–C) Tg(MHu2M-P101L)69/Prnp$^{0/0}$ mouse developing CNS dysfunction spontaneously.

(A) Section of hippocampal CA1 region stained with hematoxylin and eosin.

(B) Reactive astrocytic gliosis is shown in a serial section of the hippocampal CA1 region stained for GFAP by immunoperoxidase.

(C) PrP immunoreactivity in another serial section demonstrated by hydrolytic autoclaving.

(D–F) Tg(MHu2M-P101L)69/Prnp$^{0/0}$ mouse developing CNS dysfunction after inoculation with brain homogenate from a patient who died of GSS and carried the P102L mutation.

(D) Section of hippocampal CA1 region stained with hematoxylin and eosin.

(E) Reactive astrocytic gliosis is shown in a serial section of the hippocampal CA1 region stained for GFAP by immunoperoxidase.

(F) PrP immunoreactivity in another serial section demonstrated by hydrolytic autoclaving.

Magnification, 400×.

P102L)Prnp$^{0/0}$ mice will develop a CNS illness spontaneously is currently under study.

Transmission of GSS Human Prions to Tg(MHu2M-P101L) Mice

Although the Tg(MHu2M-P101L)Prnp$^{0/0}$ mice eventually develop a spontaneous neurologic disorder, we asked whether illness might appear more rapidly after inoculation. These mice were inoculated at ~70 days of age with brain extract from a GSS patient with the P102L mutation or from two sporadic CJD cases. The mice inoculated with GSS prions died after ~171 days (Figure 2) at a mean age of 247 days, which was >100 days earlier than the age at which uninoculated controls developed CNS dysfunction. Although the Tg(MHu2M-P101L) mice inoculated with prions from sporadic CJD cases have a mean incubation time of 259 days, these mice were 350 days of age at the time of death. The age of these mice prevented us from concluding whether they became ill from the inoculated prions or spontaneously as a result of expression of the MHu2M PrP-P102L mutant protein.

Our findings demonstrate that human prions from the GSS patient carrying the point mutation homologous to that in the transgene caused disease more rapidly than did wild-type human prions from sporadic cases of CJD. Conversely, the human prions from the GSS patient have failed to produce disease >310 days after inoculation in Tg(MHu2M)Prnp$^{0/0}$ mice (Table 4, bottom), whereas human prions containing wild-type PrP^{Sc} cause disease in Tg(MHu2M)Prnp$^{0/0}$ mice at ~190 days (Table 4, middle). The onset of illness in the GSS-inoculated mice was relatively synchronous, with a range of 30 days, while the onset was less uniform in the spontaneously ill and CJD-inoculated Tg(MHu2M-P101L)Prnp$^{0/0}$ mice, with ranges of 210 and 157 days, respectively.

Tg(MHu2M-P101L) mice inoculated with GSS prions exhibited spongiform degeneration and reactive astrocytic gliosis similar to uninoculated Tg(MHu2M-P101L) mice that developed CNS dysfunction spontaneously (Figures 3D–3F). However, the inoculated mice showed more neuronal loss and more intense and widespread glial fibrillary acidic protein (GFAP) immunostaining than uninoculated, spontaneously ill mice. PrP accumulation was more intense in some gray matter regions, such as the hippocampus, in the Tg(MHu2M-P101L) mice inoculated with GSS prions than in the uninoculated animals exhibiting spontaneous illness.

Uninoculated Tg(MHu2M-P101L)Prnp$^{0/0}$ mice that developed CNS dysfunction did not have detectable protease-resistant PrP (PrP 27-30) (Figure 4, lanes 8 and 10), similar to Tg(MoPrP-P101L) mice (Hsiao et al., 1994). Likewise, the brain of the GSS patient from which the inoculum was derived contained little or no detectable PrP 27-30, even though numerous PrP amyloid plaques were found (Hsiao et al., 1989) (Figure 4, lane 6). In addition, Tg(MHu2M-P101L)Prnp$^{0/0}$ mice inoculated with brain homogenate from the GSS patient had no PrP 27-30 at the time of sacrifice after development of CNS dysfunction (Figure 4, lanes 12 and 14). The relatively short incubation times (Figure 2) recorded in the Tg(MHu2M-P101L)Prnp$^{0/0}$ mice argue that the GSS brain contained high prion titers, even if PrP 27-30 was difficult to detect. From these results, we conclude that PrP^{Sc} containing the P102L mutation is probably less protease resistant than wild-type PrP (Figure 4, lane 4) or PrP carrying other mutations.

Transmission of Familial CJD(E200K) Human Prions to Tg(MHu2M) Mice

Having found that homology between PrP^{C} and PrP^{Sc} in the central domain profoundly affects prion transmission

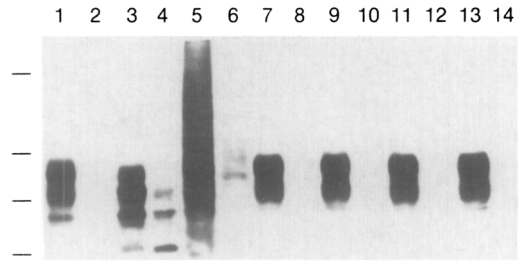

Figure 4. Western Blot of Brain Homogenates from Transgenic Mice Expressing Chimeric PrP with the P101L Mutation That Causes GSS

Tg(MHu2M-P102L)69/Prnp$^{0/0}$ mice developed neurologic dysfunction spontaneously or after inoculation with brain homogenate from a GSS patient with the P102L mutation. Aliquots of brain homogenates from ill mice were either untreated (odd-numbered lanes) or digested with 20 μg of proteinase K for 60 min at 37°C (even-numbered lanes). Samples were resolved by SDS–polyacrylamide gel electrophoresis and analyzed by Western blot. The blot was exposed to anti-PrP 3F4 MAb, developed using enhanced chemiluminescence (Amersham Corporation), and exposed to X-ray film. Lanes 1 and 2, normal human brain; lanes 3 and 4, sporadic CJD; lanes 5 and 6, GSS patient with a codon 102 proline to leucine PrP mutation; lanes 7–10, Tg(MHu2M-P102L)69/Prnp$^{0/0}$ mice developing neurologic dysfunction spontaneously; lanes 11–14, Tg(MHu2M-P102L)69/Prnp$^{0/0}$ mice developing neurologic dysfunction after inoculation with brain homogenate from a GSS patient with the P102L mutation. The positions of protein molecular mass markers are shown to the left of the blot and correspond to molecular masses of (from top to bottom) 45, 31, 21, and 14 kDa.

and that PrP^{C} appears to interact through residues between 215 and 230 with protein X, we examined the effect of an amino acid substitution within the region separating these two domains. Brain extracts were prepared from three patients who carried the E200K mutation and died of CJD (Gabizon et al., 1993) and inoculated into Tg(MHu2M) Prnp$^{0/0}$ mice. Two extracts produced disease in ~175 days, which is as rapid as those from sporadic CJD cases (Table 4). At the time of writing, a third CJD(E200K) case had not transmitted to Tg(MHu2M)Prnp$^{0/0}$ mice after >290 days. It is notable that the two cases that transmitted are M/M at codon 129, while the third is M/V with the E200K mutation on the allele encoding M at codon 129.

Discussion

The concept of the species barrier for prion transmission was introduced three decades ago based on transmission studies of experimental scrapie in sheep, goats, and rodents (Pattison, 1965). The initial molecular studies of the prion species barrier were performed using Tg(SHaPrP) mice (Prusiner et al., 1990; Scott et al., 1989). The SHaPrP transgene rendered mice susceptible to Syrian hamster prions, implying that the species barrier between Syrian hamsters and mice is due to one or more of the 16 amino acid substitutions that distinguish SHaPrP from MoPrP. Like Syrian hamster prions, human prions inoculated into nontransgenic mice produced disease infrequently after a prolonged incubation period. Based on our experience with Tg(SHaPrP) mice, we produced Tg(HuPrP) mice, but, surprisingly, they remained refractory to human prions. When the Tg(HuPrP) mice were crossed with Prnp$^{0/0}$ mice in which the MoPrP was disrupted, the resulting Tg(HuPrP) Prnp$^{0/0}$ mice became susceptible to infection with human prions (Table 3). This indicates that MoPrP^{C} inhibited the conversion of HuPrP^{C} into HuPrP^{Sc}. These findings and others described here make it likely that besides PrP^{C} and PrP^{Sc} a third component participates in the formation of nascent PrP^{Sc}. We presume that this third component is a macromolecule and that it is a protein; although it remains as yet unidentified, we have provisionally designated this third component protein X.

The site at which PrP^{C} binds to protein X must be within the mouse-encoded residues of chimeric PrP^{C}, since Tg(MHu2M) mice were found to be susceptible to human prions irrespective of the presence of endogenous MoPrP^{C} (Table 4). We interpret these results to mean that the mouse sequences in chimeric PrP^{C} enable it to compete effectively with MoPrP^{C} for binding to protein X.

We envision that during the propagation of prions a complex of homotypic PrP^{Sc} and PrP^{C} binds to protein X. This pairwise interaction of PrP^{Sc} with PrP^{C} and the stoichiometry of the conversion reaction makes it doubtful that PrP^{Sc} itself is protein X. Although the function of protein X in the formation of PrP^{Sc} is unknown, it seems likely that protein X acts in some manner to facilitate the formation of nascent PrP^{Sc}.

PrP^{Sc} Formation

The formation of nascent PrP^{Sc} is a posttranslational process (Borchelt et al., 1990; Taraboulos et al., 1995) that seems to occur after PrP^{C} reaches the cell surface (Caughey and Raymond, 1991; Stahl et al., 1987). Transgenetic studies argue that PrP^{C} and PrP^{Sc} form a complex during the conversion of PrP^{C} into nascent PrP^{Sc} (Prusiner et al., 1990; Scott et al., 1993). Although SHa transgenes provided considerable information about some of the features of PrP^{Sc} formation, use of the more divergent HuPrP transgene has greatly extended our understanding. Tg(MHu2M)Prnp$^{0/0}$ mice that express only chimeric PrP^{C} were resistant to mouse prions, whereas Tg(MHu2M)FVB mice expressing both chimeric and MoPrP^{C} were susceptible to mouse prions, but the incubation time was prolonged (Telling et al., 1994). In contrast, Tg(MHu2M)Prnp$^{0/0}$ mice inoculated with either human or chimeric MHu2M prions exhibited similar incubation times (Tables 4 and 5).

To assess the specificity of PrP^{C} binding to PrP^{Sc} in the central domain delimited by codons 96 to 167, we studied the influence of pathologic mutations and a polymorphism. Tg(MHu2M) mice were resistant to human prions from a patient with GSS who carried the P102L mutation, but were susceptible to prions from patients with familial CJD who harbor the E200K mutation (Table 4, bottom). Engineering the P102L mutation into the chimeric transgene rendered the Tg(MHu2M-P101L) mice susceptible to the GSS prions from the brain of a patient who died of GSS and carried the P102L mutation (Figure 2). Studies of Tg(HuPrP)Prnp$^{0/0}$ mice expressing M or V at the polymorphic codon 129 demonstrated the influence of this residue within the central domain on prion propagation (Figure 1). When the 129 residue within the central domain was the same in PrP^{Sc} of the inoculum and PrP^{C} of the recipient

mouse, incubation times were substantially shortened. These findings demonstrate that single amino acid mismatches at codon 102 or 129 prolong the incubation time, whereas a mismatch at codon 200 does not. Although the results reported here argue that prion propagation is facilitated by homology within the central domains of $HuPrP^{C}$ and $HuPrP^{Sc}$, other investigations with SHa/MoPrP transgenes demonstrate that the requirements for sequence similarity may vary with different species and strains of prions (M. S. and S. B. P., unpublished data).

Stoichiometry of PrP and Protein X

Although much of the specificity observed in nascent prion formation involves the formation of a PrP^{C}–PrP^{Sc} complex, the putative interaction of PrP^{C} with protein X provides an additional level of specificity. Since $MoPrP^{C}$ is present at 10%–20% of the level of $HuPrP^{C}$ in the brains of Tg(HuPrP) FVB mice, this excludes any simple model in which nonproductive dimers of $HuPrP^{C}$–$MoPrP^{C}$ are formed. In fact, it argues that there is an additional component that is critical, i.e., protein X, for the conversion process and which is present at lower levels than $HuPrP^{C}$. Since $MoPrP^{C}$ can effectively inhibit the formation of $HuPrP^{Sc}$, the level of protein X is equal to or less than that of $MoPrP^{C}$. Based on the resistance of Tg(HuPrP)$Prnp^{+/0}$ mice to human prions, the level of protein X must be <50% of the level of $MoPrP^{C}$ found in wild-type mice. Mice that are hemizygous for disruption of the MoPrP gene express ~50% less $MoPrP^{C}$. Even though the level of protein X is considerably lower than that of PrP^{C}, it is still not rate limiting, since overexpression of $SHaPrP^{C}$ or $MoPrP^{C}$ increased the rate of PrP^{Sc} formation, as reflected by abbreviated incubation times in transgenic mice (Carlson et al., 1994; Prusiner et al., 1990).

Since $MoPrP^{C}$ can inhibit the formation of $HuPrP^{Sc}$ in the presence of a substantial excess of $HuPrP^{C}$, this suggests that mouse protein X has a higher affinity for $MoPrP^{C}$ than for $HuPrP^{C}$ (Table 3, middle and bottom). $MoPrP^{C}$ prevented $HuPrP^{Sc}$ formation whether the inoculum contained amorphous aggregates of PrP^{Sc} or ordered arrays of prion rods composed of PrP 27-30 molecules (Table 3, top). If $MoPrP^{C}$ had been more inhibitory when the prion rods were inoculated, then we would suppose that a single $MoPrP^{C}$ molecule could bind to multiple $HuPrP^{Sc}$ molecules and prevent the formation of nascent $HuPrP^{Sc}$, but this is not the case.

We assume that the stoichiometry of PrP^{C} and PrP^{Sc} that form a complex is approximately 1:1. Whether this complex is composed of two or more PrP molecules is uncertain. Although some investigators have argued that the formation of nascent PrP^{Sc} involves the formation of PrP amyloid fibrils (Gajdusek, 1993; Jarrett and Lansbury, 1993), there is much evidence to the contrary. Purified preparations of PrP^{Sc} possess an amorphous ultrastructure and do not form amyloid polymers except when PrP^{Sc} undergoes partial proteolysis to produce PrP 27-30 in the presence of a nondenaturing detergent (McKinley et al., 1991; Pan et al., 1993). Isolated PrP amyloid plaques contain primarily fragments of PrP (Kitamoto et al., 1991; Tagliavini et al., 1994), and several synthetic PrP peptides spontaneously polymerize into amyloids when dispersed in water (Nguyen et al., 1995; Zhang et al., 1995). In vitro conversion of PrP^{C} into a protease-resistant form presumed to be equivalent to PrP^{Sc} by mixing a >50-fold excess of PrP^{Sc} with labeled PrP^{C} has been reported (Kocisko et al., 1994). Interestingly, the binding of PrP^{C} to PrP^{Sc} was found to be dependent on the same residues that render Tg(MH2M) mice susceptible to Syrian hamster prions (Scott et al., 1993; Kocisko et al., 1995) and seems to be strain dependent (Bessen et al., 1995). Whether PrP^{C} actually undergoes a conformational change that is characteristic of PrP^{Sc}, or the binding of PrP^{C} to PrP^{Sc} renders it protease resistant without actually undergoing this conformational transition, remains to be established. With a different experimental protocol, mixing equimolar amounts of PrP^{C} and PrP^{Sc} did not result in the conversion of PrP^{C} into PrP^{Sc} (Raeber et al., 1992).

Evidence for Protein X Binding to the C-Terminus of PrP^{C}

Since truncation experiments show that the N-terminal 67 residues of mature PrP are dispensable (Rogers et al., 1993), it seems likely that the site at which PrP^{C} binds to protein X is at the C-terminal end of PrP^{C}. A comparison of predicted amino acid sequences (Westaway et al., 1987) shows sufficient variation from codon 167 to 231 between Hu and MoPrP, as well as similarity between SHa and MoPrP, to account for our results. The location of residue 215 is particularly interesting; in HuPrP it is an I, while in MoPrP it is a V and in SHaPrP a T.

In contrast with SHaPrP, HuPrP differs from MoPrP at four additional amino acids that lie C-terminal to residue 215. Any or all of these substitutions besides residue 215 could explain the difference in susceptibility between Tg(HuPrP)FVB and Tg(SHaPrP)FVB mice to human and Syrian hamster prions, respectively. Two of the four additional residues that distinguish HuPrP from MoPrP lie at positions 219 and 220. While these residues might participate in the binding of PrP^{C} to protein X, it seems unlikely that residues at 228 or 230 are involved in the binding to protein X, since they are adjacent to the glycosylphosphatidylinositol anchor that is attached to an S residue at 231. The proposed model is consistent with our findings that chimeric MHu2M PrP^{C} but not $HuPrP^{C}$ is converted into PrP^{Sc} in the presence of $MoPrP^{C}$ and that $HuPrP^{C}$ is converted into PrP^{Sc} in the absence of $MoPrP^{C}$ (Table 3).

Does PrP^{Sc} Bind Protein X?

The transmission of human and MHu2M prions into Tg(MHu2M)FVB mice yielded similar incubation times. This finding argues that the differences in the human or mouse sequences of PrP^{Sc} at the N- and C-termini have little effect on the transmission of prions to Tg(MHu2M) mice (Tables 3 and 4). Conversely, the region of PrP containing residues 96 to 167 clearly governs prion transmission to Tg(MHu2M)$Prnp^{0/0}$ mice: inoculation of human or MHu2M prions produced disease, but mouse prions did not. The apparent lack of binding of PrP^{Sc} to protein X is consistent with PrP^{Sc} being the product of the reaction and protein X facilitating the conformational change.

Is Protein X Distinct from PrP^{Sc}?

Besides the evidence delineated above, other results also argue that protein X is not PrP^{Sc}. Although Tg(HuPrP) $Prnp^{0/0}$ mice express the transgene product at levels 4- to 8-fold higher than Tg(MHu2M)$Prnp^{0/0}$ mice (Table 1), the incubation times for human prions were similar in both transgenic lines (Tables 3 and 4). Since earlier studies demonstrated an inverse relationship between the level of PrP^{C} expression and the incubation time (Prusiner et al., 1990), we conclude that chimeric PrP^{C} is substantially more efficient in supporting prion propagation than $HuPrP^{C}$. Although $HuPrP^{Sc}$ initiates the conversion of chimeric PrP^{C} into PrP^{Sc} in Tg(MHu2M) mice, $HuPrP^{Sc}$ becomes a minor fraction of the total PrP^{Sc}, the vast majority of which is MHu2M PrP^{Sc}. Thus, it is difficult to attribute these results to the higher avidity of $HuPrP^{Sc}$ for MHu2M PrP^{C} than for $HuPrP^{C}$; instead, the binding of mouse protein X to chimeric PrP^{C} with a higher avidity than to $HuPrP^{C}$ seems a more reasonable explanation.

If protein X does not exist, some alternative mechanism must also be invoked to explain the results of studies with uninoculated mice expressing PrP transgenes encoding the P102L mutation of GSS. When the P102L mutation was introduced through a mutant HuPrP transgene, the Tg(HuPrP-P102L)FVB mice did not become ill spontaneously (see Results). In contrast, introduction of the mutation through a mutant MoPrP transgene readily produced spontaneous disease in Tg(MoPrP-P101L) mice (Hsiao et al., 1990, 1994). Since no PrP^{Sc} was inoculated into the mice, we cannot use binding of this isoform as an explanation for why the Tg(HuPrP-P102L)FVB mice did not become ill and those expressing mutant $MoPrP^{C}$ did. Indeed, it seems likely that wild-type $MoPrP^{C}$ in Tg(HuPrP-P102L) FVB mice may have prevented the interaction of mutant HuPrP with mouse protein X, which in turn prevented disease.

Although the evidence speaks to the existence of protein X, only when protein X has been identified, and either the conditions defined for its functioning in vitro or the gene encoding it ablated (thereby rendering mice resistant to prions), will protein X be shown to be distinct from PrP^{Sc}. If progressive subcortical gliosis proves to be an inherited prion disease without a PrP gene mutation, then perhaps the mutant gene responsible for this disease encodes protein X (Petersen et al., 1995).

Does Protein X Function as a Molecular Chaperone?

How PrP^{C} unfolds and refolds into PrP^{Sc} is unknown (Pan et al., 1993), but the profound change in protein structure that occurs during this process is likely to be associated with a large activation barrier (Cohen et al., 1994). Whether protein X functions as a molecular chaperone that lowers this barrier remains to be established; consistent with such a role for protein X is the apparent lack of PrP^{Sc} binding. Changes in the inducibility of heat shock proteins (Hsp) as well as their subcellular distribution, some of which function as molecular chaperones (Georgopoulos and Welch, 1993), have been found in scrapie-infected cells (Tatzelt et al., 1995) and raise the possibility that protein X might be an Hsp. Notably, PrP^{Sc} itself has been suggested to function as a chaperone (Liautard, 1993). Alternative possibilities for protein X include scaffolding or assembly proteins that provide a milieu for the PrP isoforms to interact, as well as the Bcl-2 protein, which was found to bind PrP using the yeast two-hybrid system (Kurschner and Morgan, 1995). Another possibility is that protein X features in the transient or as yet undetected chemical modification of PrP^{C} that facilitates its refolding into PrP^{Sc} (Stahl et al., 1993).

New Approaches to Studies of Prions

Although many findings support the proposed mechanism for prion propagation involving protein X, it is of utmost importance to identify those proteins that bind to the PrP^{C}–PrP^{Sc} complex and mediate a conformational change in PrP^{C}. If PrP^{C} binds to protein X through a domain near the C-terminus, as our data suggest, then systematic substitution of residues in HuPrP with amino acids specified by MoPrP should facilitate identification of residues that modify susceptibility to human prions. Such experiments must consider the effects of single amino acid substitutions, which can cause conformational changes at a great distance along a polypeptide chain. Whether protein X is a single protein or a complex of proteins remains to be established. Once protein X is identified, then it may be possible to form prions in vitro and to determine the mechanism of the conformational transition that underlies the conversion of PrP^{C} into PrP^{Sc}.

While most cases of human prion disease are not readily transmitted to Tg(HuPrP)FVB mice, an exception has been noted (Telling et al., 1994). Undoubtedly other such cases will be found, since it has been reported that a group of GSS cases harboring the P102L mutation transmit CNS degeneration to nontransgenic mice in <400 days while a second group of cases with the same genotype do not (Tateishi and Kitamoto, 1995). One explanation for these cases of prion disease, which are unusual with respect to their transmission characteristics, is that they represent different strains of prions.

The concept of protein X in prion propagation, besides having practical ramifications for the bioassay of prions from humans as well as domestic animals such as cattle with bovine spongiform encephalopathy (Wells and Wilesmith, 1995), is intriguing with respect to understanding the function of PrP^{C}. If, as recently suggested, several yeast proteins induce alternative metabolic states through a prion-like mechanism (Wickner, 1994), then perhaps PrP^{C} also exists in more than one physiologic state. The transformation of PrP^{C} to an alternative metabolic isoform might be facilitated by protein X. Interestingly, one prion-like protein in yeast (Sup35) seems to require intermediate levels of the molecular chaperone (Hsp104) to undergo transformation to [PSI^{+}] (Chernoff et al., 1995). Might protein X function as a chaperone that mediates a conformational change in PrP^{C} that alters its cellular function in the absence of PrP^{Sc} or a pathologic PrP gene mutation? In the presence of PrP^{Sc}, protein X might catalyze the conversion of PrP^{C} into PrP^{Sc}. Whatever the mechanism of PrP^{Sc}

formation, this process seems to be unprecedented in biology, and its elucidation promises to have implications far beyond the prion diseases.

Experimental Procedures

Production of Transgenic Mice

The HuPrP-M129 and MHu2M-P101L transgenes were constructed by the same procedures described for the HuPrP-V129 and MHu2M PrP transgenes (Telling et al., 1994). Purified fragments containing the PrP ORF were ligated to the SalI-cut cos.SHa.Tet cosmid expression vector (Scott et al., 1992). NotI fragments, recovered from large-scale DNA cosmid preparations, were used for microinjection into the pronuclei of fertilized oocytes from FVB/N or $Prnp^{0/0}$ mice as previously described (Scott et al., 1989). By crossing Tg(HuPrP)152/FVB, Tg(HuPrP)440/FVB, and Tg(MHu2M PrP)5378/FVB mice with $Prnp^{0/0}$ mice (Büeler et al., 1992) and subsequent backcrossing of these mice, Tg(HuPrP)$Prnp^{0/0}$ and Tg(MHu2M PrP)$Prnp^{0/0}$ mice were produced. Genomic DNA isolated from tail tissue of weanling animals was screened for the presence of incorporated transgenes using a probe that hybridizes to the 3′ untranslated region of the SHaPrP gene contained in the cos.SHa.Tet vector (Scott et al., 1992) or for the ablated MoPrP gene by PCR (Prusiner et al., 1993).

Genotyping of Patients

Genomic DNA was extracted from frozen brains of autopsied patients or the leukocyte fraction from venous blood collected during life. To determine the codon 129 genotype, we performed allele-specific amplification by running separate reactions using one of two sense strand primers matched to either M or V by a single nucleotide change at the 3′ end (GCCTTGGCGGCTACA for M and GCCTTGGCGGCTACG for V). The antisense primer used in both reactions (AAGAATTCTCTGACATTCTCCTCTTCA) lies within the ORF. A 500 bp product results from annealing of the sense strand primer, and no product results if annealing does not occur because of mismatch. Alternatively, DNA sequencing was used.

Preparation of Brain Homogenates

Homogenates (10% [w/v]) of mouse brain were prepared by repeated extrusion through an 18 gauge syringe needle followed by a 22 gauge needle in PBS lacking Ca^{2+} and Mg^{2+}. The same procedure was used to prepare human brain homogenates, except that thawed brain tissue was initially disrupted with a sterile disposable homogenizer. Purified human prions were prepared using a protocol previously developed for Syrian hamster prions (Prusiner et al., 1983).

Prion Inocula

Human brain specimens were collected from patients dying of sporadic, inherited, or infectious prion disease. The iatrogenic CJD case denoted 364 was provided by Dr. John Collinge. The RML isolate from Swiss mice (Chandler, 1961) was provided by Dr. William Hadlow and was passaged in Swiss CD-1 mice obtained from Charles River Laboratories (Wilmington, MA).

Measurement of Incubation Times

Samples were diluted 10-fold in PBS prior to intracerebral inoculation of 30 μl. Criteria for diagnosis of scrapie in mice have been described elsewhere (Carlson et al., 1986). When CNS dysfunction appeared, the mice were examined daily. Histopathology was performed to confirm the clinical diagnosis in selected cases.

Immunoblotting

Immuno dot blots for the determination of the relative levels of PrP expression in transgenic mouse brains were performed (Scott et al., 1993). Enhanced chemiluminescent (ECL) detection (Amersham, Arlington Heights, IL) was used for Western blots. 3F4 MAb in ascites fluid (Kascsak et al., 1987) was used at a dilution of 1:5000.

Immunohistochemistry

To enhance PrP immunoreactivity, the sections were immersed in 1.3 mM HCl and autoclaved at 121°C for 10 min (Muramoto et al., 1992). Staining was performed using the 3F4 MAb (Hecker et al., 1992). Immunohistochemistry with antibodies to GFAP was used to evaluate the extent of reactive astrocytic gliosis.

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