Heritable disorder resembling neuronal storage disease in mice expressing prion protein with deletion of an α-helix

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Mice were constructed carrying prion protein (PrP) transgenes with individual regions of putative secondary structure deleted. Transgenic mice with amino-terminal regions deleted remained healthy at >400 days of age, whereas those with either of carboxy-terminal α-helices deleted spontaneously developed fatal CNS illnesses similar to neuronal storage diseases. Deletion of either C-terminal helix resulted in PrP accumulation within cytoplasmic inclusions in enlarged neurons. Deletion of the penultimate C-terminal helix resulted in proliferation of rough endoplasmic reticulum. Mice with the C-terminal helix deleted were affected with nerve cell loss in the hippocampus and proliferation of smooth endoplasmic reticulum. Whether children with the human counterpart of this malady will be found remains to be determined.

During the course of transgenetic studies of prion protein (PrP) structures that feature in formation of the scrapie isoform, we produced a new illness in mice. In the brains of animals expressing PrP with either of the carboxy-terminal α-helices deleted, the mutated PrP accumulated in nerve cell bodies results in a disorder that resembles neuronal storage diseases. The pathogenesis of this fatal CNS disorder differs from that described previously for the infectious, inherited and sporadic forms of the prion diseases[1-5].

Molecular modeling studies predicted that PrP^C contains four regions of α-helical structure designated H1, H2, H3 and H4 (ref. 6). Thirteen different point mutations have been identified that cause the inherited human prion diseases, and each lies within or adjacent to these regions of putative secondary structure[6]. In contrast, species variations in the amino acid residues of PrP generally spare these regions[7,8]. The prediction of an all α-helical structure for PrP^C was supported by optical spectroscopy of PrP^C purified from the brains of Syrian hamsters (SHa)[9,10]. Nuclear magnetic resonance (NMR) studies of a large N-terminal PrP synthetic peptide and C-terminal fragments of PrP synthesized in *Escherichia coli* have provided physical evidence for some aspects of the model (ref. 11, 12, and James *et al.*, manuscript in preparation).

To assess the role of each region of putative secondary structure as well as those adjacent and intervening segments, we created a series of seven recombinant PrPs lacking specific domains which vary in length from 13 to 66 residues and expressed each of these in transgenic (Tg) mice.

Mice expressing mutant PrP transgenes

We performed studies using the epitope-tagged PrP, which consisted of mouse (Mo) PrP with two SHaPrP residues at positions 108 and 111 forming the epitope for the α-PrP 3F4 monoclonal antibody; this chimeric Mo/SHa PrP was designated MHM2 (ref. 13, 14). Since PrP^C encoded by MHM2 can propagate prions, it could be used to assess the effect of selective deletions of specific domains within the PrP molecule[15,16] (Fig. 1). Because limited proteolysis truncates the N terminus of PrP^{Sc} to form PrP 27–30 without loss of infectivity and MHM2 PrP^C lacking residues 23–88 can be converted into PrP^{Sc} in scrapie-infected neuroblastoma (ScN2a) cells[17], our studies utilize the chimeric MHM2 molecule in which the N-terminal region consisting of residues 23–88 had been deleted. Recently, transgenic mice deficient for wild-type MoPrP ($Prnp^{0/0}$) but expressing a 160-residue, N-terminal deletion (32–80) have been shown to support prion formation[18]. Another SHa epitope tag, Met138, was created in constructs where deletions eliminate the epitope tag for α-PrP 3F4 monoclonal antibody so that we could perform parallel studies with ScN2a cells (Fig. 1)[19]. The Met138 epitope tag binds to the α-PrP 13A5 monoclonal antibody[14,20].

Chemical analyses showed that both PrP^C and PrP^{Sc} possess a disulfide bond[21], and molecular modeling studies predicted that this disulfide bond between Cys residues 178 and 213 stabilizes the H3 and H4 α-helices in both PrP isoforms[6,22]. NMR studies of PrP(121–231) demonstrate this disulfide bond between H3 and H4 appears to function in stabilizing the hydrophobic core of the protein[12]. To evaluate the importance of this disulfide bond on PrP^{Sc} formation, we substituted Ala for Cys at position 178 (Fig. 1).

Transgenic mouse lines were generated for all these truncated PrPs in FVB mice deficient for PrP (ref. 23). Two or more lines were established for all constructs except for the H2 deletion (Table 1). Tg(MHM2)294 mice produced from (C57BL × SJL)F_2 oocytes were inoculated with brain homogenate prepared from normal CD-1 mice and did not develop CNS dysfunction with a life span of 592 ± 35 days (n = 20). When these Tg(MHM2)294 mice were inoculated with Rocky Mountain Laboratory (RML) prions passaged in CD-1 mice, they developed scrapie at 119 ± 3.3 days (mean ± s.e.m., n = 14) after inoculation[16]. On second passage through Tg(MHM2)294/$Prnp^{0/0}$ mice, the incubation time was 125 ± 5.2 days (n = 10).

CNS dysfunction in transgenic mice with H3 or H4 deletion

Like the Tg(MHM2) mice, Tg[MHM2 PrP (del 23–88)] mice expressing N-terminally truncated MHM2 PrP^C have remained

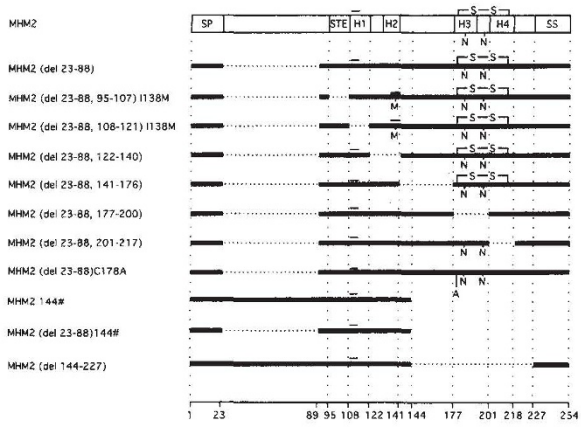

Fig. 1 Scheme of PrP transgenes with various deletions and mutations. MHM2 PrP was modified as shown. SP, signal peptide (residues 1–22); STE, stop transfer effector region (residues 95–107); H1, residues 108–121; H2, residues 128–140; H3, residues 177–190; H4, residues 201–217; SS, signal sequence for anchoring to glycolipid (residues 232–254). –S–S–, a disulfide bond between Cys178 and Cys213; –N, locations of Asn-linked glycosylation; –M, Met138; –A, Ala178. Thin bar above H1, location of epitope for mAb 3F4; thick bar above H2, location of epitope for mAb 13A5. Horizontal dotted lines, locations of deletions. The amino acid positions bordering deletions are shown at the bottom. All numbers of residues are based on MHM2 molecule.

healthy for more than 500 days. Mice expressing additional PrP deletions corresponding to the stop transfer effector region (STE) (95–107), H1(108–121), or the 36-residue loop between H2 and H3 also remained well (Table 1). Five lines with high copy numbers of the transgene encoding MHM2 PrP (del 23–88) with the H2 deletion were produced, but all failed to express the protein. The founders of these lines remained well but were killed at 232 days of age. The pathology of their brains was not remarkable. Five founders expressing MHM2 PrP (del 23–88) with the C178A substitution remained well at more than 200 days of age. In contrast, mice with PrP transgenes in which the H3 (177–200) or H4 (201–217) α-helices were deleted developed signs of CNS dysfunction and neuropathological changes characteristic of a neuronal storage disease (Table 1). Signs of clinical illness included tremor, ataxia, bradykinesia and kyphosis as well as poor grooming of the hair coat and weight loss. Between 2 and 3 weeks after onset of neurological dysfunction, the mice became terminally ill and were humanely killed. Although disease in the mice with the H4 deletion appears to be completely penetrant, it shows incomplete penetrance in mice with the H3 deletion, with the disease seen only in males, to date (Table 1). One female with the H3 deletion was found dead, but unfortunately pathological examination was not done.

Cytoplasmic inclusions composed of PrP

Bielschowsky silver stain of brain sections from ill transgenic mice expressing PrP with an H3 or H4 deletion revealed that many, but not all, nerve cell bodies in multiple brain regions contained golden-brown, round to ovoid cytoplasmic inclusions (data not shown). The cell bodies of neurons were enlarged by these cytoplasmic masses. Inclusions with an H3 or H4 deletion were also identified by the periodic acid-Schiff stain (data not shown). The inclusions with the H3 deletion were about half the size of the nucleus of a pyramidal cell of Ammon's horn, smaller than those with the H4 deletion, which were frequently larger than the nucleus. Single inclusions were common with the H3 deletion but multiple with H4.

The cytoplasmic inclusions and PrP immunostaining colocalized when compared on serial sections (Fig. 2). However, the intensity and appearance of PrP immunostaining differed for the H3 and H4 deletions. For the H4 deletion, the entire inclusion was uniformly and intensely stained (Fig. 2, *a–c*). In contrast, immunostaining of the inclusions with the H3 deletion was overall less intense, but contained punctate foci of a higher intensity (Fig. 2*d*). PrP accumulation was not seen in all nerve cells; no accumulations were found in the nerve cells of the dentate gyrus (Fig. 2*a*).

Other neuropathological changes

In transgenic mice with the H3 deletion, there appeared to be no nerve cell loss or reactive astrocytic gliosis in the gray matter (Fig. 2*g*). The other substantial neuropathological change was the relatively small number of axons in white matter tracts (data not shown); the corpus callosum was 30-μm-thick in cross sections compared with 90 μm with the H4 deletion, which was normal in size, and the myelinated fiber tracts crossing through

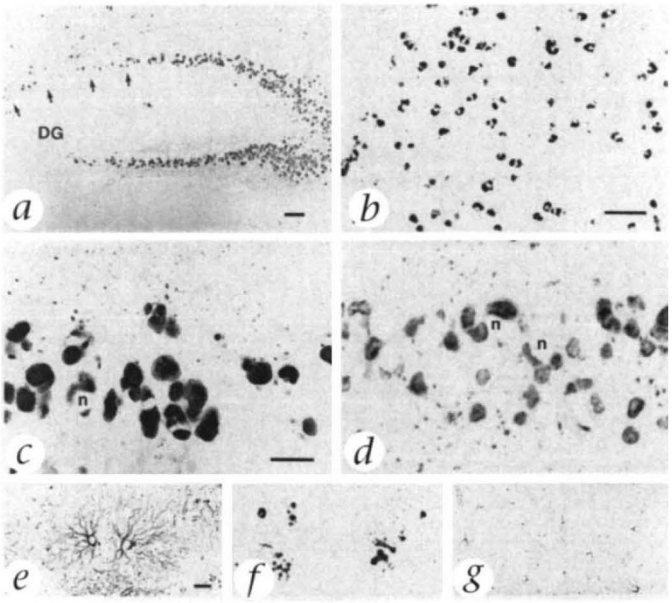

Fig. 2 Neuropathology of transgenic mice with H3 or H4 deletion. Transgenic mice expressing PrP with H3 (*d* and *g*) or H4 (*a–c, e, f*) deletion. Immunohistochemistry of PrP (*a–d, f*) or GFAP (*e, g*). *a*, Low magnification of the hippocampus shows most of the pyramidal neurons of Ammon's horn are filled with PrP with H4 deletion. Arrows indicate the region of CA-1 with degeneration and loss of neurons. Neurons of the dentate gyrus (immediately below "DG") are unaffected. *b*, Medium magnification of the caudate nucleus shows most of its nerve cells are filled with PrP with H4 deletion. *c*, High magnification of the hippocampus shows that the nerve cell bodies of Ammon's horn are enlarged and their cytoplasm filled with PrP with H4 deletion. Their nuclei (n) do not contain the mutant PrP. The location of PrP immunopositivity corresponds to the inclusions positive for Bielschowsky silver here and in *d*. *d*, High magnification of the hippocampus shows PrP with H3 deletion localized to the inclusions in nerve cell bodies. No PrP is located in the nucleus (n). *e*, GFAP immunohistochemistry of transgenic mice expressing PrP with H4 deletion reveals giant, bizarre astrocytes, with diameters as large as 100 μm measured across the arbors of their processes that are scattered in relatively small numbers throughout the brain (see Fig. 4). *f*, PrP immunohistochemistry indicates that the bizarre astrocytes are composed of PrP with H4 deletion. *g*, No reactive astrocytes are found in the gray matter of transgenic mice expressing PrP with H3 deletion construct with GFAP immunohistochemistry. Scale bars in *a*, 50 μm; in *b*, 50 μm; in *c*, 25 μm and applies to *d*; in *e*, 50 μm and applies to *f* and *g*.

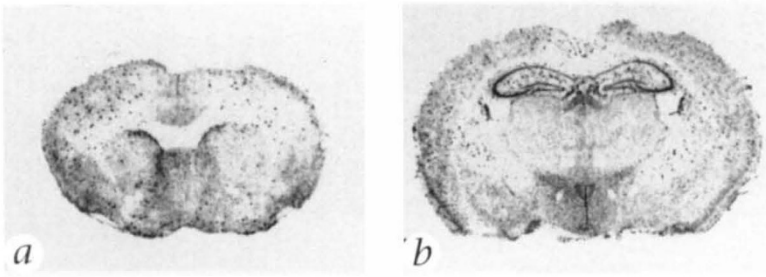

Fig. 3. Histoblot analysis of frozen brain sections of transgenic mice expressing PrP with H4 deletion. *a*, Coronal section through the caudate nucleus and septum. *b*, Coronal section through the hippocampus and thalamus. The number, size and distribution of the punctate immunopositive foci scattered throughout both sections correspond to the location and distribution of the giant, bizarre astrocytes (see Fig. 2, *e* and *f*). Deposition of PrP with H4 deletion in nerve cell bodies of Ammon's horn and the dentate gyrus of the hippocampus are also seen in *b*. The gray speckled appearance of other gray matter regions represents immunopositive neurons within them.

the caudate nucleus had a maximum diameter of 30 μm compared with a maximum of 90 μm with the H4 deletion, which was also normal. There was a mild reactive astrocytic gliosis in the white matter of the H3 deletion mice.

In transgenic mice expressing the H4 deletion, there was extensive nerve cell loss in the CA-1 region of the hippocampus (Fig. 2*a*). Another characteristic finding was the presence of bizarre, giant astrocytes scattered throughout the cerebral hemispheres (Fig. 2*e*). The cross-sectional diameters of the astrocytes were as large as 100 μm when measured across their arborization. These astrocytes also contained PrP with the H4 deletion (Fig. 2*f*), which was confirmed with double-immunostaining for PrP and glial fibrillary acidic protein (GFAP) (data not shown).

The histoblots of brain sections from transgenic mice with the H4 deletion show the distribution and frequency of these unusual astrocytes, as they are of large enough size to be seen by this method (Fig. 3). The pyramidal cell bodies of Ammon's horn and neurons of the dentate gyrus in the hippocampus are also visible in the histoblot, probably because they are several layers thick and packed tightly together. The relatively small size and low density of the PrP immunopositive neurons resulted in a speckled gray appearance in other gray matter regions.

Ultrastructural studies

The cytoplasmic inclusions observed in mice with the H3 deletion are composed of closely packed particles of variable size that are often arranged in a circular pattern (Fig. 4). The electron density, morphology and size of the larger particles were indistinguishable from ribosomes in the adjacent cytoplasm. The circular arrangement of the particles suggests they are arranged around a membrane; however, no definitive or continuous membrane was identified. The inclusions were neither membrane-bound nor localized to lysosomes. They most likely represent fragmented rough endoplasmic reticulum (RER) with ribosomes.

The large cytoplasmic inclusions found in mice with the H4 deletion consisted of large masses of membranous structures, which resembled the smooth endoplasmic reticulum (SER) (Fig. 5, *b* and *c*). These inclusions seem to localize to the same sites as PrP immunoreactivity and account for the enlargement of neuronal cell bodies. Such structures are not present in normal, nontransgenic mice (Fig. 5*a*). The ultrastructure of these inclusions resembles the proliferation of SER in hepatocytes induced by phenobarbital or prednisone[24], but they differ from the drug-induced SER proliferation in that they are not dilated tubules.

Table 1 Transgenic mice expressing mutant prion proteins

PrP transgenes and designations for founders[a]	PrP transgene expression (fold)[b]	F_1[a,c]	F_2[a,c]
MHM2 (del 23–88)			
D9308[d] (18 mon)	0.5	3 (601)	
D9381[d] (18 mon)	2–4	3 (709), 2 (601)	
MHM2 (del 23–88, 95–107)I138M			
E5538 (476)	8	2 (392), 1 (328)	
E5547 (476)	2–4	1 (392), 3 (347)	
E5556 (473)	4	5 (216)	
MHM2 (del 23–88, 108–121)I138M			
E5299 (484)	4	2 (396), 2 (345)	
E5306 (483)	4–8	2 (341)	
MHM2 (del 23–88, 122–140)			
No lines expressing detectable amounts of PrP have been established.			
MHM2 (del 23–88, 141–176)			
E4280 (511)	1–2	1 (421), 2 (367), 1 (321)	
E4281 (511)	0.5	2 (413), 7 (349)	
E4290 (509)	8	1 (367)	
MHM2 (del 23–88, 177–200)			
E14011 (311)	0.5	3[e] (49), 3 (207)	3[e] (45), 3 (122)
E14012 (311)	ND	1[e] (46), 1 (227)	
MHM2 (del 23–88, 201–217)			
E5116[e] (170)	1–2	5[e] (165)	1[e] (190)
E5124[e] (190)	1	1[e] (67), 1[e] (107)	1[e] (24), 4[e] (90)
MHM2 (del 23–88)C178A			
5 founders (215–221)	ND	F1 being bred	
MoPrP (144#)			
C10643	0	19 (>480)	
C10643[f] (RML)	20 (>470)		
C10644	0	20 (>480)	
C10644[f] (RML)	10 (>690)		

[a]Numbers in parentheses indicate periods (in days, unless otherwise indicated) for which animals have been asymptomatic since birth.
[b]Level of PrP transgene expression in the brain was measured by serially diluting brain homogenates, followed by western immunoblot with α-PrP 3F4 or 13A5 monoclonal antibodies. Each sample was compared with PrP^C in Syrian hamster brain. For the MoPrP (144#) construct, expression was compared with PrP^C levels in mouse CD-1 brain by using α-PrP polyclonal antiserum RO73.
[c]Numbers not in parentheses indicate numbers of animals observed. Only animals that were characterized for transgene expression level were counted. There are more F_1 animals from each founder whose expression levels are not characterized, and all of them have been asymptomatic. Numerous F_2 animals are available for all lines except for MHM2 (del 23–88, 177–200), MHM2 (del 23–88, 201–217) and MHM2 (del 23–88)C178A, and all remain healthy.
[d]Animals that developed illness after the periods shown in parentheses. The illnesses of D9380 and D9381 were not accompanied by any neurological symptoms. For D9380, pathological examination was not done. The pathological examination of D9381 brain revealed only mild age-related spongiosis and astrocytic gliosis in the white matter.
[e]Mice with H3 or H4 deletion developed signs of CNS dysfunction after the periods shown in parentheses.
[f]Mice were also inoculated with RML where indicated.

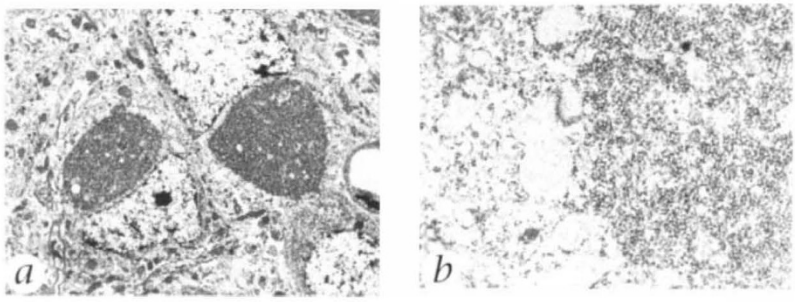

Fig. 4 Ultrastructure of neurons in the CA-1 region of the hippocampus in a transgenic mouse with H3 deletion showing proliferation of the rough endoplasmic reticulum. *a*, Inclusions in two neurons of the CA1 region of the hippocampus (original magnification, ×5,000). The inclusions are about the same size as the neuronal nucleus. *b*, One edge of an inclusion which fills the right half of electron micrograph shows that it contains electron dense particles whose size and density are the same as the free ribosomes in the left half (×39,000, original magnification). The ribosomal particles within the inclusion are mixed with smaller particles which tend to be arranged in circles suggesting that they rim a membranous structure; however, a definitive membrane is not identified.

Definitive evidence that these inclusions are SER awaits application of enzyme histochemistry for SER-specific factors. Of note, the Golgi stacks appeared to be normal.

Insoluble PrP

Western blots of the fractions from the brain tissues of ill mice with the H3 or H4 deletion that were insoluble in detergent showed that a substantial fraction of the PrP carrying the H3 or H4 deletion was insoluble, like PrP^{Sc} in a scrapie mouse brain, but readily digestible with proteinase K (Fig. 6).

Deletion of both H3 and H4

A patient was reported with Gerstmann-Sträussler syndrome (GSS) in whom the Tyr residue at codon 145 was mutated to a stop codon[25,26]. We produced the equivalent amber mutation in MoPrP designated MoPrP(144#) (Fig. 1). In addition, a doubly-mutated construct containing the 144 amber mutation, as well as a deletion encompassing the codons for amino acids 23–88 of MoPrP denoted MoPrP(del 23–88, 144#), was made. To assess these constructs in cultured cells, we engineered the mutations into the MHM2 PrP. When stably expressed in ScN2a cells, neither mutant PrP form was detectable by immunoblot analysis of cell lysates or by immunofluorescence analysis of transformed cell lines with the 3F4 monoclonal antibody, whereas the control MHM2 PrP was readily detectable[15].

Transgenic mice for MoPrP(144#) were produced in FVB mice deficient for PrP and referred to as Tg(MoPrP144#) (Table 1). Although high copy number transgenic lines were established, no PrP was detected in brain extracts of transgenic offspring by using a polyclonal antiserum that recognizes MoPrP. Uninoculated Tg(MoPrP144#) mice and those inoculated with mouse RML prions did not develop symptoms of neurodegenerative disease after over 600 days[18].

We reasoned that since the 144# mutation deleted the region responsible for glyophosphatidylinositol(GPI) anchorage of PrP on the cell surface, the mutant protein might be secreted into the medium and rapidly degraded[17]. We engineered an additional construct in which the last sense codon (aspartate at codon 143) of MoPrP144# was directly fused to the C-terminal 26 amino acids of MoPrP, which start with glycine at residue 227 and include the signal sequence for the GPI anchor (Fig. 1). We engineered this mutation into the MHM2PrP and then stably expressed it in ScN2a cells. However, we also failed to detect expression of this mutant construct in transformed cells.

Discussion

Our report describes for the first time a heritable PrP disorder in mice that resembles neuronal storage disease. This α-helix deletion disease produces CNS dysfunction and numerous neurons with intracellular accumulations of PrP, which are insoluble in nondenaturing detergents, a property that distinguishes it from wild-type PrP^{C} (ref. 27, 28). That the same fatal illness was observed in independent mouse lines with deletions of H3 or H4 establishes that the disease phenotype is due to expression of the mutant transgene (Table 1). None of the controls expressing transgenes in which the H3 and H4 α-helices were both present have developed CNS dysfunction, to date.

Domains of secondary structure. Why should the deletion of H3 or H4 but not H1, the STE domain, or the 36-residue segment between H2 and H3 produce disease? Deletion of either H3 or H4 presumably destabilizes PrP by eliminating a critical segment of the hydrophobic core of the protein as well as the disulfide bond[6,12]. The intact H3–H4 domain may be important in stabilizing the conformation of the H1–H2 domain through interactions on the surfaces of these α-helices. Since the H1 and H2 segments are not stabilized by a disulfide bond; interactions between helices may be essential for stability of the H1–H2 domain. Thus, deletion of H3 or H4 would be expected to destabilize the H1–H2 domain. Our inability to produce mice with the H2 deletion is due either to technical difficulties or the toxicity of H2-deleted PrP during embryogenesis. Expression of the H2-deleted construct under

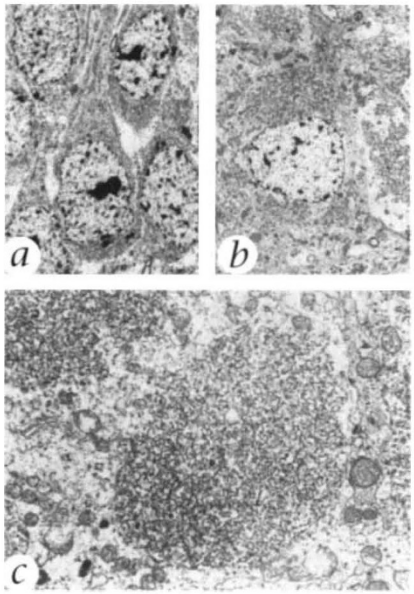

Fig. 5 Ultrastructure of neurons in the CA-1 region of the hippocampus in a transgenic mouse with H4 deletion showing proliferation of the smooth endoplasmic reticulum. *a*, Normal pyramidal cells in the CA-1 region of the hippocampus of nontransgenic wild-type mice (original magnification, ×5,000). *b*, Enlarged pyramidal cell in the CA-1 region in transgenic mice expressing PrP with H4 deletion. There are single and multiple masses of smooth endoplasmic reticulum (SER) (original magnification, ×5,000). *c*, Proliferated SER in a neuron (original magnification, ×25,000). Normal appearing Golgi stacks are seen in the lower left side of the micrograph.

control of an inducible promoter[29,30] may obviate this difficulty if the problem is one of toxicity in the embryo. Of note, MoPrP(121–231) with deletion of both the STE region and H1 in transgenic mice caused cerebellar atrophy and death in the neonatal period [D. Shmerling, *et al. 29th Annual Meeting of the Union of the Swiss Societies for Experimental Biology, Geneva, March 20–21,* (Abstr.) A46; 1997]. It is interesting that our results indicate that addition of either the STE region or H1 renders MoPrP(121–231) nonlethal.

The insolubility of PrPs with an H3 or H4 deletion seems likely to feature in the pathogenesis of the neurologic diseases found in the transgenic mice. It is possible that elimination of the H1, STE region, or 36-residue region did not produce disease because the structure of the H3–H4 domain may not be significantly affected by these deletions. Since five founders expressing the C178A mutation that disrupts the disulfide bond in the absence of any deletion are well at more than 200 days of age, it may be argued that the α-helix deletions are unlikely to produce disease solely through prevention of the disulfide bond formation. It will be of interest to determine the structural perturbations in PrP(90–231), which can be expressed in *E. coli* and purified in large quantities[31], when H3 or H4 is deleted.

The proposed structural model for PrP^{Sc} posits that the H1 and H2 regions acquire β-sheet conformation[22]. Should this model prove useful, then deletion of H3 or H4 would be expected to distort the conformation of H1 or H2. Destabilizing H1 or H2 might favor conversion to a PrP^{Sc}-like molecule that is insoluble but not resistant to digestion by proteinase K. Whether this insolubility in detergents reflects intermediate structures that are on the pathway to PrP^{Sc} formation remains to be determined[28,32]. These postulates are in accord with studies of ScN2a cells, in which deletion of any putative helical region or the STE domain prevented PrP^{Sc} formation, whereas deletion of the region between H2 and H3 was compatible with PrP^{Sc} formation[19].

An alternative explanation for these diseases is that H3 becomes pathogenic when H4 is deleted and vice versa. If such a hypothesis were correct, then deletion of both H3 and H4 should not produce disease. When we produced Tg(MoPrP144#)$Prnp^{0/0}$ mice with a transgene encoding PrP(23–144) corresponding to a human amber mutation in codon 145 (ref. 25), which results in a C-terminal truncation, no PrP(23–144) was detected in the brains of two transgenic lines by immunoblotting, which raises the possibility that the lack of Asn-linked oligosaccharides increased its degradation. Consistently, the mice remained healthy for up to ~700 days. The lack of measurable PrP with both H3- and H4-deleted in transgenic mice argues that testing this hypothesis will be difficult. It is noteworthy that the clinical phase of the illness spanned more than two decades in the GSS patient, who was heterozygous for the codon 145 mutation, and that C-terminal portions of wild-type PrP were found within PrP amyloid deposits in the CNS (ref. 26). The long clinical phase, the widespread deposition of PrP amyloid, and the expression of both the mutant and wild-type PrP genes makes comparison of this patient with our Tg(MoPrP144#)$Prnp^{0/0}$ mice problematic.

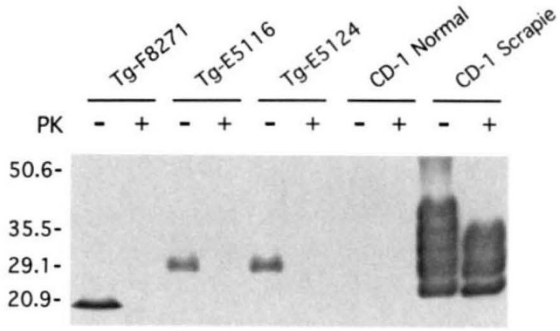

Fig. 6 Solubility and digestion with proteinase K of MHM2 PrP (del 23–88) with H3 or H4 deletion in brains from transgenic mice.

PrP^{C} from normal CD-1 brain was not found in the pellet fraction, whereas PrP^{Sc} from scrapie-infected CD-1 brain was. PrP molecules were found in the pellet fractions of brain homogenates prepared from transgenic mouse F8271 brain with H3 deletion and from transgenic mice E5116 and E5124 with H4 deletion. PrP from H3 or H4 deleted mice have M_r values of 17–18K or 26–27K. Mutant PrPs were digested by proteinase K (PK) (–) undigested or (+) digested.

Disorder resembling neuronal storage disease. The neuropathological features of these α-helix deletion diseases resemble those of neuronal storage disorders. Cell bodies of many but not all neurons in most brain regions had enlarged and rounded perikarya with accumulations of mutant PrP. Ultrastructurally, enlarged nerve cell bodies were filled with a proliferation of the endoplasmic reticulum (ER) suggesting that mutant PrP may accumulate in the ER. Such findings raise the possibility that the absence of H3 or H4 prevents trafficking of nascent PrP^{C} molecules from the ER into the Golgi apparatus and then to the nerve cell surface. It is possible that altered glycosylation of mutant PrP due to the H3 or H4 deletion affected the trafficking of PrP (ref. 33, 34). However, it is unlikely that only altered glycosylation can make PrP pathogenic, because transgenic mice expressing SHaPrPs with point mutations to block Asn-linked glycosylation(s) did not produce any of the pathological findings reported here (F. Yehiely, M. Rogers, S.J.D., S.B.P., manuscript in preparation). It is noteworthy that deletion of H3 or H4 did not create a KDEL sequence in PrP, which would act as a signal for retention in the ER (ref. 35). In the absence of a KDEL sequence, it seems likely that misfolded PrP with an H3 or H4 deletion either binds to other proteins or forms aggregates in the ER that do not enter transport vesicles[36]. It will be of interest to produce transgenic mice expressing full-length PrP with a KDEL sequence at the C terminus in place of the GPI anchor addition signal sequence. To our knowledge, no such inclusions as described here have been reported in any neuronal storage, metabolic, or toxic disease of humans or animals.

Do PrP storage diseases exist in humans? Whether the α-helical deletion disease described here is transmissible is unknown. Our findings described here strongly argue that the pathogenesis of this disorder differs considerably from that of the prion diseases. To date, no human PrP mutations corresponding to deletion of either the H3 or H4 regions have been recorded; however, little attention has been given to the molecular genetics of PrP in childhood CNS diseases, since prion diseases usually present in adults. Based on the discoveries recorded here, it would seem prudent to immunostain brain sections for PrP in CNS storage diseases of children where the etiology is unclear and to sequence the open reading frames of PrP alleles from these patients.

Methods

All PrP constructs as well as their open reading frame (ORF) cassettes with *BglII*/*XhoI* termini were described elsewhere[14,15,17,19]. The PrP *BglII*/*XhoI* cassettes were modified to be the *SalI*/*XhoI* cassettes as described[15]. They were then ligated to *SalI*-digested cosSHa.Tet vector and used to construct transgenic mice[37]. Breeding and screening of transgenic mouse lines were carried out as described[37].

Formalin-fixed paraffin-embedded brain sections were stained with the hematoxylin-eosin, the periodic acid-Schiff (PAS), the Bielschowsky silver and the Luxol fast blue-PAS stains and were also used for immunohistochemistry. Sections stained with the Bielschowsky (for axons) or the Luxol

fast blue-PAS (for myelin) stain were used to evaluate the white matter lesions in mouse brains. For PrP immunostaining, sections were pretreated with hydrolytic autoclaving as described and probed with monoclonal antibody 3F4 (ref. 38).

Double-immunolabeling was used to verify accumulation of PrP with H4 deletion in astrocytes. In brief, formalin-fixed, paraffin-embedded tissue sections were immunostained first for PrP (H4 deletion) by the hydrolytic autoclaving technique by using the α-PrP rabbit antiserum RO73, a biotinylated anti-rabbit secondary antibody, a peroxidase-conjugated avidin-biotin kit (ABC Kit PK4000, Vector Laboratories, Burlingame, CA), and diaminobenzidine as the marker. Following color development, the bound antibodies were removed from the section by treatment with 100 mM glycine-HCl (pH 2.2) for 20 min. To identify the astrocytes, a second immunostaining step for glial fibrillary acidic protein (GFAP) was performed by using α-bovine-GFAP rabbit antiserum (Dako Corp., Carpinteria, CA), a biotinylated anti-rabbit secondary antibody, an alkaline phosphatase-conjugated avidin-biotin kit (ABC-AP Kit AK5000, Vector Laboratories) and the Vector Red kit (SK5100, Vector Laboratories) for color development.

Histoblots were performed as described[39]. Coronal sections of brain (10 μm) were blotted onto nitrocellulose paper and pretreated with NaOH before PrP immunohistochemistry. The blot was probed with the monoclonal antibody (mAb) 3F4.

Animals were anesthetized with phenobarbital. The vascular system was flushed with warm saline via the heart, followed by perfusion with warm 3% glutaraldehyde, 1% paraformaldehyde in phosphate buffer, pH 7.2. The brain was removed, sectioned coronally, and immersed in ice-cold fixative for 24 h. Ammon's horn of the hippocampus was dissected, post-fixed in 2% osmium tetroxide for 1–2 h, and embedded in Araldite. Ultrathin sections (800 Å thick) were cut, mounted on copper grids and stained with 2% uranyl acetate and lead citrate. The sections were viewed in a Jeol 100S electron microscope (Jeol, Chicago, IL) at 80 kev.

Ten percent brain homogenates were prepared in PBS from an ill F_2 mouse F8271 with the H3 deletion and from founders E5116 and E5124 with the H4 deletion. To the homogenates, 0.5% Nonidet P-40 and 0.5% sodium deoxycholate were added before low-speed centrifugation[40]. The level of PrP transgene expression was 50% in F8271, 12.5–25% in E5116 and E5124 in comparison with PrP^c in normal SHa brain. After adjusting protein concentration in the low-speed supernatant fraction to 3 mg/ml, *N*-laurylsarcosine (Sarkosyl) was added to 0.8 %. Aliquots of the extracts were then digested by proteinase K (20 μg/ml, 37 °C, 1 h). The digested extracts and undigested controls were then centrifuged (100,000*g*, 20 °C, 1 h). Pellets were resuspended in 10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1% Sarkosyl, centrifuged again and probed on western immunoblots with the α-PrP RO73 antiserum.

The mutant MoPrP constructs to be assessed in cultured cells were engineered into the MHM2 PrP and then cloned into the SPOX.II neo vector. Stably transformed neomycin-resistant ScN2a cell lines were established in medium containing G418 as described[15].

Acknowledgments

The authors thank Daniel Friend at Harvard University for help interpreting the electron micrographs. This work was supported by grants from the National Institutes of Health (NS 14069, AG 08967, AG 02132, NS 22786 and AG 10770) and the American Health Assistance Foundation, as well as by gifts from the Sherman Fairchild Foundation, Leila and Harold G. Mathers Foundation, Bernard Osher Foundation, and Centeon Inc. T.M. was supported by the International Human Frontier Science Program and the French Foundation for Alzheimer Research.

RECEIVED 10 MARCH; ACCEPTED 22 MARCH 1997

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