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The Absence of Prion-Like Infectivity in Mice expressing Prion Protein-Like Protein

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ABSTRACT

Cellular prion protein, PrP^{C}, undergoes pathogenic structural conversion into the proteinase K (PK)-resistant isoform, PrP^{Sc}, to constitute a nucleic acid-free infectious agent, so called a prion. To determine whether a recently identified PrP-like protein, named PrPLP/Dpl, could also be transformed to a prion-like protein, we intracerebrally inoculated a mouse-adapted Fukuoka-1 prion into Ngsk and Zrch I mice either homozygously (*Prnp*$^{0/0}$) or heterozygously (*Prnp*$^{0/+}$) devoid of PrP^{C}. Only the former expressed PrPLP/Dpl ectopically in the brains, particularly in neurons. Ngsk *Prnp*$^{0/+}$ and Zrch I *Prnp*$^{0/+}$ mice similarly developed the disease. The diseased Ngsk *Prnp*$^{0/+}$ mice transmitted the disease to the mice expressing PrP^{C} but not to the mice expressing PrPLP/Dpl, showing abundant accumulation of PrP^{Sc} but not PK-resistant PrPLP/Dpl in the brains. Moreover, the inoculated Ngsk *Prnp*$^{0/0}$ mice neither developed the disease nor produced any infectivity transmissible to PrPLP/Dpl-expressing mice. These results indicate that PrPLP/Dpl have no potential to undergo pathogenic conversion to form a prion-like infectious particle.

Key words: Prion protein, prion protein-like protein, prion, knockout mice

INTRODUCTION

Transmissible spongiform encephalopathies, or prion diseases including Creutzfeldt-Jakob disease in humans and scrapie in animals, are infectious neurodegenerative disorders characterized by deposition of an abnormally folded, proteinase K (PK)-resistant isoform of prion protein, PrP^{Sc}, in the central nervous system (Prusiner, 1998). PrP^{Sc} is a highly fibrinogenic protein generated by the structural conversion of the normal PK-sensitive PrP (PrP^{C}), a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein abundantly expressed by neurons (Prusiner, 1998). Mice

devoid of PrP^{C} (*Prnp*$^{0/0}$) were resistant to the diseases without accumulation of PrP^{Sc} or propagation of infectious agents, or prions, in the brain (Bueler et al., 1993, Prusiner et al., 1993, Sakaguchi et al., 1995), strongly arguing for the "protein-only" hypothesis (Prusiner, 1998). This hypothesis postulates that a prion is mainly composed of PrP^{Sc} and propagates via the pathogenic conversion of PrP^{C} into PrP^{Sc} (Prusiner, 1998). Intermolecular interaction of these two molecules is thought to be essential in the conversion (Prusiner, 1998).

The gene designated *Prnd* encoding a PrP-like protein, PrPLP/Doppel (Dpl), was recently identified 16 kb downstream of the murine PrP gene, *Prnp* (Li et al., 2000, Moore et al., 1999). PrPLP/Dpl is also a GPI-anchored glycoprotein of the raft membrane and shares 23% identical amino acids with PrP (Li et al., 2000, Moore et al., 1999). Its protein structure is also extremely similar to that of the C-terminal two-third of PrP^{C}, comprising three α-helices and two short β-strands (Mo et al., 2001). These topological and structural similarities of the two proteins raised the question of whether PrPLP/Dpl could modify the pathogenesis of prion diseases by interfering with the interaction between PrP^{C} and PrP^{Sc}. Moore et al. and Tuzi et al. recently showed that PrPLP/Dpl had no potential to affect pathogenesis by demonstrating that mice expressing PrPLP/Dpl ectopically in the brains developed the disease with incubation periods and pathologies indistinguishable from those in control mice (Moore et al., 2001, Tuzi et al., 2002). However, another intriguing question remains to be addressed, that is, whether PrPLP/Dpl itself can undergo pathogenic conversion and become an infectious isoform that can propagate like a prion.

In the present study, we inoculated a mouse-adapted Fukuoka-1 prion into Ngsk *Prnp*$^{0/0}$ and Ngsk *Prnp*$^{0/+}$ mice, both ectopically expressing PrPLP/Dpl in the brain due to an unusual intergenic RNA splicing conducted on the Ngsk targeted allele (Li et al., 2000), and then examined whether PrPLP/Dpl-associated infectivity could be generated in these mice by inoculating the brain homogenates of these mice into Ngsk *Prnp*$^{0/0}$ mice expressing PrPLP/Dpl only.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Japan SLC. Ngsk *Prnp*$^{0/0}$ and Zrch I *Prnp*$^{0/0}$ mice were generated as previously described (Bueler et al., 1992, Sakaguchi et al., 1995). To produce Ngsk *Prnp*$^{0/+}$ and Zrch I *Prnp*$^{0/+}$ mice, Ngsk *Prnp*$^{0/0}$ and Zrch I *Prnp*$^{0/0}$ mice were intercrossed with C57BL/6 mice, respectively. The mice were handled in accordance with the Guidelines of Animal Experimentation of Nagasaki University.

Prion Inoculation

10% (w/v) brain homogenates of mice suffering from terminal disease after inoculation of a Fukuoka-1 prion (kindly provided by Dr. Tateishi) were prepared in phosphate-buffered saline (PBS), and an aliquot (20 μl) of the homogenates was intracerebrally inoculated into each mouse under anesthetization by the inhalation of diethyl ether (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Western Blotting

Total proteins extracted from mouse brains in a buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA) were treated with or without 20 μg/ml PK (Wako Pure Chemical Industries) at 37˚C for 30 min, and then boiled in the presence of Laemmli's buffer for 10 min to halt the PK digestion. The proteins were separated on 12% SDS-polyacrylamide gel and electrically transferred onto a nitrocellulose membrane (Millipore Corporation, Bedford, MA). After 1 hr blocking at room temperature (RT) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20) containing 5% dry skim milk, the membrane was incubated with an indicated first antibody in TBST containing 1% dry skim milk for 2 hr at RT. Anti-PrP and anti-PrPLP/Dpl sera were raised against recombinant mouse PrP23-231 in Ngsk *Prnp*$^{0/0}$ mice and recombinant mouse PrPLP/Dpl24-154 in rabbit, respectively. The immunocomplexes were detected by using horseradish peroxidase-conjugated antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK) and an ECL system (Amersham Pharmacia Biotech).

Immunohistochemistry

Deparaffinized sections were digested with 1 mg/ml trypsin for 15 min at 37˚C, and then placed in 3% H_2O_2 in methanol for 30 min at room temperature to eliminate endogenous peroxidase activity. After treatment with normal rabbit serum for 30 min, the tissue sections were incubated overnight at 4˚C with anti-GFAP (1:50 [Dako, Kyoto, Japan]). To detect the glial fibrillary acidic protein (GFAP) immunoreactivity, we used the Polymer-Immuno Complex method in accordance with the manufacturer's recommendations (Dako). The antibody-bound peroxidase was revealed with 0.04 % diamino-benzidine (Sigma Chemical Co., St. Louis, MO).

RESULTS

A mouse-adapted Fukuoka-1 prion was inoculated intracerebrally into Ngsk *Prnp*$^{0/0}$, Ngsk *Prnp*$^{0/+}$, and Zrch I *Prnp*$^{0/+}$ mice. The former two mouse lines expressed PrPLP/Dpl in the brains, particularly in neurons, but the latter did not (Li et al., 2000). In keeping with our previous report (Sakaguchi et al., 1995), none of the Ngsk *Prnp*$^{0/0}$ mice exhibited the disease-specific neurological symptoms for up to 600 days post-inoculation (p.i.) (Table 1). By contrast, all of the Ngsk *Prnp*$^{0/+}$ and Zrch I *Prnp*$^{0/+}$ mice developed the disease with similar incubation times of 282.3±22.5 and 286.4±27.0 days p.i., respectively (Table 1), and with similar clinical symptoms such as body weight loss, greasy yellowish hair, kyphosis, and flaccid paralysis of legs. Comparable levels of PrP^{Sc} with the same patterns of glycosylation were detected in the brains of these diseased mice on Western blotting (Figure 1). Histological findings in the affected brain tissues were also indistinguishable between the two lines of mice: the brains were markedly atrophic (data not shown) and many vacuoles were detectable mainly in the cerebral cortex and hippocampus (Figure 2, upper panels). Moreover, hypertrophic astrocytes with strong immunoreactivities of GFAP were similarly infiltrated in the same brain regions of these mice on immunohistochemical examinations (Figure 2, lower panels).

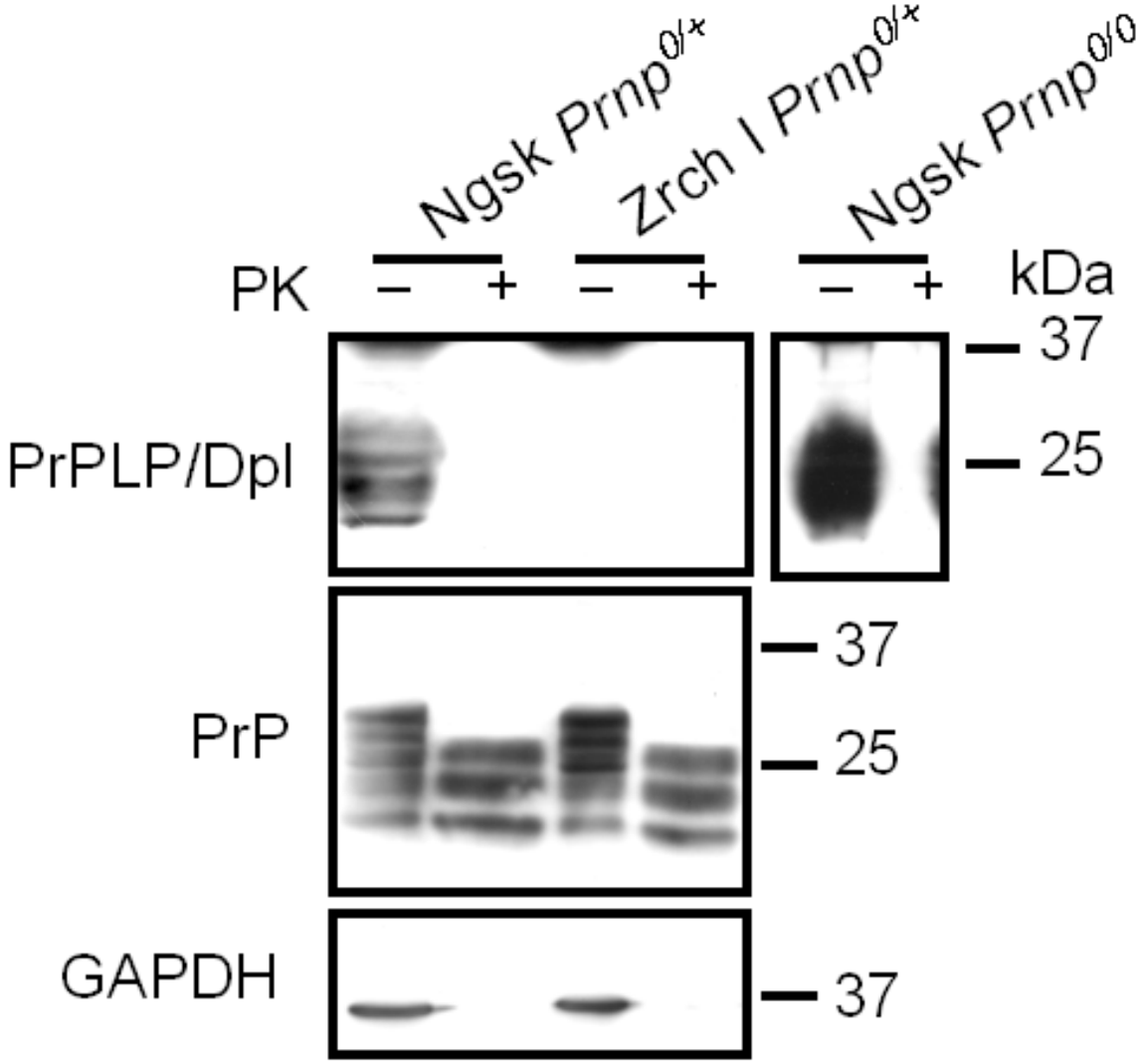

Figure 1: Western blotting of the brains of Ngsk *Prnp*$^{0/+}$ and Zrch I *Prnp*$^{0/+}$ mice with neurological symptoms and of Ngsk *Prnp*$^{0/0}$ mice 360 days after inoculation with the Fukuoka-1 prion. The brain homogenates treated with (+) or without (-) proteinase K (PK) were probed by rabbit antiserum against recombinant mouse PrPLP/Dpl, mouse antiserum against recombinant mouse PrP, and mouse monoclonal antibody against rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH; HyTest, Turku, Finland).

PrP^{C} undergoes conformational conversion into a PK-resistant PrP, PrP^{Sc}, and mediates the prion transmission. It has been suggested that PrP^{C} is also converted into a PK-sensitive but infectious intermediate form of PrP, termed PrP*, and that it supports the prion propagation (Aguzzi and Weissmann, 1997). To determine whether PrPLP/Dpl could be similarly transformed into a PK-resistant or a PK-sensitive but infectious intermediate isoform, we first subjected the brains of both the diseased Ngsk *Prnp*$^{0/+}$ and the Ngsk *Prnp*$^{0/0}$ mice sacrificed at 360 days p.i. to Western blotting. On Western blotting,

anti-PrP antibodies detected PK-resistant PrP^{Sc} abundantly accumulated in the brains of Ngsk *Prnp*$^{0/+}$ mice (Figure 1), but not in the Ngsk *Prnp*$^{0/0}$ mice (data not shown). By contrast, the PrPLP/Dpl detectable in the brains of these mice by anti-PrPLP/Dpl antibodies raised against recombinant mouse PrPLP/Dpl in rabbit was digested to an undetectable level by the PK treatment (Figure 1).

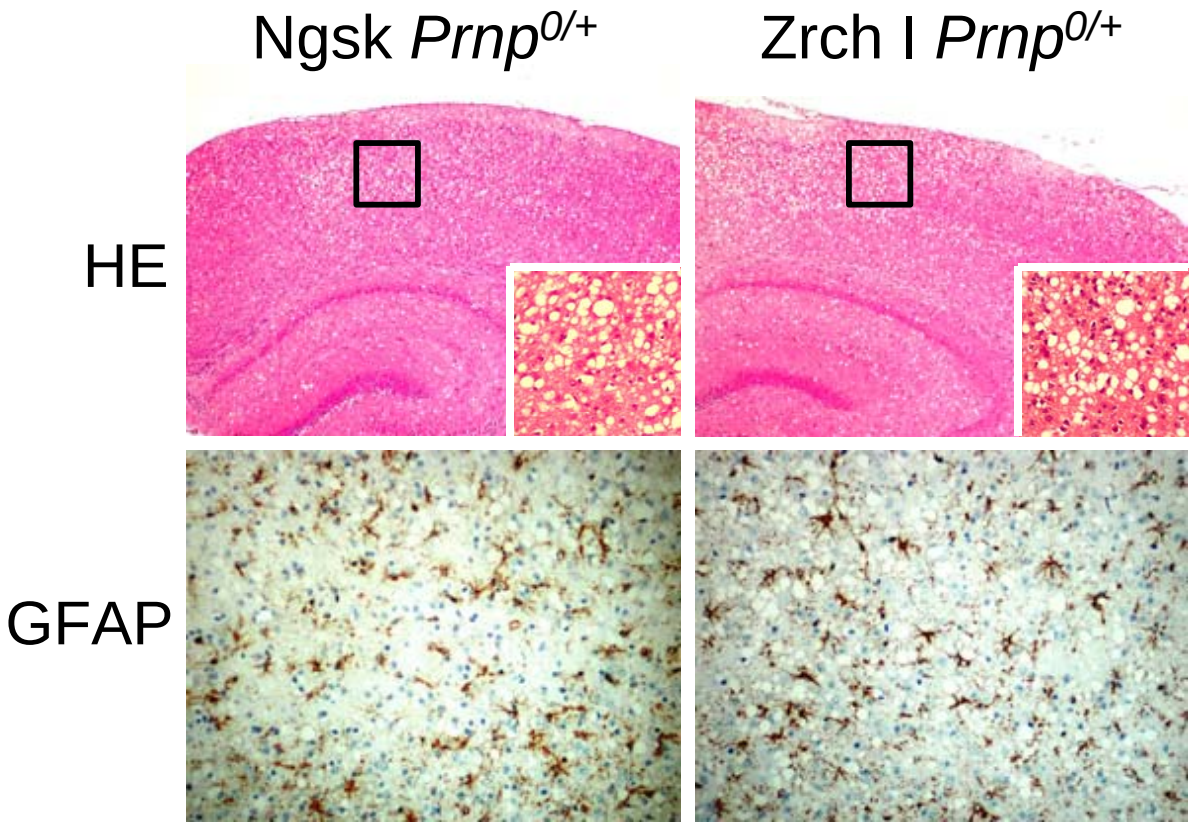

Figure 2: Hematoxylin-eosin staining (upper panel) and GFAP immunohistochemical staining (lower panel) of the brains of Ngsk *Prnp*$^{0/+}$ and Zrch I *Prnp*$^{0/+}$ mice with neurological symptoms after inoculation with the Fukuoka-1 prion. (Magnification, ×12.5; Inset magnification, ×50).

We next inoculated the brain homogenate of the diseased Ngsk *Prnp*$^{0/+}$ mice intracerebrally into wild-type, Ngsk *Prnp*$^{0/+}$ and Ngsk *Prnp*$^{0/0}$ mice to determine whether PrPLP/Dpl could be converted into a prion-like infectious protein. All of the wild-type and Ngsk *Prnp*$^{0/+}$ mice developed the disease at 161±11 and 269±27 days p.i., respectively (Table 1). The clinical symptoms in these mice were indistinguishable from those of the corresponding mice inoculated by the brain homogenates of infected wild-type mice (data not shown). By contrast, none of the Ngsk *Prnp*$^{0/0}$ mice exhibited disease-specific symptoms for up to 600 days p.i. (Table 1). Additionally, we inoculated the brain homogenate from the Ngsk *Prnp*$^{0/0}$ mice sacrificed at 112 weeks p.i. into indicator Ngsk *Prnp*$^{0/0}$ mice. However, no disease-specific neurological symptoms could be detected in the indicator mice for up to 600 days p.i. (Table 1).

Table 1. No PrPLP/Dpl-associated infectivity in Ngsk *Prnp*$^{0/+}$ and Ngsk *Prnp*$^{0/0}$ mice

donor mouse	recipient mouse	diseased mice /inoculated mice	incubation time (mean±SE; days)
wild-type (diseased)	Ngsk *Prnp*$^{0/0}$	0/5	>600
	Ngsk *Prnp*$^{0/+}$	5/5	282.3±22.5
	Zrch I *Prnp*$^{0/+}$	5/5	286.4±27.0
Ngsk *Prnp*$^{0/+}$ (diseased)	wild-type	7/7	161.4±10.9
	Ngsk *Prnp*$^{0/+}$	8/8	296.3±12.1
	Ngsk *Prnp*$^{0/0}$	0/5	>600
Ngsk *Prnp*$^{0/0}$ (112-wk p.i.)	Ngsk *Prnp*$^{0/0}$	0/5	>600

DISCUSSION

In the present study, we first showed that Ngsk *Prnp*$^{0/+}$ mice expressing PrPLP/Dpl ectopically in neurons succumbed to the disease after the infection of a Fukuoka-1 prion with incubation periods identical to that of Zrch I *Prnp*$^{0/+}$ mice expressing no PrPLP/Dpl. PrP^{Sc} was similarly accumulated in the brains of both lines of *Prnp*$^{0/+}$ mice, and pathological changes such as vacuolation and astrocyte infiltration patterns were indistinguishable between them. These findings are consistent with the results reported previously by Moore et al. (Moore et al., 2001) and Tuzi et al. (Tuzi et al., 2002), indicating that PrPLP/Dpl has no potential to affect the pathogenesis of prion disease. By contrast, Ngsk *Prnp*$^{0/0}$ mice were free of the disease for up to 600 days p.i. with no accumulation of PrP^{Sc} in the brains after inoculation of the prion, confirming that PrP^{C} is crucial for the pathogenesis of the diseases via its conversion into PrP^{Sc}.

The pathogenesis of prion diseases is yet to be elucidated. *Prnp*$^{0/0}$ mice were shown to spontaneously develop neurological abnormalities similar to those often observed in prion diseases, including impairment of memory and learning, alteration of circadian rhythms, and demyelination in the spinal cord and peripheral nerves (Collinge et al., 1994, Nishida et al., 1999, Tobler et al., 1996), strongly suggesting that the functional loss of PrP^{C} is involved in the pathogenesis of the diseases at least in part. Unlike the accumulation of PrP^{Sc}, the constitutive conversion of PrP^{C} into PrP^{Sc} is thought to cause the reduction of PrP^{C} in the affected brain, which in turn might lead to the functional impairment of PrP^{C}. It was recently shown that PrPLP/Dpl ectopically expressed in neurons impairs the function of PrP^{C}, causing neuronal cell death, particularly in Purkinje cells, granule cells, and hippocampal pyramidal cells (Anderson et al., 2004, Moore et al., 2001, Yamaguchi et al., 2004). In the present study, no exacerbation of prion pathogenesis was demonstrated in Ngsk *Prnp*$^{0/+}$ mice expressing PrPLP/Dpl in neurons as compared with Zrch I *Prnp*$^{0/+}$ mice

expressing no PrPLP/Dpl. This result seems to refute the idea that some aspects of the prion pathogenesis are attributable to the functional loss of PrP^{C}. We cannot, however, rule out the possibility that PrP^{C} is functionally inactivated in prion diseases in a way different from that of PrPLP/Dpl.

We next showed that, in contrast to PrP^{Sc} abundantly accumulated in the brains of the diseased Ngsk *Prnp*$^{0/+}$ mice, no PK-resistant PrPLP/Dpl could be detected in the brains of these mice nor in the Ngsk *Prnp*$^{0/0}$ mice sacrificed at 360 days p.i. These results were consistent with those of Tuzi et al. (Tuzi et al., 2002). We further showed that intracerebral inoculation of the brain homogenates of the diseased Ngsk *Prnp*$^{0/+}$ mice resulted in the disease only in mice expressing PrP^{C}, such as wild-type and Ngsk *Prnp*$^{0/+}$ mice, but not in Ngsk *Prnp*$^{0/0}$ mice which express PrPLP/Dpl but are devoid of PrP^{C}. Similarly, no disease-specific symptoms were detected in indicator Ngsk *Prnp*$^{0/0}$ mice, which were intracerebrally inoculated with the brain homogenates of the Ngsk *Prnp*$^{0/0}$ mice sacrificed at 112 weeks p.i.. The homologous combination of PrP^{C} and PrP^{Sc} is important for the efficient transmission of prions. Thus, taken together with the finding that PK-resistant PrPLP/Dpl were undetected in the diseased Ngsk *Prnp*$^{0/+}$ mice and the Ngsk *Prnp*$^{0/0}$ mice sacrificed at 360 days p.i., this unsuccessful transmission of the disease from these mice into Ngsk *Prnp*$^{0/0}$ mice strongly indicates that PrPLP/Dpl does not undergo pathogenic conversion to a prion-like infectious protein. On the contrary, the disease transmission is dependent on the expression of PrP^{C} in the recipient mice and the presence of PrP^{Sc} in the inocula, reaffirming the central role of PrP in prion diseases.

Behrens et al. previously showed that PrPLP/Dpl is not indispensable to either the pathogenesis or the PrP^{Sc} generation by demonstrating that scrapie prions induced typical features of prion pathology including PrP^{Sc} accumulation in neuronal grafts derived from embryonic stem cells homozygous for a disrupted *Prnd* (Behrens et al., 2001). PrPLP/Dpl is the first identified host protein exhibiting high similarities in amino acid composition and protein conformation to PrP. No other PrP-like proteins have been reported to date. Together with these results, our findings strongly indicate that prion diseases are disorders specifically associated with the pathogenic conformation of PrP.

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