Lethal recessive myelin toxicity of prion protein lacking its central domain

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PrP^C-deficient mice expressing prion protein variants with large amino-proximal deletions (termed PrP_{ΔF}) suffer from neurodegeneration, which is rescued by full-length PrP^C. We now report that expression of PrP_{ΔCD}, a PrP variant lacking 40 central residues (94–134), induces a rapidly progressive, lethal phenotype with extensive central and peripheral myelin degeneration. This phenotype was rescued dose-dependently by coexpression of full-length PrP^C or PrP^C lacking all octarepeats. Expression of a PrP variant lacking eight residues (114–121) was innocuous in the presence or absence of full-length PrP^C, yet enhanced the toxicity of PrP_{ΔCD} and diminished that of PrP_{ΔF}. Therefore, deletion of the entire central domain generates a strong recessive-negative mutant of PrP^C, whereas removal of residues 114–121 creates a partial agonist with context-dependent action. These findings suggest that myelin integrity is maintained by a constitutively active neurotrophic protein complex involving PrP^C, whose effector domain encompasses residues 94–134.

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Introduction

PrP^{Sc} is the only known constituent of mammalian prions (Prusiner, 1982), the infectious agents causing transmissible spongiform encephalopathies. PrP^{Sc} is an isoform of the cellular prion protein PrP^C (Oesch *et al*, 1985), which is

expressed in a broad range of vertebrate tissues (Bendheim *et al*, 1992). PrP^C is secreted into the endoplasmic reticulum and processed by removal of its amino-terminal signal peptide, N-linked glycosylation at two sites, and replacement of its carboxy-terminal tail by a glycosyl phosphatidyl inositol (GPI) group, which anchors it to the cell surface. Ablation of *Prnp*, the gene encoding PrP^C, abrogates prion replication (Bueler *et al*, 1993) and scrapie pathogenesis (Brandner *et al*, 1996).

The physiological function of PrP^C is unclear (Aguzzi and Polymenidou, 2004). *Prnp*^{0/0} mice enjoy a normal life expectancy (Bueler *et al*, 1992). Changes in circadian rhythms, alterations of hippocampal function, and behavioral abnormalities were reported in *Prnp*^{0/0} mice, but their molecular basis has not been clarified. PrP^C may have antiapoptotic (Kuwahara *et al*, 1999; Bounhar *et al*, 2001; Chiarini *et al*, 2002; Zanata *et al*, 2002) or proapoptotic properties (Paitel *et al*, 2002, 2003, 2004). A possible superoxide dismutase activity of PrP^C (Brown *et al*, 1997) has not been confirmed *in vivo* (Hutter *et al*, 2003). Unsurprisingly, a unified view of the above observations is still missing.

Transgenic expression of PrP variants carrying amino-proximal interstitial deletions (Δ32–121, termed PrP_{ΔE}, and Δ32–134, termed PrP_{ΔF}) causes ataxia, progressive cerebellar granule cell degeneration, leukoencephalopathy, and death of *Prnp*^{0/0} mice at 3–4 months of age (Shmerling *et al*, 1998; Radovanovic *et al*, 2005). Toxicity was associated only with PrP versions whose deletions extended into the highly conserved hydrophobic core (HC) domain (112–133), which is situated between the flexible amino-proximal tail of PrP^C and its globular carboxy-proximal domain (Wuthrich and Riek, 2001). A similar phenotype was detected in mice overexpressing Dpl (Moore *et al*, 1999; Weissmann and Aguzzi, 1999; Rossi *et al*, 2001), whose structure resembles that of PrP_{ΔF}. In each case, the pathological phenotype was rescued by substoichiometric coexpression of full-length PrP^C, either from the endogenous *Prnp* locus or as a transgene driven by various promoters. Those results suggested that PrP_{ΔF} and Dpl interfere with a physiological function of PrP^C in the brain.

PrP_{ΔF} lacks the octarepeat region (OR) and a central domain (CD) that comprises a charge cluster (CC, residues 95–110) and a hydrophobic core (HC, residues 112–134) (Figure 1A and B). To analyze the relative contribution of these domains to the phenotype, we generated *Prnp* transgenes lacking the entire CD (henceforth termed PrP_{ΔCD}) or part of the HC domain (termed PrP_{ΔPHC}). Whereas PrP_{ΔPHC} was innocuous even in the absence of full-length PrP^C, expression of PrP_{ΔCD} caused a drastic neuropathological phenotype and death within 20–30 days. This phenotype was partially counteracted by high levels of PrP^C and, to a lesser extent, by a PrP mutant lacking the octarepeats. While PrP_{ΔPHC} behaved neutrally in the absence of PrP^C, it partially rescued the toxic effects of PrP_{ΔF}, whereas it accentuated those of PrP_{ΔCD}.

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Figure 1 (A) Hydrophobicity plot of the full-length murine prion protein. SP: secretory signal peptide, cleaved after sorting of the precursor to endoplasmic reticulum; repeats: five repeats of eight amino acids; CC: charge cluster; HC: hydrophobic core; CD: central domain; H1, H2, H3: α-helices 1, 2, 3 of the globular carboxy-proximal domain; MA: membrane anchor of precursor protein, replaced during maturation with glycosyl phosphatidyl inositol anchor. (B) Scheme of wild-type prion protein (WT PrP) and deletion mutants utilized for generation of transgenic mice. Antibody POM1 recognizes a carboxy-proximal epitope, whereas POM11 binds to octarepeats, and POM3 binds to the charge cluster. (C) Southern blot analysis of PrP_{ΔCD} (lines Tg1046, Tg1047, and Tg1050) and PrP_{ΔPHC} transgenic mice (line Tg902). Genomic DNA was digested with *EcoRI* before loading. PrP_{ΔCD} transgenic band: 2916 bp; PrP_{ΔPHC}: 3015 bp; *Prnp*^{0/0}: 2747 bp; *Prnp*^{+/+}: 2148 bp. The lanes denoted phgΔCD and MoPrP-ΔH1 contain the plasmid constructs used for pronuclear injection. Numbers below the respective lanes indicate the numbers of transgenic copies per haploid genome, as determined by quantitation of ³²P Southern blotting signals against the respective *Prnp*^{+/+} genomic band. Line Tg1050 contained the largest number of transgenic copies. (D) Transgenic PrP transcription. RNA was isolated from PrP^{wt}, PrP_{ΔCD} (Tg1047), and PrP_{ΔPHC} (Tg902) brains, reverse-transcribed, and PrP-containing cDNA was amplified with specific primers. Wild-type brain and original plasmid served as molecular weight standards. DNase-treated, non-retrotranscribed samples were loaded for control.

Results

Characterization of interstitially deleted PrP species in transgenic mice

All PrP mutants described below are based on the 'half-genomic' pPrPHG backbone containing a redacted murine *Prnp* gene from which intron #2 was deleted, and flanked by 6 and 2.2 kb of 5' and 3' genomic regions, respectively (Borchelt *et al*, 1996; Fischer *et al*, 1996). PrP_{ΔC} lacks residues 32–93 within the OR region, and PrP_{ΔC} mice do not display any pathological phenotype (Shmerling *et al*, 1998). The deletions in PrP_{ΔE} and PrP_{ΔF} extend into the HC domain (aa 32–121 and 32–134; Figure 1A and B) and induce neuropathological lesions in *Prnp*^{0/0} mice. These lesions may be caused by the ablation of CD, by the combined ablation of octarepeat and CD, or by the disruption of HC. We tested these alternatives by creating additional PrP mutants with interstitial deletions of amino acids 94–134 (termed PrP_{ΔCD}) and 114–121 (termed PrP_{ΔPHC}; Figure 1B).

PrP_{ΔCD} was injected into *Prnp*^{+/0} zygotes derived from crosses between *Prnp*^{0/0} and C57BL/6 mice. Three of 35 offspring were found to carry the transgene. Surprisingly, all three pups died between 10 and 23 days of age, suggesting perinatal toxicity. Further microinjections into hybrid B6D2F₁ *Prnp*^{+/+} zygotes resulted in eight PrP_{ΔCD} and four PrP_{ΔPHC} founders carrying diploid *Prnp* alleles (henceforth termed

PrP_{ΔCD}^{+/+} and PrP_{ΔPHC}^{+/+}, with superscripts defining the *Prnp* allelic status and subscripts denoting the respective hemizygous transgenes).

PrP_{ΔCD} founder mice 1046, 1047, 1048, and 1050, as well as PrP_{ΔPHC} founder mice 630, 642, and 902 exhibited undistorted Mendelian transmission of the transgene. Four PrP_{ΔCD} lines (Tg1046, Tg1047, Tg1048, Tg1050) and one PrP_{ΔPHC} line (Tg902) were chosen for further analyses. Quantitative Southern blotting revealed that the number of transgenic copies varied between 1 and 5 per haploid genome (Figure 1C). Each line was crossed twice to *Prnp*^{0/0} mice, and F₂ offspring (PrP_{ΔCD}^{0/0} and PrP_{ΔPHC}^{0/0}) were analyzed for transgenic transcription (Figure 1D) and protein expression (Figure 2).

In all transgenic lines, brain expression of PrP mutants was lower than that of endogenous PrP^C (Figure 2C) and paralleled the number of integrated transgenic copies. Tg1046 PrP_{ΔCD} and Tg902 PrP_{ΔPHC} expressed <20% of *Prnp*^{+/0}, whereas Tg1047, Tg1048, and Tg1050 expressed ca. 35, 80, and 75% of *Prnp*^{+/0}, respectively (Figure 2D). The anatomical distribution of the transgenic proteins resembled that of PrP_{ΔF} (Figure 2E–S). No pathological aggregates were detected by PrP immunohistochemistry with antibody SAF84 (data not shown).

Upon PNGase-F treatment, PrP_{ΔCD}, PrP_{ΔPHC}, and PrP^C displayed enhanced electrophoretic motility similar to PrP^C

Figure 2 Expression of transgenic proteins. **(A)** Similar glycosylation patterns of full-length PrP, PrP^{ΔCD}, and PrP^{ΔpHC}. Brain homogenates were subjected to PNGase F treatment as indicated and analyzed by Western blotting using anti-PrP antibody POM1 (Polymenidou *et al*, 2005). Arrowhead: C1 fragment. **(B)** Density gradient analysis of detergent-resistant membrane (DRM) preparations from the brains of wild-type, PrP^{ΔCD}, and PrP^{ΔpHC} transgenic mice. Fractions 4–16 of the step gradient were analyzed by Western blotting. Full-length PrP, PrP^{ΔCD}, and PrP^{ΔpHC} were detectable to a similar extent in the upper fractions, indicating that they all localize to DRMs similar to flotillin (48 kDa). Non-DRM contaminations were controlled for by probing blots with antibodies to GAPDH (lowest panel, 35 kDa). Non-buoyant PrP may indicate raft disruption or may represent immature protein fractions. **(C, D)** Expression levels of PrP^{ΔCD} and PrP^{ΔpHC}. Total brain homogenates were digested with PNGase F treatment and analyzed using the monoclonal antibody POM11 (*PrP^{wt} band, **PrP^{ΔCD} band). **(D)** Densitometric quantification of protein expression after Western blotting. Each column represents the average of at least three individual mice from each transgenic strain coexpressing wild-type PrP from one haploid genomic allele. **(E–S)** POM1-immunostained brain sections. **(E–I)** Overview; **(J–N)** higher magnification of hippocampus and corpus callosum; **(O–S)**, cerebellum. Similar spatial expression patterns for PrP^C and its variants in wild-type, PrP^{ΔCD}, PrP^{ΔpHC}, and PrP^{ΔF} mice. No immunoreactivity was visible in Prnp^{0/0} mice. Scale bar: **(E–I)** 10 mm; **(J–N)** 100 μm; **(O–S)** 10 μm.

(Figure 2A), suggesting that each protein was N-glycosylated to a similar extent. PrP^C displayed a significant amount of C1 fragment (Figure 2A, arrowhead), yet C1 formation was reduced in PrP^{ΔpHC} and absent from PrP^{ΔCD}, suggesting abnormal endoproteolysis of these proteins. We then analyzed detergent-resistant membranes from PrP^C, PrP^{ΔCD}, and PrP^{ΔpHC} brains by step density-gradient centrifugation. The buoyancy of PrP^{ΔCD} and PrP^{ΔpHC} was similar to that of PrP^C and flotillin (Figure 2B), suggesting that they all reside in similar membrane microdomains. Therefore, most aspects of PrP^{ΔCD} and PrP^{ΔpHC} biogenesis appear similar to those of PrP^C.

Pathological phenotypes of mice expressing interstitially deleted PrP species

All PrP^{ΔCD} transgenic lines (Tg1046, Tg1047, Tg1048, and Tg1050) were maintained in the Prnp^{+/+} background. Founders Tg1046, Tg1047, and Tg1050 remained healthy, whereas founder Tg1048, which displayed the highest expression of the transgene, developed behavioral abnormalities at 120 days of age (Supplementary movie). However, its entire transgenic PrP^{ΔCD} offspring died at 20 days of age. As incipient paraparesis of founder Tg1048 precluded natural breeding, sperm was used for *in vitro* fertilization of Prnp^{+/+}

Table 1 Scoring of neurological signs in mice expressing PrP variants

Score	Neurological signs	Cage grid test	Action
0	No detectable signs of abnormal movement		
1	Limp tail, abnormal gait, hind limb weakness	Occasionally positive grid test	Provide food on the cage floor
2	Unilateral partial hind limb paresis	One leg consistently falls through the grid	Provide food and water on the cage floor, observe twice a week
2.5	Bilateral partial hind limb paresis	Bilateral impairment of cage grid test	Observe every second day
3	Complete bilateral hind limb paralysis		Observe daily; kill if persisting for >7 days
3.5	Bilateral hind limb paralysis and forelimb paresis		Kill if persisting for >4 days
4	Tetraplegia		Kill immediately
5	Animal found dead		

C57BL/6 oocytes. This resulted in a total of 117 *Prnp*^{+/+} pups, of which 46 carried the PrP_{ΔCD} transgene. The gender distribution of pups was mendelian (58 ♂; 59 ♀), but transgenic males were marginally underrepresented (17 ♂; 29 ♀; χ^2 $P < 0.03$). By 50 days of age, all (46/46) Tg1048 PrP_{ΔCD}^{+/+} animals had developed ataxia, which progressed over 20–30 days, eventually leading to the inability to stride on cage grids. By the age of 80 days, 40/46 animals had developed spastic paraparesis, which by 95 days progressed to full paraplegia. A four-degree clinical score was developed for monitoring and animal welfare purposes (Table 1). Mice were euthanized within 3 days of reaching a score of 3.5, or within 1 day of developing a score of 4 (Figure 3E and Supplementary Figure 1). By the age of 99 days all 46 animals had to be euthanized (Figure 3A). All transgenic Tg1050 PrP_{ΔCD}^{+/+} mice developed a disease similar to that of Tg1048, but progression was slower. First signs became noticeable at 110 days and progressed to terminal disease at 160 days (Figure 3B). Tg1046 and Tg1047 PrP_{ΔCD}^{+/+} mice, which expressed lower levels of transgenic protein, did not experience spontaneous disease before 450 days of age.

Genetic interactions between PrP^C and mutated PrP

In order to probe genetic interaction between PrP_{ΔCD} and PrP^C, we removed *Prnp* through sequential crosses of each transgenic line with *Prnp*^{0/0} mice. Tg1050 PrP_{ΔCD}^{+/0} offspring developed clinical signs by 29 days and reached clinical scores of >3 by 45 days (Figure 3B). Similarly, Tg1047 PrP_{ΔCD}^{+/0} mice developed neurological disease at 75–80 days (Figure 3C) and were consistently underweight despite facilitated access to food and water (Figure 3F). Only 20% of Tg1046 PrP_{ΔCD}^{+/0} offspring developed disease within 500 days (Figure 3D), yet PrP_{ΔCD}^{0/0} mice developed clinical signs at 17–21 days and progressed to terminal disease by 23–28 days. The most dramatic phenotype was seen in Tg1050 PrP_{ΔCD}^{0/0} mice, which developed perinatal ataxia and terminal disease before 10 days of age (Figure 3B). We conclude that reduction or removal of PrP^C results in greatly exacerbated phenotypes.

We then tested whether overexpression of PrP^C antagonizes PrP_{ΔCD} toxicity. We crossed Tg1050 PrP_{ΔCD}^{0/0} to *tga20* mice, which overexpress PrP^C six-fold. In order to circumvent the early lethality of Tg1050 PrP_{ΔCD}^{0/0} mice, we first introduced the *tga20* allele into PrP_{ΔCD}^{+/+} mice and subsequently removed the *Prnp* wild-type alleles by crossing to *Prnp*^{0/0} mice. The resulting PrP_{ΔCD}^{0/0}*tga20* mice stayed healthy for >300 days (Figure 3B). Therefore, the severity of the disease is attenuated by overexpression of PrP^C (Figure 3G). As these phenomena were confirmed in four independently generated

lines of transgenic mice, we conclude that both the PrP^C-dependent attenuation and the *Prnp*⁰-dependent exacerbation of the phenotype are independent of the site of transgene integration.

Tg902 PrP_{ΔPHC}^{+/+} mice developed normally and transmitted the transgene in an undistorted Mendelian manner (data not shown), although PrP_{ΔPHC} expression was similar to that of PrP_{ΔCD} in Tg1046 mice. Tg902 mice were then crossed to *Prnp*^{0/0} mice. Over 150 Tg902 PrP_{ΔPHC}^{0/0} mice were monitored, yet did not develop disease during an observation period of >450 days. Therefore, expression of PrP_{ΔPHC} is not intrinsically toxic in the absence of PrP^C.

Neuropathological changes

Histological examination of the brains from terminally sick Tg1047 PrP_{ΔCD}^{+/0} mice revealed vacuolar degeneration and astrogliosis of several white matter tracts, most pronouncedly in the internal capsule and the pyramidal tracts (Figure 4B), the white matter areas of the brainstem, and, to a lesser degree, in the cerebellum. No degeneration, however, was present in the cerebellar cortex including the cerebellar granule cell layer (Figure 4D). There were no changes in the cerebral cortex or in the basal ganglia. No changes were found in age-matched non-transgenic littermates (Figure 4A and C).

Coarse vacuolar degeneration was found in both descending and ascending fiber tracts of the spinal cord (Figure 4E and G). In contrast to non-transgenic *Prnp*^{+/0} littermates (Figure 4F and H), myelinated fiber tracts in the spinal cord displayed axonal loss, which was associated with enlarged degenerating fibers with thinning of their myelin sheaths (Figure 4I). There was no onion bulb formation. Evidence for degeneration of myelinated fibers, including axonal breakdown and segmentation of myelin into ellipsoids ('digestion chambers'), was obtained in the sciatic nerve (Figure 4K). Analysis of semithin sections revealed axonal loss, enlarged 'vacuolated' fibers, as well as degenerating myelinated fibers (Figure 4M). No such changes were found in sciatic nerves from control littermates (Figure 4J and L). Electron microscopy revealed axonal breakdown and hypomyelinated fibers, as well as demyelinated fibers and myelin-digesting macrophages (Figure 4N–P). These findings are indicative of widespread axomyelinic degeneration with minor neuronal degeneration. Because axons build functional units with their myelin sheaths, and both compartments underwent degeneration, it is difficult to establish whether primary damage was to axons or to myelin sheaths. We proposed earlier that myelin toxicity is the primary cause of damage, as the white

Figure 3 (A–D) Survival of *PrP^{ΔCD}* mice. Longevity was dependent on their *Prnp* status, with *Prnp^{+/+}* transgenic mice surviving longer than *Prnp^{+/o}* mice and *Prnp^{o/o}* mice showing the shortest survival times. Each line represents ≥ 5 individuals. (E) Typical clinical phenotypes at terminal stage: kyphosis (left), ataxia (middle), and clamping when held head-down at the tail (right). See also Supplement movie online. (F) Several litters from a *Tg1047* breeding pair were monitored for their weight. Transgenic mice (*PrP^{+/o}ΔCD*) were constantly underweight as compared to their non-transgenic littermates (*PrP^{+/+}*). (G) The ratio between the expression of *PrP^{ΔCD}* and full-length *PrP^C* determines the life expectancy of mice. The black bars depict the ratios of steady-state level *PrP^{ΔCD}*/*PrP^{wt}*, as determined by quantitative Western blot analysis of brain homogenates of at least four transgene-positive individuals of the indicated line. The gray bars represent the mean life expectancy of ≥ 4 transgenic individuals of each line.

matter disease in Shmerling mice was rescued by oligodendrocyte-restricted (but not neuron-restricted) expression of *PrP^C* (Radovanovic *et al*, 2005).

Direct comparisons showed similar myelin damage in *PrP^{ΔCD}* and *PrP^{ΔF}* mice. However, *PrP^{ΔCD}* mice lacked the extensive cerebellar granule cell degeneration found in *PrP^{ΔF}* mice (Shmerling *et al*, 1998; Radovanovic *et al*, 2005). Myelin damage in *Prnp^{Nsk/Nsk}* and *Prnp^{o/o}* mice was associated with concentric ‘onion bulb’ figures indicative of multiple episodes of demyelination and remyelination, which was not a feature in *PrP^{ΔCD}* mice. The latter discrepancy may be related to the higher toxicity of *PrP^{ΔCD}*, which may preclude reparative remyelination.

Genetic interactions between *PrP^{ΔF}* and *PrP^{ΔCD}*

The toxicity of both *PrP^{ΔF}* and *PrP^{ΔCD}* is counteracted by overexpression of *PrP^C* and enhanced by its absence, suggesting that both toxic mutants act through the same molecular mechanism. If so, expression of *PrP^{ΔF}* should not rescue the toxicity of *PrP^{ΔCD}*, and/or vice versa. We tested this prediction by crossing *Tg1046 PrP^{ΔCD}* and *TgF35 PrP^{ΔF}* mice ($n = 33$ mice). All *PrP^{o/o}ΔCDΔF* mice developed a phenotype identical to that of parental *PrP^{ΔCD}* mice. Coexpression of *PrP^{ΔF}* and *PrP^{ΔCD}* in *Prnp^{+/o}* mice (*PrP^{+/o}ΔCDΔF*) resulted in fully penetrant neurological disease starting at 70 days of age ($n = 9$), and by 300 days 50% had reached a clinical score of ≥ 3.5 (Figure 5A). When *PrP^{ΔCD}* and *PrP^{ΔF}* were coexpressed in

Figure 4 White matter pathology and peripheral neuropathy in terminally sick PrP^{ACD} mice. Age-matched non-transgenic $\text{Prnp}^{+/+}$ littermates served as controls for all histological investigations. GFAP-stained pyramidal tract showed vacuolar degeneration and associated astrogliosis in a sick PrP^{ACD} mouse (B) but not in a control mouse (A). GFAP-stained cerebellum in PrP^{ACD} mice revealed moderate astrogliosis but preserved cerebellar granule cell layer (D). The same region in an age-matched control littermate (C). Luxol-Nissl-stained cross-section through the mid-thoracic spinal cord showed coarse vacuolar degeneration in descending and ascending fiber tracts (E, G). No such changes were observed in control mice (F). Paraphenylenediamine-stained semithin section of a descending myelinated fiber tract in a PrP^{ACD} mouse revealed axonal loss together with some enlarged 'vacuolated' myelinated fibers with thinning of their myelin sheaths (asterisk) (I). Age-matched control littermate (H). Luxol-Nissl-stained longitudinal section through the sciatic nerve of a control (J) and a PrP^{ACD} mouse (K). Axonal breakdown and segmentation of myelin into digestion chambers (arrows). Axonal loss, hypomyelinated fibers, and degeneration of myelinated fibers with collapsed myelin sheaths (arrow) were observed in paraphenylenediamine-stained semithin sections of the sciatic nerve in PrP^{ACD} mice (M) but not in control mice (L). Electron microscopic analysis of the sciatic nerve showed demyelinated fibers (N) associated with macrophages filled with myelin debris (O). Some fibers showed collapsed axons surrounded by abnormal condensed multilamellar myelin sheaths (P). Normal myelinated fibers in a control mouse (Q). Scale bar: (A–E) 100 μm ; (F, G, J, K) 20 μm ; (H, I, L, M) 10 μm ; (N–Q) 2 μm .

the presence of diploid Prnp ($\text{PrP}^{\text{ACD}/\Delta\text{F}}$), only two of six aged mice developed disease (Figure 5A). We conclude that neither $\text{PrP}^{\Delta\text{F}}$ nor PrP^{ACD} antagonizes the toxicity of each other. This reinforces the suggestion that $\text{PrP}^{\Delta\text{F}}$ and PrP^{ACD} compete with PrP^{C} by the same molecular mechanism. Interestingly, PrP^{ACD} fully imposed its phenotype onto $\text{PrP}^{\text{ACD}/\Delta\text{F}}$ mice, although $\text{PrP}^{\Delta\text{F}}$ was much more abundant than PrP^{ACD} (Figure 5E).

Tg1047 or Tg1050 $\text{PrP}^{\text{ACD}/+}$ mice were then mated to PrP^{AC} mice, which express a PrP mutant devoid of all ORs (line TgC4 ; Shmerling *et al*, 1998). The longevity of $\text{PrP}^{\text{ACD}/+}$ offspring ($n=37$) was 290 and 120 days of age, respectively, which is three-fold more than PrP^{ACD} mice from the same lines ($n=22$; Figure 5B). As PrP^{AC} does not contain any OR, we conclude that the rescuing ability of PrP^{C} does not require this structural motif, as was shown in coexpression experiments of PrP^{AC} and $\text{PrP}^{\Delta\text{F}}$ (Flechsigs *et al*, 2000; Flechsigs and Weissmann, 2004). PrP^{AC} expression was higher than that of PrP^{C} and PrP^{ACD} (Figure 5F), suggesting that the rescuing potency of PrP^{C} was lower than that of PrP^{C} .

$\text{PrP}^{\Delta\text{PHC}}$ did not elicit any pathological phenotype in $\text{Prnp}^{0/0}$ mice. However, Western blots (Figure 5G) revealed that $\text{PrP}^{\Delta\text{PHC}}$ was ca. 20% endogenous PrP^{C} . We therefore reasoned that any potential effect of $\text{PrP}^{\Delta\text{PHC}}$ may become apparent in intercrosses to $\text{PrP}^{\Delta\text{F}}$ mice. Whereas TgF35 $\text{PrP}^{\Delta\text{F}/0}$ mice developed progressive neurological signs and terminal disease at 106 ± 23 days (Figure 5C), we found that double transgenic $\text{PrP}^{\Delta\text{F}/\Delta\text{PHC}}$ mice survived to 157 ± 27 days. Therefore, $\text{PrP}^{\Delta\text{PHC}}$ expression extends the lifespan of $\text{PrP}^{\Delta\text{F}}$ mice ($n=4$; Mann–Whitney test: $P=0.064$).

We then tested whether $\text{PrP}^{\Delta\text{PHC}}$ rescues the toxicity of PrP^{ACD} by crossing Tg1047 or Tg1050 $\text{PrP}^{\text{ACD}/+}$ to Tg902 $\text{PrP}^{\Delta\text{PHC}/0}$ mice. Unexpectedly, we observed significantly reduced latency of first clinical signs and reduced longevity in $\text{PrP}^{\text{ACD}/\Delta\text{PHC}}$ mice (Figure 5D). Tg1047 $\text{PrP}^{\text{ACD}/\Delta\text{PHC}}$ mice ($n=19$) developed a clinical phenotype 15–20 days earlier and had to be euthanized 35 days earlier than their $\text{PrP}^{\text{ACD}/+}$ littermates ($n=24$). Similarly, crossing of Tg902 $\text{PrP}^{\Delta\text{PHC}/0}$ and Tg1050 $\text{PrP}^{\text{ACD}/+}$ mice ($n=10$) resulted in a reduced life

Figure 5 Survival of compound transgenic mice. (A–D) Survival curves of mice expressing $PrP^{\Delta CD}$ (Tg1046), $PrP^{\Delta F}$ (TgF35), or both, in the absence or presence of full-length PrP^C . Each line comprises ≥ 5 individuals. (B) Mice expressing $PrP^{\Delta CD}$ (Tg1047) or $PrP^{\Delta C}$ (TgC4), or both, in the presence of one wild-type $Prnp$ allele. Each line comprises ≥ 7 individuals. (C) Mice expressing $PrP^{\Delta PHC}$ (Tg902), $PrP^{\Delta F}$ (TgF35), or both, in the absence or presence of PrP^C . Each line represents ≥ 3 individual mice. (D) Mice expressing $PrP^{\Delta CD}$ (Tg1047) or $PrP^{\Delta PHC}$ (Tg902), or both, in the presence of one wild-type $Prnp$ allele. Each line represents ≥ 7 individuals. (E–G) Brain expression of PrP^C and transgenic PrP. (E) $PrP^{\Delta F}$ has a higher steady-state level than PrP^{wt} and $PrP^{\Delta CD}$. Brain homogenates were optionally treated with PNGase F and Western blots were decorated with the antibody POM1. The arrows refer to the molecular weight of each protein after deglycosylation. (F) $PrP^{\Delta C}$ expression is stronger than that of PrP^{wt} and $PrP^{\Delta CD}$. Brain homogenates were treated with PNGase F and Western blots were decorated with antibodies POM1, POM3, and POM11 recognizing different domains of PrP. (G) $PrP^{\Delta PHC}$ expression is lower than that of PrP^C and $PrP^{\Delta F}$, but similar to that of some affected $PrP^{\Delta CD}$ lines. All homogenates were treated with PNGase F.

expectancy ($PrP^{+/o} \Delta CD \Delta PHC$ 30 days versus $PrP^{+/o} \Delta CD$ 40 days, data not shown). Therefore, although non-toxic to $Prnp^{o/o}$ and wild-type mice, $PrP^{\Delta PHC}$ mitigates the toxic effect of $PrP^{\Delta F}$ and enhances the toxicity of $PrP^{\Delta CD}$.

Discussion

We found that prion proteins lacking solely a highly conserved sequence of 40 amino acids (the ‘central domain’, CD)

are potent neurotoxins. In the absence of full-length PrP^C , even small amounts of $PrP^{\Delta CD}$ induce early postnatal death. Toxicity was strictly dose-dependent, with doubling of expression resulting in a decrease of longevity by 10 days (Figures 2D and 3A–D).

The clinical signs of $PrP^{\Delta CD}$ mice were associated with widespread central and peripheral neuropathy similar to mice overexpressing Dpl or $PrP^{\Delta F}$ (Shmerling *et al*, 1998; Rossi *et al*, 2001). Myelin damage, rather than neuronal loss, was

the primary cause of lethality in these mice (Radovanovic *et al*, 2005). A similar, but milder, myelin phenotype was described in aged *Zrch-I Prnp^{0/0}* mice (Nishida *et al*, 1999). Therefore, rather than inducing an entirely artificial disease, PrP_{ΔCD}, PrP_{ΔE}, PrP_{ΔF}, and Dpl appear to greatly aggravate a pathology caused by a defect in PrP^C function. This intriguing observation suggests that maintenance of myelin integrity represents a major physiological role of PrP^C.

What may be the mechanism of PrP_{ΔCD} neurotoxicity? Any interstitial deletions may destabilize proteins, resulting in cytotoxicity. However, as most of the CD domain is intrinsically unstructured, its deletion is unlikely to dramatically destabilize PrP. Secondly, toxicity may result from subcellular mislocalization (Kaneko *et al*, 1997; Ma *et al*, 2002) or inappropriate membrane topology of PrP (Hegde *et al*, 1998). However, these scenarios are unlikely as (1) PrP_{ΔCD} undergoes glycosylation, processing, and transport to detergent-resistant membrane microdomains, similar to full-length PrP, and (2) full-length PrP^C does not antagonize the toxicity of cytosolic PrP (Ma *et al*, 2002).

We tested whether the ΔCD, ΔE, and ΔF deletions increase the aggregation propensity of PrP^C. This is unlikely, as PrP_{ΔCD} was fully sensitive to proteinase K digestion and did not form immunohistochemically detectable aggregates in transgenic mice (data not shown). Finally, formation of the carboxy-proximal C1 fragment of PrP was significantly impaired in PrP_{ΔCD} mice, suggesting abnormal endoproteolysis of PrP_{ΔCD}. Although the molecular basis of this phenomenon is unclear, it is unlikely to account for toxicity, as healthy PrP_{ΔPHC} mice also displayed a similarly reduced C1 fragment.

The pathogenetic mechanisms of many human hereditary diseases, as well as many bacterial toxins, include inappropriate signaling events. As PrP^C may transduce signals (Mouillet-Richard *et al*, 2000; Mattei *et al*, 2004; Solfrosi *et al*, 2004), the neurotoxicity of PrP mutants may rely on similar mechanisms. The pathological phenotype of PrP_{ΔCD}, PrP_{ΔE}, and PrP_{ΔF} mice was counteracted by overexpression of full-length PrP^C (Shmerling *et al*, 1998) and exacerbated by removal of the endogenous *Prnp* gene, suggesting that PrP_{ΔCD}, PrP_{ΔE}, and PrP_{ΔF} compete with PrP^C for a common signaling molecule. This suggestion is strengthened by crossing experiments showing that neither mutant protein ameliorates the phenotype induced by the other. The phenotype was solely determined by the stoichiometric ratio of full-length versus redacted PrP, regardless of whether PrP^C was expressed from its endogenous *Prnp* locus or as a transgene.

All toxic mutants displayed disruption of the charge cluster (CC, residues 95–110) and a part of the hydrophobic core (HC, residues 111–121) of PrP^C. Toxicity was ameliorated by coexpressing PrP variants with intact CC and HC, even if these variants—as in the case of PrP_{ΔC}—lacked the OR. We therefore posit that PrP^C exerts a myelin-maintaining activity by signaling through the CD domain to an unknown receptor (tentatively termed PrP_R). In all paradigms investigated, the phenotype was determined by the stoichiometry of mutant to full-length PrP, strongly suggesting that PrP^C, the various mutant PrP moieties, and PrP_R form hetero-oligomeric complexes (Figure 6). The mild pathological phenotype of *Prnp^{0/0}* mice suggests that myelin integrity is supported by residual PrP_R activity, whereas disruption of the CD domain sequesters PrP_R in a dominant-negative state. Complex stability could be influenced by domains distinct from those involved

Figure 6 A model for the positive and negative effects of PrP^C and its variants. (A) PrP (black) consists of a globular C-terminal domain (hexagon) and an N-terminal flexible tail (arch) encompassing the ORs (circle). The model rests on the following assumptions: (1) PrP^C activates a hitherto unidentified receptor (PrP_R), which transmits myelin maintenance signals (flashes); (2) in the absence of PrP^C, PrP_R exerts some residual activity, either constitutively or by recruiting a surrogate ligand; (3) the activity of PrP^C and its mutants requires homo- or heterodimerization, and induces dimerization of PrP_R; and (4) PrP dimers containing PrP_{ΔCD} or PrP_{ΔCD} trap PrP_R in an inactive dominant-negative state. Finally, (5) the OR region stabilizes the interaction between PrP and PrP_R, but does not contribute directly to signaling. (B) By increasing complex affinity to PrP_R through its ORs, PrP_{ΔPHC} may displace PrP_{ΔCD} homodimers, thereby ameliorating the phenotype of compound PrP_{ΔPHC} transgenic mice. However, as PrP_{ΔCD} also contains ORs, it may more efficiently compete with PrP_{ΔPHC} for PrP_R and thus prevents any phenotypic improvement.

in executing signal transduction: the context-dependent toxicity of PrP_{ΔPHC} implicates the OR as one such domain (Figure 6). Although deletion of 40 amino acids produced a powerfully toxic molecule, ablation of eight amino acids within this domain (PrP_{ΔPHC}) was innocuous to both wild-type and *Prnp^{0/0}* mice. Crossing experiments show that PrP_{ΔPHC} is not functionally equivalent to PrP^C. The toxicity of PrP_{ΔF} was diminished, yet that of PrP_{ΔCD} was augmented by coexpression of PrP_{ΔPHC}. In the frame of the signaling model, the deletion in PrP_{ΔPHC} may affect the interaction between PrP^C and PrP_R.

Verification of the model presented above requires the physical identification of PrP_R. Towards that goal, it will be crucial to enumerate the cellular constituents—which may not necessarily all consist of protein—binding differentially to PrP^C and to the various mutants described here. Functional assays of such constituents may finally shed light on the physiological function of the prion protein, and may eventually help understanding neurodegeneration in transmissible spongiform encephalopathies.

Materials and methods

Construction of transgenes

The coding sequence of the murine *Prnp* gene was analyzed using DNAMAN software (Lynnon BioSoft, Canada) and a hydrophobicity plot was generated using a window of nine amino-acid residues. The regions identified in this plot were used to define the CC, CD, and HC domains. Based on pPrPHG (Fischer *et al*, 1996), a *PmeI*/*NheI* subclone was generated in the pMECA backbone. To create the *Prnp_{ΔCD}* cDNA, this plasmid was used as template to obtain two

PCR fragments with primer sets RAMP fw (5'-CTA TCA GTC ATC ATG GCG AAC C)/RAMP 93 ex-rc (5'-CCA AAA TGG ATC ATG GGC CTA CCC CCT CCT TGG CCC CAT C) and RAMP 93-134 fw (5'-GAT GGG GCC AAG GAG GGG GTA GGC CCA TGA TCC ATT TTG G)/RAMP rc (5'-CAT CAT CTT CAC ATC GGT CTC G). The resulting PCR products were purified, mixed in stoichiometric amounts, and annealed by slowly decreasing the temperature from 95 to 40°C. The flanking primers RAMP fw and RAMP rc were then added and the fusion product was amplified. This product was purified, digested with *XmaI*/*Bst*II, and cloned into the respective sites of the pMECA PrP subcloning vector. The subcloned construct was then cloned into the *PmeI*/*NheI* sites of the pPrPHG plasmid. Plasmid MoPrP-ΔH1 coding for PrP_{ΔH1} was constructed by inserting the cDNA encoding moPrPΔ114-121, recovered from plasmid pUC-PrPΔ114-121 (Holscher *et al*, 1998) by restriction with *AatII* and *SacI*, followed by blunting into plasmid MoPrP.XhoI, a derivative of pPrPHG (Borchelt *et al*, 1996; Fischer *et al*, 1996) upon restriction with *XhoI* and blunting.

Generation, identification, and maintenance of transgenic mice

For construction of PrP_{ACD} and PrP_{PHC}, the pPrPHG plasmids containing the PrP coding sequences were propagated in *Escherichia coli* XL1 blue, the minigene excised with *NotI* and *Sall*, processed as described (Fischer *et al*, 1996), and injected into fertilized *Prnp*^{+/+} oocytes (C57BL/6 × DBA/2 cross and C3H × C57BL/6) by standard procedures (Rulicke, 2004). Transgenes on a *Prnp*^{0/0} background were identified by PCR using the exon 2 primer pE2 + (5'-CAA CCG AGC TGA AGC ATT CTG CCT) and the exon 3 primer Mut217 (5'-CCT GGG ACT CCT TCT GGT ACC GGG TGA CGC). In order to outbreed the *Prnp*⁺ allele, PCR analysis was carried out using primers P10 (*Prnp* exon 3, 5'-GTA CCC ATA ATC AGT GGA ACA AGC CCA GC), 3'NC (non-coding region at 3' of exon 3, 5'-CCC TCC CCC AGC TGA GAC CAC GA), and P3 (neoR gene, 5'-ATT CGC AGC GCA TCG CCT TCT ATC GCC); P10 and 3'NC gave a 560-bp signal for the *Prnp*⁺ allele and P3 and 3'NC gave a 362-bp product for the *Prnp*⁰ allele. Alternatively, to test for the presence or absence of the *Prnp*⁺ allele, an additional PCR was performed using primers P2 (*Prnp* int 2, 5'-ATA CTG GGC ACT GAT ACC TTG TTC CTC AT) and P10rev (reverse complementary of P10 5'-GCT GGG CTT GTT CCA CTG ATT ATG GGT AC) giving a product of 352 bp for the *Prnp*⁺ allele.

Southern blot analysis

Mouse tail biopsies were kept on ice and digested in lysis buffer (100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 20 mM NaCl, and 100 μg/ml Proteinase K) overnight at 55°C under constant agitation. Purified genomic DNA (20 μg) was digested overnight (*EcoRI*, 100 U and separated on 0.7% agarose gels (GIBCO BRL ultra Pure agarose). Completeness of the restriction digest and DNA separation was checked by ethidium bromide staining. Genomic DNA was transferred by vacuum blotting at 50–55 mbar with a Vacu Gene Pump and a Gene XL blotting apparatus (Pharmacia) using solutions according to the manufacturer's manual (Pharmacia). The blot was marked, washed for 5 min in 2 × SSC, UV-crosslinked and prehybridized according to Church and Gilbert (1984). After hybridization (overnight at 65°C) with a radioactively labeled probe (PrP open reading frame), the blot was washed and membranes were subjected to autoradiography using a Phosphor-Imager (Molecular Dynamics). Images were scanned and signals were quantified with Aida 2.41 imaging software.

For probe labeling, template DNA (50 ng) was diluted in sterile water and random primers (nonamers or hexamers) were added (Prime-IT[®] II; Random Primer labeling kit cat# 300385; Stratagene). After gentle mixing, incubation was performed at 95°C for 5 min. Brief centrifugation was followed by 3 min incubation on ice. This was followed by addition of 5 × primer buffer (–dCTP), radioactively labeled [α -³²P]-dCTP (corresponding to 25 μCi; Amersham Biosciences), and Klenow 5 U/μl (Prime-IT[®] II; Random Primer labeling kit cat# 300385). For primer elongation, the probe was incubated at 37°C for 75 min. For purification, the QIAquick PCR purification kit was used according to the manufacturer's manual (Quiagen).

mRNA analysis

Total brain RNA was isolated in Trizol (Life Technologies), purified, and DNase-treated according to the manufacturer's manual (Roche). After reverse transcription (Geneamp; Roche), cDNA was used for PCR using primers Taq3'PrPa (5'-CAA CCG AGC TGA AGC

ATT CTG CCT) and Taq3'PrPa (5'-GAT CTT CTC CCG TCG TAA T). As control for the PCR procedure and possible DNA contamination, DNase-treated RNA from wild type and transgenic mice that had not been reversely transcribed was used.

Western blot analysis

Brain hemispheres were homogenized in 7 vol PBS, 0.5% Nonidet P-40, and 0.5% deoxycholate and the solution was centrifuged for 5 min in an Eppendorf centrifuge. For deglycosylation, up to 50 μg of denatured total protein was incubated at 37°C for 4 h with 500 U PNGase F (New England Biolabs) according to the manufacturer's instructions. The protease inhibitors Pefabloc (1 mg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (1 μg/ml) (all from Boehringer, Mannheim), and 0.5 mg/ml EDTA were added. After electrophoresis of protein samples through 12% SDS-polyacrylamide gels, samples were transferred to nitrocellulose membranes (Schleicher & Schuell) and incubated with mouse monoclonal anti-PrP antibodies POM1, POM3, and POM11 (Polymenidou *et al*, 2005), followed by incubation with peroxidase-labeled anti-mouse antiserum (1:2500; Amersham) and developed with the ECL detection system (Pierce). Antibody incubations were performed in 1% Top Block (Juro) in Tris-buffered saline-Tween (TBS-T) for 1 h at room temperature or overnight at 4°C.

Flotation assays

Flotation of detergent insoluble complexes was performed as described (Naslavsky *et al*, 1997). Appropriate brain homogenates were extracted for 2 h on ice in cold lysis buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100; total protein: 1 mg in 1.6 ml). Extracts were mixed with two volumes (3.2 ml) of 60% Optiprep (Nycomed) to reach a final concentration of 40%. All lysates were loaded at the bottom of Beckman ultracentrifuge tubes. A 5–30% Optiprep[®] step gradient in TNE (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 5 mM EDTA) was then overlaid onto the lysate (8.4 ml of 30% Optiprep[®] and 3.6 ml of 5% Optiprep[®]). Tubes were centrifuged for 24 h at 4°C in a TLS55 Beckman rotor at 100 000 g. Fractions (1 ml) were collected from the top of the tube and processed for immunoblotting and visualization with anti-PrP antibody POM1 (Polymenidou *et al*, 2005), anti-flotillin 1, and anti-GAPDH antibody (both from BD Transduction Laboratories).

Clinical scoring and observation

Mice were examined once weekly for clinical signs, including weight loss, kyphosis, rough hair coat, paresis, ataxia, and decreased motor activity. In addition, mice were placed on a metal grid and monitored for 5 min for their walking proficiency. The incidence of slipping between bars was assessed and integrated with all parameters mentioned above into a clinical score (Table I). Mice were euthanized when they reached a score of 3.5 or higher. Statistical significance was assessed using Mann-Whitney test.

Morphological analysis

Brains, spinal cords, and sciatic nerves were removed and fixed in 4% formaldehyde in PBS, pH 7.5, paraffin embedded, and cut into 2–4 μm sections. Sections were stained with hematoxylin and eosin (H&E), Luxol-Nissl (myelin and neurons), and commercial antibodies to GFAP (glial fibrillary acidic protein; activated astrocytes), MBP (myelin basic protein), NF200 (neurofilament 200), IBA1 (microglia), SAF84 (PrP^{Sc} aggregates), and POM1 (soluble cellular PrP). For semi-thin sections and electron microscopy, mice were perfused with ice-cold 4% PFA/2% glutaraldehyde. Spinal cord and sciatic nerve tissues were removed, immersed in the same solutions, and kept at 4°C until processing. Tissues were embedded in Epon, and semi-thin sections were stained with toluidine blue and paraphenylene diamine or used for electron microscopy.

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