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Chapter II

ROLES OF PRION PROTEIN AND PRION PROTEIN-LIKE PROTEIN IN NEURODEGENERATION: IMPLICATION IN THE PATHOGENESIS OF PRION DISEASES

*Suehiro Sakaguchi**
Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8523, Japan,
PRESTO, Japan Science and Technology Agency, Saitama, Japan.

ABSTRACT

The cellular isoform of prion protein, PrP^C, is a glycoprotein tethered to the cell membrane by a glycosylphosphatidylinositol anchor moiety. The conformational conversion of PrP^C into the abnormally folded, amyloidgenic prion protein (PrP^{Sc}) is pivotally involved in the pathogenesis of prion diseases. Indeed, mice devoid of PrP^C (PrP-/-) were resistant to prions, neither accumulating PrP^{Sc}, nor developing the diseases, or propagating prions. On the other hand, PrP-/- mice spontaneously developed various neurological abnormalities, which are similar to those in prion diseases, strongly suggesting the possible involvement of the functional loss of PrP^C in the pathogenesis of the diseases. We and others recently isolated the first PrP-like protein, termed PrPLP/doppel (Dpl), which is toxic to neurons, especially to Purkinje cells, when ectopically expressed in neurons. Interestingly, the neurotoxicity of PrPLP/Dpl could be neutralized by PrP^C. Elucidation of the neuroprotective function of PrP^C and the neutotoxicity of PrPLP/Dpl might be useful for further understanding of the molecular pathogenesis of prion diseases.

* Correspondence concerning this article should be addressed to Suehiro Sakaguchi, Division of Molecular Cytology, Institute for Enzyme Research, The University of Tokushima, 3-18-15 Kuramoto-cho, 770-8503 Tokushima, Japan. E-mail: sakaguch@ier.tokushima-u.ac.jp; Tel: +81-88-633-7438; Fax: +81-88-633-7440.

Keywords: prion protein, prion protein-like protein, neurodegeneration, Purkinje cell, knockout mice, prion disease, intergenic splicing

INTRODUCTION

Prion diseases are a group of transmissible neurodegenerative disorders including Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), kuru, and fatal familial insomnia (FFI) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals [1]. Most cases of CJD are sporadic with unknown etiologies [2]. About 10% of cases are an inherited disease, carrying mutations of the prion protein (PrP) gene [2], and the remaining cases were iatrogenically transmitted via prion-contaminated materials, such as electroencephalogram electrodes, human growth hormone preparations, dura matter and corneal grafts [3-6]. It is also highly suspected that BSE could be orally infected to humans via contaminated food, causing about more than 150 cases of a new variant CJD in young people, especially in England [7,8]. GSS and FFI are an inherited prion disease and kuru is a disease spread via ritualistic cannibalism [1].

The infectious agents of these diseases are the so called prions, mainly consisting of the abnormally folded, amyloidgenic isoform of PrP, designated PrP^{Sc} [1]. PrP^{Sc} is produced by the conformational conversion of the normal cellular isoform of PrP, PrP^{C} [1]. The exact nature of the conversion remains unknown. However, accumulating lines of evidence indicate that the conversion plays pivotal roles in the pathogenesis of the diseases. Due to the constitutive conversion, PrP^{Sc} is markedly accumulated in the infected neurons. In contrast, PrP^{C} is reduced in these neurons, suggesting that PrP^{C} is functionally deficient in the infected neurons.

Gene targeting technology in mice using homologous recombination in embryonic stem cells is very useful to investigate the physiological functions of a gene. We and others produced independent lines of mice devoid of PrP^{C} (PrP-/-) using different targeting strategies [9-13]. PrP-/- mice were resistant to prion diseases [11,14-16], indicating that PrP^{C} is necessary for the development of the diseases and the propagation of prions. Spontaneous abnormal phenotypes were reported in some lines of the mutant mice [17-21]. These neurological abnormalities are markedly similar to those often observed in prion diseases, supporting the idea that the functional loss of PrP^{C} could be involved in the pathogenesis. Very intriguingly, marked phenotypic discrepancies were observed among different lines of PrP-/- mice, some lines of the mice developing and growing normally but the others exhibiting progressive ataxia and Purkinje cell degeneration [9,10,12,13,18]. We and others identified the first PrP-like protein, termed PrPLP/doppel (Dpl), and found that PrPLP/Dpl was ectopically expressed in the neurons of PrP-/- mice showing Purkinje cell degeneration [12,18]. We and others also recently confirmed that the ectopic expression of PrPLP/Dpl was toxic to Purkinje cells deficient in PrP^{C} [22-24]. We do not know so far whether or not PrP^{Sc} might have a PrPLP/Dpl-like neurotoxic potential. However, it is interesting to postulate that PrP^{Sc} exerts neurotoxicity in a manner similar to that of PrPLP/Dpl. I will discuss the roles of PrP^{C} and PrPLP/Dpl in neurodegeneration and its implication in the molecular pathogenesis of prion diseases.

GENERATION AND CHARACTERIZATION OF KNOCKOUT MICE

1. PrP

Human and mouse PrP genes, *Prnp*, consist of two and three exons, located on chromosomes 20 and 2, respectively, and the protein coding sequence is present only in the last single exon [25]. *Prnp* is normally expressed in the central nervous system (CNS), especially in neurons and glial cells, and to a lesser extent in other non-neuronal tissues including the spleen, kidney, lung, and heart [25,26].

PrP^C is a membrane glycoprotein anchored to the cell surface via a glycosyl-phosphatidyl-inositol (GPI) moiety [25]. Mouse PrP^C is first translated as a precursor protein consisting of 254 amino acids (Figure 1). The N-terminal 22 and C-terminal 23 hydrophobic amino acids are removed as a signal peptide and a GPI-anchor signal sequence, respectively, upon the biosynthesis of PrP^C (Figure 1) [25]. The N-terminal half of PrP^C is highly flexible and lacks identifiable secondary structure [27]. This part includes the so called octapeptide repeat region (Figure 1), which is very specific to PrPs and is considered to bind to Cu^{2+} via the histidine residues [28]. However, the exact function of this region remains to be elucidated. The C-terminal half of PrP^C forms a globular structure with three α-helices and two short antiparallel β-strands (Figure 1) [29]. This part contains two N-type glycosylation sites and one disulfide bond between the second and the third helices (Figure 1) [25].

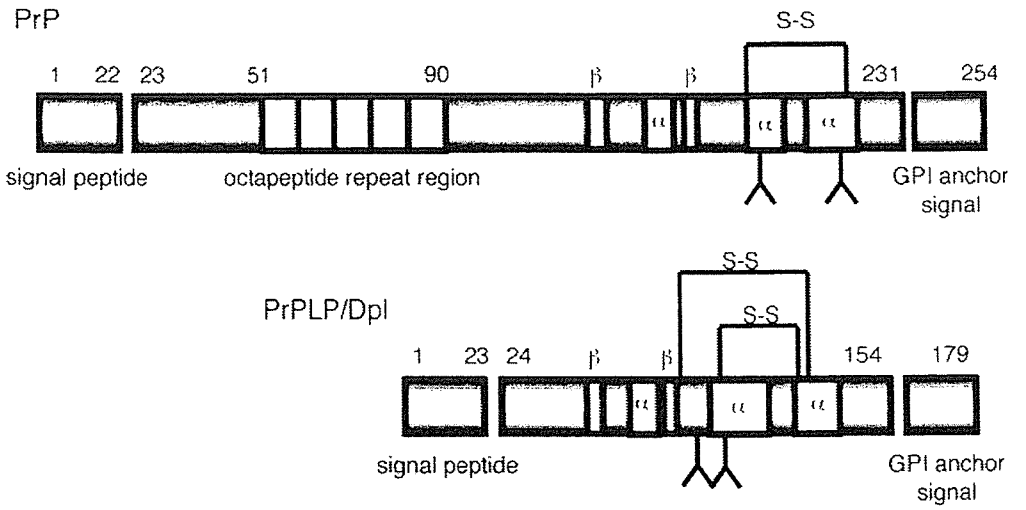

Figure 1. Protein structures of PrP and PrPLP/Dpl. α and β indicate α-helix and β-strand, respectively. S-S and Y indicate a disulfide bond and N-glycosylation, respectively. Arabic figures indicate amino acid positions.

2. Abnormal Brain Functions in PrP Knockout Mice

Büeler et al. produced the first line of PrP-/- mice, Zrch I PrP-/-, in which PrP codons 4-187 of a total of 254 codons were replaced with the neomycin phosphotransferase (neo) gene with the herpes simplex virus thymidine kinase promoter [9]. These mutant mice were born in

accordance with Mendel's law with no obvious congenital deficits [9], indicating that PrP^C could be dispensable for mammalian embryogenesis.

PrP^C is abundantly expressed in pyramidal neurons of the hippocampus [30], in which learning and memory processes are integrated. To investigate the role of PrP^C in these functions, Zrch I PrP-/- mice were subjected to different behavioral tasks, such as a swimming navigation test and a Y-maze discrimination test [9]. The swimming navigation test is thought to be valuable in evaluating spatial learning abilities [31,32], and the Y-maze test in assessing discrimination abilities [33]. The mutant mice performed these tasks as well as control mice [9]. Therefore, Büeler et al. concluded that PrP-null mice have no defects in learning and memory [9]. However, Collinge et al. subsequently reported an intriguing result that long-term potentiation (LTP) was impaired in the hippocampal CA1 neurons of Zrch I PrP-/- mice on electrophysiological studies [17]. LTP is a form of synaptic plasticity that is thought to important for memory formation. Loss of LTP was also reported in a second line of PrP-knockout mice, Npu PrP-/-, which was produced by simple insertion of the neo gene under the control of the mouse metallothioneine promoter into a unique *Kpn* I site in the PrP-coding sequence [10,34]. These results indicate that PrP^C might be involved in the processes of learning and memory.

We produced a third line of PrP-/- mice, Ngsk PrP-/-, by replacement of a 2.1-kb genomic DNA segment including 0.9-kb of intron 2, 10-bp of 5' untranslated region (UTR) of exon 3, the entire PrP open reading frame, and 0.45-kb of 3' UTR with the neo gene under the control of the mouse phosphoglycerate kinase promoter [11]. Ngsk PrP-/- mice were also born normally with no obvious deficits [11]. However, these mutant mice showed poor performance on other behavioral tests, including a water-finding test and a conditioned passive-avoidance test [20]. The water finding test was developed to evaluate latent learning [35], and the passive-avoidance task investigates short- and long-term memory [36]. Taken together with that Zrch I PrP-/- mice normally behaved in a swimming navigation test and a Y-maze discrimination test, these results indicate that PrP^C might be involved in certain types of learning and memory. Therefore, the electrophysiological abnormalities detected in Zrch I PrP-/- and Npu PrP-/- mice might be related to the abnormal behaviors in Ngsk PrP-/- mice. However, no electrophysiological abnormalities were reported in the hippocampus of Zrch I PrP-/- mice by other investigators [37].

Tobler et al. found abnormal sleep and circadian activity in both Zrch I PrP-/- and Npu PrP-/- mice [19], and also demonstrated that these abnormalities were rescued by re-introduction of a transgene encoding PrP^C [19]. The mutant mice slept much more fragmentedly than wild-type mice [19]. The period of circadian activity was 23.3 h in wild-type mice whereas the mutant mice exhibited much longer activity period of 23.9 h under the condition of constant darkness [19]. These results indicate that PrP^C is involved in the regulation of circadian rhythm.

3. Neuropathological Abnormalities in PrP Knockout Mice

We found interesting abnormal phenotypes in Ngsk PrP-/- mice [18,21,38]. These mutant mice spontaneously developed progressive ataxia from around 70 weeks old and showed

poor performance in a rotorod motor coordination test [18]. The cerebellum of the mutant mice was markedly atrophic, compared with those of wild-type mice and Ngsk PrP+/- mice, which are hemizygous for the Ngsk disrupted PrP allele [18]. In these atrophic cerebella, the number of Purkinje cells was dramatically decreased due to degenerative death of the cells (Figure 2) [18]. The molecular layer became much thinner in these mice than in wild-type mice, as a result of the loss of the dendritic trees of Purkinje cells (Figure 2) [18]. We subsequently demonstrated that the ataxia and Purkinje cell degeneration in Ngsk PrP-/- mice could be successfully rescued by re-introduction of multiple copies of a cosmid transgene encoding mouse PrP^C (Figure 2) [21], indicating that the ataxia and Purkinje cell degeneration in Ngsk PrP-/- mice are attributable to loss of PrP^C. The same abnormal phenotypes were also reported in other independent lines of PrP-/- mice, Rcm0 PrP-/- and Zrch II PrP-/- [12,13]. Rcm0 PrP-/- mice were generated by the targeting strategy similar to that in Ngsk PrP-/- mice [12]. Zrch II PrP-/- mice were produced by replacement of 0.27-kb of intron 2, the entire exon 3, and 0.6-kb of the 3' flanking DNA sequence with a specific 34-bp *loxP* sequence [13]. No significant microscopic abnormalities could be detected in the cerebrum of these mice, particularly the cerebral cortex, hippocampus, putamen, thalamus, and hypothalamus [18].

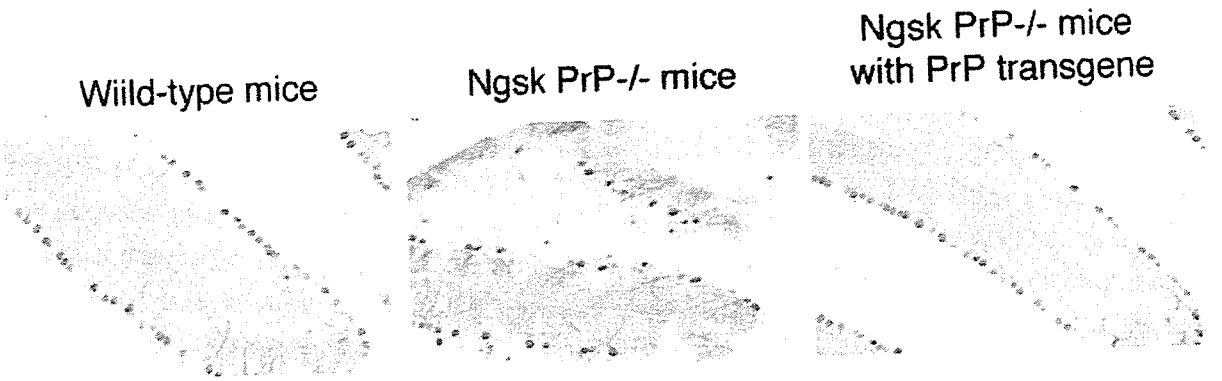

Figure 2. Purkinje cell degeneration in Ngsk PrP-/- mice and rescued Purkinje cells in Ngsk PrP-/- mice reconstituted with PrP transgenes. Purkinje cells were immunohistochemically stained using anti-calbindin antibodies.

Purkinje cells were normally developed in Ngsk PrP-/- mice [18]. The cerebellar atrophy was not evident in Ngsk PrP-/- mice until about 40 weeks after birth [21]. No Purkinje cell loss was obvious in younger Ngsk PrP-/- mice [20]. Therefore, it is conceivable that PrP^C is important for the long-term survival of Purkinje cells. In Ngsk PrP-/- mice aged 43 weeks old, torpedo-like varicosities composed of a swollen disorganized myelin sheath became detectable along the axons of Purkinje cells in spite of the seemingly normal cell bodies and dendritic trees [21]. PrP^C is expressed along the axons and at the axonal terminals of Purkinje cells, but not in the cell bodies and dendrites [30,39]. It is therefore conceivable that loss of PrP^C could cause defects in the axonal organization of Purkinje cells, consequently leading to the degeneration of the cells. In marked contrast to Ngsk PrP-/- mice, Zrch I PrP-/- and Npu PrP-/- mice were reported to grow without any neurological abnormalities, including ataxia and Purkinje cell degeneration [9,10]. We and others recently resolved this puzzling phenotypic discrepancy by identifying that PrPLP/Dpl is ectopically overexpressed in the

brains of ataxic lines of PrP-/- mice, including Ngsk PrP-/-, Rcm0 PrP-/-, and Zrch II PrP-/- mice, but not in non-ataxic lines of Zrch I PrP-/- and Npu PrP-/- mice [12,40], as described below.

We also detected another discrepant phenotype between Ngsk PrP-/- and Zrch I PrP-/- mice [38]. Ngsk PrP-/- mice but not Zrch I PrP-/- mice showed marked activation of glial cells, including astrocytes and microglia, both in the cerebrum and in the cerebellum in an age-dependent manner [38]. As in the case of the ataxia and Purkinje cell degeneration, re-introduction of a cosmid transgene encoding mouse PrP^C could rescue Ngsk PrP-/- mice from this glial activation [38], indicating that the glial activation in Ngsk PrP-/- mice is attributable to the loss of PrP^C and the ectopic expression of PrPLP/Dpl. Astrocytes and microglia have previously been shown to express PrP^C abundantly [41,42].

Demyelination in the spinal cord and peripheral nervous system was a phenotype commonly detected in Ngsk PrP-/- and Zrch I PrP-/- mice [21]. Ngsk PrP-/- mice aged 31 weeks showed many vacuoles in the white matter of spinal cord due to marked demyelination [21]. Most of the vacuoles were surrounded by an enlarged myelin sheath, but in some cases splits within a myelin sheath formed vacuoles [21]. Similar pathologies were observed in the peripheral sciatic nerves of aged Ngsk PrP-/- mice [21]. In these affected nerves, large myelinated fibers were markedly reduced in number and remaining axons were thinly myelinated, in addition to many similar vacuoles [21]. We also found onion bulbs, a multiple layer of myelin sheath formed by multiple episodes of demyelination and remyelination, in the affected nerves [21]. This demyelination in Ngsk PrP-/- mice could be successfully rescued by expressing transgenic mouse PrP^C [21], and the same demyelination was observed in the sciatic nerves of Zrch I PrP-/- mice [21]. These results clearly indicate that PrP^C is involved in the organization of a myelin sheath. Oligodendrocytes and Schwann cells, known to express PrP^C on the surface, form myelin sheaths in the CNS and the peripheral nervous system, respectively [41,43]. It is therefore possible that PrP^C functions as an adhesion molecule within a myelin sheath and/or between a myelin sheath and an axon to form a tightly compacted myelin sheath. Alternatively, PrP^C could be a trophic factor for these glial cells.

PrP-LIKE PROTEIN AND PURKINJE CELL DEGENERATION

1. PrP-like Protein

Prnd encoding PrPLP/Dpl is located about 16-kb downstream of *Prnp* [12]. Mouse *Prnd* consists of three exons and the coding sequence is present in the second single exon [12]. PrPLP/Dpl mRNA is expressed in various tissues of adult wild-type mice, including the testis, heart, spleen and skeletal muscle but not the brain [44].

PrPLP/Dpl is also a GPI-anchored glycoprotein [45]. The precursor protein consists of 179 amino acids and undergoes several modifications on the biosynthesis of PrPLP/Dpl (Figure 1) [45]. The N-terminal 23 and C-terminal 25 hydrophobic residues are removed as a signal peptide and a GPI-anchor signal, respectively (Figure 1) [45]. PrPLP/Dpl is a structural homologue of the C-terminal globular domain of PrP^C, sharing ~23% identical amino acids

comprising three α-helices and two short antiparallel β-strands (Figure 1) [46]. PrPLP/Dpl is N-glycosylated at two sites and contains two disulfide bonds (Figure 1) [45]. However, PrPLP/Dpl lacks the corresponding N-terminal part of PrP^C, which includes the octapeptide repeat region (Figure 1) [12].

2. Physiological Functions of PrP-Like Protein

In contrast to undetectable expression of PrPLP/Dpl in the brains of adult wild-type mice, we found substantial expression of PrPLP/Dpl mRNA in the brains of neonatal mice, preferentially in endothelial cells [44]. PrPLP/Dpl was already expressed 1 day after birth, peaked at about 1 week, and then gradually decreased to an undetectable level by at least 8 weeks [44]. This developmental expression of PrPLP/Dpl in the brain endothelial cells suggests that PrPLP/Dpl is involved in the development of brain blood vessels and/or blood-brain barrier.

On immunohistochemical studies of mouse testes, Behrens et al. demonstrated the specific expression of PrPLP/Dpl in spermatids [47]. Peoc'h et al. also showed that in human testes, spermatozoa and Sertoli cells expressed PrPLP/Dpl [48]. Behrens et al. produced mice devoid of PrPLP/Dpl, the designated $Prnd^{neo/neo}$ mice [47]. No significant abnormalities were observed in the brain endothelial cells of these mutant mice [47]. However, male mutant mice were sterile but female mutant mice were fertile [47]. The testes in these mutant mice seemed developmentally normal [47]. However, the number of spermatozoa and the motility of mutant sperm were significantly decreased [47]. Moreover, the mutant sperm exhibited abnormal morphologies [47]. The authors also showed that the acrosome function was impaired in the mutant spermatozoa [47]. These results clearly indicate that PrPLP/Dpl is involved in spermatogenesis.

3. Ectopic Expression of PrP-Like Protein in PrP Knockout Mice

PrPLP/Dpl was ectopically overexpressed in the brains of PrP-/- mice developing ataxia and Purkinje cell degeneration [12,44]. Newly synthesized pre-mRNAs usually undergo several modifications within the framework of a single gene, such as capping at the 5' end, splicing out intronic sequences, and cleavage and polyadenylation at the 3' end, to convert into mature translatable mRNAs. In wild-type mice, the PrP pre-mRNA is normally cleaved and polyadenylated at the end of *Prnp*, producing the PrP^C-encoding mRNA (Figure 3). However, ataxic lines of PrP-/- mice expressed aberrant chimeric mRNA consisting of the remaining non-coding *Prnp* exons 1 and 2 and the PrPLP/Dpl-coding exons, probably due to the lack of the 3' part of intron 2 including a splice acceptor (Figure 3) [12,40]. The pre-mRNA transcribed from the *Prnp* promoter could not efficiently undergo cleavage/polyadenylation at the end of *Prnp* [12,40], elongated until the last exon of *Prnd*, and subjected to intergenic splicing between the residual *Prnp* exon 2 and the PrPLP/Dpl-coding exon (Figure 3) [12,40]. Therefore, *Prnd* became regulated under the control of the *Prnp* promoter, leading to the ectopic expression of PrPLP/Dpl in the brains of ataxic lines of

PrP-/- mice, especially in neurons and glial cells where the *Prnp* promoter is very active [12,40]. Indeed, PrPLP/Dpl mRNA could be detected in nearly all of neurons including Purkinje cells in these ataxic PrP-/- mice by in situ hybridization [40].

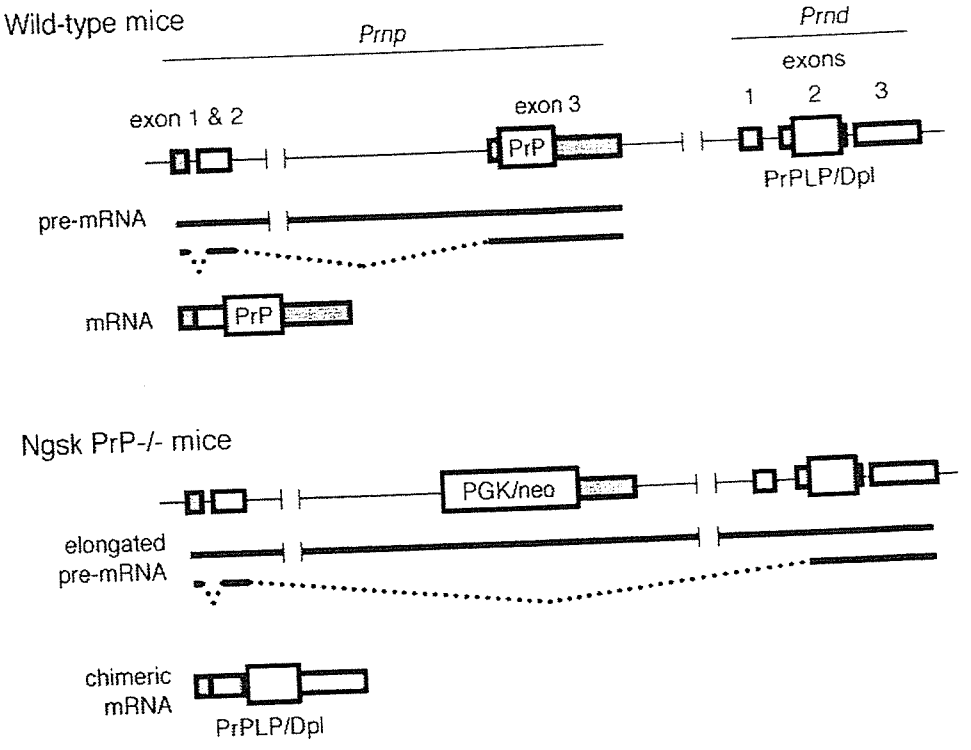

Figure 3. Mechanisms of the generation of the PrPLP/Dpl-encoding chimeric mRNAs via intergenic splicing taking place between *Prnp* and *Prnd* in Ngsk PrP-/- mice.

4. Neurotoxicity of Ectopically Expressed PrP-Like Protein

To investigate whether or not the ectopic expression of PrPLP/Dpl could be involved in the ataxia and Purkinje cell degeneration in ataxic lines of PrP-/- mice, we generated two different types of transgenic (tg) mice, designated tg(N-PrPLP/Dpl) and tg(P-PrPLP/Dpl) [24]. Tg(N-PrPLP/Dpl) mice expressed PrPLP/Dpl specifically in nearly all neurons including Purkinje cells under the control of the neuron-specific enolase promoter, and tg(P-PrPLP/Dpl) mice expressed PrPLP/Dpl only in Purkinje cells under the control of the Purkinje cell protein-2 promoter. Tg(N-PrPLP/Dpl) mice lines 25 and 32 and tg(P-PrPLP/Dpl) mice lines 26 and 27 were crossed with non-ataxic Zrch I PrP-/- mice, and each tg line of mice carrying the Zrch I PrP-/- background was produced [24]. All of the tg(N-PrPLP/Dpl)25 and 32 mice on the Zrch I PrP-/- background developed ataxia at 359±52 and 58±15 days, respectively (Figure 4) [24]. The tg(P-PrPLP/Dpl)26 and 27 mice also developed similar ataxia at 268±28 and 167±13 days, respectively, in the absence of PrP^C (Figure 4) [24]. These ataxic tg mice showed marked degeneration of Purkinje cells [24]. In contrast, neither ataxia nor Purkinje cell degeneration was observed in these tg mice on the wild-type genetic background [24]. These results clearly indicate that PrPLP/Dpl ectopically overexpressed on Purkinje cells is itself neurotoxic to Purkinje cells, and that PrP^C neutralizes

the neutotoxicity of PrPLP/Dpl. Moore et al. reported a similar result that PrPLP/Dpl transgenically driven by the *Prnp* promoter rendered Zrch I PrP-/- mice ataxic due to marked Purkinje cell loss [22]. Anderson et al. also reported that the targeted expression of PrPLP/Dpl to Purkinje cells caused ataxia and Purkinje cell degeneration in Zrch I PrP-/- mice [23].

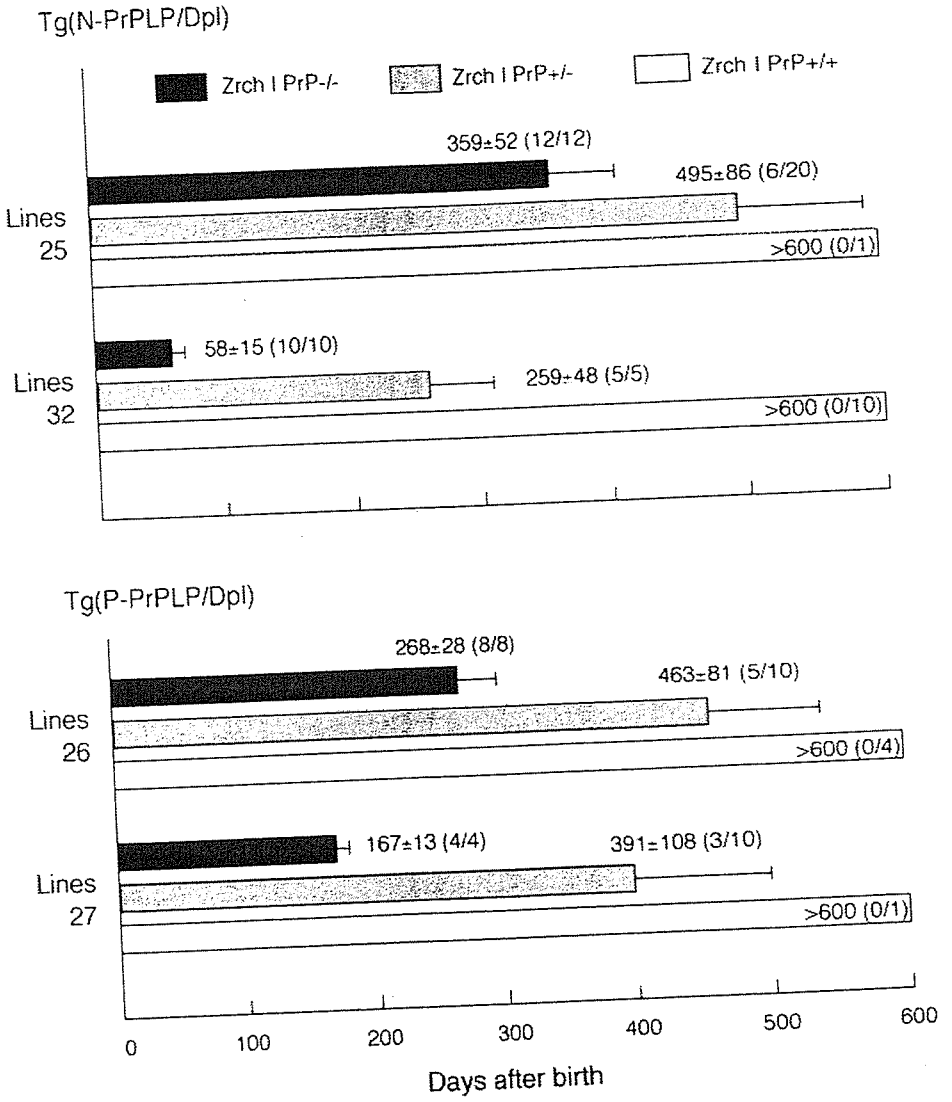

Figure 4. Onset of ataxia in tg(N-PrPLP/Dpl) and tg(P-PrPLP/Dpl) mice carrying the different dosage of Zrch I PrP allele. Numbers in the parenthesis indicate number of ataxic mice /total mice.

We also found that PrPLP/Dpl functionally antagonizes PrP^C to cause ataxia and Purkinje cell degeneration in a stoichiometric manner [24]. Tg(N-PrPLP/Dpl)25 mice expressed PrPLP/Dpl in the cerebrum and cerebellum at a level less than a quarter that of Ngsk PrP-/- mice on Western blotting, and developed ataxia at 359±52 days on the Zrch I PrP-/- background (Figure 4) [24]. Tg(N-PrPLP/Dpl)32 mice expressed PrPLP/Dpl in the cerebrum and cerebellum at a level about 2-3 and 1-2 times more than that of Ngsk PrP-/- mice, respectively, and succumbed to the disease much earlier at 58±15 days on the Zrch I PrP-/- background (Figure 4) [24]. A similar correlation between the onset of ataxia and the

expression levels of PrPLP/Dpl was detected in tg(P-PrPLP/Dpl) mouse lines. Tg(P-PrPLP/Dpl)26 mice, exhibiting weak signals for PrPLP/Dpl in most Purkinje cells on in situ hybridization, developed ataxia at 268±28 days on the Zrch I PrP-/- background, while tg(P-PrPLP/Dpl)27 mice with Purkinje cells much more abundantly expressing PrPLP/Dpl got sick at 167±13 days on the Zrch I PrP-/- background (Figure 4) [24]. This inverse correlation of the incubation time of ataxia and Purkinje cell degeneration to the expression levels of PrPLP/Dpl was also demonstrated by other investigators [13,22]. In contrast, the onset of the ataxia in each line of tg mice with the Zrch I PrP+/- background was greatly prolonged, compared with that of the same line of mice with the Zrch I PrP-/- background [24], indicating the time to ataxia and Purkinje cell degeneration correlates with the expression levels of PrP^C. Tg(N-PrPLP/Dpl)32 mice developed ataxia at 259±48 days on the Zrch I PrP+/- background, and 6/20 tg(N-PrPLP/Dpl)25 mice exhibited similar symptoms at 495±86 days on the Zrch I PrP+/- background (Figure 4) [24]. 5/10 tg(P-PrPLP/Dpl)26 mice and 3/10 tg(P-PrPLP/Dpl)27 mice became ataxic at 463±81 and 391±108 days, respectively, on the Zrch I PrP+/- background (Figure 4) [24].

5. Reverse Genetic Studies on Neuroprotective Function of PrP

Successful rescue of the ataxia and Purkinje cell degeneration in Ngsk PrP-/- mice by introduction of a transgene encoding mouse PrP^C made it possible to perform the reverse genetic studies of PrP^C for its neuroprotectional function against PrPLP/Dpl. We transgenically introduced PrP carrying a familial prion disease-associated mutation (E199K) into Ngsk PrP-/- mice [49], showing that the mutant PrP could fully neutralize the PrPLP/Dpl-induced neurotoxicity to prevent the ataxia and Purkinje cell degeneration. This result suggests that other disease-associated mutant PrPs are also functionally competent. We also introduced PrP with a deletion of the N-terminal residues 23-88 (MHM2.del23-88) into Ngsk PrP-/- mice [49]. Interestingly, Ngsk PrP-/- mice expressing the deletion mutant developed ataxia and Purkinje cell degeneration on a time course identical to that of non-transgenic Ngsk PrP-/- mice [49], clearly indicating that PrP^C neutralizes the neurotoxicity of PrPLP/Dpl through its N-terminal residues 23-88. The deleted part contains the Cu^{2+}-binding octapeptide repeat region, strongly suggesting that this octapeptide repeat region could be important for PrP^C to protect Purkinje cells from PrPLP/Dpl-induced neurodegeneration.

Ischemic Brain Damage in PrP Knockout Mice

We demonstrated that male but not female Zrch I PrP-/- mice were very susceptible to transient forebrain ischemia compared to control C57BL/6 wild-type mice, developing marked apoptotic neuronal death in the hippocampal CA1 region (Figure 5) [50]. McLennan et al. also reported that permanent occlusion of the middle cerebral artery increased the infarction volume in male PrP-null mice [51]. These results indicate that PrP^C is involved in neuroprotection against brain ischemia. No increased susceptibility in female Zrch I PrP-/- mice suggests that the neuroprotective function of PrP^C is masked by female-specific

neuroprotective factor(s). Interestingly, we also showed that, in contrast to the case of Zrch I PrP-/- mice, both male and female Ngsk PrP-/- mice exhibited severe ischemic damage to CA1 neurons (Figure 5) [50]. These results strongly suggest that PrPLP/Dpl counteracts the female-specific neuroprotective function observed in Zrch I PrP-/- mice, increasing the susceptibility of PrP^C-deficient neurons to ischemic insults.

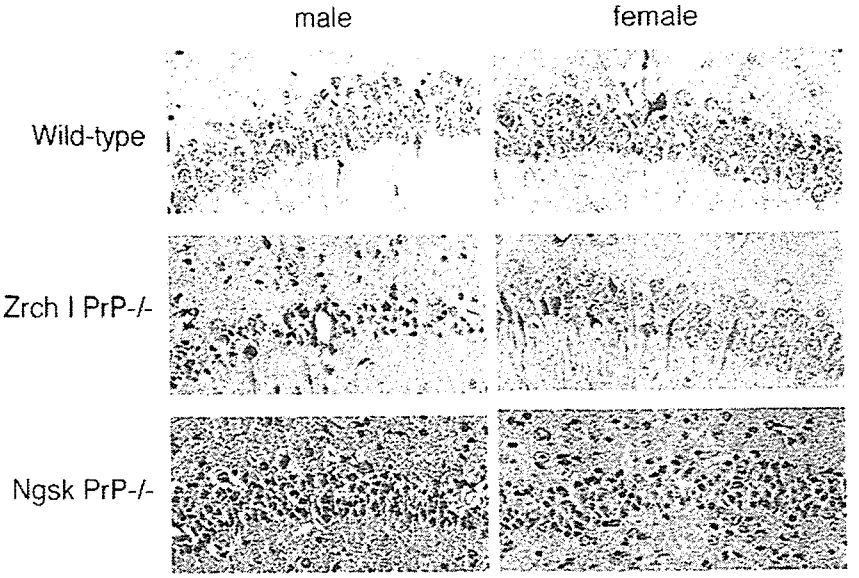

Figure 5. Neuronal cell death in wild-type, Zrch I PrP-/-, and Ngsk PrP-/- mice due to 10-min transient brain ischemia.

ROLES OF PRP AND PRP-LIKE PROTEIN IN NEURODEGENERATION

The C-terminal part of PrP^C, a homologous part to PrPLP/Dpl, was shown to be markedly neurotoxic similarly to PrPLP/Dpl. Shmerling et al. first reported that the N-terminally truncated PrPs, PrPΔ32-121 and PrPΔ32-134, lacking the N-terminal residues 32-121 and 32-134, respectively, caused ataxia and cerebellar degeneration characterized by marked granule cell death in Zrch I PrP-/- mice [52]. Both PrPΔ32-121 and PrPΔ32-134 lack the octapeptide repeat and central hydrophobic region. The neurotoxicity of these truncated PrPs was antagonized by the expression of full-length of mouse PrP^C [52]. However, in contrast to Ngsk PrP-/- mice, these mice showed no Purkinje cell degeneration [52]. This is probably because the truncated PrPs were not expressed in Purkinje cells due to the limited activity of the promoter they used [53]. Indeed, Flechsig et al. subsequently demonstrated that ataxia and Purkinje cell loss could be induced by the targeted expression of PrPΔ32-134 to Purkinje cells of Zrch I PrP-/- mice [53]. The exact mechanism of how PrPLP/Dpl and the truncated PrPs induce Purkinje cell degeneration and how PrP^C antagonizes the neurotoxicity of PrPLP/Dpl and the truncated PrPs has not been understood.

Weissmann and colleagues have proposed a hypothesis [52,54]. They hypothesized two conjectural molecules. One is another PrP-like protein, named protein π, and the other is a cognate ligand for PrP^C. PrP^C and protein π elicit a signal necessary for Purkinje cell survival

upon interaction with the ligand [52,54]. In non-ataxic PrP-/- mice, the protein π signaling remains intact and therefore Purkinje cells are healthy. However, PrPLP/Dpl and the N-terminally truncated PrPs compete with protein π for the ligand and disturb the signal, resulting in Purkinje cell death. This hypothesis can be verified only when the putative molecules are identified. It was demonstrated that copper could bind not only to PrP^C but also to PrPLP/Dpl [55-57]. Hence, copper might be a candidate for the hypothetical ligand.

An alternative explanation could be possible. In contrast to PrPΔ32-121 and PrPΔ32-134, PrPΔ23-88 has never shown neurotoxicity in Zrch I PrP-/- mice [58], indicating that the residues between 88 and 121 are important for PrP not to become PrPLP/Dpl-like neurotoxic protein. This region, overlapping with the hydrophobic region, forms part of the binding sites for the heat shock protein, stress-inducible protein 1 [59], and the extracellular matrix constituent, glycosaminoglycans [60]. It is therefore conceivable that interaction of PrP^C with these associating molecules might be important for PrP^C not to be neurotoxic, and that PrPLP/Dpl and the truncated PrPs are unable to interact with these molecules, therefore resulting in neurotoxic proteins. However, PrPΔ23-88 has no potential to antagonize PrPLP/Dpl [49], indicating that these associating molecules are unlikely involved in the neuroprotecive function of PrP^C.

It is also possible that PrPLP/Dpl could produce oxidative stress toxic to Purkinje cells and that PrP^C could detoxify it. PrP^C is suggested to regulate antioxidant activity by activating Cu^{2+}-dependent antioxidant enzymes such as superoxide dismutase via transfer of the octapeptide repeat-bound Cu^{2+} to the enzymes [28]. Indeed, the primary cultured cerebellar neurons from non-ataxic Npu PrP-/- mice were more sensitive to oxidative stress than those from wild-type mice [61]. Wong et al. reported that oxidative stress was much more elevated in the brains of ataxic Rcm0 PrP-/- mice than in those of non-ataxic Npu PrP-/- mice [62]. Interestingly, Cui et al. showed that recombinant PrPLP/Dpl is toxic to the cultured cerebellar neurons of non-ataxic Zrch I PrP-/- mice inducing apoptotic cell death and concomitantly increased the expression of inducible and neuronal NO synthases and heme oxygenase-1, molecular markers for oxidative stress [63]. They also demonstrated that a pharmacological inhibitor of NO synthases, L-*N*-acetyl methyl ester (NAME), could protect the neurons from the apoptosis [63]. These results suggest that PrPLP/Dpl is involved in increase in oxidative stress.

It is also conceivable that PrP^C is an anti-apoptotic protein while PrPLP/Dpl is pro-apoptotic. Kuwahara et al. reported that hippocampal neuronal cells from ataxic PrP-/- mice easily undergo apoptosis after withdrawal of serum, and that the apoptosis could be prevented by either re-introduction of PrP^C or by expressing an anti-apoptotic protein, Bcl-2, exogenously [64]. Bounhar et al. showed that PrP^C protected human primary neurons from the apoptosis induced by the pro-apoptotic protein Bax [65], and that PrP lacking the octapeptide repeat region completely lost this neuroprotective potential [65]. This result seems to be consistent with the unsuccessful rescue of the ataxia and Purkinje cell degeneration by PrP(MHM2.del23-88) [49].

It is further possible that loss of PrP^C renders neurons susceptible to apoptosis via dysregulating homeostasis of intracellular Ca^{2+}, and that the ectopic expression of PrPLP/Dpl increases the intracellular Ca^{2+} load, enhancing the susceptibility of the neurons to the apoptosis. Dysregulated excess of intracellular Ca^{2+} has been shown to be critically involved

in the apoptosis of neurons [66]. Abnormal Ca^{2+}-activated K^+ currents were reported in hippocampal pyramidal neurons of Zrch I PrP-/- mice and the mutant mice showed altered intracellular Ca^{2+} in cerebellar granule cells [67,68]. We showed that the T- and L-type Ca^{2+}-antagonist, flunarizine, significantly reduced the ischemic neuronal apoptosis in Ngsk PrP-/- mice [50], suggesting that the Ca^{2+}-dependent signaling is involved in the PrPLP/Dpl-mediated neurotoxicity.

CONCLUSION

There has been a strong argument that the conformational conversion of PrP^C to PrP^{Sc} plays an essential role in the pathogenesis of prion diseases [11,14-16,69], but the exact nature of this role has not been fully understood. The constitutive conversion causes the accumulation of PrP^{Sc} in the affected brain. In contrast, the conversion reduces PrP^C in the affected neurons. Indeed, Yokoyama et al. showed that the PrP^C-specific immunoreactivity was decreased in the brain regions where PrP^{Sc} had accumulated in experimentally infected mice [70].

Neurological abnormalities, including impairment of LTP, alteration in sleep and circadian rhythm, demyelination in the spinal cord and peripheral nervous system, were identified in PrP-/- mice without the ectopic expression of PrPLP/Dpl [17,19,21]. LTP is a form of synaptic plasticity that is thought to underlie memory formation. Memory loss or dementia is a common symptom in prion diseases [69]. Thus, it is conceivable that the functional loss of PrP^C might be relevant to the dementia seen in prion diseases. Alteration in sleep and circadian rhythms is also a symptom characteristic for the inherited human prion disease, fetal familiar insomnia [69]. Demyelinating peripheral neuropathy has been reported in some cases of inherited prion disease associated with the E200K mutation of PrP [71,72]. It is therefore strongly suggested that the functional loss of PrP^C is involved in the pathogenesis of the diseases.

The ectopic expression of PrPLP/Dpl, PrPΔ32-121, or PrPΔ32-134 further caused other similar neurological abnormalities in PrP-/- mice, such as ataxia, degeneration of Purkinje cells and granule cells, and gliosis [22,53]. Ataxia is one of the major neurological symptoms, and Purkinje cells and granule cells are markedly degenerative in human prion diseases [69]. Gliosis is invariably observed in prion diseases [69]. However, PrPLP/Dpl was undetectable in the brains affected by experimental prion diseases [73], indicating that PrPLP/Dpl itself is unlikely involved in the pathogenesis of prion diseases. PrP^{Sc} is markedly accumulated in the affected neurons. Forloni et al showed that an amyloidgenic PrP peptide (PrP106-126) was highly toxic to primary cultured neurons, arguing that PrP^{Sc} could itself be neurotoxic [74]. Therefore, it might be possible that the accumulated PrP^{Sc} or its fragmented products, which are often observed in the affected brains [75], possess a neurotoxic potential equivalent to that of PrPLP/Dpl, PrPΔ32-121, or PrPΔ32-134. Elucidation of the molecular mechanism for the Purkinje cell degeneration detected in ataxic lines of PrP-/- mice could be useful for further understanding of the molecular pathogenesis of prion diseases.

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