Dominant-negative Effects of the N-terminal Half of Prion Protein on Neurotoxicity of Prion Protein-like Protein/Doppel in Mice*

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From the † Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. The abbreviations used are: PrP, prion protein; Dpl, doppel; OR, octapeptide repeat (1–124) of prion protein and doppel in the pre-octapeptide repeat (cis part of prion protein has a neuroprotective potential acting both days in a background devoid of prion protein. In addition, the neurons failed to develop any neurological signs for up to 730 days in a background devoid of prion protein. In addition, the fusion protein prolonged the onset of ataxia in mice expressing exogenous doppel. These results suggested that the N-terminal part of prion protein has a neuroprotective potential acting both cis and trans on doppel. We also show that prion protein lacking the pre-octapeptide repeat (Δ25–50) or octapeptide repeat (Δ51–90) region alone could not impair the antagonistic function against doppel.

Prion protein-like protein/doppel is neurotoxic, causing ataxia and Purkinje cell degeneration in mice, whereas prion protein antagonizes doppel-induced neurodegeneration. Doppel is homologous to the C-terminal half of prion protein but lacks the amino acid sequences corresponding to the N-terminal half of prion protein. We show here that transgenic mice expressing a fusion protein consisting of the N-terminal half, corresponding to residues 1–124, of prion protein and doppel in neurons failed to develop any neurological signs for up to 730 days in a background devoid of prion protein. In addition, the fusion protein prolonged the onset of ataxia in mice expressing exogenous doppel. These results suggested that the N-terminal part of prion protein has a neuroprotective potential acting both cis and trans on doppel. We also show that prion protein lacking the pre-octapeptide repeat (Δ25–50) or octapeptide repeat (Δ51–90) region alone could not impair the antagonistic function against doppel.

The normal prion protein (PrPSc) is a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein expressed most abundantly in the central nervous system, particularly in neurons, and to a lesser extent in non-neuronal tissues, including the heart, lung, spleen, and kidney (1, 2). It is well known that conformational conversion of PrPSc into the abnormally folded amyloidogenic isoform, PrPSc, plays a pivotal role in the pathogenesis of transmissible spongiform encephalopathies or prion diseases, including Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy in cattle (1, 3). However, the physiological function of PrPC remains largely unknown.

We and others identified a novel gene, Prnd, that encodes a GPI-anchored PrP-like protein, termed Doppel (Dpl), 16 kb downstream of the murine PrP gene Prnp (4, 5). Dpl is expressed in the testis, heart, kidney, and spleen of wild-type mice but not in the brain where PrPSc is actively expressed. Intriguingly, some lines of mice devoid of PrPSc (Prnp0/0), including Ngsk, Rcm0, and Zrch II, ectopically expressed Dpl in their brains, particularly in neurons, because of an unusual intergenic splicing between Prnp and Prnd, developed ataxia, and Purkinje cell degeneration (5, 6). However, others, such as Zrch I and Npu, neither ectopically expressed Dpl nor exhibited ataxia and Purkinje cell degeneration (4, 5). It was finally confirmed that Dpl is neurotoxic, and PrPSc antagonizes the neurotoxicity of Dpl by a demonstration that transgenically expressed Dpl caused ataxia and Purkinje cell degeneration in nonataxic Zrch I Prnp0/0 mice but not in wild-type mice (7–9). However, the exact mechanism of the antagonistic interaction of PrPSc and Dpl remains unknown.

Dpl shares 23% identity in amino acid composition with PrP (4, 5) and bears conformational similarity to the C-terminal globular domain of PrPSc, both comprising three α-strands and two short β-strands (10). However, Dpl lacks the amino acid sequences corresponding to the N-terminal half of PrPSc (4, 5). Interestingly, it was shown that PrP with truncated N-terminal residues 32–121 or 32–134, termed PrPΔ32–121 or PrPΔ32–134, respectively, exhibited neurotoxicity similarly to that of Dpl, causing ataxia and cerebellar neurodegeneration in nonataxic Zrch I Prnp0/0 mice but not in wild-type mice (11, 12). Therefore, it might be possible that the neurotoxicity of Dpl is attributable to lack of the corresponding N-terminal part of PrPC. However, this remains to be elucidated.

We previously showed that the N-terminal residues 23–88 of PrPSc are involved in the antagonistic function of PrPSc against the Dpl neurotoxicity by demonstrating that PrP lacking the residues 23–88 completely lost the ability to rescue Ngsk Prnp0/0 mice from Dpl-induced Purkinje cell degeneration (13). Residues 23–88 include most of the PrP-specific octapeptide

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‡ The abbreviations used are: PrP, prion protein; Dpl, doppel; OR, octapeptide repeat; GPI, glycosylphosphatidylinositol; tg, transgenic; dtg, double transgenic; SOD, superoxide dismutase; PNGase, peptide:N-glycosidase.

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repeat (OR) region, which includes residues 51–90. Recent lines of evidence from cell culture experiments show that the OR may be involved in the neuroprotective function of PrPC (14–16). However, the biological relevance of OR in the neuroprotective function of PrPC against Dpl is not yet understood in vivo.

In this study, we generated transgenic (tg) mice, tg(PrPΔOR) and tg(PrPΔpreOR), expressing PrP lacking OR and Dpl fused with the N-terminal half of PrPC, respectively. We also produced tg(PrPΔpreOR) mice expressing PrP without the pre-OR region. By intercrossing these tg mice with mice transgenically overexpressing Dpl in neurons on the genetic background of nonataxic Zrch I Prnp0/0, we investigated whether or not these mutant molecules could antagonize Dpl neurotoxicity, rescuing mice from ataxia and Purkinje cell degeneration.

**Experimental Procedures**

Construction of Transgenes—A DNA fragment corresponding to the N-terminal residues 1–124 of PrP was first amplified by PCR with primers a and c (5’-cttcacaatgaa-3’; the underlined and italic sequences correspond to residues 58–179 of Dpl together with 1–24 of PrP was first amplified by PCR with primers a and d (5’-cttcacaatgaa-3’; the underlined and italic sequences correspond to DNA encompassing residues 91–95 of PrP at the 3’ site) using PrP cDNA as a template. The resulting DNA fragment corresponding to residues 91–95 of PrP at the 3’ site was then utilized as a 5’ primer to amplify another DNA fragment corresponding to residues 91–254 of PrP together with primer g using PrP cDNA as a template, resulting in amplification of a DNA fragment for the deletion protein PrPΔOR consisting of residues 1–50 and 91–254 of PrP.

After DNA sequence confirmation of the amplified fragment, it was inserted into a unique Sall site of the Syrian hamster PrP cosmid vector, CosSHa.tet (InPro Biotechnology, Inc.), to construct the PrPΔOR transgene.

Generation of Transgenic Mice—The plasmid-derived sequences were removed from each of the transgene constructs, and the resulting DNAs were injected into the zygotes of C57BL/6 mice to generate tg mice as described elsewhere (17, 18).

Expression Vectors for Wild-type PrPΔC, PrPΔpreOR, PrPΔOR, and PrPΔ23–88—The DNA fragments encoding wild-type mouse PrPΔC and PrPΔ23–88 were amplified by PCR with a sense primer (5’-cttcacaatgaa-3’; the underlined sequence corresponds to a BamHI site; the boldface sequence corresponds to a start codon) and an antisense primer (5’-cttcacaatgaa-3’; the underlined sequence corresponds to an XbaI site; the boldface sequence corresponds to a stop codon) using mouse genomic DNA extracted from wild-type mice and PrPΔOR consisting of residues 1–124 of PrP and residues 58–179 of Dpl. After DNA sequence confirmation of the amplified fragment, it was inserted into a unique Sall site of the Syrian hamster PrP cosmid vector, CosSHa.tet (InPro Biotechnology, Inc. South San Francisco, CA), to construct the PrPΔOR transgene.

A DNA fragment corresponding to the N-terminal residues 1–24 of PrP was first amplified by PCR with primers a and c (5’-cttcacaatgaa-3’; the underlined sequences correspond to DNAs encompassing residues 51–55 and 20–24 of PrP, respectively) using PrP cDNA as a template. The resulting DNA fragment containing the DNA sequence corresponding to residues 51–55 of PrP at the 3’ site was then utilized as a 5’ primer to amplify another DNA fragment corresponding to residues 51–254 of PrP together with primer g (5’-cttcacaatgaa-3’; the underlined sequence corresponds to the HindIII and Xhol sites, and the boldface sequence corresponds to a stop codon) using PrP cDNA as a template, resulting in amplification of a DNA fragment for the deletion protein PrPΔpreOR consisting of residues 1–24 and 51–254 of PrP. After DNA sequence confirmation of the amplified fragment, it was inserted into an unique Sall site of the Syrian hamster PrP cosmid vector, CosSHa.tet (InPro Biotechnology, Inc.), to construct the PrPΔpreOR transgene.

A DNA fragment corresponding to the N-terminal residues 1–50 of PrP was first amplified by PCR with primers a and d (5’-atgagtagacagctctctctttttggttcgagggcc-3’, the underlined and italic sequences correspond to DNAs encompassing residues 91–95 and 46–50 of PrP, respectively) using PrP cDNA as a template. The resulting DNA fragment containing the DNA sequence corresponding to residues 91–95 of PrP at the 3’ site was then utilized as a 5’ primer to amplify another DNA fragment corresponding to residues 91–254 of PrP together with primer g using PrP cDNA as a template, resulting in amplification of a DNA fragment for the deletion protein PrPΔOR consisting of residues 1–50 and 91–254 of PrP. After DNA sequence confirmation of the amplified fragment, it was inserted into a unique Sall site of the Syrian hamster PrP cosmid vector, CosSHa.tet (InPro Biotechnology, Inc.), to construct the PrPΔOR transgene.
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mals were cared for in accordance with the Guidelines for Animal Experimentation of Nagasaki University.

**Diagnosis of Ataxia**—The behavior of mice was inspected at least every 3 days evaluating difficulties for walking straight or trembling in their hindquarters on initiation of movement and during walking. When mice showed such abnormal behaviors, they were subjected to a second inspection at least 3 days later. At this time, if the same or exacerbated symptoms were obvious, mice were diagnosed with ataxia, and the date of the first recognition of the abnormal behaviors was registered as the onset of the ataxia. If the symptoms were trivial or difficult to diagnose as ataxia by an investigator, another investigator also inspected the mice to confirm the symptoms. In this case, mice were not diagnosed as ataxia until the two investigators independently confirmed the symptoms.

**Western Blotting**—Homogenates (10%, w/v) were prepared in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, and protease inhibitor mixture (Nakalai Tesque Co., Kyoto, Japan) and centrifuged at low speed. Protein concentrations of the resulting supernatant were determined using the BCA protein assay kit (Pierce). Total proteins were electrophoresed through a 12% SDS-polyacrylamide gel and electrically transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp.). The membrane was immersed in 5% nonfat dry milk containing TBST (0.1% Tween 20, 100 mM NaCl, 10 mM Tris-HCl, pH 7.6) for 1 h at room temperature and incubated with M20 goat polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), SAF32 mouse monoclonal antibody (SPI-BIO, Montigny le Bretonneux, France), or FL176 rabbit polyclonal antibodies against human Dpl (Santa Cruz Biotechnology, Santa Cruz, CA) expressed under the control of the hamster PrP promoter (Fig. 1A). Therefore, to evaluate the effects of the N-terminal region of PrP on Dpl in cis, the PrP N-terminal residues 1–124 were fused to the Dpl residues 58–179 to make the PrP N-Dpl transgene (Fig. 1A). The PrPΔpreOR and PrPΔOR, PrP deletion mutants lacking the N-terminal residues 25–50 and 51–90, respectively, were also constructed to examine the involvement of each region in protection from the Dpl-induced neurodegeneration (Fig. 1A). Therefore, to evaluate the effects of the N-terminal region of PrP on Dpl in cis, the PrP N-terminal residues 1–124 were fused to the Dpl residues 58–179 to make the PrPΔpreOR and PrPΔOR transgenes. All of these founders successfully transferred the transgenes into their offspring. These tg mice were successively intercrossed with nonatxoid Zrch1 Prnp<sup>0/0</sup> mice to eliminate endogenous PrP<sup>0</sup>.

RESULTS

**Generation and Characterization of tg(PrPN-Dpl), tg(PrPΔpreOR), and tg(PrPΔOR) Mice**—The amino acid alignment of PrP and Dpl depicts the homology between the C-terminal regions of the two proteins, corresponding to the residues 125–254 of PrP and 51–179 of Dpl, both of which form a neurotoxic globular structure with three α-helices and two β-strands (Fig. 1A). Therefore, to evaluate the effects of the N-terminal region of PrP on Dpl in cis, the PrP N-terminal residues 1–124 were fused to the Dpl residues 58–179 to make the PrPN-Dpl transgene (Fig. 1A). The PrPΔpreOR and PrPΔOR, PrP deletion mutants lacking the N-terminal residues 25–50 and 51–90, respectively, were also constructed to examine the involvement of each region in protection from the Dpl-induced neurodegeneration (Fig. 1A). We introduced each corresponding DNA into the Syrian hamster PrP cosmig vector, CosSha.6itet (20), allowing each of the mutant proteins to be expressed under the control of the hamster PrP promoter (Fig. 1B). These transgenes were then microinjected into fertilized eggs of C57BL/6 mice, yielding four founders from the PrPN-Dpl transgene and two from each of the PrPΔpreOR and PrPΔOR transgenes. All of these founders successfully transferred the transgenes into their offspring. These tg mice were successively intercrossed with nonatxoid Zrch1 Prnp<sup>0/0</sup> mice to eliminate endogenous PrP<sup>0</sup>.
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The expression of mutant proteins was confirmed in 1/2 Ig(PrP ΔpreOR)/Prnp$^{0/0}$, 2/2 Ig(PrPΔOR)/Prnp$^{0/0}$, and 1/4 Ig(PrPN-Dpl)/Prnp$^{0/0}$ mice by Western blotting. As shown in Fig. 2A, goat M-20 antibodies against the C-terminal PrP peptide visualized bands in the cerebellar tissue homogenates from Ig(PrPΔpreOR)/Prnp$^{0/0}$ and Ig(PrPΔOR)/Prnp$^{0/0}$ mice (left panel, lanes 4 and 5). These bands migrated slightly faster than authentic PrPC of wild-type mice. But M20 antibodies did not detect any immunoreactivities in Ig(PrPN-Dpl) mice (Fig. 2A, lane 7). On the other hand, SAF32 anti-OR antibodies revealed signals in the cerebellum of Ig(PrPΔpreOR)/Prnp$^{0/0}$ and Ig(PrPΔOR)/Prnp$^{0/0}$ mice, but not in Ig(PrPΔOR)/Prnp$^{0/0}$ mice (Fig. 2A, right panel). In situ hybridization showed that each transgene was ubiquitously expressed over the brain, with the strongest signals being detectable in Purkinje cells (Fig. 2B) and hippocampal neurons (data not shown). In Ig(PrPN-Dpl) mice, some Purkinje cells were faintly stained because of non-specific hybridization of the probe because no PrP could be detected by Western blotting (Fig. 2, A, lane 1, and B).

We also performed immunohistochemical analysis of cerebella from these Ig mice with the Zrch 1 Prnp$^{0/0}$ background using two different antibodies, rabbit polyclonal IBL-N and mouse monoclonal ICSM-18 antibodies, which are directed against residues 24–37 and 146–159 of murine PrP, respectively. Both antibodies showed no immunoreactivities in the cerebella of Zrch 1 Prnp$^{0/0}$ mice (Fig. 3, E–H). In contrast, the molecular and granule cell layers of normal C57BL/6 mice were clearly stained with both antibodies (Fig. 3, A–D). However, there seemed to be no immunoreactivity in the Purkinje cell layer (Fig. 3, A–D). These staining patterns of PrPC in the cerebellum of normal mice were consistent with previous reports (21–23). PrPΔpreOR mutant protein was expressed in the cerebellum of Ig(PrPΔpreOR)/Prnp$^{0/0}$ mice indistinguishably from PrPC in C57BL/6 mice, detectable in the molecular and granule cell layers but not in the Purkinje cell layer (Fig. 3, K and L). PrPΔOR and PrPN-Dpl mutant proteins were also expressed in the molecular and granule cell layers of Ig(PrPΔOR)/Prnp$^{0/0}$ and Ig(PrPN-Dpl)/Prnp$^{0/0}$ mice, respectively (Fig. 3, M–R). However, the mutant proteins were more abundant in the granule cell layer than in the molecular layer (Fig. 3, M–R). Moreover, in Ig(PrPΔOR)/Prnp$^{0/0}$ mice, the Purkinje cell layer was...
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FIGURE 3. Cytological distribution of PrPΔpreOR, PrPΔOR, and PrPN-Dpl in the cerebella of tg mice. The cerebellar sections from C57BL/6 (A–D), Zrch 1 Prmp0/0 mice (E–H), tg(PrPΔpreOR)/Prnp0/0 mice (I–L), tg(PrPΔOR)/Prnp0/0 mice (M–P), and tg(PrPN-Dpl)/Prnp0/0 mice (Q–T) were subjected to immunohistochemistry using IBL-N and ICSM-18 antibodies, which are directed against PrP residues 24–37 and 146–159, respectively.

devoid of the signal, but the basolateral area surrounding some but not all Purkinje cells was strongly stained (Fig. 3, M and N and Fig. 7). In tg(PrPN-Dpl)/Prnp0/0 mice, the cell bodies of Purkinje cells appeared positive, and some cells scattered in the granule cell layer were strongly stained in the cell bodies (Fig. 3, Q and R). These cells are currently unidentified. Moreover, cortical neurons of tg(PrPN-Dpl)/Prnp0/0 mice but not wild-type and tg(PrPΔOR)/Prnp0/0 mice were positively stained in the cell bodies by IBL-N antibodies (data not shown).

PrPN-Dpl Delays Onset of Dpl-induced Ataxia and Purkinje Cell Degeneration in Mice—No tg(PrPN-Dpl)/Prnp0/0 mice showed any abnormal symptoms, including ataxia, up to 730 days after birth, at the time of writing (Fig. 4A). Purkinje cells were also unaffected in these mice (data not shown). The signals visualized by anti-Dpl antibodies on a Western blot of brain homogenates from tg mice was about 35% that of Ngsk Prnp0/0 mice, ectopically expressing Dpl in neurons under the control of the PrP promoter (Fig. 2C). These results indicate that, unlike wild-type Dpl, the fusion protein PrPN-Dpl might be nontoxic to Purkinje cells even in the absence of PrP C, although we could not completely rule out the possibility that the lack of neurotoxicity of PrPN-Dpl is because of its lower expression.

We next generated dtg mice by intercrossing tg(PrPN-Dpl)/Prnp0/0 mice with tg(Dpl32)/Prnp0/0 mice, expressing the full-length Dpl in neurons, including Purkinje cells under the control of the neuron-specific enolase promoter at a level higher than that in Ngsk Prnp0/0 mice (Fig. 2C), which develop ataxia because of Purkinje cell degeneration 99 ± 20 days after birth (Fig. 4A, Table 1, and Fig. 5). The times of onset of ataxia in tg(Dpl32)/Prnp0/0 mice were slightly prolonged compared with those reported previously (8). This is probably because we employed more strict criteria for diagnosis of ataxia in this study. The resulting dtg(PrPN-Dpl)(Dpl32)/Prnp0/0 mice eventually suffered from ataxia, but their onsets were significantly delayed to 200 ± 52 days after birth (Fig. 4A and Table 1). Consistent with this, immunohistochemistry using antibodies against calbindin, a Purkinje cell-specific marker, revealed well preserved Purkinje cells in the dtg mice 90 days after birth (Fig. 5), when Purkinje cells had been significantly lost in tg(Dpl32)/Prnp0/0 mice (Fig. 5). No decreased expression of Dpl could be detected in the brains of dtg(PrPN-Dpl)(Dpl32)/Prnp0/0 mice, compared with tg(Dpl32)/Prnp0/0 mice (Fig. 6). These results indicate that the fusion protein PrPN-Dpl antagonizes the Dpl-induced neurotoxicity, similar to PrP C.

PrPΔpreOR and PrPΔOR Inhibit Dpl-induced Ataxia and Purkinje Cell Degeneration in Mice—To evaluate the potential of PrPΔpreOR and PrPΔOR to antagonize the neurotoxicity of Dpl, tg(PrPΔpreOR)/Prnp0/0 and tg(PrPΔOR)/Prnp0/0 mice were intercrossed with tg(Dpl32)/Prnp0/0 mice. We previously showed that the onset of ataxia by Dpl-induced Purkinje cell degeneration depended on the expression levels of wild-type PrP C, and neither ataxia nor Purkinje cell degeneration occurred in tg(Dpl32) mice on the wild-type (Prnp +/− ) background (8). The expression levels of PrPΔOR and PrPΔpreOR in each of the dtg mouse lines, dtg(PrPΔOR)(Dpl32)/Prnp0/0 and dtg(PrPΔpreOR)(Dpl32)/Prnp0/0, were 1.7- and 0.4-fold, respectively, of the level of PrP C in wild-type mice (Fig. 2A). The dtg(PrPΔOR)(Dpl32)/Prnp0/0 mice showed no ataxic symptoms up to 500 days after birth (Fig. 4B and Table 1). On the other hand, dtg(PrPΔpreOR)(Dpl32)/Prnp0/0 mice developed ataxia 385 ± 47 days after birth, which was very delayed compared with the onset in tg(Dpl32)/Prnp0/0 mice (99 ± 20 days).
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| PrP genetic background | Expression level of mutant or wild-type forms of PrP* (fold) | No. of ataxic mice/No. of total mice | Times to the onset of ataxiaa (days) | p value log rank test |
|------------------------|-------------------------------------------------------------|------------------------------------|------------------------------------|----------------------|
| Zrch 1 Prnp0/0         | 0                                                           | 24/24                              | 99 ± 20                           | <0.0001              |
| Zrch 1 Prnp0/0         | 0.5                                                         | 11/15a                             | 387 ± 25f                         | 0.016                |
| Zrch 1 Prnp0/0         | 0.21                                                        | 6/7                                | 200 ± 52                          | <0.0001              |
| Zrch 1 Prnp0/0         | 0.42                                                        | 6/7                                | >730                              | <0.0001              |
| Zrch 1 Prnp0/0         | 1.7                                                         | 0/6                                | >500                              | <0.0001              |

Results indicate that PrPΔpreOR and PrPΔOR preserve the potential to protect from Dpl-induced Purkinje cell degeneration.

PrPΔ23–88 Is Expressed in the Cerebellum of Mice and on the Surface of Cultured Cells Similarly to Wild-type PrP overturn. We previously showed that PrPΔ23–88 was incompetent to rescue Ngsk Prnp0/0 mice from the Dpl-induced Purkinje cell degeneration, indicating that the region comprising the residues 23–88 is important for PrPΔC to be protective against Dpl. To further gain insights into the role of the residues 23–88 in the neuroprotective function of PrPΔC, we investigated cytological expression of PrPΔ23–88 in the cerebellum of mice. The cerebella from tg(PrPΔ23–88) mice on the Ngsk Prnp0/0 background as well as from Zrch 1 Prnp0/0 and tg(PrPΔOR)/Prnp0/0 mice were subjected to immunohistochemistry using IBL-N and ICSM-18 antibodies. Consistent with the results shown in Fig. 3, no signals could be detected in Zrch 1 Prnp0/0 mice, and tg(PrPΔOR)/Prnp0/0 mice showed abundant expression of PrPΔOR in the molecular and granule cells layers but not in the Purkinje cell layer (Fig. 7). PrPΔ23–88 was detected in the molecular and granule cell layers but not in the Purkinje cell layer (Fig. 7), similarly to PrPΔOR (Fig. 7) and wild-type PrPΔC (Fig. 3, A–D). We also investigated the cell surface expression of PrPΔ23–88 using cultured cells in comparison with that of wild-type PrPΔC and two other neuroprotective mutants, PrPΔpreOR and PrPΔOR. COS-7 monkey kidney cells were transiently transfected with each expression vector and then subjected to flow cytometry analysis using SAF61 monoclonal antibodies against PrP-(142–160) residues. PrPΔ23–88 was detected on the cell surface of COS-7 cells similarly to that of wild-type PrPΔC, PrPΔpreOR, and PrPΔOR (Fig. 8). These results indicate that lack of the residues 23–88 neither alter cell types for PrPΔ23–88 to be expressed in the cerebellum of mice nor impair the cell surface expression of PrPΔ23–88.

FIGURE 4. A, rescue from ataxia in dtg(PrP-N-Dpl)/(Dpl32)/Prnp0/0 mice. No ataxic symptoms were observed in tg(PrP-N-Dpl)/Prnp0/0 mice for up to at least 500 days after birth. In contrast, tg(Dpl32)/Prnp0/0 mice developed ataxia 99 ± 20 days after birth. The PrP-N-Dpl transgene delayed the onset of ataxia in tg(Dpl32) mice to 200 ± 52 days, as observed in dtg(PrP-N-Dpl)/(Dpl32)/Prnp0/0 mice. B, rescue of the ataxia in dtg(PrPΔpreOR)/(Dpl32)/Prnp0/0 and dtg(PrPΔOR)/(Dpl32)/Prnp0/0 mice. No ataxic symptoms were observed in dtg(PrPΔOR)/(Dpl32)/Prnp0/0 mice for up to at least 500 days after birth. dtg(PrPΔpreOR)/(Dpl32)/Prnp0/0 mice developed delayed onset of ataxia at 385 ± 47 days after birth similarly to tg(Dpl32)/Prnp0/0 mice, those developing ataxia at 387 ± 25 days after birth.

TABLE 1

Antagonistic effects of mutant proteins on Dpl-induced neurotoxicity in tg mice

| tg or dtg lines | PrP genetic background | Expression level of mutant or wild-type forms of PrP* (fold) | No. of ataxic mice/No. of total mice | Times to the onset of ataxiaa (days) | p value log rank test |
|-----------------|------------------------|-------------------------------------------------------------|------------------------------------|------------------------------------|----------------------|
| Zrch 1 Prnp0/0  | 0                      | 24/24a                                                      | 99 ± 20                            | <0.0001                            |
| Zrch 1 Prnp0/0  | 0.5                    | 11/15a                                                     | 387 ± 25f                          | 0.016                              |
| Zrch 1 Prnp0/0  | 0.21                   | 6/7                                                        | 200 ± 52                           | <0.0001                            |
| Zrch 1 Prnp0/0  | 0.42                   | 6/7                                                        | >730                               | <0.0001                            |
| Zrch 1 Prnp0/0  | 1.7                    | 0/6                                                        | >500                               | <0.0001                            |

DISCUSSION

Accumulating evidence indicates a neuroprotective role for PrPΔC. For instance, Prnp0/0 mice are highly sensitive to ischemic or traumatic brain damage, developing more severe pathological changes than in wild-type mice (24–27). In contrast, Dpl, the first identified structural homologue of the C-terminal domain of PrPΔC, is neurotoxic causing ataxia and Purkinje cell degeneration in mice (7–9). Interestingly, PrPΔC functionally antagonizes the neurotoxicity of Dpl, preventing the neurode-
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In this study, we also showed that PrPΔOR, PrP lacking the OR alone, rescued mice from the ataxia and Purkinje cell degeneration induced by Dpl. This clearly indicates that the OR is unnecessary for PrPΔC to antagonize the neurotoxicity of Dpl in mice. Interestingly, Shmerling et al. (11) described that the OR is also unnecessary for PrPΔC to antagonize the neurotoxicity of truncated PrPs. They showed that granule cell death induced by PrPΔ32–134 could be abrogated by PrPΔ32–93, which lacks the entire OR and about 2/3 of the pre-OR in mice (11). In contrast, in primary cultures of granule cells from Zrch 1 Prnp0/0 mice, apoptotic cell death induced by transient overexpression of Dpl could be successfully rescued by wild-type PrPΔC but not by PrP lacking the OR (16). Dpl was preferentially toxic to Purkinje cells and not to granule cells in mice (8, 28, 29). Therefore, Dpl toxicity may vary in primary cultured granule cells and mouse models. However, why PrP lacking the OR has differential activity against Dpl in primary cultured granule cells and mice is unknown.

Kuwahara et al. (31) showed that hippocampal neuronal cell lines established from Prnp0/0 mice easily succumbed to apoptosis after serum withdrawal. Furthermore, expression of the anti-apoptotic molecule Bcl-2 could rescue cell lines from apoptosis (31). Bouhkar et al. (14) also showed that PrPΔC prevented human primary neurons from Bax-induced apoptosis. This suggests that the neuroprotective function of PrPΔC might involve anti-apoptotic activities. Interestingly, PrP lacking OR failed to rescue the cells from serum withdrawal- and Bax-induced apoptosis, indicating that the OR plays an important role in the anti-apoptotic function of PrPΔC (14, 32). Furthermore, our present results showing that PrPΔOR antagonized Dpl in mice clearly indicates that neuroprotection by PrPΔC against Dpl is not associated with OR-mediated anti-apoptotic activities.

The anti-apoptotic activity of PrPΔC may also be associated with anti-oxidative responses (32, 33). Binding of PrPΔC to copper may be important for the anti-oxidative function of PrPΔC by either chelating copper or by activating anti-oxidant enzymes, such as Cu,Zn-superoxide dismutase, via transfer of the bound copper to the enzymes, or both (34–36). Six conserved histi-
dine residues have been identified as copper-binding sites in human PrP<sup>C</sup>, with four in the OR and two at positions 96 and 111 (37). As PrP<sub>ΔOR</sub> blocked Dpl-mediated neurotoxicity, OR-mediated copper binding might not be involved in the neuroprotection of PrP<sup>C</sup> against Dpl. In addition, our previous result that PrP<sub>Δ23–88</sub>, in which two other histidine residues are preserved, failed to rescue mice from ataxia and Purkinje cell degeneration, indicate that copper binding at these sites might not be relevant to the antagonistic function of PrP<sup>C</sup> against Dpl. Taken together, these suggest that the copper binding-mediated function of PrP<sup>C</sup>, including anti-oxidative activity, is not associated with its neuroprotective function against Dpl. However, we cannot rule out copper binding to all histidine residues simultaneously for PrP<sup>C</sup> to have anti-oxidative function.

**N-terminal Residues and the Neuroprotective Function of PrP<sup>C</sup> against Dpl in Mice**—In this study, we also showed that PrP<sub>ΔpreOR</sub>, PrP lacking residues 25–50, prevented Dpl-induced ataxia and Purkinje cell degeneration in mouse as efficiently as PrP<sub>ΔOR</sub>. This indicates that N-terminal residues 25–50 are not required for PrP<sup>C</sup> to antagonize Dpl in mice. The two deletions, Δ25–50 and Δ51–90, almost entirely cover the region deleted in PrP<sub>Δ23–88</sub>, which failed to rescue mice from the neurotoxicity of Dpl (13). PrP<sub>Δ23–88</sub> is a chimeric protein of mouse and hamster PrPs, containing two methionines at 108 and 111 in mouse PrP instead of leucine and valine. No such substitutions were present in PrP<sub>ΔpreOR</sub> and PrP<sub>ΔOR</sub>. However, we previously showed that Ngsk Prnp<sup>0/0</sup> mice were successfully rescued from ataxia and Purkinje cell degeneration by full-length chimeric PrP with these methionine substitutions (13), clearly indicating that the incompetence of PrP<sub>Δ23–88</sub> to antagonize Dpl is because of lack of residues 23–88 and not to the amino acid substitutions. We also showed here that PrP<sub>Δ23–88</sub>, PrP<sub>ΔpreOR</sub>, and PrP<sub>ΔOR</sub> were similarly expressed in the cerebellum of mice, consistent with these mutant molecules being expressed under the control of the same hamster PrP promoter/enhancer. Moreover, in this study, we used tg(Dpl32)/Prnp<sup>0/0</sup> mice for the rescue experiments instead of Ngsk Prnp<sup>0/0</sup> mice because tg mice develop ataxia and Purkinje cell degeneration on the Zrch I Prnp<sup>0/0</sup> background much earlier than Ngsk Prnp<sup>0/0</sup> mice because of higher expression of Dpl in their brains (8). Dpl was expressed in tg(Dpl32) mice from the neuron-specific enolase promoter and in Ngsk Prnp<sup>0/0</sup> mice from the residual PrP promoter (4, 8). However, Dpl was similarly expressed in neurons of tg(Dpl32) mice and Ngsk Prnp<sup>0/0</sup> mice with the highest expression in Purkinje cells and hippocampal neurons (4, 8). Therefore, Dpl is toxic to Purkinje cells in the same way in both tg (Dpl32)/Prnp<sup>0/0</sup> mice and Ngsk Prnp<sup>0/0</sup> mice. Taken together, these results indicate that PrP<sub>ΔpreOR</sub> and PrP<sub>ΔOR</sub> but not PrP<sub>Δ23–88</sub> can antagonize Dpl neurotoxicity in mice.

PrP<sub>ΔpreOR</sub> and PrP<sub>ΔOR</sub> but not PrP<sub>Δ23–88</sub> have the N-terminal two amino acids (residues 23 and 24) conserved adjacent to the junction with the signal peptide. Thus, the two amino acids may be important for the neuroprotection of PrP<sup>C</sup> against Dpl. This may be consistent with the observation that PrP<sub>Δ32–93</sub> protected against the truncated PrPs (11). Interestingly, in PrP<sub>ΔpreOR</sub> the two amino acids are followed by residues starting from 51, generating a new N-terminal sequence (KKPQGGTWG), which is very similar to the N-terminal 9 residues (KKRPKPGGW) of wild-type PrP<sup>C</sup> and PrP<sub>Δ32–93</sub>. Six out of 9 of these amino acids are identical. Therefore, this new N-terminal sequence might mimic the function of wild-type PrP<sup>C</sup>. In PrP<sub>ΔOR</sub>, the N-terminal sequence is intact. Thus, these N-terminal residues might be important for the neuroprotection of PrP<sup>C</sup> against Dpl. However, it is possible that the antagonistic function of PrP<sup>C</sup> against Dpl is impaired only by a large deletion of the N-terminal domain with or without the N-terminal residues, as observed in PrP<sub>Δ23–88</sub>.

Interestingly, PrP with only the central residues 105–125 or 94–134 deleted was reported to be neurotoxic, causing cerebellar degeneration or demyelination in mice, respectively (38, 39). These results suggest that these central residues are essential for PrP<sup>C</sup> to be neuroprotective. However, PrP<sub>Δ23–88</sub> contains these central residues but has no protective activity against Dpl (13). Therefore, the central residues alone might not be enough for PrP<sup>C</sup> neuroprotection, and other region(s), present among the N-terminal residues 23–88, may also be necessary for neuroprotection. These region(s) might be located in the N-terminal 2 or 9 residues. However, unrelated region(s) to the N-terminal 2 or 9 residues may also be necessary.

**Possible Mechanisms for N-terminal Region Neuroprotectivity of PrP<sup>C</sup> against Dpl**—There are reports showing that the N-terminal domain is involved in the subcellular trafficking of PrP<sup>C</sup> (40–44). In this study, we found that PrP<sub>Δ23–88</sub>, PrP<sub>ΔpreOR</sub>, and PrP<sub>ΔOR</sub> were expressed in the molecular and granule cell layers of the cerebellum and on the cell surface of COS-7 monkey kidney cells similarly to that in wild-type PrP<sup>C</sup>.
Antagonistic Role of N Terminus of PrP against Doppel

![Cell surface expression of PrP mutants](image)

This indicates that the cellular expression and cell surface transport of these mutant molecules may be unchanged. It is therefore unlikely that the cell surface localization of PrPΔ23–88 is different from that of PrPΔpreOR and PrPΔOR because of the large deletion of the N-terminal domain, thus impairing the neuroprotective function of PrP<sup>C</sup>. The N-terminal part is also involved in efficiency of PrP<sup>C</sup> endocytosis. PrPΔ23–90 and PrPΔ48–93, which lacks the OR region, were shown not to be efficiently internalized in mouse neuroblastoma N2a cells (44), indicating that lack of the OR alone might affect the internalization of PrP<sup>C</sup>. However, we showed here that PrPΔOR was neuroprotective against Dpl in mice, indicating that the internalization may not be relevant to the neuroprotective activity of PrP<sup>C</sup>. Recently, Santuccione et al. (45) showed that PrP<sup>C</sup> activates p59<sup>Fyn</sup> to enhance neurite outgrowth via recruitment of the neuronal cell adhesion molecule to lipid rafts, indicating that the proper localization at lipid rafts could be important for PrP<sup>C</sup> function. Interestingly, PrPΔ23–90 but not PrP lacking the OR region was not properly targeted to lipid rafts (44). Thus, PrPΔ23–88 but not PrPΔOR and PrPΔpreOR may not properly localize at lipid rafts either because of lack of the N-terminal 2 or 9 residues or because of large scale deletion of the N-terminal domain with or without the N-terminal residues, resulting in unsuccessful rescue of mice from Dpl neurotoxicity.

Alternatively, the N-terminal region may be involved in the neuroprotective function of PrP<sup>C</sup> by eliciting a neuroprotective signal through an associated molecule, as in the models proposed so far (11, 30, 38, 39, 46). Among them, Weissmann and Aguzzi (46) proposed that PrP<sup>C</sup> binds to an as yet unidentified molecule and elicits a Purkinje cell survival signal through the N-terminal domain. Dpl can bind to the molecule but cannot generate the signal because of lack of the N-terminal domain, resulting in Purkinje cell degeneration. However, PrP<sup>C</sup> competes with Dpl for the molecule, thereby preventing Dpl-induced Purkinje cell degeneration. The results showing that PrPN-Dpl, PrPΔpreOR, and PrPΔOR but not PrPΔ23–88 antagonize the neurotoxicity of Dpl suggests that the former three molecules bind the molecule and produce the survival signal through the N-terminal domain of PrP<sup>C</sup>, preventing neurodegeneration. This may be because they have a part of or the whole N-terminal domain. It might be also possible that Dpl itself may bind to its own unidentified cognate molecule to elicit a neurotoxic signal and PrP<sup>C</sup>, PrPN-Dpl, PrPΔpreOR, and PrPΔOR but not PrPΔ23–88 may compete for the molecule via a part of or the whole N-terminal domain, thereby preventing Dpl-mediated neurotoxicity. However, these models can be verified only if the hypothetical molecules are identified.

In this study, we showed that the N-terminal domain mediates the neuroprotective function of PrP<sup>C</sup> against Dpl in trans and cis and that the OR region and residues 25–50 (pre-OR) are dispensable for the neuroprotective function of PrP<sup>C</sup>. However, to understand the exact molecular mechanism how the N-terminal domain is involved in the neuroprotective function of PrP<sup>C</sup>, further studies are required.

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