Deletion of N-terminal Residues 23–88 from Prion Protein (PrP) Abrogates the Potential to Rescue PrP-deficient Mice from PrP-like Protein/Doppel-induced Neurodegeneration*

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Accumulating evidence has suggested that prion protein (PrP) is neuroprotective and that a PrP-like protein/Doppel (PrPPL/Dpl) is neurotoxic. A line of PrP-deficient mice, Ngsk Prnp0/0, ectopically expressing PrPPL/Dpl in neurons, exhibits late-onset ataxia because of Purkinje cell death that is prevented by a transgene encoding wild-type mouse PrP. To elucidate the mechanisms of neurodegeneration in these mice, we introduced five types of PrP transgene, namely one heterologous hamster, two mouse/hamster chimeric genes, and two mutants, each of which encoded PrP lacking residues 23–88 (MHM2.del23–88) or with E199K substitution (Mo.E199K), into Ngsk Prnp0/0 mice. Only MHM2.del23–88 failed to rescue the mice from the Purkinje cell death. The transgenic mice, MHM2.del23–88/ Ngsk Prnp0/0, expressed several times more PrP than did wild-type (Prnp+/+) mice and PrPPL/Dpl at an equivalent level to Ngsk Prnp0/0 mice. Little difference was observed in the pathology and onset of ataxia between Ngsk Prnp0/0 and MHM2.del23–88/Ngsk Prnp0/0 mice. No detergent-insoluble PrPPL/Dpl was detectable in the central nervous system of Ngsk Prnp0/0 mice even after the onset of ataxia. Our findings provide evidence that the N-terminal residues 23–88 of PrP containing the unique octapeptide-repeat region is crucial for preventing Purkinje cell death in Prnp0/0 mice expressing PrPPL/Dpl in the neuron.

Prion protein (PrP) is a membrane glycoprotein expressed on the neuronal cell surface, but its physiological function is not fully understood. However, the complete resistance of PrP-deficient mice to prion infection indicates an essential role in the development of prion diseases and replication of prion (1, 2). In affected brains, the constitutive structural conversion of PrP results in the accumulation of a detergent-insoluble and proteinase K-resistant PrP isoform, namely PrPSc (3). Although accumulated PrPSc is thought to play a central role in the pathogenesis, molecular mechanisms for the neurodegeneration in prion diseases remain to be elucidated.

We previously established a line of PrP-deficient mice, designated Ngsk Prnp0/0, that exhibited late-onset ataxia because of cerebellar Purkinje cell degeneration (4), which was rescued by the mouse wild-type PrP gene (Prnp) (5). Subsequently, these results were reproduced in two independent lines, Rcm0 and Zrch II Prnp0/0 mice (6, 7). In contrast, two other independent lines, Zrch I and Edgb Prnp0/0, have never revealed such a phenotype (8, 9). Recent studies have provided evidence that the ectopic expression of a PrP-like protein/Doppel (PrPPL/Dpl) in the neuron of ataxic Prnp0/0 mouse lines but not non-ataxic lines could explain the discrepancy between phenotypes (6, 10). Disruption of a part of the Prnp intron 2, including its splicing acceptor, in all the ataxic lines leads to unusual intergenic splicing between Prnp and the downstream gene, designated Prnd, encoding PrPPL/Dpl. As a consequence, the PrPPL/Dpl gene comes under the control of the Prnp promoter, leading to the ectopic expression of PrPPL/Dpl in the neuron. In the physiological situation, PrPPL/Dpl mRNA is expressed at a high level in the testis and heart but is not detectable in the brain except for transient expression by brain endothelial cells around 1 week after birth (11). A study using PrPPL/Dpl-deficient mice has suggested a physiological role for PrPPL/Dpl in spermatogenesis (12). Recent reports demonstrated that introduction of a transgene encoding PrPPL/Dpl rendered Zrch I Prnp0/0 mice capable of reproducing the Purkinje cell degeneration observed in the Ngsk Prnp0/0 mice (13). This is direct evidence confirming that both ectopic expression of PrPPL/Dpl and functional loss of PrP are required for the neurodegeneration in Ngsk Prnp0/0 mice.

PrPPL/Dpl, a glycoprotein expressed on the cell surface with a glycosylphosphatidylinositol moiety (14), consists of 179 amino acids with 23% identity to PrP in the primary structure but lacking the unique octapeptide-repeat region and hydrophobic region present in the N-terminal half of PrP (10). An NMR study revealed similarities in the tertiary structure between PrPPL/Dpl and the C-terminal half of PrP, both of which are composed of two short β-strands and three α-helices (15). Interestingly, expression of a transgene encoding truncated PrP lacking N-terminal residues 32–121 or 32–134 in non-ataxic Zrch I Prnp0/0 mice also resulted in severe cerebellar degeneration, which was abrogated by reintroduction of the wild-type mouse PrP gene (16). These results have suggested that PrPPL/Dpl and N-terminal-truncated (del32–121 or 32–134) PrP are toxic to neurons through similar mechanisms and that wild-type PrP may have a neuroprotective function.

In the present study, to further elucidate mechanisms of the

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Purkinje cell death, we generated five different transgenic mouse lines expressing, respectively, heterologous, chimeric, and mutant PrPs on the Ngsk Prnp0 background. A transgene encoding PrP-lacking N-terminal residues 23–88 failed to rescue the Ngsk Prnp0 mice from Purkinje cell degeneration, indicating a crucial role for the residues. The roles of PrPPLP/Dpl aggregation and impaired metabolism of reactive oxygen species in the brain tissues of Ngsk Prnp0 mice were also evaluated.

**EXPERIMENTAL PROCEDURES**

**Mice**—The mouse PrP (Mo.PrP) transgenic (Tg) mice with the Ngsk Prnp0 background were described previously (5). In the current study, we introduced five types of PrP transgene into Ngsk Prnp0 mice by mating them with the transgenic mouse lines, each harboring the corresponding transgene with the Zrch I Prnp0 background (8). Drs. P. Tremblay and S. B. Prusiner, University of California, San Francisco, CA kindly provided the Tg mouse lines. The transgenes included a Syrian hamster wild-type PrP gene (SHa); two SHa/mouse chimeric PrP genes (MH2M and MHM2), MHM2 lacking residues 23 to 88 (MHM2.del23–88); and Mo.PrP gene with E199K substitution (Mo.E199K) (17–20). They were constructed in a cosSha.Tet. vector, encompassing the entire SHa Prnp locus but not containing the PrPPLP/Dpl inhibitor (Prnd) (6). MHM2 PrP has two amino acid substitutions from Mo.PrP, L108M and V111M, which are included in the epitope recognized by 3F4 anti-PrP antibody. MHM2 PrP has five amino acid substitutions (L105M, V111M, 113SM, Y154N, and S169N) from Mo.PrP. The E199K substitution in Mo.E199K PrP corresponds to that found in patients with familial Creutzfeldt-Jakob disease (21). Offspring carrying the transgenes were identified by PCR of tail DNA as described previously (5). To distinguish Ngsk from Zrch I Prnp0 genotypes, both a Zrch I-specific primer pair (MP685S, 5'-AACCTTACCGAGACTGAT and MP215A, 5'-GAAAACCTTGGCTGATGTA) and a Ngsk-specific primer pair (PGK468S, 5'-CGATGT and MP215A, 5'-AACTTCACCGAGACTGAT) were used in the PCR. The F2 offspring carrying transgens with Ngsk Prnp0 background, TgNgsk Prnp0, were subjected to analysis.

**Antibodies**—The anti-PrP polyclonal mouse antiserum used was described previously (5). We produced anti-PrPLP/Dpl polyclonal rabbit antiserum using full-length (27 kDa) PrPLP/Dpl aggregation and impaired metabolism of reactive oxygen species in the brain tissues of Ngsk Prnp0 mice were also evaluated.

**RESULTS**

**Rescue of Ngsk Prnp0 Mice from Neurodegeneration by Mo.E199K but Not MHM2.del23–88 PrP Mutants**—We introduced five types of PrP transgene (SHa, MH2M, MHM2, MHM2.del23–88, and Mo.E199K) into Ngsk Prnp0 and evaluated their effects on the phenotype. All the TgNgsk Prnp0 mice developed normally and initially showed no neurological symptoms. At about 13 months after birth, however, TgMHM2.del23–88/Ngsk Prnp0 mice, as well as Ngsk Prnp0 mice lacking a PrP transgene, began to exhibit progressive symptoms of ataxia, such as tremor and gait disturbance (Fig. 1A). Analysis of hind footprints confirmed that the ataxic mice could not walk in a straight line, and the length of their steps was shorter than that of the wild-type mice (Fig. 1B). Age at onset of Ngsk Prnp0 and Tg-MHM2.del23–88/Ngsk Prnp0 mice was about 68.4 ± 6.5 weeks (Fig. 1A, 1B, and Tg-MHM2/Ngsk Prnp0 mice showed no Purkinje cell loss at 24 months of age, indicating that SHa, MH2M, MHM2, and MoE199K transgenes, but not MHM2.del23–88, were able to rescue the Ngsk Prnp0 mice from ataxia.

**Immunostaining with anti-spot 35 (calbindin) antibody of the brain sections derived from 20-month-old Ngsk Prnp0 and Tg-MHM2.del23–88/Ngsk Prnp0 mice revealed an extensive loss of Purkinje cells throughout most of the cerebellar vermis (Fig. 2, B and G). No difference was found in the extent of Purkinje cell loss between the two mouse lines. In contrast, the Tg-SHa, Tg-MH2M, Tg-MHM2, and Tg-Mo.E199K/Ngsk Prnp0 mice showed no Purkinje cell loss (Fig. 2, A, C, D, E, and F).**

**Expression of Transgene-encoded PrP in the Brain Tissues of TgNgsk Prnp0 Mouse Lines**—Expression of the transgene products, PrPs, in the brain tissues of 4-month-old Tg mice was examined by Western blotting using polyclonal anti-PrP mouse serum (Fig. 3) and SAF-mix anti-PrP monoclonal antibody (data not shown). Two lines, Tg-MH2M and Tg-MHM2.del23–88/Ngsk Prnp0, overexpressed PrP about four to eight times more than wild-type mice. The expression levels of the remaining three, Tg-SHa, Tg-MH2M, and Tg-Mo.E199K/Ngsk Prnp0, appeared to be equivalent to that of wild-type mice. As shown in Fig. 4A, after deglycosylation by PNGase F, SAF-mix visualized two clear bands of 18 and 27 kDa, and Tg-Mo.E199K/Ngsk Prnp0 mice showed no Purkinje cell loss (Fig. 4, A and C). Expression of transgenic-encoded PrP in the brain tissues of TgNgsk Prnp0 Mouse Lines—Expression of the transgene products, PrPs, in the brain tissues of 4-month-old Tg mice was examined by Western blotting using polyclonal anti-PrP mouse serum (Fig. 3) and SAF-mix anti-PrP monoclonal antibody (data not shown). Two lines, Tg-MH2M and Tg-MHM2.del23–88/Ngsk Prnp0, overexpressed PrP about four to eight times more than wild-type mice. The expression levels of the remaining three, Tg-SHa, Tg-MH2M, and Tg-Mo.E199K/Ngsk Prnp0, appeared to be equivalent to that of wild-type mice. As shown in Fig. 4A, after deglycosylation by PNGase F, SAF-mix visualized two clear bands of 18 and 27 kDa, and a faint band of 21 kDa in the brain homogenates from Tg-SHa and Tg-MH2M/Ngsk Prnp0 mice, but 3F4 monoclonal antibody failed to detect an 18-kDa product (Fig. 4B). It has been demonstrated that PrPC undergoes physiological proteolytic cleavage at amino acid residues 110/111 or 111/112, leading to the production of a C-terminal fragment, C1 (23). Because the cleavage occurs within the linear 3F4 epitope, C1 is recognized by SAF-mix but not by 3F4. The 18-kDa band is thus most
likely to correspond to C1. The remaining 27- and 21-kDa bands are likely to be the full-length PrP and another C-terminal minor product, C2, respectively. The brain tissues from MHM2.del23–88/Ngsk Prnp0/0 mice also expressed an 18-kDa band that reacted with SAF-mix but not 3F4, in addition to its full-length product of 20 kDa (lane 6 in Fig. 4, A and B).

Expression Levels and Detergent Insolubility of PrPLP/Dpl in the Brain Tissues of Tg/Ngsk Prnp0/0 Mouse Lines—PrPLP/Dpl expression levels in the brains were examined by Western blotting with polyclonal anti-PrPLP/Dpl rabbit antiserum. Although the brain tissues of Prnp+/+ mice expressed no detectable PrPLP/Dpl, the Tg-Ngsk Prnp0/0 mouse lines all exhibited PrPLP/Dpl expression at levels equivalent to those of Ngsk Prnp0/0 mice (Fig. 5). The expression levels remained stable during aging, even after the onset of ataxia (data not shown).

Accumulating evidence has indicated that certain neurodegenerative conditions are caused by aggregation of detergent-insoluble proteins, such as PrPSc, in the central nervous system (24). To evaluate the possibility of PrPLP/Dpl aggregation in the brain tissues, we examined the detergent-insolubility of PrPLP/Dpl in the brains of ataxic Ngsk Prnp0/0 and Tg-SHa/Ngsk Prnp0/0 mice at 98 weeks of age. As shown in Fig. 6A, PrPLP/Dpl was partially insoluble in the buffer containing 0.5% Triton X-100 and 0.5% sodium deoxycholate but completely solubilized by 1% sarcosyl. The levels of Triton X-100-insoluble fraction of PrPLP/Dpl were similar between Ngsk Prnp0/0 and Tg-SHa/Ngsk Prnp0/0 mice even at 98 weeks of age and did not increase during aging (Fig. 6B).

DISCUSSION

We previously demonstrated that wild-type Mo.PrP has the potential to prevent PrPLP/Dpl-induced neurodegeneration in Ngsk Prnp0/0 mice (5). The present study showed that Mo.E199K PrP, as well as heterologous hamster and hamster/mouse chimeric PrPs, but not MHM2.del23–88 PrP, could rescue Ngsk Prnp0/0 mice from Purkinje cell death. Mo.E199K represents the E200K substitution in human PrP, which is commonly found in patients with familial Creutzfeldt-Jakob disease (21). The successful rescue by Mo.E199K strongly indicates that the mutant retains the major aspects of normal PrP function and thus argues against a dominant-negative role for the mutant. The solution structure of recombinant human PrP 90–231 with E200K was reported to be nearly identical to that of wild-type PrP (25). This finding also supports the notion that the amino acid substitution does not disrupt the structure critical for the normal function of PrP.

Although various hypothetical models have been proposed, precise mechanisms for the neurodegeneration seen in Ngsk Prnp0/0 mice remain to be elucidated. In addition to the structural similarities between PrP and PrPLP/Dpl, the previous finding that a recombinant C-terminal PrP (121–231) fragment could convert into a β-sheet-rich structure at acidic pH in vitro (26) prompted us to examine the involvement of abnormal PrPLP/Dpl aggregation in the neurodegeneration. However, we failed to detect detergent-insoluble PrPLP/Dpl accumulation in the central nervous system of Ngsk Prnp0/0 mice even after the onset of ataxia. PrPLP/Dpl contains two intramolecular disulfide bonds at the C-terminal portion (14), in contrast to PrP with a single disulfide bond, which has been shown to be necessary for conversion to the pathogenic isoform, PrPSc. The structures determined by intramolecular disulfide bonds may account for the difference between the two proteins in the potential for structural conversion.

It is noteworthy that the MHM2.del23–88 transgene, which encoded a product lacking almost the entire unique octapeptide-repeat region, failed to rescue Ngsk Prnp0/0 mice from neurodegeneration. Little difference was observed in the pathology and onset of ataxia between Ngsk Prnp0/0 and Tg-MHM2.del23–88/Ngsk Prnp0/0 mice. The PrP expression level of Tg-MHM2.del23–88/Ngsk Prnp0/0 mice was higher than that of Tg-SHa and Tg-MHM2/Ngsk Prnp0/0 but similar to that of Tg-MHM2/Ngsk Prnp0/0 mice. This strongly indicates that the N-terminal part of PrP is essential to the rescue from Purkinje...
cell death. In contrast, MHM2.del23–88 PrP was shown to retain the potential to convert into the pathogenic isoform in prion-infected cell cultures (27) and brain tissues of Tg-MHM2.del23–88 mice (19). The N-terminal region could thus be a key factor for determining whether physiological function is maintained but is likely to be less important in pathogenic structural conversion.

Because the unique octapeptide-repeat region has the potential to bind copper (28), PrP may function in the transport or metabolism of copper in the neuron (29–31). Copper is involved in various aspects of reactive oxygen species metabolism (32). It could thus be possible that impaired copper transport may result in reactive oxygen species accumulation, leading to cell death. Indeed, the reduced activity of Cu/Zn-SOD in the brains of Zrch I Prnp0/0 mice has been reported (33). However, along with other contradictory reports (34), the present study failed to show reduced activities of Cu/Zn-SOD, Mn-SOD, and Gpx in the brains of Ngsk Prnp0/0 mice. The fact that Cu/Zn-SOD-deficient mice have never developed any neurological signs such as ataxia (35) also argues against a causal relationship between reduced activity of Cu/Zn-SOD and Purkinje cell death.

Interestingly, Tg mice expressing PrP.del32–121 or 32–134 on the Zrch I Prnp0/0 background also developed cerebellar cell death, but those with wild-type Prnp background did not (16). The two mutant PrPs lack a part of or most of the hydrophobic region (111–134), in addition to the octapeptide-repeat region, which are also absent in PrPLP/Dpl. The structural similarities between PrP.del32–121/32–134 and PrPLP/Dpl strongly suggested that a common mechanism is involved in the neurodegeneration in the Tg mice and Ngsk Prnp0/0 mice. Behrens and Aguzzi (36) have hypothesized an as yet unidentified ligand, which harbors affinities to both PrP and PrPLP/Dpl or the N
terminus-truncated PrPs. In the absence of PrP, binding of the putative ligand to PrPLP/Dpl or PrP.del32–121/32–134 may trigger an apoptotic signal or prevent a survival signal. On the other hand, in the presence of PrP, the binding would be impeded because of much lower affinity. If this is the case, the failure of MHM2.del23–88 PrP in the phenotype rescue suggests that residues 23–88 include a site critical for ligand binding or functional transduction of ligand signals. Recent studies have demonstrated that copper binds not only to the octapeptide-repeat region but also to the C-terminal PrP-(91–231/121–231) fragments (37, 38) and PrPLP/Dpl (39). Hence, copper could be one candidate for this hypothetical ligand, acting to raise a neurotoxic signal or prevent a survival signal through binding to PrPLP/Dpl or PrP.del32–121/32–134.

We demonstrated that MHM2.del23–88 PrP, as well as full-length PrP, underwent proteolytic cleavage leading to the production of C1. This indicated that loss of the N-terminal residues 23–88 did not affect the cleavage, and C1 was not necessary for the phenotype rescue. It has also been shown that a N-terminal cleavage product, PrP-(23–110/111), designated...
N1, was released into the supernatant of cultured cells (40). An intriguing hypothesis is that N1 may play a neuroprotective role outside of the cells. For instance, it may bind to copper or other molecules to produce a neuroprotective signal. The N1 derived from MHM2.del23–88 PrP lacking the octapeptide-repeat region is unlikely to preserve this function. It would be of value to examine the potential of N1 to rescue Ngsk Prnp0 mice from the Purkinje cell death.

Another model for the neurodegeneration to be evaluated is that the absence of PrP might make cerebellar cells vulnerable to a constitutive weak apoptotic signal transduced by PrPPL/Dpl (36). PrP could raise an anti-apoptotic signal independently and protect from the PrPPL/Dpl-induced apoptosis. Accumulating evidence has supported an anti-apoptotic role of PrP. For instance, overexpression of PrP protected human primary neurons from Bax-mediated apoptosis (41). The protective effect was abolished by deletion of the octapeptide-repeat region, residues 56–88, of human PrP. Serum withdrawal-induced apoptosis of immortalized hippocampal cells derived from Prnp0 mice was also prevented by overexpressed PrP or Bcl-2 (42). Chiariini et al. (43) recently demonstrated evidence that a peptide capable of binding to residues 113–128 of PrP protected developing retina neurons from apoptosis via activation of cAMP-dependent protein kinase. The same group has protected developing retina neurons from apoptosis via activation of cAMP-dependent protein kinase.

In prion diseases, the constitutive conversion of PrP into the pathoisoform, PrPSc, is likely to result in the functional loss of normal PrP (45). If PrPSc mimics the neurotoxic role of PrPPL/Dpl or PrP.del32–121/32–134, Ngsk Prnp0 mice lacking PrP but ectopically expressing PrPPL/Dpl could provide a valuable model for the neurodegeneration. Alternatively, it is also possible that the mechanisms of neurodegeneration in mice are distinct from those of prion diseases, based on the finding that no detectable-in-soluble PrPPL/Dpl was detected in the brain tissue. Exudation of the molecular mechanisms responsible for the neurodegeneration in Ngsk Prnp0 mice would provide new insights into the understanding of the pathogenesis of prion diseases and other neurodegenerative conditions, as well as the physiological function of PrP.

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