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Tissue- and cell type-specific modification of prion protein (PrP)-like protein Doppel, which affects PrP endoproteolysis

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ABSTRACT

A prion protein (PrP)-like protein, Doppel (Dpl) is a homologue of cellular PrP (PrP^C). Immunoblotting revealed heterogeneous glycosylation patterns of Dpl and PrP^C in several cell lines and tissues, including brain and testis. To investigate whether the glycosylation and modification of Dpl and PrP^C could influence each other, PrP gene (*Prnp*)-deficient neuronal cells, transfected with *Prnp* and/or the Dpl gene (*Prnd*), were analyzed by deglycosylation with peptide *N*-glycosidase F. The modification of Dpl was not influenced by PrP^C, whereas an N-terminally truncated fragment of PrP^C was reduced by Dpl expression. These results indicated that Dpl was glycosylated in a cell type- and tissue-specific manner regardless of PrP^C, while PrP^C endoproteolysis was modulated by Dpl expression.

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1. Introduction

Prion diseases are fatal neurological disorders that include Creutzfeldt–Jakob disease and Gerstmann-Sträussler Scheinker syndrome in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in cattle [1]. The pathological characteristics of the diseases encompass neuronal cell loss, vacuolation, astrocytosis, and amyloid plaques in the brain [1]. After prion infection, cellular prion protein (PrP^C) is converted into abnormal prion protein (PrP^{Sc}), which has been proposed to be responsible for the disease [1]. Therefore, PrP^C is hypothetically required to induce the disease and eventually provoke neural damage.

PrP^C is encoded by the prion protein (PrP) gene (*Prnp*), whereas Doppel (Dpl), a PrP-like protein, is encoded by the gene *Prnd* which is located 16 kb downstream of *Prnp* in mice [2]. Interestingly, recent biophysical studies revealed that Dpl could bind to PrP [3], although the functional significance and the site of this binding remained unclear. Furthermore, although some lines of *Prnp*-deficient mice (ZrchII, Ngsk, Rcm0, and Rikn) exhibited late-onset ataxia, possibly due to the ectopic expression of Dpl [4], the ataxic phenotype observed in the $Prnp^{-/-}$ mouse lines could be rescued by crossing the mice with those PrP over-expressing wild-type mouse [5]. This also supported that PrP could interact with Dpl for a specific function, and the absence of PrP could essentially cause Dpl neurotoxicity.

Previously, we established *Prnp*-deficient immortalized hippocampal neuronal cell lines (HpL3-4 and HpL3-2) derived from Rikn $Prnp^{-/-}$ mice [6]. In this study, we investigated the hypothesis whether the posttranslational modifications were involved in the interaction between PrP and Dpl. To test the possibility, PrP and/or Dpl were overexpressed in an HpL3-4 cell line. The electrophoretic migration of Dpl was not affected by PrP expression, whereas the endoproteolytic fragment of PrP was decreased by Dpl expression, suggesting that the endoproteolysis of PrP was modulated by Dpl.

2. Materials and methods

2.1. Cell cultures and animals

Murine *Prnp*-deficient neuronal cell lines (HpL3-4 and HpL3-2) [6] and their transfectants [7] were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO), supplemented with 10% fetal calf serum (FCS). The PT67 fibroblast cell line (Clontech, Palo Alto, CA) and its transfectants [7] were maintained in 10% FCS-DMEM (Sigma). The N2a neuroblastoma cell line and its transfectants were cultured in 10% FCS-minimum essential medium (MEM) (Sigma) at 37 °C in a humidified 5% CO_2 incubator. Male Rikn $Prnp^{-/-}$ mice [8] and C57BL/6CrSlc ($Prnp^{+/+}$) mice purchased from Nippon SLC (Shizuoka, Japan) were analyzed.

2.2. Plasmid construction and gene transfer

A part of *Prnp*/*Prnd* chimeric cDNA was amplified by the polymerase chain reaction (PCR) using PRPe11f (5′-GGCAGGATCCT GGGCGCTGCGTCGCATCGGTGGCAG-3′) (the *Bam*HI linker is

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underlined) and DPL-STOP-SR (5′-CTCGTCGACCTCTGTGGCTGC CAGCTTCATTGA-3′) (the *Sal*I linker is underlined), subcloned into the vector pT7Blue, and cloned into pIREShyg (Clontech) in the sense orientation relative to the cytomegalovirus promoter. The resulting construct was named pIREShyg-chPrPDpl. The cloned fragment was confirmed to be composed of *Prnp* exons 1 and 2 and intergene exon 2 and *Prnd* exon 2 by sequencing. Similarly, pIREShyg-Dpl was constructed using MPRNDCLO-F (5′-CGGGATCC CGCAGCCTTTCCCTTGCCGATTCAC-3′) (the *Bam*HI linker is underlined, and the exon 2a splice acceptor site is altered, as in a previous paper [2]) and MPRNDCLO-R (5′-CGGGATCCCGACTGGGCT ACCTCTGTCTACCT-3′) (the *Bam*HI linker is underlined). The pIREShyg constructs were transfected into HpL3-2 cells by lipofectamine (Gibco/BRL). N2a transfectants were established by virus-mediated transduction with a Dpl-expressing vector, pMSCVhyg-EGFP-Dpl, or the control vector, pMSCVhyg-EGFP [7]. Selection was performed for more than 10 days in complete medium containing 400 μg/ml hygromycin (WAKO, Osaka, Japan).

2.3. Antibodies

For the detection of mouse Dpl, a rabbit anti-Dpl antiserum (kindly provided by Dr. Shu G. Chen, Case Western Reserve University), which was generated by immunizing rabbits with recombinant human Dpl 24–152 [9], was used. To detect mouse PrP^C, the 6H4 antibody (Prionics, Zürich, Switzerland), which recognized residues 144–152 of mouse PrP, was used [10].

2.4. Peptide N-glycosidase F (PNGase F) digestion

For the cleavage of *N*-glycans, cell or tissue lysates were treated with PNGase F (New England Biolabs, Beverly, MA). Protein samples (60 μg) were denatured at 100 °C for 10 min after the addition of a 0.1 volume of 10× denaturing buffer [5% sodium dodecyl sulphate (SDS), 10% 2-mercaptoethanol]. After boiling, a 0.1 volume of 10% Nonidet P-40 and a 0.1 volume of 0.5 M sodium phosphate buffer (pH 7.5) were added. Aliquots (1–20 μl) of PNGase F (1 unit/μl in 50 mM sodium phosphate buffer, pH 7.5) were added to the reaction mixture and incubated for 2 h at 37 °C. An equal quantity of 2× SDS gel-loading buffer [90 mM Tris/HCl (pH 6.8), 10% mercaptoethanol, 2% SDS, 0.02% bromophenol blue, 20% glycerol] was added to stop the reaction with boiling, and samples were analyzed by Western blotting.

2.5. Western blot assay

The procedures for extracting protein from tissues and cells using radio-immunoprecipitation assay (RIPA) buffer and subsequent polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-Dpl antiserum or anti-PrP monoclonal antibody 6H4 were previously described [7]. Equal quantities of protein (60 μg for PrP and 60 μg for Dpl) were used for Western blotting.

3. Results

3.1. Tissue-specific isoforms of Dpl

First, we analyzed the expression of Dpl in various tissues of adult C57BL/6 mice. As previously reported, *Prnd* mRNA levels were high in the testis and moderate in the heart, but low in other organs, including the brain [2]. Using Western blotting, Dpl was detected in only the testis in $Prnp^{+/+}$ mice under our experimental conditions (Supplementary Fig. 1A). Dpl levels in brain and heart were below the detectable limit. A band observed at 17 kDa in both $Prnp^{+/+}$ and $Prnp^{-/-}$ mouse brains was cross-reactive, because its intensity did not decrease from the preincubation with recombinant Dpl. We compared the biochemical profiles of the different isoforms of Dpl in the $Prnp^{+/+}$ testis and $Prnp^{-/-}$ brain. Western blotting demonstrated that the electrophoretic migration of Dpl differed between the $Prnp^{+/+}$ testis and $Prnp^{-/-}$ brain in the absence or presence of PNGase F (Fig. 1A). PNGase F deglycosylation of brain Dpl in $Prnp^{-/-}$ mice resulted in the lowest band, 14 kDa, whereas additional faint bands representing products of either the partial deglycosylation or degradation of Dpl could also be observed. Deglycosylation of Dpl from testis of $Prnp^{+/+}$ mice produced the lowest band, 12 kDa. These results suggested that brain and testis Dpl revealed not only different *N*-linked glycans, but also probably a different polypeptide backbone, because their glycosylated and deglycosylated products differed in their molecular weight. Dose-dependent digestion of *N*-glycans showed two *N*-linked glycosylation sites of Dpl in both the brain and testis (Fig. 1B), confirming above results.

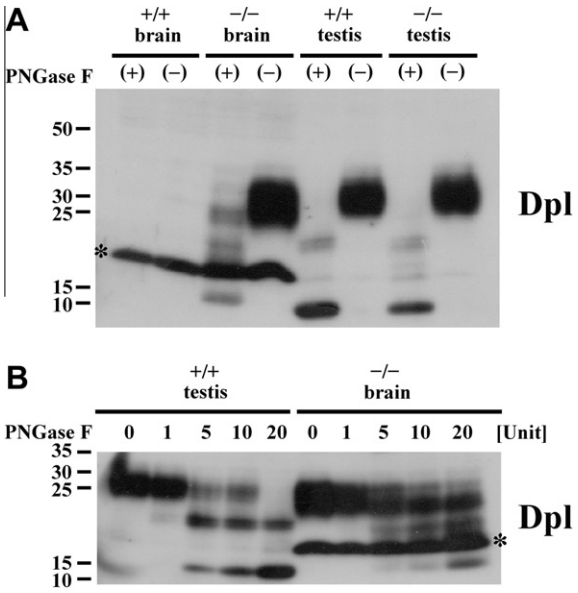

Fig. 1. Modification of Dpl was different between the brain and testis. (A) $Prnp^{+/+}$ mouse brain (+/+ brain), Rikn $Prnp^{-/-}$ mouse brain (−/− brain), $Prnp^{++}$ mouse testis (+/+ testis), and Rikn $Prnp^{-/-}$ mouse testis (−/− testis) were analyzed by Western blotting with anti-Dpl rabbit antiserum before (−) and after (+) PNGase F treatment. (B) Dose-dependent deglycosylation of Dpl by PNGase F (0–20 Unit) showed two *N*-glycosylation sites in Dpl. The asterisk indicated a nonspecific band, which was observed in both $Prnp^{+/+}$ and Rikn $Prnp^{-/-}$ mouse brain. The molecular weight marker (kDa) was shown on the left.

3.2. Prnp/Prnd chimeric and Prnd mRNA

Prnd mRNA was expressed from a promoter upstream of exon 1 of *Prnd* at high levels in the testis [2], while *Prnp*/*Prnd* chimeric mRNA originating from the *Prnp* promoter was present in the brain of ataxic *Prnp*-knockout mice Ngsk and Rcm0, but not in ZrchI [2]. The late-onset ataxia was observed from Rikn $Prnp^{-/-}$ mice, as in Ngsk $Prnp^{-/-}$ mice [4]. Northern analysis demonstrated *Prnp*/*Prnd* chimeric mRNAs (4.4 kb and 2.7 kb) in the brain of Rikn $Prnp^{-/-}$ mice, as in other ataxic *Prnp*-knockout mice (Supplementary Fig. 1B). In $Prnp^{+/+}$ mouse testis, bands of 4.0 and 2.4 kb were observed. These RNA sizes were slightly different from the previous reports [2], perhaps because of incompatibility of the size determinations.

3.3. Function of intergene exon 2 in Prnp/Prnd chimeric mRNA

Prnp/*Prnd* chimeric mRNA was composed of *Prnp* exons 1 and 2, *Prnd* exons 2 and 3, and intergene exons of unknown function.

Three *Prnp*/*Prnd* chimeric mRNAs were produced from the intergenic splicing. Interestingly, intergene exon 2 contained ATG, as already reported [2]. To investigate the effect of intergene exon 2 on the translation of Dpl, *Prnp*/*Prnd* chimeric cDNA and *Prnd* cDNA were cloned using the primers described in Materials and Methods. The resulting plasmids, pIREShyg-chPrPDpl and pIREShyg-Dpl, were transfected into HpL3-2 cells and selected for the resistance against the toxicity of hygromycin, producing HpL3-2-chPrPDpl and HpL3-2-Dpl, respectively. As a control, pIRES-hyg was transfected into HpL3-2, resulting in HpL3-2-hyg. HpL3-2-chPrPDpl and HpL3-2-Dpl cells expressed Dpl of the same size in the presence or absence of PNGase F, suggesting that the modification of Dpl was not affected by the presence of intergene exon 2 (Fig. 2A–C). Because the sequence around the ATG of intergene exon 2 was far from the Kozak consensus (consensus GCCGCCa/gCCATGG; intergene exon 2 TTCTGGATCATGA) for the initiation of eukaryotic mRNAs, which may not function as the initiation codon. As the sequence around the ATG codon of exon 2a of *Prnd* (AGATTCACCATGA) was conserved and matched the Kozak consensus sequence [2], the translation of Dpl was suggested to be initiated from exon 2a of *Prnd*.

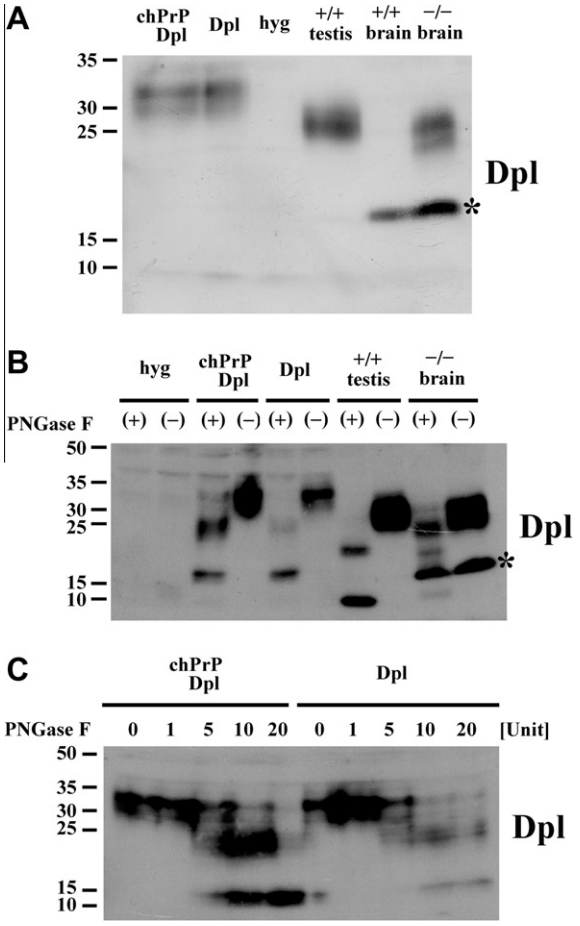

Fig. 2. Similar modification of Dpl in *Prnp*-deficient cells expressing *Prnp*/*Prnd* chimeric mRNA and *Prnd* mRNA. (A) Dpl in *Prnp*-deficient cells expressing a *Prnp*/*Prnd* chimeric mRNA (HpL3-2-chPrPDpl) and *Prnd* open reading frame (ORF) mRNA (HpL3-2-Dpl) was analyzed by Western blotting. Empty vector-transfected cells (HpL3-2-hyg), $Prnp^{+/+}$ mouse testis (+/+ testis), $Prnp^{+/+}$ mouse brain (+/+ brain), and Rikn $Prnp^{-/-}$ mouse brain (−/− brain) were also included as a control. (B) Dpl in the above cells and tissues was treated with PNGase F (+) or not (−). (C) Dpl in the above cells treated with PNGase F (0–20 Unit) in dose-dependent manner. Western blotting for Dpl was performed with anti-Dpl rabbit antiserum. The asterisk indicated a nonspecific band, which was observed in both $Prnp^{+/+}$ and Rikn $Prnp^{-/-}$ mouse brain. The molecular weight marker (kDa) was shown on the left.

3.4. Comparative analysis of PrP and Dpl protein expression patterns in several cell lines

We compared the glycosylation patterns of PrP and Dpl in PrP- or Dpl-over-expressing HpL3-4, N2a, and/or PT67 cells, respectively. Equal amounts of total proteins were extracted with RIPA buffer, separated by 12% SDS–PAGE, and analyzed using the 6H4 or anti-Dpl antibodies. A complex pattern of PrP and Dpl was observed in all samples (Fig. 3). HpL3-4-PrP and N2a cells displayed similar patterns with multiple bands, representing glycosylated and non-glycosylated forms of PrP. Similarly, HpL3-4-Dpl and N2a-Dpl cells displayed similar banding patterns of Dpl. On the other hand, PrP and Dpl showed less electrophoretic mobility in PrP- and Dpl-over-expressing PT67 cells than HpL3-4-PrP and HpL3-4-Dpl cells, respectively (Fig. 3A and B). To verify that the differences in electrophoretic mobility were indeed due to

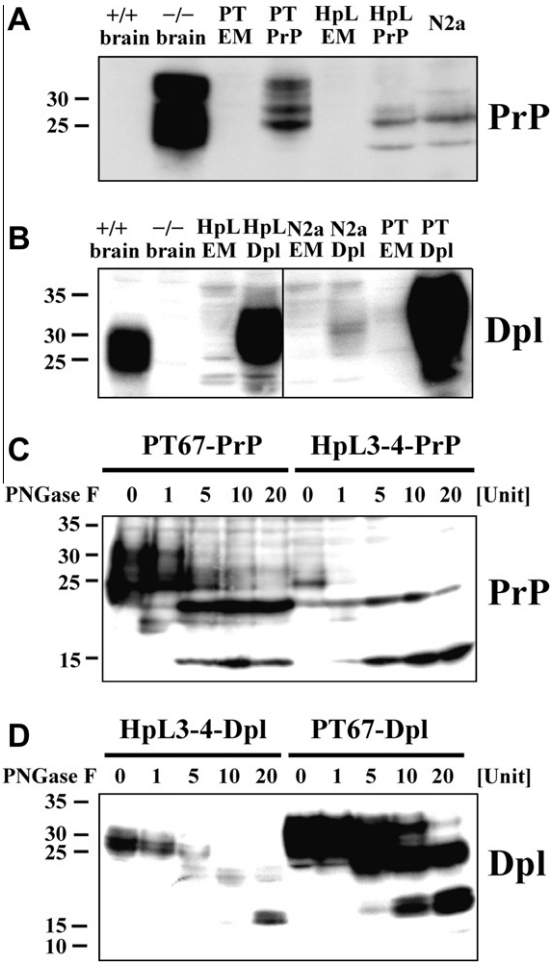

Fig. 3. Different modifications of PrP and Dpl in cell lines. (A) PrP in cell lines transduced with the *Prnp*-expressing vector pMSCVpuro-PrP (PTPrP: PT67-PrP; HpLPrP: HpL3-4-PrP) or empty vector pMSCVpuro (PTEM: PT67-EM; HpLEM: HpL3-4-EM), or the non-transfected cell line (N2a), as described in Materials and Methods, was analyzed by Western blotting with the anti-PrP antibody 6H4. $Prnp^{+/+}$ mouse brain (+/+ brain) and Rikn $Prnp^{-/-}$ mouse brain (−/− brain) were included as a control. (B) Dpl in cell lines transfected with the *Prnd*-expressing vector pMSCVhyg-EGFP-Dpl (HpLDpl: HpL3-4-Dpl; PTDpl: PT67-Dpl; N2aDpl: N2a-Dpl) or empty vectors pMSCVhyg-EGFP (HpLEM: HpL3-4-EM; PTEM: PT67-EM; N2aEM: N2a-EM) were analyzed by Western blotting with anti-Dpl rabbit antiserum. $Prnp^{++}$ mouse brain (+/+ brain) and Rikn $Prnp^{-/-}$ mouse brain (−/− brain) were included as a control. (C) PrP in PT67-PrP and HpL3-4-PrP cells was dose-dependently treated with PNGase F (0–20 Unit). Western blotting for PrP was performed with 6H4. (D) Dpl in HpL3-4-Dpl and PT67-Dpl cells was dose-dependently treated with PNGase F (0–20 Unit). Western blotting for Dpl was performed with anti-Dpl rabbit antiserum. The molecular weight marker (kDa) was shown on the left.

glycosylation, we performed specific *N*-deglycosylation with PNGase F treatment (Fig. 3C and D). Dose-dependent deglycosylation of PrP and Dpl was observed with an increasing concentrations of PNGase F. Treatment with 20 units of PNGase F revealed the deglycosylated forms of PrP and Dpl to be similar between HpL3-4 and PT67 transfectants, suggesting that the *N*-glycosylated forms revealed a similar protein backbone with different glycan chains, according to the specific cell types.

3.5. Endoproteolytic C1 fragment of PrP was decreased by Dpl, whereas another endoproteolytic fragment was increased by prion infection

By Western blotting, we investigated the modification of PrP and Dpl in HpL3-4 transfectants. HpL3-4-EMDpl and HpL3-4-PrPDpl produced a similar sized Dpl, suggesting that the expression of PrP did not change the electrophoretic migration of Dpl in HpL3-4 cells (Fig. 4A). This tendency was also observed in the presence of PNGase F. After the deglycosylation of murine PrP, full-length PrP (21 kDa) and a 13-kDa endoproteolytic fragment (C1) were detected using the 6H4 antibody, as already reported [11]. The molecular weight of the proteolytic fragment in our study was slightly different from that reported [11], perhaps because of incompatibility in molecular weight determinations or the 3F4 epitope tag, used in the previous study. Full-length and endoproteolytic C1 fragments of PrP^C were detected to a similar extent in cells expressing PrP^C only (HpL3-4-PrPEM), whereas cells expressing both PrP^C and Dpl (HpL3-4-PrPDpl) showed a decreased amount of the C1 fragment (Fig. 4B). These results indicated that Dpl-expression decreased the endoproteolytic fragmentation of PrP. In contrast to PrP, the endoproteolytic fragment of Dpl was not detected in *Prnp*$^{+/+}$ testis, Rikn *Prnp*$^{-/-}$ brain, and Dpl-over-expressing HpL3-4 cells, because the completely deglycosylated Dpl under higher concentrations of PNGase F would show only one band, as previously reported [12]. Next, PrP and Dpl proteins in the brain of *Prnp*$^{+/+}$, Rikn *Prnp*$^{-/-}$, and Rikn *Prnp*$^{+/-}$ mice were analyzed. Correspondingly, the migration of Dpl was not affected by PrP from the brain in the presence or absence of PNGase F, whereas the endoproteolytic fragment of PrP (C1) was reduced in Rikn *Prnp*$^{+/-}$ mouse brain expressing PrP and Dpl in comparison with *Prnp*$^{+/+}$ mouse brain expressing PrP, but not Dpl (Fig. 4C). Finally, PrP in the prion-infected and uninfected brains was analyzed by Western blotting using the anti-PrP antibody SAF83 after deglycosylation with PNGase F. The amount of the endoproteolytic fragment of PrP, whose molecular weight was slightly higher than that of C1, increased after the infection with prions such as the Chandler and Obihiro strains (Supplementary Fig. 2).

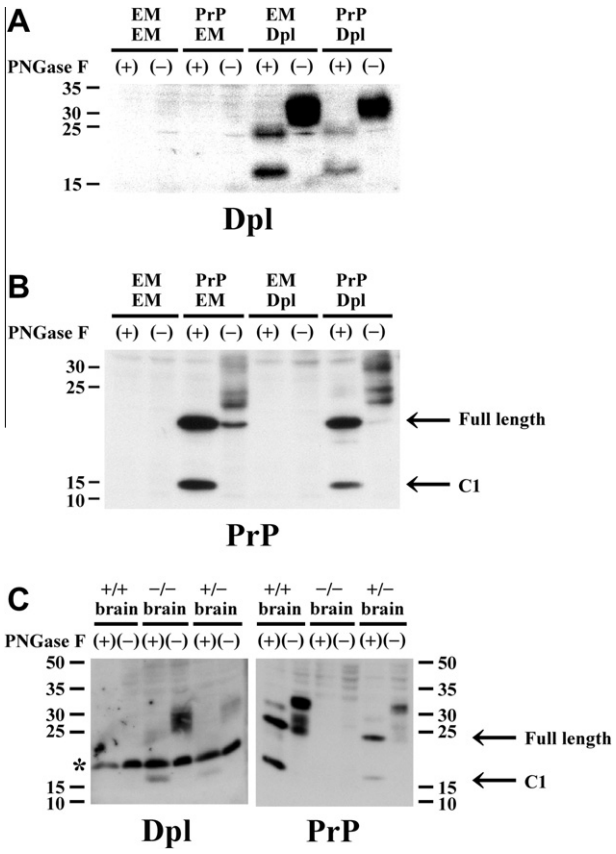

Fig. 4. Reduced endoproteolysis of PrP by Dpl expression. Dpl in HpL3-4 cells transfected with pMSCVpuro + pMSCVhyg-EGFP (EMEM: HpL3-4-EMEM), pMSCVpuro-PrP + pMSCVhyg-EGFP (PrPEM: HpL3-4-PrP), pMSCVpuro + pMSCVhyg-EGFP-Dpl (EMDpl: HpL3-4-Dpl) or pMSCVpuro-PrP + pMSCVhyg-EGFP-Dpl (PrPDpl: HpL3-4-PrPDpl) treated with PNGase F (+) or not (–) was analyzed by Western blotting with anti-PrP antibody (A) or anti-Dpl antibody (B), as described in Materials and Methods. Levels of the C1 PrP fragment were low in HpL3-4-PrPDpl cells compared to HpL3-4PrPEM cells, suggesting that Dpl-expression decreased levels of the C1 fragment. (C) Dpl and PrP in *Prnp*$^{+/+}$ mouse brain (+/+ brain), Rikn *Prnp*$^{-/-}$ mouse brain (–/– brain), and *Prnp*$^{+/-}$ mouse brain (+/– brain) were analyzed by Western blotting with anti-Dpl antibody or anti-PrP antibody after treatment with PNGase F (+) or not (–). Full-length PrP and the C1 fragment were indicated. The asterisk indicated the nonspecific band observed in both *Prnp*$^{+/+}$ and Rikn *Prnp*$^{-/-}$ mouse brain. The molecular weight marker (kDa) was shown.

4. Discussion

The expression patterns of the glycoforms of PrP and Dpl from various cell types were compared. In the mouse neuroblastoma cell line N2a, the electrophoretic migrations of PrP and Dpl were similar to those in HpL3-4 cells. In the mouse fibroblast cell line PT67, glycosylated PrP and Dpl displayed less mobility in SDS–PAGE than the corresponding Dpl in N2a and HpL3-4 cells. These results were consistent with the notion that the type of HpL3-4 cells was similar to N2a cells, but different from PT67 cells. When *N*-linked glycans were removed by digestion with PNGase F, the migration patterns of PrP and Dpl from all three cell lines (HpL3-4, N2a and PT67) was similar. These results suggested that carbohydrate modifications, including the composition and length of oligosaccharide chains, caused the differences in mobility in PrP and Dpl from different cell types.

Different modifications of Dpl also occurred in tissues. While the predicted size of mouse Dpl with the removed signal peptides from the N- and C-termini was 14.9 kDa [12], Dpl in Rikn *Prnp*$^{-/-}$ brain was 21–27 kDa and 14 kDa long in the absence and the presence of PNGase F. Dpl in the testis was 24–28 kDa long in the absence and 12 kDa in the presence of PNGase F. Brain Dpl was 1 kDa smaller, while testis Dpl was 2 kDa smaller than the predicted size of Dpl. As the putative size of mature Dpl was calculated to be larger, the Dpl in brain and testis may be digested with tissue-specific proteases, whose expression was probably regulated in a tissue-specific manner; however, the presence of N- or C-terminal truncation was not fully determined. Several studies reported that Dpl in the testis was translated from *Prnd* mRNA, and Dpl in the brain of ataxic *Prnp*$^{-/-}$ mice, such as Rcm0, ZrchII, Ngsk and Rikn, was translated from *Prnp*/*Prnd* chimeric mRNA. Two *Prnp*/*Prnd* chimeric mRNAs contained intergene exon 2, which included ATG, while *Prnd* mRNA did not contain intergene exon 2 [2]. However, such a difference did not influence the modification of Dpl. Because the existence of intergene exon 2 did not change the electrophoretic migration of Dpl in HpL3-4 cells, it suggested that ATG in intergene exon 2 did not act as a translation initiation codon. Therefore, differences in electrophoretic migration patterns between the brain and testis may not

be due to differences in mRNA species; however, as we amplified a portion of *Prnd* mRNA and *Prnp*/*Prnd* chimeric mRNA, it may not be excluded that 5′ or 3′ end sequences were important to initiate the translation of Dpl.

Nishida et al. demonstrated that Dpl neurotoxicity, which occurred in the absence of PrP, was inhibited by the PrP transgene [13]. The mechanisms of the neurotoxicity and its inhibition by PrP were not determined; however, the present study suggested that the change of posttranslational modifications (especially glycosylation) of Dpl was not involved in the inhibition of toxicity by PrP, because the expression of PrP did not change the electrophoretic migration of Dpl. On the other hand, Dpl-expression decreased the levels of the endoproteolytic C1 fragment of PrP. It should be noted that the phenomenon did not appear to be related to the pathogenesis of prion disease, because prion infection did not affect levels of the C1 fragment (Supplementary Fig. 2). By contrast, another endoproteolytic fragment with higher molecular weight than C1, resembling the C2 fragment [14], increased after prion infection. Hence, C2 not C1 may contribute to the pathogenesis. Recent studies revealed that after the proteolysis, the C1 fragment of PrP involved a disintegrin and metalloprotease family 10 (ADAM10), whose activity was mediated by ADAM9 [15]. Therefore, investigating the mechanism of reduced Dpl expression proceeding PrP digestion may elucidate the regulation of ADAM9 and ADAM10. Interestingly, our recent studies indicated that PrP could specifically stabilize the copper homeostasis, where the oxidative conditions could perturb, while Dpl expression could prevent the PrP function in copper homeostasis. The results suggested the potential role of Dpl by preventing the role of PrP in the copper homeostasis [7]. Therefore, further study will be necessary to determine whether inhibition of the endoproteolytic cleavage of PrP by Dpl influences the function of PrP.

In summary, we provided evidence that the modification (especially glycosylation) of Dpl was tissue- and cell-type dependent. More importantly, we found that PrP expression did not modulate the modification of Dpl, whereas endoproteolytic cleavage of PrP was decreased by Dpl expression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.016.

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