Prion Proteins with Insertion Mutations Have Altered N-terminal Conformation and Increased Ligand Binding Activity and Are More Susceptible to Oxidative Attack*

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We compared the biochemical properties of a wild type recombinant normal human cellular prion protein, rPrPc, with a recombinant mutant human prion protein that has three additional octapeptide repeats, rPrP8OR. Monoclonal antibodies that are specific for the N terminus of rPrPc react much better with rPrP8OR than rPrPc, suggesting that the N terminus of rPrP8OR is more exposed and hence more available for antibody binding. The N terminus of rPrPc contains a glycosaminoglycan binding motif. Accordingly, rPrP8OR also binds more glycosaminoglycan than rPrPc. In addition, the divalent cation copper modulates the conformations of rPrPc and rPrP8OR differently. When compared with rPrPc, rPrP8OR is also more susceptible to oxidative damage. Furthermore, the abnormalities associated with rPrP8OR are recapitulated, but even more profoundly, in another insertion mutant, which has five extra octapeptide repeats, rPrP10OR. Therefore, insertion mutants appear to share common features, and the degree of abnormality is proportional to the number of insertions. Any of these anomalies may contribute to the pathogenesis of inherited human prion disease.

All prion diseases are believed to share the same pathogenic mechanism based on the conversion of the normal cellular prion protein (PrPc) into the infectious and pathogenic scrapie prion protein (PrPSc) (1–4). Approximately, 10–15% of human prion disease is caused by mutations in the gene coding for the germ line prion gene, PRNP.

The mechanisms by which a mutant prion protein (PrP) causes neurodegeneration are not completely understood. It is thought that the mutant protein is inherently unstable leading to self-association to produce an oligomeric structure (5, 6). This structure acts as a “seed” recruiting additional mutant PrP, eventually leading to the formation of PrPSc. The accumulation of PrPSc in the central nervous system is then thought to impair function, induce structural damage, and cause disease.

At least 20 different pathogenic mutations in the human PRNP gene have been identified (7). These are either insertion or point mutations. Insertion mutation occurs solely in the octapeptide repeat region, which is involved in the binding of divalent cations such as copper and zinc. Human PrP has five octapeptide repeats. In pathogenic mutations, the number of additional octapeptide repeats ranges from one to nine (8, 9). A transgenic mouse line with nine additional octapeptide repeats spontaneously develops neurodegeneration but does not produce infectious PrPSc (10, 11).

Although it is clear that mutation in the prion gene causes human prion diseases, very little is known about the mechanisms by which the mutant protein causes disease. Prion proteins with pathogenic mutations may cause disease because of gain of toxic functions, loss of normal physiologic functions, or both.

Recently, we identified a novel insertion mutation in a Chinese family, whose PRNP has three additional octapeptide repeats (12). Subsequently, another family with the identical mutation was identified in Europe (13). In this study, we report studies comparing the conformation of insertion mutant recombinant protein with wild type recombinant protein using a panel of well characterized anti-PrP monoclonal antibodies. We also studied the effects of the mutation on binding to glycosaminoglycan (GAG) and copper, two known ligands of PrPc, as well as susceptibility to oxidative insults. Finally, we investigated whether our findings with the three extra octapeptide repeat mutant proteins are applicable to another insertion mutant protein with five extra octapeptide repeats.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The wild type rPrPc cDNA gene corresponding to the putative mature fragment (23–231) and the human pathogenic mutant rPrP8OR with three extra octapeptide repeats encoding residues (23–255) were amplified by polymerase chain reaction (PCR) using human genomic DNA templates (12). The primers were 5’-ATCCATATGAGAGGCCGCGAGGCTG-3’ (forward sequence) and 5’-ACCGGAAATTCTCTGACTGATCCCTCTCCTGG-3’ (reverse sequence). The PCR product was cloned between restriction sites NdeI and EcoRI of the vector pET42(+) (Novagen) termed pET-rPrPc and pET-rPrP8OR. A similar procedure was also carried out to obtain pET-rPrP10OR, which contains five extra octapeptide repeats, using the human prion insertion mutant DNA template (14). A deletion mutant of rPrPc, which lacks the N-terminal GAG binding motif (resi...
dues 23–35) (15), designated as rPrP<sup>Δ23–35</sup> was constructed from pET-rPrP<sup>p</sup> using QuikChange<sup>®</sup> site-directed mutagenesis kit. Primers were 5'-TACATATGGGCAGCGCATACCC-3' (forward sequence) and 5'-CAGGTACGCGTGCATAGTATATCTCC-3' (reverse sequence). The insertion sequences were verified by using an Applied Biosystems 373A automated sequencer.

**Generation and Purification of Recombinant Human rPrP<sup>c</sup>, rPrP<sup>8OR</sup>, and rPrP<sup>10OR</sup>**—The purification and refolding process was carried out on a nickel ion-charged Sepharose column (Amersham Biosciences) as described previously (16). Briefly, freshly transformed *Escherichia coli* BL21 (DE3) (Novagen) containing the plasmid pET-rPrP<sup>p</sup> or pET-rPrP<sup>ΔOR</sup> was transferred to 1 liter of Luria broth (LB) medium supplemented with 50 μg/ml kanamycin at 37 °C until the A<sub>600</sub> reached 1.0 and induced overnight with 1 mM isopropyl β-D-thiogalactoside. Bacteria were harvested by centrifugation at 4,000 × g for 15 min at 4 °C, resuspended in 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml lysozyme, and incubated at 21 °C for 2 h before further lysis by sonication. Samples were centrifuged at 13,000 × g for 20 min, and the protein pellets were extensively washed using 1% Triton X-100 and then resuspended in 20 mM Tris/HCl (pH 8.0), 8 M urea, and 10 mM 2-mercaptoethanol. The inclusion bodies were purified and refolded on a nickel ion-charged Sepharose column by decreasing urea gradient concentrations. rPrP was eluted by 50 mM sodium acetate and refolded on a nickel ion-charged Sepharose column by decreasing urea gradient concentrations. rPrP was eluted by 50 mM sodium acetate (pH 4.0) followed by dialysis against PBS (pH 7.4). A similar procedure was used to obtain rPrP<sup>ΔOR</sup> and rPrP<sup>Δ23–35</sup>. Protein concentration was determined with a Bio-Rad protein assay kit.

**Antibodies**—Nine affinity-purified anti-PrP mAbs were used in this study (17, 18). mAb 8B4 recognizes an epitope at residues 35–45; 5B2 recognizes residues 34–52; SAF32 reacts with residues 63–94 covering the octapeptide repeat sequences (19); 7A12 interacts with residues 115–130 covering the octapeptide repeat region (Fig. 1); 7A12 interacts with helix A between residues 143–155; 7H6 recognizes residues 130–140; 11G5 reacts with residues 115–130 covering β-sheet 1; 2C2 reacts with residues 153–165 covering β-sheet 2; 8H4 recognizes residues 175–185 of helix B; and 8F9 reacts with residues 220–231. mAbs 8B4, 5B2, SAF32, 7A12, 2C2, 8H4, and 8F9 are IgG<sub>1</sub>, whereas mAbs 11G5 and 7H6 are IgG<sub>2b</sub>. Biotinylation of mAbs was performed using the EZ-linked sulfo-NHS-biotin kit (Pierce Endogen) according to the manufacturer’s recommendation.

**SDS-PAGE and Immunoblotting**—For SDS-PAGE, samples were mixed with 2× SDS loading buffer (160 mM Tris, 4% SDS, 4% 2-mercaptoethanol, 20% glycerol, and 0.04% bromphenol blue, pH 6.8), heated for 10 min at 95 °C, and then loaded onto SDS-polyacrylamide gels (Bio-Rad). After electrophoresis, 12% SDS-PAGE slabs were electroblotted onto nitrocellulose membranes and immunoblotted as described (20). Transferred rPrP was detected using anti-PrP mAbs in combination with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG F<sub>c</sub>-specific antibody as the secondary antibody. rPrP visualization was performed using the chemiluminescence blotting system (Roche Applied Science).

**Capture ELISA**—Capture antibody (8H4 or 11G5) was coated at 50 ng/well in flat-bottomed, 96-well Costar plates (Corning) overnight at 4 °C. Excess antibody was removed, and wells were blocked with phosphate-buffered saline (PBS) containing 3% BSA (Sigma) for 3 h at room temperature. Plates were washed three times with PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T), and recombinant human rPrP<sup>p</sup>, rPrP<sup>ΔOR</sup> or rPrP<sup>Δ10OR</sup> (1 μg/ml) in PBS was added in triplicate to respective wells overnight at 4 °C. Plates were washed three times with PBS-T, and appropriate dilutions of biotinylated mAbs were added to designated wells for further 6 h at room temperature. Plates were washed three times with PBS-T, and HRP-conjugated streptavidin at 1:10,000 was added for 1 h. ELISA plates were washed twice with PBS-T before adding the substrate p-nitrophenyl phosphate at 0.5 mg/ml. ELISA plates were read at 405 nm on a Beckman Coulter AD340 micro-ELISA plate reader.

To study the effects of divalent copper on the mAb binding profiles to rPrP<sup>p</sup> or rPrP<sup>ΔOR</sup>, ELISA plates were coated overnight with 50 ng/well mAb 8H4, and unbound sites were blocked using 3% BSA. After washing three times, rPrP<sup>p</sup> or rPrP<sup>ΔOR</sup> (1 μg/ml) was added for 6 h. Plates were washed three times, and different concentrations of CuSO<sub>4</sub> were added for overnight incubation at room temperature with gentle agitation (21). The plates were washed with PBS-T, and biotinylated SAF32, 11G5, 2C2, or 7A12 (0.1 μg/ml) was added for 6 h. After extensive washing, HRP-conjugated streptavidin was added for reading at 405 nm. Antibody binding is given as a percentage of the increased binding signal compared with untreated sample.

**Detection of rPrP Binding to GAG**—GAG binding experiments were performed as described previously (15). Briefly, chondroitin sulfate (from bovine trachea), heparin sulfate (from porcine intestinal mucosa), and heparin (from porcine intestinal mucosa) (all from Sigma) were coated on plates at 10 μg/well at 4 °C overnight and blocked with 3% BSA in PBS at room temperature for 3 h. Plates coated with BSA were used as controls. Appropriate dilutions of rPrP<sup>p</sup>, rPrP<sup>ΔOR</sup>, or rPrP<sup>Δ10OR</sup> were incubated with the coated plates for 2 h. After three washes with PBS-T, bound rPrP was detected with mAb 8H4. All experiments were carried out in triplicate and repeated at least three times.

**Detection of Carbonyl Groups on rPrP**—Protein oxidation was measured by assaying carbonyl groups on proteins using the OxyBlot<sup>™</sup> protein oxidation detection kit (Intergen, Purchase, NY) as described (22). To produce oxidative damages, equal amounts of rPrP<sup>p</sup>, rPrP<sup>ΔOR</sup>, or rPrP<sup>Δ10OR</sup> (1 μg) was incubated with different concentrations of H<sub>2</sub>O<sub>2</sub> at room temperature for 30 min. Samples were then derivatized to 2,4-dinitrophenyl (DNP) by reaction with 2,4-dinitrophenyl hydrazine (DNPH) at room temperature for 30 min before loading onto SDS-polyacrylamide gels. The proteins were electrotransferred, probed with a rabbit antiserum against DNP-modified carbonyl groups, and visualized using the chemiluminescence blotting system (Roche Applied Science). No immunoreactivity was detected in non-DNPH-modified samples.

**RESULTS**

**Characterization of Recombinant Wild Type Prion Protein (rPrP<sup>p</sup>) and Mutant Prion Protein (rPrP<sup>ΔOR</sup>)**—We analyzed the two recombinant proteins by SDS-PAGE under either non-reducing or reducing conditions. In both conditions, rPrP<sup>p</sup> appears as a single band with a molecular mass of approximately 23 kDa, which is the expected molecular mass of full-length rPrP<sup>p</sup> protein. rPrP<sup>ΔOR</sup> also appears as a single band but migrates a bit slower, with a molecular mass of 25 kDa, reflecting the addition of 24 amino acids in the octapeptide repeat region (Fig. 1A). Neither rPrP<sup>p</sup> nor rPrP<sup>ΔOR</sup> preparation contains larger molecular species. To further characterize the two recombinant proteins, we immunoblotted them with three different anti-PrP mAbs. The binding epitopes of these mAbs are presented diagrammatically in Fig. 1B. The three mAbs, 8B4 (residues 35–45), 8H4 (residues 175–185), and 8F9 (residues 220–231), reacted equally with rPrP<sup>p</sup> and rPrP<sup>ΔOR</sup> (Fig. 1C).

**Conformational Differences between rPrP<sup>p</sup> and rPrP<sup>ΔOR</sup>; N terminus of rPrP<sup>ΔOR</sup> Is More Available for mAb Binding**—We next studied the conformations of rPrP<sup>p</sup> and rPrP<sup>ΔOR</sup> by comparing their mAb binding profiles, using a panel of mAbs in a capture ELISA format.
mAb 8H4 (Fig. 2, A–G) or mAb 11G5 (Fig. 2H) was immobilized on ELISA plates to capture either rPrPc or rPrP\(^{\text{8OR}}\). Biotinylated anti-PrP mAbs with dissimilar specificities were then added in different concentrations to react with the captured proteins. Two biotinylated mAbs, 8B4 and 5B2, with epitopes at the N terminus reacted much stronger with rPrP\(^{\text{8OR}}\) than rPrPc (Fig. 2, A and B). mAb SAF32 reacts with an epitope within the octapeptide repeat region. At lower concentrations, biotinylated SAF32 reacted stronger with rPrPc than rPrP\(^{\text{8OR}}\) (Fig. 2C). Five other biotinylated mAbs, 7A12, 7H6, 11G5, 2C2, and 8H4, which react with epitopes in either the central region or the C terminus, reacted similarly with rPrPc and rPrP\(^{\text{8OR}}\) (Fig. 2, D, E, F, G, and H, respectively). Overall, these results
suggest that the N terminus of rPrP8OR is more exposed and thus more available for binding of N terminus-specific mAbs. The addition of three octapeptide repeats also altered the conformation of the octapeptide repeat region but in a more subtle way.

Binding of rPrPc and rPrP8OR to GAGs—All mammalian PrPc contains a GAG binding motif, KKRPK, the first five amino acids at the N terminus (15). If the N terminus of rPrP8OR is more exposed, it should bind better to GAG. To test this hypothesis, ELISA plates were coated with three different GAGs: chondroitin sulfate B (Fig. 3A), heparin sulfate (Fig. 3B), and heparin (Fig. 3C). After extensive washing, different amounts of rPrPc, rPrP8OR, or a deletion mutant protein, rPrP10OR, lacking the N-terminal GAG binding motif, were added. mAb 8H4 was then used to detect bound proteins. It is clear that rPrP8OR binds the three GAGs much better than rPrPc. On the other hand, rPrP10OR, which lacks the KKRPK motif, failed to bind any of the GAGs (Fig. 3). These results provide additional support for our conclusion that the N terminus of rPrP8OR is more exposed and thus more available for ligand binding. Furthermore, these results provide conclusive evidence that the N terminus of PrPc contains the predominant GAG binding motif.

Cu2+ Has Different Effects on the Conformations of rPrPc and rPrP8OR—Addition of Cu2+ increases the binding of mAbs 11G5 and 2C2 to brain-derived mouse PrPc suggesting that binding of Cu2+ results in conformational changes in PrPc (21). We therefore investigated whether Cu2+ also alters the binding profiles of these mAbs to rPrPc and rPrP8OR (Fig. 4). Similar to our findings with native, brain-derived mouse PrPc, the addition of Cu2+ also significantly increased the binding of mAbs 11G5 and mAb 2C2 (Fig. 4, B and C) but not the binding of mAb SAF32 or mAb 7A12 to rPrPc (Fig. 4, A and D). In contrast, adding Cu2+ did not increase the binding of mAb 11G5 or mAb 2C2 to rPrP8OR (Fig. 4, B and C).

We next investigated whether Cu2+ also influences the conformation of the N terminus. In the presence of Cu2+, rPrPc but not rPrP8OR binds more mAb 8B4 and GAG in a Cu2+ concentration-dependent manner (Fig. 4, E and F). These results suggest that Cu2+ affects the conformation of rPrPc but rPrP8OR differently.

rPrP8OR Is More Susceptible to Oxidative Damage—Protein oxidation results in the generation of carbonyl groups that can be chemically modified with DNPH. The degree of DNPH modification correlates with the level of protein oxidation (23). Histidine residues in rPrPc are highly susceptible to oxidation (24). Therefore, the additional histidine residues in the octapeptide repeat region of rPrP8OR may render the mutant protein more susceptible to oxidation. We determined whether rPrP8OR is indeed more prone to oxidation. Equal amounts of rPrPc and rPrP8OR were modified with DNPH, separated by SDS-PAGE, and then immunoblotted with an anti-DNP antibody (22). Anti-DNP immunoreactivity was detected only on rPrP8OR but not on rPrPc (Fig. 5A, lanes 1 and 5). Therefore, rPrP8OR contains carbonyl groups, which are signatures of prior protein oxidation.

We next investigated whether rPrP8OR is also more susceptible to the oxidation agent, H2O2. Equal amounts of rPrPc or rPrP8OR was incubated with different concentrations of H2O2 ranging from 1 to 100 μM and subjected to DNPH modification. Subsequently, each sample was divided into three aliquots: one was immunoblotted with anti-DNP antibody; one was immunoblotted with anti-PrP mAb 8B4; and the third one was immunoblotted with anti-PrP mAb 7A12. DNPH immunoreactivity was detected only on rPrP8OR and in an H2O2 concentration-dependent manner (Fig. 5A, lanes 6–8). All samples reacted equally with anti-PrP mAbs 7A12 and 8B4 indicating that they had comparable amounts of protein (Fig. 5, B and C).

Selective Modification of the N terminus by High Concentrations of H2O2—In subsequent experiments, we found that mAb 8B4 was unable to bind rPrP8OR when rPrP8OR was exposed to very high concentrations of H2O2 (such as 1 μM) (Fig. 6A). Under identical conditions, the epitopes of mAbs 7A12 and 8F9, which were located at the central region and the C terminus respectively, remained intact (Fig. 6, B and C). Similar treatment did not alter the binding of mAb 8B4 to rPrPc (Fig. 6A). These results are consistent with our interpretation that the N terminus of rPrP8OR is more exposed and thus is more susceptible to oxidation.

Findings with rPrP8OR Are Applicable to rPrP10OR—We next sought to determine whether some of the aberrant features found in rPrP8OR are applicable to another insertion mutant protein that has five extra octapeptide repeats, rPrP10OR. When immunoblotted with mAb 8B4, rPrP10OR migrates a bit slower than rPrP8OR, reflecting the addition of more octapeptide repeats (Fig. 7A). Similar to rPrP8OR, rPrP10OR also reacts more strongly with N terminus specific mAb 8B4 in captured ELISA (Fig. 7B), binds more GAG (Fig. 7C), and is more susceptible to oxidative damage (Fig. 7D). In all cases, the levels of abnormality are more profound in rPrP10OR than in rPrP8OR. At 1 μM H2O2, aggregates with higher molecular masses were detected in rPrP10OR but not in rPrP8OR suggesting that aggregate formation may depend on the num-

![FIGURE 3. rPrP8OR binds more GAG than rPrPc.](image-url)
number of octapeptide inserts. Collectively, these findings suggest that all prion proteins with insertion mutations share common features, and the degrees of abnormality are proportional to the number of insertions.

**DISCUSSION**

In this study, we describe four new findings on the biochemical properties of a recombinant human prion protein with three extra octapeptide repeats, rPrP8OR. The mutant protein shows the following aberrant features: 1) its N terminus is more exposed; 2) it binds better to GAG; 3) it behaves differently after binding to Cu²⁺/H₁₁₀₀₁; and 4) it is more susceptible to oxidative attack. Importantly, some of the aberrant properties associated with rPrP8OR are also observed in another insertion mutant prion protein with five extra repeats, rPrP₁₀OR, and the aberrations are even more profound in rPrP₁₀OR.

**FIGURE 4.** Copper has differential effects on the mAb binding profiles rPrP⁵ and rPrP₁₀OR. One hundred ng/well either rPrP⁵ or rPrP₁₀OR was captured on ELISA plates coated with 50 ng/well mAb BH₄. After washing, the bound rPrPs were incubated with PBS or various concentrations of CuSO₄ in PBS, and plates were incubated overnight at room temperature. Bound rPrPs were detected using different biotinylated mAbs: SA632 (A); 11G5 (B); 2C2 (C); 7A12 (D); and 8B4 (E). For binding to GAG (F), plates were coated with heparin (10 μg/ml), and bound rPrPs were detected using mAb BH₄ followed by HRP-conjugated goat anti-mouse IgG Fc-specific antiserum. Antibody binding is given as an increased percentage of optical density values. Under PBS-treated conditions, the mean optical density values for rPrP⁵ and rPrP₁₀OR were 2.18, 2.26 (A); 0.86, 0.67 (B); 0.44, 0.56 (C); 1.04, 0.98 (D); 1.23, 2.54 (E); and 0.46, 0.73 (F), respectively.

**FIGURE 5.** rPrP₁₀OR is more susceptible to oxidative damage than rPrP⁵. A, 1 μg of rPrP⁵ and rPrP₁₀OR was incubated with 0, 1, 10, and 100 μM H₂O₂ at room temperature for 30 min. The oxidative marker carbonyl groups in rPrP samples were derivatized to DNP by reaction with DNPH and subjected to Western blot using anti-DNP antibody. Aliquots of the above samples were also subjected to Western blot using mAbs 8B4 (B) and 7A12 (C).

**FIGURE 6.** The mAb 8B4 reactive epitope on rPrP₁₀OR is selectively eliminated in the presence of 1 mM H₂O₂. 1 μg of rPrP⁵ or rPrP₁₀OR was incubated with 0, 10, 100, and 1,000 mM H₂O₂ at room temperature for 30 min. Samples were separated by SDS-PAGE and immunoblotted with mAb 8B4 (A), 7A12 (B), and 8F9 (C). In A, at 1 mM H₂O₂, the mAbs 8B4 reactive epitope was no longer available for antibody binding for rPrP₁₀OR but remained intact for rPrP⁵. Under the same conditions, there were no effects on 7A12 (B) and 8F9 (C) binding epitopes for both rPrP⁵ and rPrP₁₀OR.
NMR analyses of rPrPc at pH 4.5 and 5.5 show that the N terminus is highly flexible and unstructured; in contrast, the C-terminal region has a well-structured globular domain (25, 26). The finding that half of PrPc lacks considerable secondary and tertiary structure is unusual and intriguing. Earlier transgenic mouse studies suggested that the N terminus of PrPc is not required for pathogenesis (27). However, more recent studies suggest that the N terminus of PrPc is important in the pathogenesis of prion diseases. The N terminus modifies disease phenotypes (28) and influences the conformations of protease-resistant PrPSc generated in vivo and in vitro (29, 30) and PrPc aggregation (31). Furthermore, it has been reported that the N terminus of PrP is structured if the studies are carried out at pH values between 6.5 and 7.8, i.e., the pH at cell membrane (32). Therefore, the roles the N terminus plays in the pathogenesis of prion diseases are complex and not completely understood.

Our finding that the N termini of rPrP8OR and rPrP10OR are more available for mAb 8B4 binding suggests that the octapeptide repeat region of PrPc also influences the conformation of the N terminal. The N-terminal end that contains the KKRPK motif is the predominant GAG binding site on PrPc. The reason that rPrP8OR and rPrP10OR bind GAG better is because the N termini of these two proteins are more exposed and thus more available for GAG binding. We propose a model in which the octapeptide repeat region is the “neck” of PrPc. As the length of this region increases, the N-terminal “head” increasingly protracts from the C-terminal globular domain, rendering it more available for ligand binding.

Whether binding of PrP to GAG is important in prion disease is not known. PrPSc particles in vivo contain GAG (35). In vitro, GAG facilitates the conversion of PrP to PrPSc (36). Cell surface GAG has also been reported to be the receptor for PrPSc (37). GAG may function as a scaffold for concentrating PrP, creating a reservoir of PrP for conversion. Because rPrP8OR and rPrP10OR bind GAG better, they will be more prone to be concentrated in the scaffold. Our findings with recombinant prion proteins may have implications for pathogenesis. It has been reported that patients with more octapeptide repeats have earlier disease onset and shorter disease duration (9). Interestingly, by using the N terminus-specific mAb 8B4, we found that the amounts of full-length PrP species are greatly increased in the brain of patients as well as in mice with prion diseases (38). Presumably, these full-length PrP species are able to bind GAG.
Aberrant Features of Insertion Mutant Prion Proteins

The octapeptide repeat region of PrP<sup>C</sup> binds divalent cations such as Cu<sup>2+</sup> and Zn<sup>2+</sup> (39, 40). However, whether such interactions are important in the pathogenesis of prion disease is not clear. We reported earlier that binding of Cu<sup>2+</sup> to native mouse rPrP<sub>C</sub> changes the binding profiles of two anti-PrP mAbs, 11G5 and 2C2, which react with epitopes that are downstream of the octapeptide repeat region (21). In this study, we provided new evidence that binding of Cu<sup>2+</sup> to rPrP<sub>C</sub> also alters the conformation of the N terminus, rendering the N terminus more available for antibody and GAG binding. However, these changes do not occur when Cu<sup>2+</sup> binds to rPrP<sub>D</sub>. Although the physiologic significance of these findings is not clear, it is obvious that this modulation is non-operational in rPrP<sub>D</sub>. The failure to modulate the conformation of the PrP molecule is not because rPrP<sub>D</sub> binds more Cu<sup>2+</sup> than rPrP<sub>C</sub>. Irrespective of the number of octapeptide repeats, the maximum number of Cu<sup>2+</sup>-ions a PrP molecule binds is five.<sup>3</sup>

Oxidative stress is caused by an imbalance in the levels of reactive oxygen species, which are the byproducts of normal cellular metabolism (41). Oxidative stress causes modification of amino acids and fragmentation and/or aggregation of proteins, eventually leading to cell death. The brain is very sensitive to reactive oxygen species because of the amount of oxygen it consumes. Oxidative stress has been speculated to play an important role in neurodegenerative diseases such as prion diseases and Alzheimer disease (42). Whether PrP<sup>C</sup> is directly involved in regulating reactive oxygen species remains controversial (43). However, it is clear that both rPrP<sub>C</sub> and rPrP<sub>D</sub> are more prone to oxidative damages. An increase in oxidative damage can be observed with 1 μM H<sub>2</sub>O<sub>2</sub>, a level that is physiologically relevant (44).

Oxidative stress cross-links amino acids, resulting in aggregation of proteins (45). When incubated with 1 mM H<sub>2</sub>O<sub>2</sub>, rPrP<sub>D</sub> but not rPrP<sub>C</sub> begins to form aggregates of various sizes. Therefore, a minimum number of octapeptide repeats is required for aggregate formation under these conditions. The increase in the number of histidine residues within the octapeptide repeat region is the most likely explanation why rPrP<sub>C</sub> and rPrP<sub>D</sub> are more susceptible to oxidative attack.

The epitope of mAb 8B4 is no longer available for antibody binding when rPrP<sub>D</sub> is incubated in 1 mM H<sub>2</sub>O<sub>2</sub> (46). The reasons that mAb 8B4 fails to bind rPrP<sub>8OR</sub> under this condition cannot rule out the possibility that the failure of mAb 8B4 to bind to rPrP<sub>D</sub> is caused by conformational changes in the other regions of the molecule, resulting in sequestration of the epitope.

Based on these findings, we hypothesize that an increase in the number of octapeptide repeats renders the PrP molecule more susceptible to oxidative attack and that conformational changes at the N terminus enhance the binding of mutant PrP to GAG, which further promotes PrP aggregation. Because these aberrant features are proportional to the number of the insertions, our findings provide a biochemical explanation for the observation that patients with more octapeptide repeat insertions have earlier disease onset and shorter disease duration (9).

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