Human prion proteins with pathogenic mutations share common conformational changes resulting in enhanced binding to glycosaminoglycans

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Mutation in the prion gene *PRNP* accounts for 10–15% of human prion diseases. However, little is known about the mechanisms by which mutant prion proteins (PrPs) cause disease. Here we investigated the effects of 10 different pathogenic mutations on the conformation and ligand-binding activity of recombinant human PrP (rPrP). We found that mutant rPrPs react more strongly with N terminus-specific antibodies, indicative of a more exposed N terminus. The N terminus of PrP contains a glycosaminoglycan (GAG)-binding motif. Binding of GAG is important in prion disease. Accordingly, all mutant rPrPs bind more GAG, and GAG promotes the aggregation of mutant rPrPs more efficiently than wild-type recombinant normal cellular PrP (rPrP^C). Furthermore, point mutations in *PRNP* also cause conformational changes in the region between residues 109 and 136, resulting in the exposure of a second, normally buried, GAG-binding motif. Importantly, brain-derived PrP from transgenic mice, which express a pathogenic mutant with nine extra octapeptide repeats, also binds more strongly to GAG than wild-type PrP^C. Thus, several rPrPs with distinct pathogenic mutations have common conformational changes, which enhance binding to GAG. These changes may contribute to the pathogenesis of inherited prion diseases.

genetics | structure

Prion diseases are a group of rare but fatal neurodegenerative disorders that affect both humans and animals (1–3). Approximately 85% of human prion disease cases are sporadic, and most of the remaining cases are inherited as autosomal dominant traits (1–3). In addition, several hundred cases of human prion diseases have been shown to be acquired by infection, which may occur from medical manipulations, such as tissue implants, neurosurgical procedures, and hormone treatments as in iatrogenic Creutzfeldt–Jakob disease, or consumption of contaminated foods, as in kuru and variant Creutzfeldt–Jakob disease. All three forms of prion disease, sporadic, familial, and infectious, are believed to share the same pathogenic mechanism, which is based on the conversion of the normal cellular prion protein (PrP^C) into the infectious and pathogenic scrapie prion protein (PrP^{Sc}) (4–6). The accumulation of PrP^{Sc} in the CNS impairs function, induces structural damage, and causes disease.

More than 30 pathogenic mutations in the human *PRNP* gene have been identified (1–3). They are either insertion mutations, which occur solely in the octapeptide repeat region, or point mutations, which occur along the entire *PRNP* gene. It is thought that the mutant proteins are inherently unstable, leading to self-association to produce an oligomeric structure, which then acts as a “seed” to recruit additional prion protein (PrP), eventually resulting in the formation of the pathogenic PrP^{Sc} (7–9). Although it is clear that mutations in the coding region of the *PRNP* cause prion disease, the underlying mechanisms of how a mutant PrP causes neurodegeneration are not understood.

Bacterially produced rPrPs as well as mammalian cell lines transfected to express PrPs with pathogenic mutations have been used as model systems to study the pathogenic mechanisms of inherited prion diseases (10–14). Although the results from these studies have been informative, they fail to identify common features in the pathogenic mechanisms. Thermodynamic stability and NMR structural studies of recombinant PrPs (rPrPs) with pathogenic mutations suggest that in general destabilization of mutant PrP does not underlie the pathogenesis (15–17). It is unlikely that each pathogenic mutation has a unique pathogenic mechanism.

Wild-type PrP^C has five octapeptide repeats. Recently we found that a human rPrP with the pathogenic mutation of three additional insertions, rPrP^{8OR}, has a more exposed N terminus, binds more strongly to glycosaminoglycans (GAG), and is more susceptible to oxidative attack than wild-type rPrP^C. These aberrant properties are even more profound in rPrP with five extra repeats, rPrP^{10OR} (18). Therefore, insertion mutants share common features, and the degree of abnormality is proportional to the number of inserts. It is known that patients with larger inserts experience earlier disease onset and shorter disease duration (2, 19).

Here we studied whether the aberrant features found in insertion mutants are shared by other mutant rPrPs, such as P102L, P105L, G114V, H187R, T188R, E196K, F198S, and E200K. We also investigated whether our findings are applicable to a transgenic mouse line that expresses a PrP with an insertion mutation.

Results

Characterization of rPrPs with Pathogenic Point Mutations. Freshly prepared wild-type rPrP^Cs representing the 129 polymorphism: one with a Met, rPrP^{C(129M)}; another with a Val, rPrP^{C(129V)}; and mutant rPrPs (rPrP^{P102L}, rPrP^{P105L}, rPrP^{G114V}, rPrP^{H187R}, rPrP^{T188R}, rPrP^{E196K}, rPrP^{F198S}, rPrP^{E200K}, rPrP^{8OR}, and rPrP^{10OR}) were immunoblotted with PrP-specific mAbs. The epitopes of the mAbs are shown in Fig. 1A. mAb 8H4 reacted with all rPrPs except rPrP^{H187R}

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Abbreviations: GAG, glycosaminoglycan; PrP, prion protein; PrP^C, normal cellular PrP; PrP^{Sc}, scrapie PrP; rPrP, recombinant PrP.

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and with lower affinity compared with rPrPC (Fig. 3B). Therefore, mutant rPrPs acquire higher GAG-binding activity by having a more exposed N terminus and/or by exposing normally hidden GAG-binding motifs.

rPrPs with Pathogenic Point Mutations Also Share Common Conformational Changes at the C-Terminal Domain. To verify that the point mutations indeed alter PrP conformation in the regions of residues 53–93 and/or 110–128, we scanned the topology of these

Fig. 4. rPrPs with pathogenic point mutations also share common conformational changes at the C-terminal domain. For the epitope scanning assay, 100 ng of rPrP^{C(129M)} or mutant rPrPs per well was captured on an ELISA plate that had been previously coated with 50 ng of mAb 8H4 per well. After washing, various concentrations of biotinylated mAbs SAF32 (A), 3F4 (B), and 12A3 (C) were added to the respective wells. The binding of biotinylated mAbs to rPrP^{C(129M)} or mutant rPrPs is presented as OD₄₀₅ values.

two regions using mAbs that are specific for epitopes in these regions. All tested rPrPs reacted similarly to mAb SAF32, which reacts with an epitope at residues 63–94 (Fig. 4A). On the other hand, mutant rPrPs reacted less well, albeit to varying degrees, with mAb 3F4, which recognizes an epitope between 109 and 112 (Fig. 4B). In contrast, the mutant rPrPs exhibited stronger immunoreactivity with mAb 12A3, which reacts with an epitope between 120 and 136 (Fig. 4C). mAb 7H6, which reacts with an epitope between 130 and 140, and mAb 7A12, which reacts with an epitope between 143 and 155, reacted similarly with all rPrPs (Fig. 1D and E). These results support the interpretation that the region between 109 and 136, which contains a GAG-binding motif amino acids 110–128 (26), is altered as a result of the mutation.

PrP from a Transgenic Mouse Line Bearing Nine More Octapeptide Repeats Also Binds More Strongly to GAG. Tg(PG14) is a transgenic mouse line that expresses a PrP with nine extra octapeptide repeats, PrP^{140R}, and develops neurodegeneration spontaneously (28, 29). We investigated whether brain-derived PrP^{140R} binds an increased amount of GAG when compared with wild-type PrP^C from control transgenic mouse. We first determined the amounts of soluble PrP in Tg(PG14) and control Tg(WT) mouse brains by using mAb 8H4 and biotinylated mAb

7A12. On average, Tg(WT) have 682.94 ± 29.2 ng/ml PrP^C ($n = 6$). Tg(PG14) mice have 155.32 ± 6.69 ng/ml soluble PrP ($n = 10$) [Fig. 5A and supporting information (SI) Fig. 6]. To quantify GAG binding, mAb 8H4 was used to capture brain-derived soluble PrP. A biotinylated heparin was then used to react with the captured PrP. Despite having much lower levels of PrP, PrP^{140R} from all Tg(PG14) mice exhibited much higher GAG-binding activity (Fig. 5B and SI Fig. 7). Therefore, our findings with rPrPs are applicable to brain-derived PrP.

Discussion

The mechanisms by which a mutant PrP causes neurodegeneration are not completely understood. Our results for the first time suggest that pathogenic rPrPs arising from point and insertion mutations, irrespective of the location, share common conformational changes at the N terminus as well as at a region between 109 and 136, one of the most conserved regions in PrP. Most importantly, these conformational changes also have biological consequence: allowing mutant rPrPs to bind more GAG.

Several lines of *in vitro* and *in vivo* evidences suggest that binding of PrP to GAG is important in the conversion process, and thus critical for pathogenesis (30–33). PrP^{Sc} *in vivo* contains GAG (34). *In vitro*, GAG facilitates the conversion of PrP^C to PrP^{Sc} (35). Cell surface GAG is a “receptor” for PrP^{Sc} (32, 33).

Fig. 5. Brain homogenates from transgenic mice Tg(PG14) have reduced amounts of PrP but increased GAG-binding activity. (A) The PrP amounts in Tg(WT) were approximately four times higher than in Tg(PG14). The estimation was based on titrations using rPrP^{C(129M)} as a standard protein (see SI Fig. 6). (B) PrP^{140R} from Tg(PG14) has higher GAG-binding activity than wild-type PrP^C. ELISA plates were coated with mAb 8H4 as described. Identical amounts of brain homogenates from individual Tg(PG14) mice ($n = 10$) or control Tg(WT) mice ($n = 6$) were added to the wells in triplicates. This amount was chosen based on a titration experiment to identify the amount of brain homogenate that gave the maximum signal (see SI Fig. 7). A biotinylated heparin was then used to react with the captured PrP species. To compare GAG-binding activity of all PrPs, we defined one GAG-binding unit as the average OD₄₀₅ value/nanograms of PrP. All PrP^{140R} from Tg(PG14) mice have higher GAG-binding activity than PrP^C.

Thus, GAG may function as a scaffold for concentrating PrP^C, creating a reservoir of PrP^C for conversion. Because mutant PrP binds increase amounts of GAG, it would be more concentrated on the scaffold, ready for conversion. This interpretation is supported by our observation that in the presence of GAG all mutant rPrPs form aggregates more readily than wild type rPrP^C (Fig. 2 C and D).

rPrPs with pathogenic point mutations studied thus far also show similar changes in other regions, resulting in decreased binding of mAb 3F4 and an increased binding of mAb 12A3. The mAbs 3F4 and 12A3 epitopes cover a continuous region from 109 to 136. It is interesting to note that the mAb 3F4 epitope is buried in infectious PrP^{Sc}, and recovering the 3F4 epitope after denaturation is the basis of a conformational dependency immunoassay for detecting PrP^{Sc} (36). In addition, the 3F4 epitope is also masked in Tg(PG14) (29). Therefore, our findings on mutant rPrPs are in good accord with the behavior of *in vivo* derived PrP^{Sc}. Most importantly, PrP^{140R} from a transgenic mouse also binds more GAG than control PrP^C. Of the pathogenic mutations we have studied so far, the insertion mutants consistently exhibit the highest GAG-binding capacity and are uniquely more susceptible to oxidative attack. When compared with all other pathogenic mutations, patients with insertion mutations have the earliest disease onset (19).

Both wild-type and mutant rPrP tend to aggregate *in vitro* (37, 38), and aggregation of rPrP is pH-, concentration-, and age-dependent. At 1 μ g/ml rPrP, the concentration we used in all capture ELISA experiments, no rPrP aggregate is detected by using the aggregation-specific ELISA (at least a dimer) (39) or by native, nondenaturing PAGE (25) (additional results not shown). Furthermore, all experiments were carried out with freshly prepared rPrP.

Our findings that all of the studied mutant rPrPs have a more exposed N terminus need to be discussed in the context of earlier structural studies. Earlier NMR analysis of bacterially produced rPrP at pH 4.5–5.5 showed that the N terminus is flexible and unstructured (40, 41). However, it was later found that the N terminus of rPrP is structured if NMR analysis is carried out at pH 6.5–7.5, i.e., the pH at cell membrane (42). Additional studies have also suggested that the N terminus is important in PrP^C function and pathogenesis. The N terminus modifies disease phenotype (43) and influences the conformation and generation of PK-resistant PrP^{Sc} (44, 45) as well as rPrP aggregation (37).

All mammalian PrP contain two potential N-glycosylation sites and a GPI anchor. Studies with model membranes suggest that N-linked glycans at the C terminus can affect the conformation of the N terminus (46). These translational modifications may also influence the binding of GAG and contribute to pathogenesis.

Approximately 30 different pathogenic mutations have been identified on human *PRNP*. Whether our findings with 10 of 30 mutations are applicable to all of the remaining pathogenic mutations requires further studies. Although all of the mutants studied here exhibited similar conformational changes and bound more GAG, it is nevertheless possible that the conformational changes have other biological effects that may be involved in prion diseases.

Finally, our findings may also have implications for other human diseases caused by gene mutations. We suggest that mutations that occur in distinct regions of a molecule can exhibit similar conformational changes with similar biological consequences. These events could not be predicted simply based on gene sequences.

Methods

Plasmid Constructions. The expression vectors for rPrP^{C(129M)} corresponding to the mature fragment (23–231) and the insertion mutants rPrP^{80R} or rPrP^{100R} have been described (18). Other

expression vectors for eight human prion pathogenic point mutations, such as pET-rPrP^{P102L}, pET-rPrP^{P105L}, pET-rPrP^{G114V}, pET-rPrP^{H187R}, pET-rPrP^{T188R}, pET-rPrP^{E196K}, pET-rPrP^{F198S}, pET-rPrP^{E200K}, and one polymorphism variant, pET-rPrP^{C(129V)}, were constructed by using the QuikChange site-directed mutagenesis kit from pET-rPrP^{C(129M)}. Deletion mutants that lack the N-terminal GAG-binding motif (residues 23–29), pET-rPrP ^{Δ KRPPK}, pET-rPrP^{P102L Δ KRPPK}, and pET-rPrP^{E200K Δ KRPPK}, were also constructed. The point and deletion mutant sequences were verified by using a 373A Automated Sequencer (Applied Biosystems, Foster City, CA).

Generation and Purification of rPrP^C and Mutant rPrPs. The purification and refolding processes were carried out on a nickel ion-charged sepharose column (Amersham Biosciences) as described (18, 47). Protein concentration was determined with a Bio-Rad Protein Assay kit.

Antibodies. Seven affinity-purified anti-PrP mAbs were used in this study (20, 21). mAb 8B4 recognizes a N-terminal epitope at residues 35–45; SAF32 reacts with residues 63–94 covering the octapeptide repeat sequences (48); 3F4 recognizes residues 109–112; 12A3 reacts with residues 120–136; 7A12 interacts with helix 1 between residues 143 and 155; 7H6 recognizes residues 130–140; and 8H4 recognizes residues 175–185 of helix 2. Biotinylation of mAbs was performed by using the EZ-linked Sulfo-NHS-Biotin kit (Pierce Endogen) according to the manufacturer's recommendation.

Mice. The production of transgenic mice expressing PrP^C and PrP^{140R} has been reported (28). Transgenic mice lines used in this study are Tg(PG14) A2, A3/PrP^{-/-} (hemizygous for the transgene array) ($n = 10$) and Tg(WT) E1/PrP^{-/-} (homozygous) ($n = 6$) (29). The age of the mice ranges from 45 to 300 days, and both female and male mice were used. To prepare 20% (wt/vol) total brain homogenate, individual entire brain samples were homogenized in ice-cold lysis buffer (PBS with 1% Nonidet P-40/0.5% sodium deoxycholate, pH 8.0) in the presence of 1 mM PMSF (Sigma, St. Louis, MO). After centrifugation by microcentrifuge at 1,000 $\times g$ for 10 min, the supernatant was stored in aliquots at -80°C .

SDS/PAGE and Immunoblotting. Protein samples were separated by SDS/PAGE (12% Tris-glycine precast gel; Bio-Rad), electrotransferred onto nitrocellulose membranes, and immunoblotted. Transferred rPrP was detected by using anti-PrP mAbs in combination with HRP-conjugated goat anti-mouse IgG Fc region-specific antibody as the secondary antibody. rPrP visualization was performed by using the chemiluminescence blotting system from Roche (Indianapolis, IN).

Capture ELISA. Capture antibody (8H4 or 7A12) was coated at 50 ng per well in flat-bottomed, 96-well plates (Costar, Corning, NY) overnight at 4°C . Excess antibody was removed, and wells were blocked with 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 human rPrP^C and mutant rPrPs (1 μ g/ml) in PBS containing 1% BSA were added in triplicate to respective wells overnight at 4°C . At 1 μ g/ml all rPrPs are present in monomeric form. Plates were washed three times with PBS-T, and appropriate dilutions of biotinylated mAbs were added to designated wells for a 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 three times 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 microELISA plate reader. All experiments were repeated at

least three times, each time with freshly prepared recombinant proteins.

Detection of GAG Binding. GAG-binding experiments were performed as described (22). Briefly, heparin (from porcine intestinal mucosa) (Sigma) was coated on plates at 10 $\mu\text{g}/\text{ml}$ 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^C or mutant rPrPs were incubated with the coated plates for 2 h. After three washes with PBS-T, bound rPrP was detected with mAb 8H4 or 7A12. All experiments were carried out in triplicate and repeated at least three times with freshly prepared recombinant proteins.

For native brain-derived PrP experiments, ELISA plates were coated with mAb 8H4 (50 ng per well) overnight at 4°C. Excess antibody was removed, and wells were blocked with PBS containing 3% BSA (Sigma) for 3 h at room temperature. Identical amounts of brain homogenates from individual Tg(PG14) mice ($n = 10$) or control Tg(WT) mice ($n = 6$) were diluted in PBS and added to the wells in triplicate overnight at 4°C. Plates were washed three times with PBS-T, and biotinylated mAb 7A12 or biotinylated heparin was added to react with the bound PrP for 3 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 three times 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 microELISA plate reader.

Turbidity Measurement. Aggregation reactions were performed as described (37) with modification (25). The assays were performed at room temperature in a volume of 100 μl in 96-well plates. A total of 230 $\mu\text{g}/\text{ml}$ rPrP^{C(129M)} or mutant rPrPs was suspended in 100 μl of PBS (pH 7.4). Heparin was added into the reactions with a final concentration of 10 $\mu\text{g}/\text{ml}$. After adding of heparin, turbidities were monitored as quickly as possible (during 15 sec) by reading the absorbance at 405 nm in a Beckman Coulter AD340 microELISA plate reader using a kinetic photometric model (interval time 30 sec, 30 cycles with 1 sec of shaking before every cycle). To study the effects of different concentrations of heparin on aggregation, various concentrations of heparin were added to 230 $\mu\text{g}/\text{ml}$ rPrP^{C(129M)} or mutant rPrPs in PBS (pH 7.4), respectively, and turbidities were monitored at 405 nm.

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