Role of N-terminal Familial Mutations in Prion Protein Fibrillation and Prion Amyloid Propagation in Vitro

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A self-perpetuating conformational conversion of the prion protein (PrP) is believed to underlie pathology and transmission of prion diseases. Here we explore the effects of N-terminal pathogenic mutations (P102L, P105L, A117V) and the residue 129 polymorphism on amyloid fibril formation by the human PrP fragment 23–144, an in vitro conversion model that can reproduce certain characteristics of prion replication such as strains and species barriers. We find that these amino acid substitutions neither affect PrP23–144 amyloidogenicity nor introduce barriers to cross-seeding of soluble protein. However, the polymorphism strongly influences the conformation of the amyloid fibrils, as determined by infrared spectroscopy. Intriguingly, unlike conformational features governed by the critical amyloidogenic region of PrP23–144 (residues 138–139), the structural features distinguishing Met-129 and Val-129 PrP23–144 amyloid fibrils are not transmissible by cross-seeding. While based only on in vitro data, these findings provide fundamental insight into the mechanism of prion-based conformational transmission, indicating that only conformational features controlling seeding specificity (e.g. those in critical intermolecular contact sites of amyloid fibrils) are necessarily transmissible by cross-seeding; conformational traits in other parts of the PrP molecule may not be “heritable” from the amyloid template.

The transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of fatal mammalian neurodegenerative disorders including scrapie of sheep, bovine spongiform encephalopathy of cattle, chronic wasting disease of cervids, and Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker disease (GSS), and fatal familial insomnia of humans (1–4). All of these disorders are associated with misfolding and aggregation of the prion protein (PrP), a plasma membrane glycoprotein of unknown function widely expressed in numerous tissues. The self-perpetuating conversion of the monomeric, soluble “cellular” PrP conformer (PrPC) to a polymeric, β-sheet-rich, protease-resistant “scrapie” conformer (PrPSc), often displaying the structural characteristics of an amyloid, is believed to be the critical molecular event in the pathogenesis of TSE disease. This “protein-only” hypothesis, bolstered by a wealth of recent data (1–9), successfully explains many striking effects in prion diseases, these findings provide novel insight into the mechanism of conformational “inheritance” between PrP amyloid fibrils.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Purification—The pSETB expression vector for wild-type Met-129 huPrP23–144 has been described previously (29). Wild-type Val-129 huPrP23–144 was prepared by site-directed mutagenesis on the background of Met-129 PrP23–144 cDNA using the QuikChange system (Stratagene). All other proteins were prepared by site-directed mutagenesis of these two variants. All proteins were expressed in Escherichia coli and purified as described (17, 29). The identity of all proteins was confirmed by matrix-assisted laser desorption ionization time-of-flight–mass spectrometry, and final purity was better than 95% as judged by SDS-PAGE. Protein
concentration was determined from UV absorbance, using a molar extinction coefficient of 42,150 M$^{-1}$ cm$^{-1}$ at 276 nm.

**Thioflavine T Fluorescence**—The time course of huPrP23–144 fibrilization was monitored using the fluorometric thioflavine T (ThT) assay described previously (17, 22). Briefly, lyophilized protein was dissolved in Milli-Q water, to which 1 M potassium phosphate (pH 6.4) was added to a final concentration of 50 mM buffer, 400 μM protein (protease inhibitors were included). Samples were incubated in a 25°C water bath, and 5-μl aliquots were withdrawn and added to 500 μl of 10 μM ThT, mixed well and measured for ThT fluorescence emission at 482 nm (450 nm excitation) on an SLM 8100 spectrofluorometer. For seeding experiments, fibrillar protein prepared as above was sonicated on ice to disrupt large aggregates, and 2% (w/w) fibrillar material was added to freshly dissolved protein. Lag phases of PrP23–144 conversion were calculated by linear extrapolation of the growth (increasing) phase of ThT fluorescence plots. As noted previously (22), mature fibrils had a tendency to clump into very large aggregates, resulting in some scattering of ThT fluorescence signal. Furthermore, after reaching its maximum, the ThT fluorescence often showed a time-dependent decrease. However, these clumping-induced effects occurred only at late stages of the reaction; they did not affect the accuracy of the measurements of lag phases. All experiments were repeated a minimum of five times.

**Atomic Force Microscopy**—Fibrillar huPrP23–144 samples were visualized using atomic force microscopy (AFM) essentially as described (23). Briefly, samples displaying a ThT response were diluted with water to ~1 mg/ml, and 5 μl of diluted solution was applied to freshly peeled mica. This substrate was then rinsed with 150 μl of distilled water and dried in an air stream. Imaging was performed in tapping mode on a Digital Instruments Multimode microscope equipped with a NanoScope IV controller and a type E scanner. All samples were imaged under ambient conditions in both height and amplitude modes, using single-beam silicon cantilever probes with a nominal tip radius of 10 nm and a nominal spring constant of 20 newton/m.

**FTIR Spectroscopy**—Samples for FTIR spectroscopy were prepared as described above, except deuterium oxide was used in place of water. Spectra were acquired in transmission mode using a Bruker IFS66 spectrometer equipped with a DTGS detector and continuously purged with dry air. For spectrum acquisition, solutions of fibrillar protein (5–7 μl) were placed between CaF$_2$ discs separated by a 50-μm spacer. For each sample, 256 scans were accumulated and averaged at a resolution of 2 cm$^{-1}$. All spectra were corrected for absorption of buffer by interactively subtracting a blank spectrum to obtain a flat baseline between 1700 and 1850 cm$^{-1}$. To resolve overlapping components of the conformation-sensitive amide I band, all spectra were Fourier self-deconvoluted in the Opus 4.0 software package, using a Lorentzian line shape and parameters equivalent to a 15 cm$^{-1}$ half-width and a resolution enhancement factor of 1.6.

**RESULTS**

**Pathogenic Mutations and the Residue 129 Polymorphism Do Not Alter PrP23–144 Conversion Kinetics and Seeding Specificity**—Since GSS-associated mutations outside the folded domain of PrPC do not affect thermodynamic properties of the protein (12–14), it has been hypothesized that these mutations may facilitate the PrPC$^{-}$PrPSc conversion by increasing the amyloidogenic potential of the N-terminal region (14). As demonstrated previously (17), human PrP23–144 spontaneously converts to amyloid fibrils through a nucleation-dependent process characterized by distinct lag and growth phases (30). Mutation-induced increase in the amyloidogenicity of PrP23–144 would result in a decrease of the lag phase (~3.5 h under present experimental conditions). However, to our surprise, none of the disease-associated mutations studied (P102L, P105L, A117V) substantially affected the kinetics of PrP23–144 conversion (Fig. 1). The results obtained using PrP23–144 variants possessing valine at position 129; all three mutant proteins converted to amyloid with a nucleation-dependent lag phase similar to that of wild-type Val-129 PrP23–144 (Fig. 1C). On the average, Val-129 variants appeared to convert to amyloid fibrils slightly faster than Met-129 variants; however, these differences are very small, within the error margin of the present experiments.

Recently we have shown that substitution of a single amino acid in a critical region encompassing residues 138 and 139 may have a dramatic effect on the properties of PrP23–144 fibrils, resulting in cross-seeding barriers (22). Prompted by this observation, we asked whether similar effects may result from any of the mutations corresponding to familial prion diseases. We therefore tested the ability of individual mutant PrP23–144 amyloid fibrils to induce (seed) conformational conversion of monomeric wild-type protein. An example of such a cross-seeding experiment, using fibrillar P102L PrP23–144 and soluble wild-type protein, is shown in Fig. 2A. Clearly, addition of a small amount (2% w/w) of preformed fibrillar P102L PrP23–144 effectively seeds the conversion of wild-type PrP23–144 (both Met-129), as evidenced by elimination of fibril growth.
the lag phase. Within the resolution limits of the present approach, the efficiency of this seeding is indistinguishable from that of a homologous reaction in which wild-type protein is seeded with wild-type PrP23–144 fibrils (Fig. 1A). An equally effective cross-seeding was observed between monomeric wild-type PrP23–144 and fibrillar P102L and A117V variants (data not shown). Furthermore, identical behavior was observed for Val-129 PrP23–144 proteins; fibrils formed by pathogenic mutants containing valine at residue 129 were capable of seeding wild-type Val-129 PrP23–144 (data not shown for brevity).

We next questioned whether the residue 129 polymorphism might itself be sufficient to introduce a seeding barrier, a possibility suggested by the influence of this polymorphism on TSE transmissibility in vivo (27). Fig. 2B, however, shows that preformed fibrils of wild-type Val-129 PrP23–144 are fully capable of seeding wild-type Met-129 PrP23–144. Wild-type Met-129 PrP23–144 fibrils are likewise able to seed the conversion of wild-type Val-129 PrP23–144 (Fig. 2C). These results hold not only for wild-type protein; each pathogenic mutant with methionine at position 129 was able to seed the same variant with valine at residue 129 and vice versa (data not shown for brevity). Collectively, these data show that neither the residue 129 polymorphism nor GSS-associated mutations alter the amyloidogenic propensity and seeding specificity of PrP23–144.

Effect of Residue 129 Polymorphism and Familial Mutations on PrP23–144 Amyloid Conformation—Conformational features of PrP23–144 fibrils were analyzed by FTIR spectroscopy, an established technique for probing protein secondary structure (31). This method is especially well suited for studying β-sheet structures, with amide I bands associated with these structures depending on factors such as molecular packing of β-strands and the strength of hydrogen bonds (31, 32). In the soluble monomeric state, the spectra of both Met-129 and Val-129 PrP23–144 display a single broad amide band centered at about 1648 cm⁻¹ (17), consistent with an unordered structure. In the amyloid state, fibrils display spectra indicative of β-sheet secondary structure (Fig. 3). Fibris of Met-129 PrP23–144 exhibit closely spaced peaks in the range 1630–1650 cm⁻¹ (Fig. 3A). To resolve these overlapping component peaks, spectra were band-narrowed using Fourier self-deconvolution. The spectrum of Met-129 PrP23–144 fibrils is consistent with our previous studies (17, 23), showing major amide I component bands at 1628, 1639, and 1649 cm⁻¹. The bands at 1628 and 1649 cm⁻¹ are characteristic of β-sheet and random coil secondary structure respectively, while the 1639 cm⁻¹ band likely represents β-sheet but may also contain unresolved random coil components (31). The spectra of amyloid fibrils formed by P102L, P105L, and A117V mutants are very similar to that of wild-type Met-129 PrP23–144 amyloid (Fig. 3B–E), indicating very similar secondary structure in amyloids of all these proteins.

The spectrum of Val-129 PrP23–144 amyloid is substantially different (Fig. 3F). Deconvolution reveals this spectrum consists of major amide I bands at 1628 and 1649 cm⁻¹. The 1639 cm⁻¹ peak, so prominent in the spectrum of Met-129 PrP23–144, is essentially absent (visible as a faint shoulder on the much stronger 1649 cm⁻¹ peak). Val-129 PrP23–144 amyloids also showed a weak but relatively well resolved band at about 1617 cm⁻¹ (the same peak can be seen as a faint shoulder in Met-129 PrP23–144 spectra, Fig. 3, B–E). Moreover, while the 1628 cm⁻¹ band is noticeably weaker than the 1649 cm⁻¹ band in Met-129 PrP23–144 amyloids, the two bands are of comparable intensity in Val-129 PrP23–144 fibrils. As with the Met-129 PrP23–144 mutants, there is no difference in FTIR spectra between wild-type and mutant Val-129 PrP23–144 fibrils. These data indicate that substitution of valine for methionine at position 129 substantially changes the secondary structure of PrP23–144 amyloid fibrils, whereas familial mutations N-terminally to residue 129 have no measurable effect on PrP23–144 amyloid fibril conformation, at least within the resolution limits of the FTIR technique.

The morphology of PrP23–144 fibrils was examined by AFM. All PrP23–144 variants formed long (>5 μm) amyloid fibers with heights of ranging from 3–6 nm (Fig. 4A). Similar distributions of fibril thickness were observed for all PrP23–144 variants. Higher magnification images (Fig. 4, B–E) revealed periodic features along the fibril axis, with a mean repeat distance of 30–35 nm, giving the fibrils a segmented or “bead-like” appearance like that observed previously for human and mouse PrP23–144 (17, 23). Importantly, this fibril morphology was observed for all PrP23–144 mutants studied, including both Met-129 and Val-129 variants. Although globular oligomers were also present in AFM images, no alternate fibril morphologies were observed for any of the PrP23–144 mutants, and in all cases the fibril dimensions and periodicity were virtually identical within the resolution limits of the technique.

Conformational Features Differentiating Met-129 and Val-129 PrP23–144 Are Not Transmissible—In a previous study (23), we demonstrated that species-specific conformational features of PrP23–144 amyloid fibrils are transmitted to PrP23–144 of a different sequence by seeding. Having observed a structural variability associated with residue
129, we questioned whether these amyloid conformations may also be transmissible by seeding. FTIR spectra of PrP23–144 amyloid fibrils seeded with the opposite polymorph are shown in Fig. 5. Clearly, these spectra do not resemble those of the material used as seed. FTIR spectra of second-generation Val-129 PrP23–144 fibrils seeded with Met-129 PrP23–144 amyloid, denoted [V129]M129 as in our previous work (22), are identical to those of Val-129 PrP23–144 fibrils formed de novo or by seeding with preformed Val-129 fibrils (Fig. 5A). Likewise, Met-129 PrP23–144 amyloids seeded with Val-129 PrP23–144 fibrils ([M129]V129) displayed FTIR spectra characteristic of self-seeded Met-129 fibrils, in which the 1639 cm⁻¹ band is present (Fig. 5B). These spectral features do not result from a “failure” of the preformed fibers to seed the soluble protein, as all cross-seeded samples (including those prepared in deuterated buffer for FTIR experiments) displayed a steady increase in ThT fluorescence beginning immediately after addition of seed (see Fig. 2). Additionally, this phenomenon was not particular to wild-type PrP23–144; mutant Met-129 PrP23–144 fibrils formed by seeding with Val-129 PrP23–144 fibrils containing the same mutation displayed FTIR spectra consistent with Met-129 PrP23–144 and vice versa. We therefore must conclude that the structural features differen-
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tiating Met-129 and Val-129 amyloid fibers, unlike species-specific amyloid conformations dictated by residues 138–139 of PrP23–144 (23), are not transmissible by cross-seeding.

DISCUSSION

Pathogenic Mutations and PrP23–144 Fibrillogenesis—While the link between PrP mutations and familial prion diseases provides strong support for the protein-only model, the mechanism by which these mutations facilitate the pathogenic process remains unclear. Especially intriguing in this context is GSS-associated mutations at residues 102, 105, and 117. Because these residues lie within the unstructured region of PrP, their effect cannot be rationalized by a model based on an increase in the population of partially folded intermediates, as proposed for mutations within the C-terminal domain (14). It has been postulated that the pathogenic effect associated with these mutations could be related to an increased amyloidogenic propensity of the unstructured N-terminal region (14). Such a hypothesis seems logical, since these mutations replace a β-sheet incompatible Pro residue (P102L, P105L) or introduce a branched hydrophobic side chain (A117V), modifications that are believed to enhance amyloid-forming capability (33–35). However, to our surprise, we found that each three PrP23–144 mutants convert to amyloid fibrils with kinetics very similar to wild-type protein. Furthermore, the fibrils thus formed are all indistinguishable in conformational and seeding properties, indicating that these mutations do not affect in any significant way the amyloidogenic properties of PrP. Moreover, preliminary experiments with the P102L variant of human PrP90–231 show that the P102L mutation has a very minimal effect on PrP conformational conversion. Thus, the pathology associated with N-terminal mutations appears to be unrelated to the amyloidogenic potential of an isolated PrP molecule. Other factors, such as mutation-dependent abnormalities in PrPC interactions with a complex cellular environment (e.g. molecular chaperones, lipids, proteoglycans (36–38)) or metabolic effects related to PrP trafficking and sorting (39–41), should be considered to explain how these familial mutations facilitate the conversion of prion protein in vivo. One must also note that the present conclusions are based on experiments with the PrP23–144 model, the obvious limitation of which is the lack of the C-terminal folded domain. As discussed previously (22), the amyloidogenic and seeding properties of the full-length PrP are undoubtedly further modulated by residues C-terminal to position 144.

M129V Polymorphism Affects Amyloid Conformation but Not Seeding Specificity—The M129V polymorphism is known to determine susceptibility to prion infection in vivo (4, 27, 42). Variant CJD, believed to have arisen in humans by infection with bovine prions (43–45), has been found only in Met-129/Met-129 homozygotes (44), and expression of Val-129 PrP has been shown to confer some resistance to prion infection (27). Since the M129V polymorphism has no detectable effect on the structure or thermodynamic properties of PrPC, it has been postulated that the observations in vivo may be explained by a “molecular complementarity” model according to which only molecules that are homologous at residue 129 can be recruited to the misfolded PrPSc oligomers (46). However, our present data in vitro with PrP23–144 failed to detect any polymorphism-dependent seeding barriers, clearly demonstrating that homology at residue 129 is not required for productive interactions between PrP molecules during the conversion/propagation process. A similar lack of seeding barriers was observed in our recent study with polymorphic variants of recombinant PrP90–231, although the latter experiments required the use of chemical denaturants. Collectively, these findings argue against the proposed scenario that residue 129 Met/Val polymorphism-dependent susceptibility to prion diseases can be accounted for by the incompatibility of different polymorphs to coexist within the same PrPSc oligomer. Clearly, other factors must be considered to explain how this polymorphism determines the genetic susceptibility of humans to both sporadic and acquired forms of prion diseases.

Although Met-129 and Val-129 PrP23–144 fibrils share full mutual seeding compatibility, these amyloids have substantially different conformations as determined from FTIR spectra; higher resolution examination, however, would be needed to precisely identify the nature of these conformational differences. Polymorphism-dependent conformational differences have also been observed in our recent study with human PrP90–231 amyloid fibrils (14). However, in the latter case, these local differences, also detected using the global technique of FTIR spectroscopy, appeared much less pronounced, likely due to a masking effect of the C-terminal domain. The present more convincing evidence that residue 129 Met/Val polymorphism indeed affects the conformation of PrP amyloid fibrils is of considerable significance, since this polymorphism appears to be one of the determinants of prion strains responsible for different phenotypes of human prion diseases (4, 44, 47). Furthermore, some familial PrP mutations (including P105L and A117V) are pathogenic only in the context of Val-129 PrP (11). Thus, direct experimental evidence that the residue 129 polymorphism can influence PrP amyloid conformation strongly supports the notion that this polymorphism may be among critical factors contributing to the generation of strain-specific PrPSc conformers in vivo.

Not All Conformational Traits Can Be Transmitted by Cross-seeding: Implications for the Mechanism of Prion Propagation and Transmissibility Barriers—One of the major challenges in prion research is to provide a molecular level explanation of the phenomenon of prion transmissibility barriers. While TSE “species barriers” are known to be closely related to differences in amino acid sequence between prion proteins in the donor and acceptor of infection, the picture is complicated by the existence of multiple prion strains within the same mammalian species (4, 48–50). Insight into the molecular basis of transmission barriers was provided by our recent studies in vitro on PrP23–144 amyloid fibrils (22, 23). Using protein from three different species (human, mouse, and hamster), this study demonstrated that seeding barriers for a given pair of proteins are fully encoded in their conformational properties and that breaching of the species (i.e. sequence-dependent) barrier depends on the ability of host PrP monomer to adopt the conformation of the donor PrP amyloid. A similar conclusion was reached in studies on the propagation of structurally unrelated yeast prions (8, 51, 52). For the mouse-hamster PrP23–144 pair, such conformational adaptation was shown to lead to the emergence of a new strain of mouse fibrils that inherited conformational characteristics of the hamster PrP seed (23).

In contrast to the “conformational inheritance” observed in our previous studies with fibrils formed by addition of mouse PrP monomers to a hamster seed, the distinct amyloid conformations associated with the M129V polymorphism (as seen in FTIR spectra) have the intriguing property of being non-transmissible by seeding. To explain this conundrum, one should consider that not all regions of the protein molecule are of equal importance for PrP23–144 amyloidogenesis and seeding specificity. Previous work has established that the critical amyloidogenic determinant of PrP23–144 maps to a relatively short region including amino acid residues 138–141 (17). Furthermore, it was found

3 E. M. Jones, K. Surewicz, and W. K. Surewicz, unpublished data.

4 A. Apetri and W. Surewicz, unpublished observations.
that the properties of PrP23–144 are fully controlled by the identity of residues 138 and 139 in this critical region. Species-mimetic substitutions of these amino acids in human or hamster PrP23–144 are sufficient to completely change the behavior of these proteins, with mutant proteins acquiring fibrillation kinetics, conformational properties, and seeding specificity of proteins corresponding to different species (22, 23). This suggests that residues 138 and 139 are likely to comprise part of a critical intermolecular contact site in growing PrP23–144 amyloid fibrils, explaining why conformational traits controlled by these residues are transmissible by cross-seeding. As shown in the present study, the Met/Val polymorphism at residue 129 also affects the final conformation of PrP23–144 fibrils. However, unlike amino acids 138/139, this polymorphism changes neither the fibrillation kinetics nor the seeding specificity of the amyloid fibrils. Thus, residue 129 appears to lie outside the critical region that determines seeding capability. This would explain why the conformational features controlled by this residue are not transmitted in the cross-seeding reaction.

The central premise of the recently proposed “conformational adaptation” model is that barriers in prion transmissibility (seeding specificities) are not so much a simple function of species-dependent differences in PrP amino acid sequence but, in reality, are a conformational property of individual prion strains. Amino acid sequence, on the other hand, dictates the spectrum of conformations that PrP of the host can adopt upon conversion to the oligomeric PrPSc state; a transmission may occur only if this spectrum includes the conformation of the donor PrPSc strain (23). The present data further advance this model by showing that the above conformational adaptation needs to take place only in a critical amyloidogenic region of the PrP molecule (Fig. 6); outside this region, conformational features may arise from inherent (sequence-based) preferences rather than from the seed. Thus, while adaptability to a particular amyloid conformation does underlie seeding specificity (and thus prion transmissibility barriers), our present results localize this adaptability to a critical region in the amyloid fibril (i.e., the portion of the PrP molecule involved in amyloid templating). This finding suggests that prion amyloid structures may be even more diverse than has been generally appreciated, perhaps even within the same strain that underlies transmissibility barriers. Apart from their significance for prion research, the present findings have also broader implications, since conformational polymorphism (or “strain variability”) appears to be a general feature of many amyloids (53, 54), a feature that might greatly affect their biological properties (55).

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