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Structural effects of the highly protective V127 polymorphism on human prion protein

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Prion diseases, a group of incurable, lethal neurodegenerative disorders of mammals including humans, are caused by prions, assemblies of misfolded host prion protein (PrP). A single point mutation (G127V) in human PrP prevents prion disease, however the structural basis for its protective effect remains unknown. Here we show that the mutation alters and constrains the PrP backbone conformation preceding the PrP β-sheet, stabilising PrP dimer interactions by increasing intermolecular hydrogen bonding. It also markedly changes the solution dynamics of the β2-α2 loop, a region of PrP structure implicated in prion transmission and cross-species susceptibility. Both of these structural changes may affect access to protein conformers susceptible to prion formation and explain its profound effect on prion disease.
Prion diseases, such as bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and Creutzfeldt-Jakob disease (CJD), kuru and Gerstmann-Sträussler-Scheinker (GSS) disease in humans, are a group of neurodegenerative disorders caused by prions, self-replicating β-sheet-rich infectious polymeric assemblies of misfolded host-encoded cellular prion protein (PrPSc)2–4. Whilst rare, prion diseases are an area of intense research interest, as it is increasingly recognised that other degenerative brain diseases, such as Alzheimer’s and Parkinson’s diseases, also involve the accumulation and spread of aggregates of misfolded host proteins through an analogous process of seeded protein polymerisation5–8. Consequently, study of ‘prion-like’ mechanisms has been recognised to have much a wider relevance to the understanding of neurodegenerative disorders9–11.

PrPSc is a cell surface, predominantly α-helical, glycosylphosphatidylinositol (GPI)-anchored glycoprotein that is sensitive to protease treatment and soluble in detergents1. In contrast, prions may acquire protease-resistance and are classically designated as PrPSc (refs. 12,13). PrPSc is found only in prion-infected tissue and is β-sheet-rich, aggregated material, partially resistant to protease treatment, and insoluble in detergents14. Transmission experiments to transgenic mice provide strong supporting evidence that alternative conformers or assembly states of PrPSc encode multiple prion strains, which differ in their pathogenic properties15. Transgenic mice expressing only human PrP with either valine or methionine at residue 129 have shown that this common human polymorphism constrains the propagation of distinct human prion conformers, and the occurrence of associated patterns of neuropathology consistent with the conformational selection model of prion propagation16–20. Heterozygosity at codon 129 is thought to confer resistance to prion disease by inhibiting homologous protein–protein interactions essential for efficient prion replication with the presence of methionine or valine at residue 129 controlling the propagation of distinct human prion strains21,22. Biophysical measurements suggest that this powerful effect of residue 129 on prion strain selection is likely to be mediated via its effect on the conformation of the disease-associated PrPSc form, or its precursors or on the kinetics of their formation, as it has no measurable effect on the structure, folding or stability of PrPC22.

The acquired prion disease kuru, which was epidemic amongst the Fore linguistic group of the Papua New Guinea highlands when first studied in the 1950’s, and which was transmitted during mortuary feasts, imposed strong genetic selection on the Fore, essentially eliminating residue 129 homozygotes23. A novel variant of prion protein, V127, unique to the affected population in the epicentre of the kuru epidemic, was also identified24. In this variant, the glycine at residue 127, which is fully conserved amongst vertebrate PrP primary structures, is substituted by valine. The V127 polymorphism was found on one copy of the PRNP gene in unaffected individuals within the population, suggesting that this polymorphism conferred resistance to prion disease, having been selected for in response to the kuru epidemic23,24. The protection afforded by this polymorphism was modelled using transgenic mice expressing human PrP25, and showed that heterozygous mice expressing both alleles containing glycine and valine at residue 127 (G/V127), echoing the human resistance genotype, exhibited profoundly reduced susceptibility to infection with kuru and classical CJD prions. Most importantly, however, and in complete contrast to the protective effect of the residue 129 polymorphism, homozygous mice expressing human PrP with solely valine at residue 127 (V127), showed total resistance to all inoculated human prion strains. A comparison of the incubation periods between hemizygous mice expressing wild-type G127 human PrP only, with heterozygous mice expressing both G127 and V127 PrP, indicated a dose-dependent dominant-negative inhibitory effect of V127 PrP on prion propagation, resulting in prolonged incubation periods and variable attack rates in heterozygotes25. These data indicated that V127 PrP is intrinsically resistant to prion propagation and can inhibit propagation involving wild-type (WT) G127 PrP. In essence, this single amino acid substitution, at a residue completely conserved in vertebrate evolution, has as potent a protective effect on the host as a null mutation. Consequently, the structural and mechanistic basis of the protective effect of the V127 mutation is of keen interest as it may provide key insights into the mechanism of prion conformational conversion and recruitment.

As a first step in characterising the effect of this protective polymorphism on PrP, we undertook a detailed investigation of the effect of the residue 127 polymorphism on the biophysical properties of the native cellular PrPC conformation using a combination of X-ray crystallography, NMR and equilibrium unfolding. We show that this mutation imposes local changes in backbone conformation which facilitate formation of intermolecular hydrogen bonds between native-state dimers and imposes conformational restrictions on this region of the protein. In addition, it significantly alters millisecond timescale conformational rearrangements in regions of PrP proposed to be important in prion transmission26–28. These effects may modulate the conversion of native PrPC to a disease-associated form or on pathway intermediates relevant to the disease process, and provide a mechanistic explanation for the protective effect of this mutant.

Results

Choice of PrP variants studied. Persons who were exposed to kuru and survived the epidemic were predominantly heterozygotes at PrP residue 12923. The V127 protective polymorphism in human PrP was always present on an M129 allele24, consequently our main interest was with the V127/M129 PrP variant. However, we took the opportunity, given the known biological effect of the residue 129 polymorphism to also study the V127 variant with valine at residue 129 (V127/V129), and both forms of wild-type PrP (G127/M129 and G127/V129) with the aim of dissecting the effects of both of these protective polymorphisms.

V127 PrP structures closely resemble wild-type G127 PrP. To determine whether the overall structure of PrPC was affected by the protective V127 variant we crystallised recombinant human PrP (residues 119–231), with valine at residue 127, (V127/M129 and V127/V129), complexed with the Fab fragment of the anti-PrP antibody ICSM18, as performed previously with G127/M129 PrP (Supplementary Table 1 and Supplementary Fig. 1)29. The crystal structures of both V127 variants (V127/M129, 2.3 Å resolution, pdb 6SV2 and V127/V129, 2.5 Å resolution, pdb 6SUZ) closely resembled that of WT G127/M129 (pdb 2W9E, Fig. 1a and Supplementary Fig. 2)29. The structured C-terminal domain (residues 125–225) comprises three α-helices (α1–α3) and a short, two-stranded, anti-parallel β-sheet (Fig. 1 and Supplementary Fig. 3). Residue 127 immediately precedes the first β-strand of the β-sheet whereas residue 129 lies within it. The residues surrounding 127 and 129 are well defined in both crystal structures (Figs. 2 and 3) and show that the side-chains of both residues are predominantly located on the protein surface. Neither the 127 nor 129 polymorphisms substantially perturb the backbone or sidechain positions, or hydrogen bonding, of residues within the β-sheet (Fig. 1b and Supplementary Fig. 2a–c). Both circular dichroism (CD) and heteronuclear NMR spectra (Supplementary Figs. 4–6) are consistent with the crystal structures accurately reflecting the solution structure of the proteins. The global stability and unfolding behaviours of the V127/M129
and V127/V129 variants (Supplementary Fig. 7 and Supplementary Table 2) are also not significantly affected by the substitution of valine for glycine at position 127, reflecting the lack of major structural perturbation.

**V127 polymorphism restricts PrP backbone conformation.**

Despite the crystal structures being mostly unperturbed by the V127 polymorphism, a number of localised differences were identifiable. The most significant area of variation is found immediately N-terminal of residue 127 (residues 125–127). This region adopts an essentially identical conformation in both the V127/M129 and V127/V129 structures, which differs significantly from WT G127/M129 PrP (Fig. 1b and Supplementary Fig. 2a–c).

In particular, the reduction in the conformational plasticity of the backbone due to the valine/glycine substitution at position 127 leads to a very different conformation at this point (V127 Phi angle = −70.5°, c.f. G127 = +106.9°), as the WT backbone conformation is in a disallowed region of conformational space for valine. Consequently, the Ca of G126 in V127/M129 PrP is displaced by 2.9 Å, and the Ca of L125 by 2.2 Å (equivalent Ca atoms of most other surrounding residues are displaced by 0.2–0.3 Å). These Ca atom positions are well defined in both V127 structures (Figs. 2 and 3).

Furthermore, the V127 polymorphism appears to reduce conformational variability at residue 127, and concomitantly from WT G127/M129 PrP (Fig. 1b and Supplementary Fig. 2a–c).
increases structural definition of the β-sheet, as implied by a comparison of relative B-factors of this region in V127/M129 PrP when compared with WT G127/M129 PrP. These lower B-factors extend from L125 to A133, beyond the end of the first strand of the β-sheet (Fig. 3). In V127/M129 PrP, the average Ca B-factors for both the N-terminus (residues 126–131; 30 Å²), and β-strand 1 (residues 128–131; 27 Å²) are lower than the average B-factor for the core secondary structure elements (31 Å²). In contrast, in wild-type G127/M129 PrP, the corresponding values for both the N-terminus (46 Å²) and β-strand 1 (42 Å²) are higher than the average B-factor (39 Å²). As the crystal structures are all isomorphous with the same crystal packing, we suggest that the reduction in B-factors is likely due to conformational restriction introduced by the valine sidechain, and by additional intermolecular hydrogen bonding found in the V127 crystals, described below.

**Fig. 2** The quality of the electron density maps for PrP in the V127/M129 PrP - ICSM-18 Fab complex at 2.3 Å resolution. Residues from the PrP β-sheet and the V127 polymorphism are shown; 2Fo – Fc map contoured at 1σ.

**Fig. 3** Thermal parameter (B-factor) distribution in human PrP. (a) V127/M129 PrP (b) G127/M129 PrP shown as “putty” representation, as implemented by PyMOL. The V127/M129 PrP Ca atom B-factors range from 22.7 Å² to 96.9 Å² with average values of 38.3 Å² for the whole protein, and 30.4 Å² for the core secondary structure elements (residues 128–131 (β-strand 1), 144–154 (α-helix 1), 160–164 (β-strand 2), 174–186 (α-helix 2) and 202–220 (α-helix 3)). The Ca B-factors are depicted on the structure in dark blue (lowest B-factor) through to red (highest B-factor), with the radius of the atom B-factors range from 22.7 Å² to 96.9 Å² with average values of 38.3 Å² for the whole protein, and 30.4 Å² for the core secondary structure elements (residues 128–131 (β-strand 1), 144–154 (α-helix 1), 160–164 (β-strand 2), 174–186 (α-helix 2) and 202–220 (α-helix 3)). The Ca B-factors are depicted on the structure in dark blue (lowest B-factor) through to red (highest B-factor), with the radius of the ribon increasing from low to high B-factor. The lowest B-factor is observed in the region of α-helix 2 (α2) and α-helix 3 (α3) where the disulphide bridge links the two α-helices at residues 179 and 214 (dark blue), with the antibody-binding epitope spanning α-helix 1 also displaying lower than average B-factors, consistent with the antibody contacts stabilising this region of PrP relative to the overall structure. The largest B-factors are observed in the loop region linking helices α2 and α3 (red) (α2–α3 loop; residues 191–199), where the electron density clearly shows more disorder than elsewhere in the structure. In contrast, the B-factors for residues in close proximity to the V127 polymorphism are not unusually high, and all of these residues are clearly observed in the electron density (see also Fig. 2).

**V127 polymorphism extends PrP intermolecular β-sheet.** Notably, dimers between crystallographically-related PrP molecules are observed in the crystals (Fig. 1c). Association is mediated by a short segment of the anti-parallel β-sheet with hydrogen bonds formed between the first β-strand (residues 128–131) of each molecule. This results in the formation of a four-stranded intermolecular β-sheet between the existing anti-parallel β-sheets of each PrP molecule, involving close homotypic contacts at L130 (Fig. 1e, b). Similar intermolecular interactions are also observed in the non-isomorphous crystal structures of sheep, rabbit, and human PrP in the absence of antibody, and in different crystallographic space groups (Fig. 4). This suggests that this interaction is not a crystal packing artefact, and may reflect a greater biological significance for prion propagation, especially as residue 129 is protective and crucial to the aetiology and neuropathology of prion disease, and residue 127, which is in close proximity to the dimer interface, can completely prevent prion propagation.

The residue 129 polymorphism is accommodated within the dimer interface without significant perturbations of surrounding amino acids (Supplementary Fig. 2c, d). In contrast, substitution by valine at residue 127 results in the formation of an additional pair of intermolecular hydrogen bonds in both V127 structures, between the backbone carbonyl and amide groups of G126 and A133 respectively (Fig. 1e, Supplementary Fig. 2d), due to the alteration in backbone conformation. This orients the G126 carbonyl group towards the dimer interface, and its hydrogen bond acceptor A133. In the WT G127/M129 PrP dimer, the corresponding G126 CO – A133 N³ distance is 7.7 Å, as the carbonyl group of G126 points away from the dimer interface (Fig. 1f). This additional hydrogen bonding with V127 extends the β-sheet dimer interface to residues 126–133, thereby encompassing V127, whereas G127 is not involved in dimer contacts in WT G127/M129 PrP. The hydrogen bond distances for these additional H-bonds in the V127 structures (2.8–2.9 Å) indicate a strong interaction. Also, the hydrogen bonds involving
Increased conformational variability in V127 PrP structures. Intriguingly, altered conformational variability is observed in key regions distant from the site of the V127 polymorphism, in particular the loop linking the second strand of the β-sheet and helix 2 (β2-a2 loop; residues 165–172; Fig. 3). This region, which has been shown to affect prion cross-species transmissibility, is adjacent to the β-sheet, packing against residues N-terminal to the first β-strand (including residue 127), the β-sheet itself, the C-terminus of helix 3, and is in close proximity to the disease-associated residue D178. The Ca B-factors for residues 169–172 within this loop are higher than the average for the rest of the protein in both V127 structures (50 vs. 38 Å² for V127/M129 & 56 vs. 45 Å² for V127/V129). This contrasts with WT G127/M129, where these residues are better defined than the average, according to their B-factors (40 vs. 42 Å²). The B-factors for the V127 structures are consistent with an alteration in the degree of conformational exchange in this loop region of the protein compared to WT PrP, possibly compensating for the reduced conformational variability observed in the β-sheet region. The remaining regions of the V127 PrPs display B-factors that are comparable to WT values.

Altered conformational variability also seen in solution. Crucially, the altered conformational variabilities are also observed in solution. The effects of the V127 and V129 polymorphisms on the dynamics of PrP were investigated using NMR relaxation data (Supplementary Fig. 8), coupled with Modelfree (Fig. 5) and reduced spectral density analyses (Fig. 6, Supplementary Fig. 9). The former uses order parameters ($S^2$) to report internal sub-nanosecond (ns) motions. $S^2$ values range from 0 for highly flexible to 1 for rigid systems. The β-sheet and helical regions of all PrP variants exhibit $S^2$ values of 0.8–0.9, typical of structured regions of folded proteins (Fig. 5). However, a number of residues in structured regions, for example E168 in V127/M129 and D178 in G127/M129 PrP display anomalously low $S^2$ values. These are subject to millisecond (ms) conformational dynamics described below (Fig. 6).

The Modelfree approach also allows a general separation for each residue of ms conformational dynamics ($R_{ex}$ values) from ns and sub-ns motions. These ms timescale motions are often associated with large-scale co-operative conformational changes and highlight residues that populate low-free energy alternative conformations. For each of the PrP variants, a number of residues exhibited $R_{ex}$ values (Fig. 6c). These are concentrated in a spatially close region, involving the β-sheet (V129/G131/R164), the β2-a2 loop (M166/E168/Q172) and the C-terminus of helix 3 (I215 T216/Y218/E219/E221; Supplementary Figs. 3, 10 and 11). The line-broadening of resonances D167, Y169, S170 and N171 beyond detection in the HSQC spectra of all three variants also likely reflect ms dynamics. The observed conformational
Fig. 6 Conformational dynamics in the PrP variants. a Reduced spectral density function J(0), describing the amplitude of zero frequency motions in the PrP variants at 800 MHz. Uncharacteristically large J(0) values, such as those exhibited by β-sheet residues (128–131 and 160–164), and G131 and R164 in V127/M129 PrP in particular, indicate ms – μs dynamics. The dotted lines in the J(0) graphs are two standard deviations greater than the mean J(0) for the N-terminus (residues 200–210) of helix 3, of the respective variants. b Effect of V127 and V129 polymorphisms on the amplitude of zero frequency J(0) motions at 800 MHz. J(0) changes relative to G127/M129 PrP. Residues which experience significant changes in J(0) due to the V127 substitution include G131, R164 and E168. V129 results in altered J(0) motions for residues 129 and 131, within the first β-strand, M166, and residue 178, located in helix 2. The observed changes are due to differential ms conformational dynamics (see c). c PrP ms dynamics (Rex) modelled in the Relax Modelfree analysis. The V127 polymorphism increases ms dynamics (Rex) within the β-sheet (G131/R164) and β-α2 loop (E168/Q172), and diminishes those at the C-terminus of helix 3. The V129 polymorphism also increases ms dynamics in the first β-strand (V129/G131), and the C-terminus of helix 3. Residues 166 and 172 at either end of the β-α2 loop are also perturbed. These Rex values are mapped onto the structure of PrP C (See Fig. 7, and also Supplementary Figs. 10 and 11).

Fig. 7 Effect of V127 polymorphism on the amplitude of PrP ms dynamics (Rex). The sidechains of residues which experience altered ms dynamics in V127/M129 PrP, relative to G127/M129 PrP are shown (Fig. 6c), with varying width of backbone and colour. Residues showing increased Rex values in the V127 variant, such as G131, R164, E168 and Q172, are coloured red, with those showing a reduction, such as Y218, are coloured blue. Those residues for which a comparison is not possible, due to absence of data are not coloured. The orientation of R164 and E168 sidechains in G127/M129 PrP are shown (Fig. 6). The introduction of the bulkier valine sidechain at residue 127 appears to sufficiently perturb the side-chain position of R164 such that the interactions with E168 are essentially removed (the equivalent distances are 3.2 and 3.8 Å; Fig. 8). Significantly, NMR chemical shift changes in the Nε signal from the R164 sidechain in V127/M129 PrP reflect this alteration in side-chain orientation, with the Nε being perturbed by the change in its proximity to the aromatic ring of Y128 and the change in hydrogen bonding of R164 NεH1. (Supplementary Fig. 6). The loss of these interactions is a likely source of the increase in the ms dynamics of both residues, which appears to be disseminated along the rest of the β-α2 loop, as residue Q172, the other visible resonance in the β-α2 loop, also displays a marked increase.

Similarly, the V129 polymorphism also affects ms dynamics, however, different residues are affected. V129 increases Rex values.

dynamics are consistent with a proposed interconversion of the β-α2 loop between a more populated 310-helix and a type I β-turn(27,30,32,40,41) (Supplementary Fig. 12). The V127 polymorphism results in large increases in ms dynamics for residues G131 and R164 in the β-sheet, and E168 and Q172 in the β-α2 loop, but decreases in the C-terminus of helix 3 (215–221; Figs. 6 and 7). In a number of WT PrP crystal structures the sidechain of Y128 and the change in hydrogen bonding of R164 NεH1. (Supplementary Fig. 6). The loss of these interactions is a likely source of the increase in the ms dynamics of both residues, which appears to be disseminated along the rest of the β-α2 loop, as residue Q172, the other visible resonance in the β-α2 loop, also displays a marked increase.

Similarly, the V129 polymorphism also affects ms dynamics, however, different residues are affected. V129 increases Rex values.
for G131 and itself (Fig. 6c, Supplementary Figs. 10 and 11). This alteration in G131 exchange dynamics has recently been observed in mouse PrP42. In addition, we also observe that the $R_{ex}$ values of D178 are markedly reduced in the V129 polymorph. This is illustrated by the reduction of line-broadening of D178 observed in the HSQC spectra of G127/V129 PrP (Supplementary Fig. 13).

This is notable as the residue 129 M/V polymorphism affects the disease phenotype of the pathogenic D178N mutation which causes inherited prion disease. D178N is associated with the clinico-pathological phenotype Fatal Familial Insomnia (FFI) when residue 129 is methionine, and CJD when it is valine42. In V127/M129 PrP the D178 HSQC resonance cannot be observed directly as it is heavily overlapped with that of V127, but an analysis of the intensity of signals in V127/M129 3D HNCO NMR spectra indicates that D178 does indeed experience ms dynamics, to a similar extent as wild-type G127/M129 PrP. This suggests that the V129 polymorphism alters PrP conformational variability independently of the 127 mutation.

A number of residues at the C-termius of helix 3 (I215, Y218, E219, R220, E221 and S222) experience altered ms dynamics in V127/M129 compared with G127/V129 PrP. Residues I215/Y218/ E221/S222 are on a face of the helix that interacts with residues in the β2-α2 loop (Fig. 7, Supplementary Fig. 11). For example, residues Y218 and S222 closely interact with M166, while I215 and Y218 interact with Q172. In particular, residues Y218 and S222 closely interact with M166, while I215 and Y218, the valine sidechains of residue 127 and 129, are in areas that do not display measureable hydrogen protection, for example the β2-α2 loop and the C-terminus of helix 3.

**Effect of V127 polymorphism on PrP in vitro fibrilisation.** The lack of major structural perturbation or altered stability of the V127 variant in comparison to WT PrP43 suggests that the polymorphism may act by primarily affecting the efficiency of conversion of PrP43 to its disease-associated aggregated form. To assess this we firstly examined the ability of the V127 variant to fibrilise under partially denaturing conditions. When agitated in 2 M GuHCl, PrP can be induced to form amyloid. Binding of the fluorescent thiazole dye thioflavin T to these β-sheet-rich fibrillar structures reports their formation, allowing a quantitative analysis of the kinetics of fibril formation44. We found that although V127/M129 PrP can be induced to fibrilise within the time scale of the experiment, it did so with a significantly longer lag-time than WT G127/M129 PrP (Fig. 9). This is particularly interesting as substitutions to valine, and other bulky hydrophobic residues typically promote β-sheet formation and self-association required for amyloid formation45. These data are however consistent with previously published data which modelled the effect of the V127 mutation on a mouse PrP background, and which indicated that the V127 variant is inherently more resistant to fibrilisation than WT PrP46.

**Amplification of protease-resistant PrPSc seed (PMCA).** Although fibrillar material can be generated using these partially denaturing conditions, the material generated in such reactions has not been shown to be reliably infectious. In contrast, the protein misfolding cyclic amplification (PMCA) technique47 has been shown to amplify infectious and PK-resistant material with high fidelity. PMCA is a cyclical process where periods of conversion of substrate PrP43 to its disease-associated aggregated form. To assess this we firstly examined the ability of the V127 variant to fibrilise under partially denaturing conditions. When agitated in 2 M GuHCl, PrP can be induced to form amyloid. Binding of the fluorescent thiazole dye thioflavin T to these β-sheet-rich fibrillar structures reports their formation, allowing a quantitative analysis of the kinetics of fibril formation44. We found that although V127/M129 PrP can be induced to fibrilise within the time scale of the experiment, it did so with a significantly longer lag-time than WT G127/M129 PrP (Fig. 9). This is particularly interesting as substitutions to valine, and other bulky hydrophobic residues typically promote β-sheet formation and self-association required for amyloid formation45. These data are however consistent with previously published data which modelled the effect of the V127 mutation on a mouse PrP background, and which indicated that the V127 variant is inherently more resistant to fibrilisation than WT PrP46.

**Discussion**

This structural and biophysical study was stimulated by the remarkable effect of the V127 polymorphism on human prion propagation. Transgenic mouse transmissions show that V127 PrP is incapable of supporting prion transmission and propagation. Transgenic mouse transmissions show that V127 PrP is incapable of supporting prion transmission and propagation, consistent with the human clinical resistance data24, and is even able to inhibit heterologous propagation of wild-type protein containing glycinic at residue 127.25. This differs from the residue 129 polymorphism, where similar studies suggest the importance...
that it confers complete resistance via the variant protein itself\(^2\), leading to the hypothesis that an altered PrP\(^{C}\) fold may be the cause of resistance to prion disease\(^3\). The profound effects that these mutations have on human prion disease pathogenesis may provide key insights into the mechanism of prion conversion, and have a wider relevance to other templated protein mis-folding diseases, where changing a single amino acid could have a similar dramatic effect, with potential significance for therapeutic strategies\(^9,10\). The structural consequences of the glycine to valine substitution at residue 127 on PrP\(^{C}\) is therefore of major and wide potential interest.

Here, we have shown that there is a close similarity in overall structure between both V127 variants studied (V127/M129 and V127/V129), and wild-type G127/M129 PrP. Solution spectra (CD and NMR) confirm that the crystal structures faithfully reflect the solution structures of the proteins, allowing detailed analysis of the structural effect of the V127 polymorphism on PrP\(^{C}\). We find little evidence for a major structural change in the \(\beta\)-sheet, or \(\alpha\)-helices, in contrast to a recent NMR structure which identified unique features caused by the V127 polymorphism\(^3\). In particular, we do not observe any displacement of amino acid side chains within the \(\beta\)-sheet, which are well defined by the electron density, and note that this crystal structure satisfies the inter-residue \(\beta\)-sheet NOE distance constraints used for the NMR structural study within 0.25 Å\(^4,49\) apart from two which are satisfied within 0.43 Å and 1.03 Å (both to the Hε of Y162).

However, distinct perturbations of key regions which affect prion transmission and propagation are observed. Specifically, the V127 substitution reduces the conformational variability of the protein backbone immediately preceding the first strand of the \(\beta\)-sheet and radically alters the local backbone conformation. This facilitates the formation of an additional two intermolecular hydrogen bonds, which stabilise the native-state dimer association observed in the crystals. This dimeric association has been observed in a number of different PrP structures crystallised in the absence of antibody\(^30-32\) (Fig. 4). In these, the PrP \(\beta\)-sheet interface is composed of two intermolecular hydrogen bonds, as in WT G127/M129 PrP\(^{29}\). The V127 \(\beta\)-dimer interface presented here is unique in both the length of the \(\beta\)-sheet interface and number of hydrogen bonds (4), and argues against the proposal that V127 disadvantages native-state dimerisation, by reducing main-chain hydrogen-bond interactions\(^33,34\).

Formation of the intermolecular \(\beta\)-sheet has been proposed as a possible initiation point for \(\beta\)-sheet-mediated oligomerisation to explain the genetic susceptibility and prion strain selection determined by the polymorphic residue 129 in human prion disease\(^23,29,30,32\). If \(\beta\)-strand intersections in this region of the protein mediate PrP interactions during PrP\(^{C}\) formation, then the packing and geometry of this segment of the chain would have a strong selective effect on conformation, and also productive prion propagation\(^29\). No displacement of the protein backbone or stabilisation of the PrP\(^{C}\) dimer interface is caused by the residue 129 polymorphism\(^22,32\), which may explain the marked effect of the V127 polymorphism on prion pathogenesis.

The PrP\(^{C}\) dimer interaction does not appear to be a crystal packing artefact as endogenous PrP\(^{C}\) dimers have been detected in N2a cells and purified brain fractions\(^30,51\), with the dimers thought to form the hydrophobic domain of PrP (residues 112–133)\(^32\). PrP\(^{C}\) dimerisation inhibits PrP\(^{C}\) accumulation and prion replication\(^33,34\), and has a dominant-negative inhibitory effect on the conversion of monomeric PrP\(^{C}\). These findings suggest that it may be possible to halt prion formation by stabilising PrP\(^{C}\) dimers. Given the strengthened dimer association seen in the V127 crystals this may be one aspect of the protective mechanism of the V127 mutant. The conformational restriction imposed by the V127 polymorphism may also be sufficient to
inhibit homotypic protein–protein contacts in heterodimers of V127 and G127 PrP, or prevent the formation of extended β-sheet structure required to convert the PrP N-terminal unstructured region into protease-resistant β-enriched forms. Alternatively, the marked alteration in local backbone conformation and increased stability of intermolecular β-sheet interactions may prevent PrP folding into a thermodynamically permissible prion assembly. As V127 PrP also inhibits the generation of infectious assemblies of wild-type PrP, this would suggest that it must be either capping nascent prion assemblies or structurally weakening infectious prion assemblies on incorporation. The PMCA data presented here indicates that V127 PrP is not a permissive substrate for amplification of protease-resistant PrP disease seed. It is possible that the polymorphism introduces a protease cleavage site, which would lead to the destruction of a polymer formed of the homomeric V127 protein. PrP V127 incorporation would also dope, with a dose-dependent effect, heteromeric polymers composed of both G127 and V127 PrP variants. Their reduced stability could increase cellular clearance, which would also explain the “dominant negative” effect of V127 on prion propagation.

In addition to these local structural perturbations, long-range sequence interactions between the protective residue 127 and 129 polymorphisms affect the conformational distribution of a spatially distinct region including the β-sheet, β2-a2 loop, the C-terminus of helix 3 and the disease-associated residue D178. These conformationally variable elements have been shown to be key determinants of prion transmission and cross-species prion susceptibility, and a number are associated with inherited forms of prion disease, for example G131V, D167N, V210I, E211Q, Q212P and Q217R. In particular, the β2-a2 loop (residues 165–172) has been proposed to be a key modulator of prion transmission and disease-associated PrP misfolding. V127 alters the structural flexibility of the β-sheet and conformational dynamics of the β2-a2 loop by disrupting the electrostatic interaction between R164 of the β-sheet and E168 within the adjacent β2-a2 loop. Loss of this interaction would disrupt hydrogen bonding and close packing between Y169, F175 and D178 and destabilise the dominant 3–helix as a result of the polymorphism.

This will likely cause altered conformational dynamics in the β2-a2 loop and adjacent regions. For example, the lack of assigned resonances for R164 by Zheng et al. may be ascribed to intermediate timescale exchange broadening these signals beyond detection. Given the pK_a of interacting residues, the pH at which structural studies are carried out might thus be very significant.

It is of great interest that both the V127 and V129 polymorphisms have long-range effects on the conformational distribution of these regions of the protein and also the β-sheet. The correlation between these conformationally variable regions of PrP and its propensity to form disease-related isoforms suggest that these regions of the protein are important in prion assembly–PrP interactions, determining efficient binding and conversion. Indeed, relatively subtle variations in the strength and orientation of monomer docking can dramatically affect the productivity of fibrillogenic interactions and determine barriers to amyloid formation. Given the effect of the V127 polymorphism on the PrP β-sheet backbone geometry and intermolecular association of PrP monomers observed here, it is tempting to speculate that dimerisation via the formation of the intermolecular PrP β-sheet may be a critical event in PrP oligomerisation and prion propagation and thus explain the exceptional effect of the residue 127 polymorphism on human prion disease.

Methods

Recombinant PrP and antibodies. Recombinant human PrP containing residues 119–231 (PrP119–231) was produced and purified as previously described. This length of construct was chosen as the PrP N-terminus up to approximately residue 125 is unstructured in full-length (residues 23–231) and truncated (residues 91–231) PrP and compromises the NMR dynamics characterisation due to its effect on the rotational tumbling of the structured domain. Removal of the N-terminal tail does not affect the structure or local structural fluctuations of the PrP structured globular domain. Removal of valine at residue 127 (V127/M129 and V127/V129), and wild-type PrP with glycine at residue 127 on both 129 methionine and valine backgrounds (G127/M129 and G127/V129) were expressed and purified for biophysical analysis. ICSM 18 was purchased from D-Gen Limited. The Fab fragment of IC8M 18 was prepared by limited papain digest of the mature antibody followed by purification using gel filtration chromatography.

Crystallisation conditions. IC8M 18-Fab and PrP were mixed at a molar ratio of 3:1 for preparation of the complex prior to crystallisation, and incubated at room temperature for 30 min before buffer-exchanging the complex into 50 mM Tris, 130 mM NaCl, pH 8.0 and filtering through a 0.22 μm membrane prior to crystallisation. Crystals of the complex were obtained by vapour diffusion technique: droplets containing 5–6 mg/mL PrP in 0.4 M and 0.75 M ammonium sulphate, 0.05 M Tris (pH 7.5 and 8.0) were equilibrated over wells containing 0.8 M and 1.5 M ammonium sulphate, 0.1 M Tris (pH 7.5 and 8.0). Crystals, round in shape, grew in 6 months to 0.05–0.2 mm diameter.

In situ data collection and analysis. Data were collected at room temperature in situ on beamline I03 at Diamond Light Source, with the crystallisation plates sealed in biohazard bags. Multiple wedges of data were collected from different parts of the same crystal, and from different crystals, and scaled together to provide a complete dataset. We typically collected 15° of data from each crystal in 0.3° oscillations. Data were integrated with XDS and then subsequently BLENDF9 was used to analyse how well the different wedges of data scaled together and the results used to decide which datasets should be scaled and merged with Aimless.

Structure determination and refinement. The structures were solved by molecular replacement using Phaser with the heavy and light chains of the Fab fragment of antibody IC8M18 and the PrP molecule used as search models (protein databank accession code 2W9E). Electron density maps were inspected and the models built using COOT followed by refinement with REFMAC5. Data collection and final refinement statistics are summarised in Supplementary Table 1. Ramachandran statistics for V127/M129 and V127/V129 structures (in parentheses) are as follows: residues in most favoured region 96.7% (96.7%), residues in additionally allowed regions 3.3% (3.3%) and residues in disallowed regions: 0.0% (0.0%). The final coordinates of the V127/M129 and V127/V129 structures have been deposited in the Brookhaven Protein Data Bank (http://www.rcsb.org), with accession numbers 6SV2 and 6SUZ respectively.

NMR sample preparation and spectroscopy. For the NMR study 2H/15N-labelled samples of PrP were prepared. Following purification, protein samples...
were either (A) buffer-exchanged into 20 mM sodium acetate, containing 1.5 mM sodium azide (NaN₃), pH 5.5, 10 mM D₂O (ν/ν) was added to the NMR samples to provide the lock signal, together with TSP as the chemical shift reference to 1H m final concentration. NMR samples were placed in Sigma FEP NMR sample tube liners (ZY19-27-1EA), held within Wilmad PP-528 NMR tubes for NMR data acquisition.

Assignment spectra for V127/M129 PrP were acquired at 303 K on Bruker DRX-600 and DRX-800 spectrometers, with 1H-N triple-resonance measurements for V127 and V129 PrP acquired at 298 K on Bruker Avance III 500 and 800 MHz spectrometers, all equipped with 5 mm 1H/15N/1H triple-resonance probes. Sensitivity-enhanced 1H-15N HSQC81,82 and standard triple-resonance experiments83 with uniformly 13C/15N-labelled protein (HNCa, HNCAc, CBCA (CO)NH and HNCO) were used to obtain V127/M129 backbone resonance assignments. Proton chemical shifts were referenced to TSP. 1H & 15N chemical shifts were calculated relative to TSP, using the gyromagnetic ratios of 1H, 15N and 13C/15N/H = 0.101329118; 1H² = 0.25149530). For residue 127, when comparing 1H chemical shifts, the difference in residue type was compensated for by subtracting residue-specific random coil shifts (glycine/valine) to generate secondary chemical shifts, which were then subtracted84, NMR data were processed and analyzed using INSIGHT II (Accelrys, San Diego), Topspin (v 3.2, Bruker) and CCPN Analysis (v. 2.3.1)85 software.

Spin relaxation measurements. Spin relaxation measurements (T₁, T₂ and 15N (1H)-NOE) were acquired on 1 mM 1H-15N-labelled PrP19–23 WT (G127/M129) PrP, V127 (V127/M129) PrP and V129 (G127/V129) PrP as described in Yip et al.90. Briefly, by using this methodology, heating compensation was improved by the incorporation of a compensation block based on the relaxation block, followed by a pre-scan 1H saturation sequence and constant length recovery period. The T₁ data were obtained using 15N relaxation delays of 50, 100, 200, 300, 500, 800, 1000, 1500, 2000, 3000, 4000 and 5000 ms. The T₂ data were obtained using 15N relaxation delays of 8.5, 17.0, 35.9, 50.9, 67.8, 101.8, 135.7, 186.6, 254.4 ms (500 MHz) and 7.8, 15.7, 31.4, 47.0, 62.7, 94.1, 125.4, 172.5 and 235.2 ms (800 MHz; the astersisks denote duplicate measurements). T₁ and T₂ datasets were recorded as pseudo 3D experiments, with randomised order of time increments. Two separate nitrogen offsets were used to reduce build-up of off-resonance artefacts during the CPMG block of the T₂ measurements. For the 1H-15N NOE measurement, two-dimensional spectra were acquired with a relaxation delay of 6 scans. Spectra were collected with t₁ acquisition times of 94.7 ms (500 MHz)/59.2 ms (800 MHz) and t₂ (direct) acquisition times of 127.8 ms (500 MHz)/91.8 ms (800 MHz). Errors for time-series T₂ and T₁ data were calculated from the overall standard deviation for duplicate data points in the series. Errors for the NOE data were estimated from measurements of the root mean-square deviation of the base-plane noise in those spectra. Non-linear least-squares (Levenberg-Marquardt) fitting of two-parameter exponential functions to decay data was performed using in-house routines using Numerical Python.

Modfree analysis. Protein dynamics were analysed by Relax (v. 3.3.1)38,39, using the T₁, T₂ and 15N [1H]-NOE spin relaxation data. Reduced spectral density mapping analysis, as implemented by the Relax default jsub mapping script mode was used to obtain Jsub values for each given field strength. A full Modfree analysis67,88 was carried out using the "auvA" protocol within RELAX. Extracted order parameters (S₂, S₅, S₆), the effective correlation time for fast internal motions (τ₁) and intermediate exchange broadening contribution (κva) values were obtained using this protocol.

Amide exchange protection experiments. Hydrogen-deuterium exchange rates (kex) were determined by adding 260 µl 20 mM sodium acetate, 1 mM sodium azide, pH 4.5, dissolved in 100% (v/v) D₂O to lyophilised PrP samples, to obtain final protein concentrations of 1 mM. A series of sensitivity-enhanced 1H-15N HSQC spectra81,82 were acquired at 293 K on a Bruker DRX-800 spectrometer. The decay curves of the 1H-15N HSQC cross-peaks were fitted to single exponential decays with offset, and protection factors (κva/kex) for observable amides were determined using intrinsic amide exchange rates88,89. Acquisition of the first experiment began ~5 min after mixing, setting a lower limit on the detection of protection factors of ~5.

Circular dichroism. Circular dichroism was measured at 25°C with a Jasco J-715 spectropolarimeter, using a 0.1 cm pathlength quartz cuvette. The sample temperature was controlled with a circulating water bath. Far-UV (amide) CD spectra were recorded between 180 nm and 300 nm with 20 µM protein (2 nm bandwidth; Data Pitch 0.5 nm). In all, 10–20 spectra were averaged.

Equilibrium unfolding measurements. For equilibrium unfolding experiments, 6 µM ps was incubated in 10 mM HEPES, 25 mM NaCl pH 7.5, and increasing concentrations of GuHCl denaturant. Molecular ellipticity (θ), degree M⁻¹ cm⁻²) was recorded at 222 nm (5 nm bandwidth; 20 s integration time). The denaturation profile for each protein was measured in three separate experiments.

Conversion to molar denaturant activity. To allow more accurate extrapolation of data to calculate folding parameters in the absence of denaturant and the free energy change of protein folding (ΔG), denaturant concentration ([GuHCl]) was converted to molar denaturant activity (D), as described in Parker et al.89, using Cₙₐ = 7.5.

Equilibrium constant between folded and unfolded states. For the two-state equilibrium unfolding transitions, data were fitted to the following equation90:

\[ K = K_{(W)} \exp(\frac{m(D)}{D}) \]  

For visual representation of the data shown, data were converted to proportion folded, as, using the following, as = (K/(1 + K)). Data fitting was carried out using GraFit (Erithacus software). The significance of the differences in free energy for folding and m values between the three variants characterised were determined by paired two-tailed Student’s t test.

Quantitative analysis of the kinetics of PrP fibril formation. Reconstituent V127/M129 and WT G127/M129 PrP (residues 119–231) was dialysed into 20 mM sodium acetate, 2 mM sodium azide, pH 6.0, and then denatured by the addition of GuHCl to a final concentration of 6 M. Denatured PrP was then diluted to a final concentration of 10 µM in 20 mM sodium acetate, 2 M guHCl, 10 mM EDTA, 100 mM Thioflavin T (ThT) and pH 6.0. All solutions were filtered through a 0.22-µm filter to remove particulates. In all, 200 µl aliquots were placed in silenced Greiner 96-well flat-bottomed plates (N65077) containing 0.5 mm diameter zirconium ceramic beads in each well to assist agitation. The plates were incubated at 37°C with constant agitation in a Tecan Infinite F200 Microplate Fluorimeter. Fibril formation was monitored through the increase in ThT fluorescence (excitation 430 nm, emission 485 nm), with readings acquired every 600 s. Five replicates were used for each PrP sample.

To determine the half- and lag-times for fibril formation, data were fitted to an empirical function described by Nielsen et al.90.

\[ F_i + F_f \left\{ 1 + \exp \left[ -\left( t - t_{\alpha} \right)/r \right] \right\} \]  

where Fi is the initial fluorescence reading, Ff is the final fluorescence reading, t is time, tα is the time taken to half maximal fluorescence and r is the reciprocal of the propagation rate during the rise phase t1/2/τ(propagation). Lag-time is defined as tα − 2r.

Formation of protease-resistant PrP by PMCA amplification. PMCA substrate homogenates were prepared from mice that had been perfused with PBS containing 5 mM EDTA at the time of death. PrP-null (Prp-null), G127/M129, Tg152 (homozygous for huPrP G127/V129) or Tg183 (homozygous for huPrP V127/M129) mouse brains25 were homogenised in cold conversion buffer (PBS containing 150 mM NaCl, 1.0% (v/v) Triton X-100, 4 mM EDTA and 1× Complete protease inhibitor (Roche Applied Science)), using a Dual tissue grinder to give a 10% (w/v) homogenate. Samples were seeded with a 1/100 dilution of vCJD (14618) 10% brain homogenate in PBS. Each reaction mixture was divided in two prior to PMCA with one half stored at −70°C as a minus PMCA control. PMCA consisted of 96 cycles of 30 s sonication every 30 min in a Misonix S3000 at 75% power output (Misonix, Farmingdale, NY), reactions were carried out with 40 µl substrate in 200-µl thin-walled PCRB tubes at 35°C.

Samples were digested with 50 µg ml⁻¹ proteinase K (PK) for 1 h at 37°C. The reaction was stopped by the addition of AEBSF in SDS loading buffer and samples were boiled for 10 min before running on 16% Tris-glycine gels. Western blotting was carried out according to Unit protocol, using 3F4 (Merck Inc, N.J., U.S.A) as the primary antibody and goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma A2179) as the secondary antibody.

Statistics and reproducibility. In the reported experiments, each protein sample was independently engineered. The sample size (n) of each experiment is provided in the corresponding figure captions in the main manuscript and supplementary information files. Sample sizes were chosen to support meaningful conclusions. All in vitro folding experiments were replicated at least three times. In vitro fibrilisation assays were replicated five times. T₁ and T₂ NMR data were recorded with randomised order of time increment and each included one duplicate dataset. Replicate experiments were successful. Investigators were not blinded during experimental measurements or data analysis.
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Author contributions

L.L.P.H., R.C., D.S., M.J.C., A.M.H., K.M., R.L.B., G.S.J., J.P.W. and J.C. designed research; L.L.P.H., R.C., M.J.C., A.M.H., K.M., R.L.B. and J.P.W. performed research; D.S. and K.M. contributed new analytic tools; L.L.P.H., R.C., M.J.C., A.M.H., K.M., R.L.B., J.P.W. and J.C. wrote the paper.

Competing interests

G.S.J. and J.C. are shareholders, and J.C. a director of D-Gen Limited, an academic spin-out company in the field of prion disease diagnosis, decontamination, and therapeutics, which provided the ICSM18 monoclonal antibody used in this study. The remaining authors share no competing interests.

Additional information

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