

Strain-specific prion-protein conformation determined by metal ions

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In animals infected with a transmissible spongiform encephalopathy, or prion disease, conformational isomers (known as PrP^{Sc} proteins) of the wild-type, host-encoded cellular prion protein (PrP^C) accumulate. The infectious agents, prions, are composed mainly of these conformational isomers, with distinct prion isolates or strains being associated with different PrP^{Sc} conformations and patterns of glycosylation. Here we show that two different human PrP^{Sc} types, seen in clinically distinct subtypes of classical Creutzfeldt–Jakob disease, can be interconverted *in vitro* by altering their metal-ion occupancy. The dependence of PrP^{Sc} conformation on the binding of copper and zinc represents a new mechanism for post-translational modification of PrP and for the generation of multiple prion strains, with widespread implications for both the molecular classification and the pathogenesis of prion diseases in humans and animals.

The transmissible spongiform encephalopathies are a group of transmissible neurodegenerative diseases that include Creutzfeldt–Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals. These diseases have attracted wide interest not only because of their unique biology, but also because of the appearance of new variant CJD¹, which appears to be caused by exposure to the causative agent of BSE^{2–4}. The diseases are associated with the accumulation in affected brains of a conformational isomer (PrP^{Sc}) of host-derived prion protein (PrP^C). According to the protein-only hypothesis of prion propagation, PrP^{Sc} is the principal or sole component of transmissible prions⁵. The formation of PrP^{Sc} from PrP^C is associated with an increase in the amount of β -sheet secondary structure in the protein⁶, and PrP^{Sc} is recognized biochemically by its acquisition of partial resistance to digestion by proteinase K. Although the structure of PrP^C has been determined⁷, the insolubility of PrP^{Sc}, which is often present in brain as amyloid fibrils and which is isolated from tissue in a highly aggregated state, has so far precluded high-resolution structural analysis of PrP^{Sc}.

The existence of multiple strains or isolates of prions, encoding distinct disease phenotypes, has been difficult to accommodate within a protein-only model of prion propagation. However, considerable evidence now indicates that the diversity of prion strains may be encoded within PrP itself. Distinct PrP^{Sc} conformations^{2,8–11} and patterns of glycosylation² are associated with different prion strains and these biochemical properties can be transmitted to experimental animals^{2,10}. The elucidation of cellular mechanisms that may influence PrP^{Sc} conformation is thus of considerable interest. Here we show that strain-specific human PrP^{Sc} molecules are isolated from diseased brain in a metal-ion-occupied form and can undergo conformational change as a result of binding metal ions. We propose that this simple post-translational mechanism may be of widespread importance in conferring strain-specific properties to distinct PrP^{Sc} conformers.

Results

Human prion strain types. We previously identified four human PrP^{Sc} types or strains that are associated with distinct forms of sporadic or acquired CJD². Type-4 PrP^{Sc} characterizes new variant CJD, which is causally related to BSE^{2–4}; however, there is no evidence for an animal origin for the prion strains causing classical, or sporadic, CJD (PrP^{Sc} types 1–3)¹². Following limited proteolysis with protein-

ase K and western blotting, these distinct types of PrP^{Sc} can be easily distinguished by their differing fragment sizes or by relative differences in intensities of the three PrP glycoforms (corresponding to amino-terminally truncated cleavage products generated from di-, mono-, or non-glycosylated PrP^{Sc})² (Fig. 1). A common PrP polymorphism (the presence of either methionine (M) or valine (V) at residue 129) contributes to genetic susceptibility to both sporadic and acquired human prion disease^{13,14}. So far, PrP^{Sc} types 1 and 4 have been found only in individuals of the MM genotype; type 2 is seen in individuals of all genotypes (MM, MV and VV); and type-3 PrP^{Sc} accompanies only the MV or VV genotypes (refs 2, 15, 16 and A.F.H., S.J. and J.C., unpublished observations).

In an earlier study¹⁷ of PrP^{Sc} types in classical CJD, only two types of PrP^{Sc} were described and these authors have argued that the types 1 and 2 that we describe correspond to their type 1, while our type-3 pattern corresponds to that of their type-2 protein¹⁸. However, these authors concede a degree of heterogeneity in their type-1 cases¹⁸. We have performed a large-scale study of PrP^{Sc} types in CJD in conjunction with the UK National CJD Surveillance Unit. Comprehensive phenotypic assessment of patients and PrP^{Sc} typing were performed blind. A detailed study will be published elsewhere, but we showed that patients classified as type 1 and type 2 using our criteria have quite distinct phenotypes (Fig. 2), confirming the validity

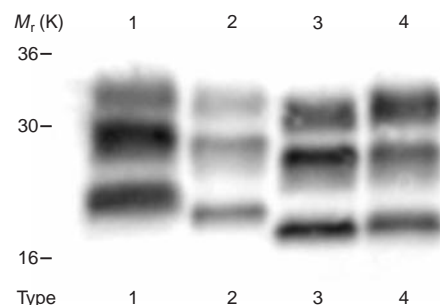


Figure 1 Western blot of human PrP^{Sc} types 1–4 following treatment with protease K, using anti-PrP monoclonal antibody 3F4 for the western blot. Lane 1, type-1 PrP^{Sc}, genotype PRNP MM; lane 2, type-2 PrP^{Sc}, PRNP MM; lane 3, type-3 PrP^{Sc}, PRNP VV; lane 4, type-4 PrP^{Sc}, PRNP MM. (M and V indicate that methionine or valine is found at position 129 of the PrP^{Sc} protein.)

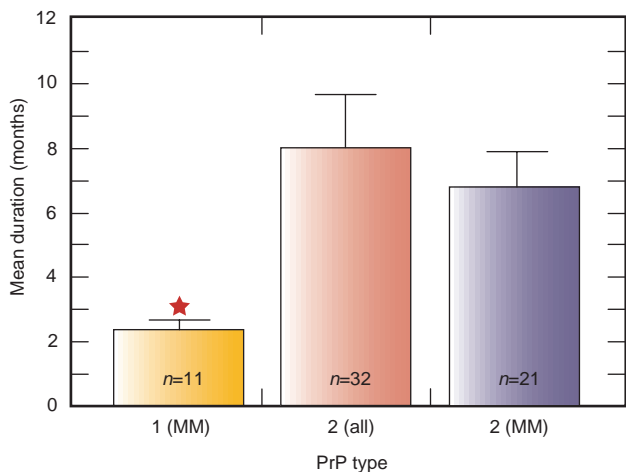


Figure 2 Mean duration of illness for CJD patients with PrP^{Sc} types 1 and 2. Duration in type-1 cases is significantly different (asterisk) from the duration in type-2 cases, regardless of whether *PRNP* encodes methionine or valine at position 129 in type-2 PrP^{Sc} ($P < 0.004$; Mann-Whitney *U*-test).

of our molecular classification. Type-1 human CJD is a distinct human prion disease with an aggressive clinical course and remarkably short clinical duration. These observations are consistent with PrP^{Sc} conformation being the foundation of prion strain diversity. **Conformations of PrP^{Sc} types 1 and 2 depend on metal ions.** In an attempt to elucidate the molecular basis of strain variation, we investigated the biochemical properties of type-1 and type-2 human PrP^{Sc}. In all patients studied, both alleles of the PrP^C-encoding gene, *PRNP*, coded for PrP with methionine at position 129 (genotype MM). When we treated type-1 and type-2 PrP^{Sc} from these patients with 20 mM of the metal-ion chelator EDTA before treatment with proteinase K, the pattern of cleavage was changed. Rather than producing their distinct patterns, both types gave indistinguishable and common fragment sizes (Fig. 3a). As these digestion products had lower relative molecular masses than the cleavage products of either type-1 or type-2 PrP^{Sc}, we designated these products type 2'. In marked contrast, treatment with EDTA did not alter the generation of characteristic cleavage products from PrP^{Sc} types 3 or 4 (Fig. 3b).

The generation of type-2' cleavage products from type-1 PrP^{Sc} typically required final EDTA concentrations in the range of 15–20 mM; no further change was elicited by higher chelator concentrations (data not shown). This effect of EDTA was fully reproducible (>60 repetitions using samples from nine type-1 patients) and occurred irrespective of the buffer in which brain homogenates were prepared (Fig. 3c). We analysed nine homogenates from type-1 and type-2 patients before and after treatment with EDTA. In each case, we detected the expected shift of type-1 or type-2 products to type-2' products. We estimated the shift in apparent relative

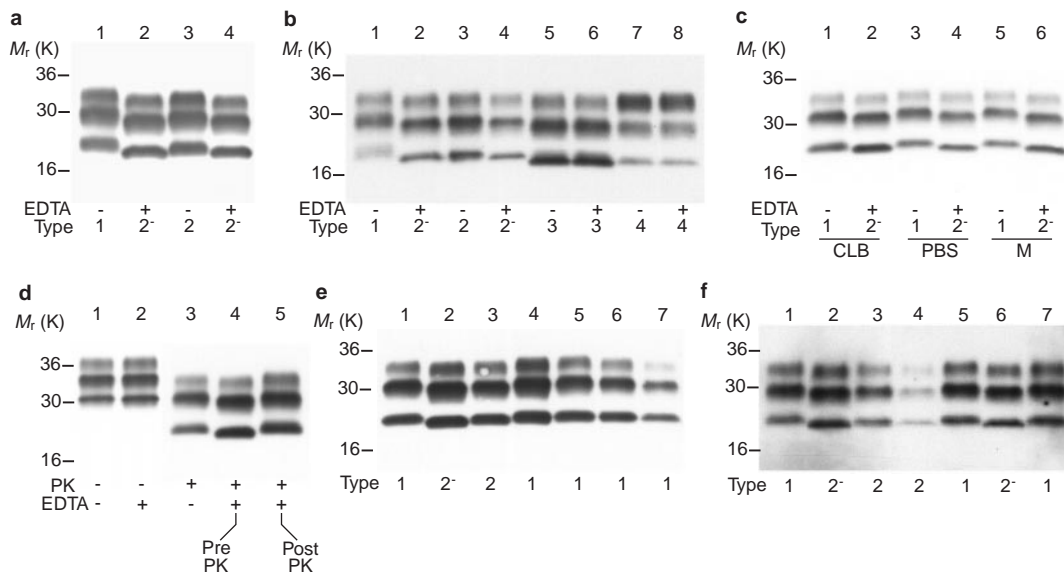


Figure 3 Digestion of human PrP^{Sc} by proteinase K in the presence of metal chelators. **a, b,** Effects of EDTA on digestion of PrP^{Sc} types 1–4. 10% w/v brain homogenates prepared in cold lysis buffer were treated with proteinase K directly (–) or after (+) adjustment of EDTA in the buffer to a final concentration of 20 mM. **a,** Lanes 1, 2, type-1 PrP^{Sc}; lanes 3, 4, type-2 PrP^{Sc} MM. **b,** Lanes 1, 2, type-1 PrP^{Sc} genotype MM; lanes 3, 4, type-2 PrP^{Sc} genotype MM; lanes 5, 6, type-3 PrP^{Sc} genotype MV; lanes 7, 8, type-4 PrP^{Sc} genotype MM. **c,** The effect of EDTA on type-1 PrP^{Sc} is consistent in different buffers. 10% w/v brain homogenates from a patient with type-1 PrP^{Sc} were prepared in cold lysis buffer (CLB; lanes 1, 2), PBS (lanes 3, 4) or *N*-ethylmorpholine buffer (M; lanes 5, 6) and were digested with proteinase K before (–) or after (+) adjustment with EDTA to a final concentration of 20 mM. **d,** EDTA exposes a new site of cleavage by proteinase K (PK) on type-1 PrP^{Sc}. Aliquots of a 10% w/v PBS brain homogenate from a patient with type-1 PrP^{Sc} were western blotted directly (no proteinase-K treatment) in the absence (lane 1) or presence (lane 2) of 25 mM EDTA. In lanes 3–5, aliquots of a 10% w/v PBS brain homogenate from a type-1

patient were treated with proteinase K in the absence (lanes 3, 5) or presence (lane 4) of 25 mM EDTA. Following proteolysis, the sample in lane 5 was boiled in SDS sample buffer and subsequently adjusted to 25 mM EDTA before electrophoresis. **e, f,** Effects of different chelators on the digestion of type-1 PrP^{Sc}. Aliquots of a 10% w/v *N*-ethylmorpholine buffer brain homogenate from a type-1 PrP^{Sc} patient were treated with proteinase K in the absence (lane 1) or presence (lanes 2–7) of different chelators. The chelators and their final concentrations were: **e,** lane 2, 20 mM EDTA; lane 3, 20 mM EGTA; lane 4, 20 mM dipicolinic acid; lane 5, 20 mM bathophenanthroline disulphonic acid; lane 6, 20 mM neocuproine; lane 7, 20 mM 1,10 phenanthroline; **f,** lane 2, 20 mM EDTA; lane 3, 20 mM EGTA; lane 4, 20 mM triethylenetetramine; lane 5, 20 mM dipicolinic acid; lane 6, 10 mM triethylenetetramine plus 10 mM dipicolinic acid; lane 7, 10 mM triethylenetetramine plus 10 mM bathophenanthroline disulphonic acid. The 'types' are the types of PrP^{Sc} cleavage products produced, that is, types 1, 2, 3, 4 or 2'.

molecular mass from type-1 to type-2⁻ products and from type-2 MM to type-2⁻ products to be $1,100 \pm 300$ (mean \pm s.d.; $n = 9$) and 650 ± 300 (mean \pm s.d.; $n = 9$), respectively. There was no significant alteration in the ratios of the three principal PrP glycoforms.

We excluded the possibility that EDTA itself directly influenced electrophoretic mobility. Without protease digestion, type-1 PrP^{Sc} samples migrated equivalently in the presence or absence of EDTA (Fig. 3d). Similarly, application of EDTA to type-1 PrP^{Sc} samples after proteolysis had no effect (Fig. 3d). These findings indicate that the respective conformations of type-1 PrP^{Sc} and type-2 PrP^{Sc} MM may depend upon the presence of metal ions and that metal-ion chelation may induce a conformational change in the protein, exposing a new proteolytic cleavage site that is apparently common to both metal-ion-depleted conformers.

Effects of metal-ion-selective chelators. As the N-terminal octapeptide repeat region of PrP binds Cu²⁺ (refs 19–24), we thought that Cu²⁺ might be involved in determining metal-ion-dependent PrP^{Sc} conformation. However, the use of various metal-selective chelating agents revealed a more complex situation. EDTA is a broad-specificity chelator with high affinity for many divalent metal ions; other, more selective chelators, including those with high affinity for Cu²⁺ (EGTA and triethylenetetramine), Cu⁺ (neocuproine), Zn²⁺ (dipicolinic acid and 1,10 phenanthroline) or Fe²⁺ (1,10 phenanthroline and bathophenanthroline disulphonic acid) were unable to mirror the effects of EDTA precisely (Fig. 3e, f). However, the effectiveness of combined application of triethylenetetramine and dipicolinic acid (Fig. 3f) indicated that chelation of both Cu²⁺ and Zn²⁺ may be required for generation of type-2⁻ cleavage products from type-1 PrP^{Sc}.

Both Cu²⁺ and Zn²⁺ interact with PrP^{Sc}. We developed an alternative method for probing the metal-ion dependency of PrP^{Sc} conformation, by washing the homogenates to strip bound metal from the

protein. Washing type-1 PrP^{Sc} homogenates with *N*-ethylmorpholine buffer (equivalent to a ~5,000-fold dilution) before digestion by proteinase K readily resulted in the formation of type-2⁻ digestion products (Fig. 4a, b). Repetition of this procedure using buffers supplemented with various metal ions at the total concentrations observed in serum²⁵ convincingly showed that the type-1 conformation is dependent on metal ions. In the maintained presence of Cu²⁺ or Zn²⁺, digestion products closely resembled those generated from untreated type-1 PrP^{Sc} (Fig. 4a); that is, the original PrP^{Sc} conformation was retained. Other metal ions, when present at their respective total concentration found in serum, had no effect, when applied either separately (data not shown) or together (Fig. 4a). We can exclude the occurrence of anomalous electrophoretic mobility of cleavage products in the presence of Cu²⁺ or Zn²⁺; exposure of metal-ion-depleted and proteinase-K-digested type-1 PrP^{Sc} samples to either Cu²⁺ or Zn²⁺ before and during electrophoresis had no effect (Fig. 4b). Together, these findings (coupled with the results obtained using metal-ion-selective chelators) implicate Cu²⁺ or Zn²⁺ as the most relevant metal ions that interact with type-1 PrP^{Sc} in prion-diseased brain. Notably, the concentrations of Cu²⁺ that we find to be effective in maintaining type-1 PrP^{Sc} conformation correlate closely with the dissociation constant of 14 μ M for binding of Cu²⁺ to recombinant full-length hamster PrP²³.

Although both Cu²⁺ and Zn²⁺ are present at much higher total concentrations in normal brain compared with in serum (discussed in refs 23, 25), these ions would exist predominantly in protein complexes rather than as free ions. Transient total extracellular concentrations of Zn²⁺ can reach as high as 300 μ M in brain during sustained neuronal activity²⁶, but the proportion that exists as free ions is uncertain. In the case of Cu²⁺, it is unlikely that micromolar levels of free ions will occur in any cell compartment in the physiological state, and it thus remains to be shown how PrP^C might

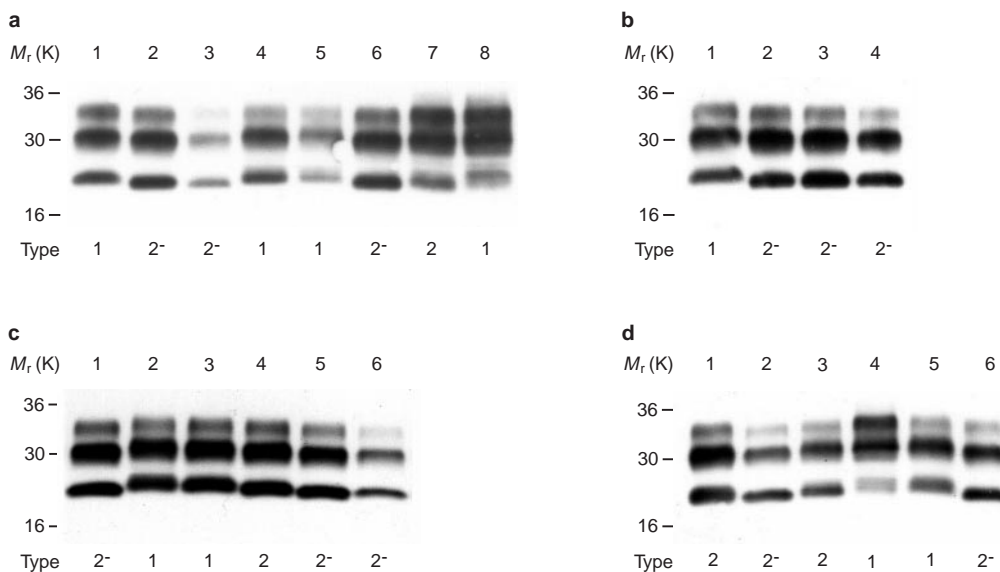


Figure 4 Both Cu²⁺ and Zn²⁺ interact with PrP^{Sc}. PrP^{Sc} types 1 and 2 (PRNP genotype MM) were washed in the presence of various metal ions. **a**, Type-1 PrP^{Sc}. A 10% w/v brain homogenate from a type-1 patient was prepared in PBS and aliquots were digested with proteinase K before (lane 1) or after washing with *N*-ethylmorpholine buffer alone (lane 2) or the same buffer containing 20 μ M FeCl₃, 1 mM MgCl₂, 1 μ M NiCl₂, 2 mM CaCl₂, 0.05 μ M MnCl₂ and 0.03 μ M CoCl₂ (lane 3); 10 μ M ZnCl₂ (lane 4); 20 μ M ZnCl₂ (lane 5); 10 μ M CuSO₄ (lane 6); 20 μ M CuSO₄ (lane 7); or 25 μ M CuSO₄ (lane 8). **b**, Type-1 PrP^{Sc}. A 10% w/v brain homogenate from a type-1 patient was prepared in PBS and aliquots were digested with proteinase K before (lane 1) or after (lanes 2–4) washing with *N*-ethylmorpholine buffer. Following proteolysis and before electrophoresis, samples in lanes 3 and 4 were washed with

N-ethylmorpholine buffer containing either 20 μ M ZnCl₂ (lane 3) or 25 μ M CuSO₄ (lane 4). **c**, Type-1 PrP^{Sc}. A 10% w/v brain homogenate from a type-1 patient was prepared in *N*-ethylmorpholine buffer and aliquots were digested with proteinase K after washing with *N*-ethylmorpholine buffer alone (lane 1) or the same buffer containing 20 μ M ZnCl₂ (lane 2), 30 μ M NiCl₂ (lane 3), 30 μ M CoCl₂ (lane 4), 30 μ M MnCl₂ (lane 5), or 30 μ M FeCl₃ (lane 6). **d**, Type-2 PrP^{Sc}. A 10% w/v brain homogenate from a type-2 patient was prepared in cold lysis buffer and aliquots were digested with proteinase K before (lane 1) or after washing with *N*-ethylmorpholine buffer alone (lane 2) or the same buffer containing 20 μ M ZnCl₂ (lane 3) or 25 μ M CuSO₄ (lane 4). Lanes 5, 6 show digestion products from a type-1 PrP^{Sc} PBS brain homogenate that was proteinase-K-treated directly (lane 5) or after addition of 25 mM EDTA (lane 6).

acquire Cu^{2+} *in vivo*. From our findings, however, the pathological relevance of metal-ion binding to PrP^{Sc} is clear: PrP^{Sc} types 1 and 2 are isolated from diseased brain in metal-ion-occupied form. These results could indicate that the concentrations of Cu^{2+} and Zn^{2+} in prion-diseased brain are grossly perturbed. This has recently been shown to be the case in Alzheimer's disease, where Cu^{2+} , Zn^{2+} and Fe^{2+} are highly concentrated within the periphery and core of senile plaque deposits²⁷. Moreover, micromolar concentrations of Cu^{2+} and Zn^{2+} can induce marked aggregation of amyloid $\text{A}\beta$ protein²⁵. In the latter study, at the total concentrations of metal ions found in serum, only Cu^{2+} and Zn^{2+} were able to induce marked aggregation of $\text{A}\beta$ protein; however, at supraphysiological concentrations (30 μM), Ni^{2+} and Co^{2+} were also effective. We have also tested the effectiveness of 30 μM Ni^{2+} , Co^{2+} or Mn^{2+} in maintaining the conformation of type-1 PrP^{Sc} ; at this concentration, only Ni^{2+} can effectively substitute for Cu^{2+} or Zn^{2+} (Fig. 4c). These results further reinforce our deduction that Cu^{2+} and Zn^{2+} are likely to be the most important metal ions that interact with PrP^{Sc} in prion-diseased brain.

Interconversion of human PrP^{Sc} types 1 and 2. Interestingly, whereas the conformation of type-1 PrP^{Sc} could be easily maintained in the presence of 10 μM Zn^{2+} , higher concentrations of Cu^{2+} were required to have the same effect. We studied a range of Cu^{2+} concentrations (10–25 μM); in the presence of 20 μM Cu^{2+} , a pattern of cleavage products was produced that migrated with a mobility similar to that of the type-2 products, that is, intermediate between type 1 and type 2 (Fig. 4a, compare lanes 6–8). This intermediate pattern could also be discerned after digestion of type-1 PrP^{Sc} in the presence of the copper-selective chelators EGTA or triethylenetetramine (Fig. 3e, f), or after washing and digestion of type-1 PrP^{Sc} in the presence of 30 μM Co^{2+} (Fig. 4c). As the mobility of these intermediate fragments resembled that of type-2 cleavage products, these results indicate that the conformations of type-1 PrP^{Sc} and type-2 PrP^{Sc} may differ principally with respect to the relative occupancy of their metal-ion-binding sites.

To explore this possibility further, we studied the effects of applying exogenous metals to type-2 PrP^{Sc} . Consistent with the results obtained for type-1 PrP^{Sc} , washing insoluble aggregates of type-2 PrP^{Sc} with buffer alone gave type-2 cleavage products (Fig. 4d). However, in the maintained presence of different concentrations of Cu^{2+} or Zn^{2+} , we observed either the original type-2 pattern of digestion products, or a new pattern of higher-molecular-mass cleavage fragments similar to those generated from untreated type-1 PrP^{Sc} (Fig. 4d). These findings indicate that the conformations of type-1 PrP^{Sc} and type-2 PrP^{Sc} may be interchangeable and depend on the level of occupancy by these metal ions.

Discussion

The demonstration that phenotypically distinct types of CJD are associated with the biochemically distinct PrP^{Sc} types 1 and 2 clarifies earlier confusion on classification of CJD subtypes¹⁸. However, the precise aetiology of sporadic CJD remains obscure. The spontaneous conversion of PrP^{C} to PrP^{Sc} in a rare stochastic event, or somatic mutation of the *PRNP* gene, resulting in expression of a pathogenic PrP mutant², are possible causes. However, epidemiological studies have not ruled out the possibility that environmental exposure to human or animal prions²⁸ causes at least some cases. Sporadic CJD may have multiple aetiologies. Our results immediately allow a more precise molecular classification of human prion disease, with important implications for epidemiological studies into the aetiology of sporadic CJD. Re-analysis of epidemiological data using these molecular subtypes may reveal important risk factors that are obscured when sporadic CJD is analysed as a single entity.

Our results also define a potential molecular mechanism for strain variation. The ability of metal ions to influence PrP^{Sc} conformation directly has widespread implications for understanding

strain diversity in human and animal prion diseases. Our demonstration of an interaction between PrP^{Sc} and Cu^{2+} not only supports recent work that indicates that Cu^{2+} may stabilize PrP^{Sc} conformation²⁹, but also provides further evidence that the neuropathology of prion diseases may be related to abnormalities in copper metabolism^{22,30–32} and raises the possibility that drugs that influence copper metabolism may have therapeutic potential in prion disease.

Methods

Western blot analysis.

All procedures were carried out in a microbiological-containment level-3 facility with strict adherence to safety protocols. 10% *w/v* brain homogenates from human brain tissue obtained at autopsy from patients with CJD were prepared in the following solutions: cold lysis buffer (10 mM Tris and 10 mM EDTA, pH 7.4, containing 100 mM NaCl, 0.5% *w/v* NP-40 and 0.5% *w/v* sodium deoxycholate); phosphate-buffered saline (PBS) (Dulbecco's sterile PBS lacking Ca^{2+} and Mg^{2+} ; Sigma); *N*-ethylmorpholine buffer (25 mM *N*-ethylmorpholine, pH 7.4, containing 0.5% *w/v* NP-40). Samples were adjusted to a final concentration of 50 $\mu\text{g ml}^{-1}$ proteinase K (Merck) and incubated at 37 °C for 1 h. Digestion was terminated by addition of an equal volume of 2 × SDS sample buffer (125 mM Tris-HCl and 20% *v/v* glycerol, pH 6.8, containing 4% *w/v* SDS, 4% *v/v* 2-mercaptoethanol, 8 mM 4-(2-aminoethyl)-benzene sulphonyl fluoride and 0.02% *w/v* bromophenol blue) and immediate transfer to a 99 °C heating block for 10 min. Samples were analysed by electrophoresis and western blotting using anti- PrP monoclonal antibody 3F4 as described³.

Chelation studies.

Chelators were added to brain homogenates as aliquots from stock solutions. EDTA was prepared as either a 100-mM or a 250-mM stock in water and titrated to pH 8.0 with NaOH. Other chelators (Fig. 3) were prepared similarly as 100-mM stock solutions, pH 8.0, with the exception of 1,10 phenanthroline and neocuproine which were prepared as 100-mM stocks in 50% *v/v* ethanol in water. All chelators were obtained from Sigma. Physical properties of the chelators used and the stability constants of complexes formed with various metal ions have been described³³.

Metal-ion-supplementation studies.

10- μl aliquots of 10% *w/v* brain homogenates were centrifuged for 10 min at 14,000 r.p.m. in a microfuge (Eppendorf), after which supernatants were removed and discarded. Pellets were thoroughly resuspended in 500 μl *N*-ethylmorpholine (pH 7.4, 25 mM) containing 0.5% *w/v* NP-40; the *N*-ethylmorpholine either lacked or contained various metal salts as described in Fig. 4. Following incubation for ~10 min at room temperature, samples were centrifuged (15 min at 14,000 r.p.m. in a microfuge), after which the supernatant was discarded. Each aspirated pellet was resuspended appropriately with the analogous solution to a final volume of 10 μl and treated with proteinase K. In some experiments samples were washed with metal solutions after digestion by proteinase K.

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