The molecular biology of prion propagation

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Prion diseases such as Creutzfeldt–Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals 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, PrP^{Sc} is the principal or sole component of transmissible prions. The conformational change known to be central to prion propagation, from a predominantly α -helical fold to one predominantly comprising β structure, can now be reproduced *in vitro*, and the ability of β -PrP to form fibrillar aggregates provides a plausible molecular mechanism for prion propagation. The existence of multiple prion strains has been difficult to explain in terms of a protein-only infectious agent but recent studies of human prion diseases suggest that strain-specific phenotypes can be encoded by different PrP conformations and glycosylation patterns. The experimental confirmation that a novel form of human prion disease, variant CJD, is caused by the same prion strain as cattle BSE, has highlighted the pressing need to understand the molecular basis of prion propagation and the transmission barriers that limit their passage between mammalian species. These and other advances in the fundamental biology of prion propagation are leading to strategies for the development of rational therapeutics.

Keywords: prion protein; scrapie; Creutzfeldt–Jakob disease; bovine spongiform encephalopathy

1. INTRODUCTION

Prion diseases of both humans and animals are associated with the accumulation in the brain of an abnormal, partially protease-resistant isoform of a host-encoded glycoprotein known as the prion protein. The disease-related isoform, PrP^{Sc}, is derived from its normal cellular precursor, PrP^C, by a post-translational process that involves a rearrangement of the protein conformation. According to the 'protein-only' hypothesis (Griffith 1967), an abnormal PrP isoform (Prusiner 1982) is the principal, and possibly the sole, constituent of the transmissible agent or prion. PrP^{Sc} is hypothesized to act as a conformational template, promoting the conversion of PrP^C to further PrP^{Sc}.

The human prion diseases have been traditionally classified into Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease and kuru, and they can be further divided into three aetiological categories: sporadic, acquired and inherited. The acquired prion diseases include iatrogenic CJD and kuru, and arise from accidental exposure to human prions through medical or surgical procedures or participation in cannibalistic feasts. Epidemiological studies do not provide any evidence for an association between sheep scrapie and the occurrence of CJD in humans (Brown *et al.* 1987). Sporadic CJD occurs in all countries with a random case distribution and an annual incidence of one per million. Around 15% of human prion disease is inherited and all

cases to date have been associated with coding mutations in the prion protein gene (*PRNP*), of which over 20 distinct types are recognized (Collinge 1997). The inherited prion diseases can be diagnosed by *PRNP* analysis and the use of these definitive genetic diagnostic markers has allowed the recognition of a wider phenotypic spectrum of human prion disease to include a range of atypical dementias and fatal familial insomnia (Collinge *et al.* 1990, 1992; Medori *et al.* 1992*a,b*). No such pathogenic *PRNP* mutations are present in sporadic and acquired prion disease. However, a common PrP polymorphism at residue 129, where either methionine or valine can be encoded, is a key determinant of genetic susceptibility to acquired and sporadic prion diseases, the large majority of which occur in homozygous individuals (Collinge *et al.* 1991; Palmer *et al.* 1991; Windl *et al.* 1996). This protective effect of *PRNP* codon 129 heterozygosity is also seen in some of the inherited prion diseases (Baker *et al.* 1991; Hsiao *et al.* 1992).

The appearance of a novel human prion disease, variant CJD (vCJD), in the UK from 1995 onwards, and the experimental evidence that this is caused by the same prion strain as that causing BSE in cattle (Collinge *et al.* 1996*b*; Hill *et al.* 1997; Bruce *et al.* 1997), has raised the possibility that a major epidemic of vCJD will occur in the UK and other countries as a result of dietary or other exposure to BSE prions (Collinge 1999). These concerns, together with those of potential iatrogenic transmission of preclinical vCJD via medical and surgical procedures, have led to intensification of efforts to understand the molecular basis of prion propagation and to develop rational therapeutics. The development of blood-based

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early diagnostic methods remains a key goal to help evaluate and manage the threat to UK public health (Collinge 1999). The lack of an agent-specific genome, or humoral immune response to what is a host protein widely expressed in the immune system, makes such research highly challenging. Indeed success may depend on understanding how prions may be replicated *in vitro* to obtain sufficient sensitivity for a useful blood-screening test.

2. GENERAL PROPERTIES OF THE PRION PROTEIN

The normal or cellular form of the prion protein (PrP^C) is expressed in many tissues, although at highest levels in the central nervous system and lymphoreticular tissue, as a glycosylated, cell-surface protein held *in situ* by a glycolipid anchor (Stahl *et al.* 1987). The mature PrP^C species consists of an N-terminal region of about 100 amino acids, which is unstructured in the isolated molecule, and a C-terminal segment, also around 100 amino acids in length, which is folded into a largely α -helical conformation and stabilized by a single disulphide bond. The disease-associated or 'scrapie' form of PrP (designated PrP^{Sc}) which accumulates in these diseases is covalently indistinguishable from PrP^C (Stahl *et al.* 1993; Pan *et al.* 1993) but has a radically different conformation (Pan *et al.* 1993) associated with self-association into stable aggregated particles. Low-resolution spectroscopic methods have shown that isolated PrP^{Sc} has a much higher proportion of β -sheet. Although PrP^{Sc} has never been purified to homogeneity, fractions enriched for infectivity contain a high proportion of PrP^{Sc} (Bolton *et al.* 1982). Little or nothing is known for certain about the molecular state of the protein that constitutes the self-propagating, infectious particle itself. A single infectious unit corresponds to around 10^5 PrP molecules (Bolton *et al.* 1982). It is unclear whether this indicates that a large aggregate is necessary for infectivity or, at the other extreme, whether only a single one of these PrP^{Sc} molecules is actually infectious. This relationship of PrP^{Sc} molecules to infectivity could simply, however, relate to the rapid clearance of prions from the brain known to occur on intracerebral challenge.

PrP^{Sc} is resistant to digestion by proteinase K and in infected brain tissue the normal PrP^C isoform can be degraded rapidly to leave only the protease-resistant core of PrP^{Sc}, known as PrP²⁷⁻³⁰ (McKinley *et al.* 1983). This resistant material is enriched by detergent solubilization and differential centrifugation electron microscopy shows it to contain fibrillar material (Prusiner *et al.* 1983), but it is by no means certain that these ordered aggregates constitute the infectious agent. In several cases there have been claims that infectivity can be separated from fibrils and infectious brain tissue has been shown to contain no detectable protease-resistant PrP (Collinge *et al.* 1995a; Wille *et al.* 1996; Lasmézas *et al.* 1997; Shaked *et al.* 1999).

3. MODELS OF PRION PROPAGATION

Despite these uncertainties about the structural characteristics of the infectious form of PrP, it is well established that prion diseases arise by one of three processes. In outline all three aetiological routes can be described with

reference to a single, general model for prion initiation and propagation. In this model, the native PrP^C molecule is envisaged as existing in equilibrium with the rare PrP^{Sc} conformational isoform. PrP^{Sc} can then be stabilized by complementary association with a like molecule or can actively convert PrP chains to a like conformation. Assembly then continues until a stable seed is formed. Such structures can continue to grow by accretion and can divide by breakage into smaller, infectious units. A mechanism involving linear polymerization has been modelled mathematically and predicts many of the observed aspects of the disease (Masel *et al.* 1999). This gross mechanism explains the observation that prion diseases occur by inherited mutations that destabilize the cellular form and therefore predispose it to conversion to PrP^{Sc} or by iatrogenic or dietary infection with PrP^{Sc}.

The cause of sporadic CJD is unknown. However, its remarkably uniform worldwide incidence of around one case per million would be consistent with involvement of a random stochastic process. Sporadic CJD might represent somatic mutation of the PrP gene (resulting in production of one of the known pathogenic mutants) or by a rare, spontaneous conversion of the wild-type protein to the PrP^{Sc} conformation. A recent study has suggested that sporadic CJD may be linked to a past history of surgery or residency on a farm (Collins *et al.* 1999) such that a small number of apparently sporadic cases may in fact be environmentally or iatrogenically acquired.

In view of the peculiar status of the prion as an infectious protein devoid of nucleic acids, it seems reasonable to ask whether some aspect of its normal role may predispose it to this unique behaviour. Mice with an ablated PrP gene (*Prnp*^{0/0}) show only a mild phenotype. Amongst these are abnormalities of synaptic physiology (Collinge *et al.* 1994; Whittington *et al.* 1995; Colling *et al.* 1996, 1997), and altered circadian rhythms (Tobler *et al.* 1996). While none of these observations defines a molecular role for PrP^C, it has been argued that PrP may act as a receptor for an, as yet, unidentified extracellular ligand. Newly synthesized PrP^C is transported to the cell surface and then cycles rapidly via a clathrin-mediated mechanism, with a transit time of around an hour, between the surface and early endosomes (Shyng *et al.* 1994). This type of behaviour is associated with other cell-surface receptors, e.g. those for transferrin and LDL. Potential partner proteins identified by two-hybrid screening include the laminin receptor (Rieger *et al.* 1997). However these are of questionable relevance due to the methodology used for identification, i.e. the prion protein does not fold in the cytoplasm of cells and thus candidates so far identified are unlikely to interact specifically with PrP.

4. THE CONFORMATION AND STABILITY OF Prp^C

The conformation of the cellular isoform was first established by NMR measurements made on the recombinant mouse protein (Riek *et al.* 1996). Since then NMR measurements on recombinant hamster (James *et al.* 1997) and human PrP (Hosszu *et al.* 1999) show that they have essentially the same conformation. However, despite strenuous efforts, no group has yet determined the three-dimensional

structure of PrP^C by crystallographic methods. As described in §2, the N-terminal region of about 100 amino acids is unstructured in the isolated molecule. However, within this region there is a segment of five repeats of an eight amino-acid sequence (the octapeptide repeat region) which has a tight binding site for a single Cu²⁺ ion with a dissociation constant (K_d) of 10^{-14} M. A second tight copper site ($K_d=10^{-13}$ M) is present upstream of the octa-repeat region but before the structured C domain (Jackson *et al.* 2001). These values for copper-binding affinity are some eight or nine orders of magnitude tighter than previously published (Brown *et al.* 1997a; Hornshaw *et al.* 1995a,b; Stockel *et al.* 1998). The remaining 100 or so amino acids at the C-terminus are folded into a series of three α -helices and a small two-strand β -sheet and are stabilized by a single disulphide bond (Riek *et al.* 1996). Molecular modelling has demonstrated that the octapeptide repeat region can adopt several different conformations with identical stabilities upon binding copper(II) ions. However, it is possible that in association with a receptor molecule a single conformation may be preferentially stabilized (Jackson *et al.* 2001). A role for PrP in copper metabolism or transport seems likely, and disturbance of this function by the conformational transitions between isoforms of PrP could be involved in prion-related neurotoxicity (Jackson *et al.* 2001).

The structured domain folds and unfolds reversibly in response to chaotropic denaturants and recent work on the folding kinetics of mouse PrP^C (Wildegger *et al.* 1999) demonstrates that there are no populated intermediates in the folding reaction and that the protein displays unusually rapid rates of folding and unfolding. These findings have been reinforced by hydrogen–deuterium exchange measurements on the human protein, which show that the overall equilibrium constant describing the distribution of folded and unfolded states is the same as the protection factor (Hosszu *et al.* 1999). This observation shows that there are no partially unfolded forms or intermediates that have a population greater than the unfolded state. The data suggest that PrP^{Sc} is unlikely to be formed from a kinetic folding intermediate, as has been hypothesized in the case of amyloid formation in other systems. In fact, on the basis of population it would be more likely that PrP^{Sc} were formed from the unfolded state of the molecule.

Inherited prion diseases may produce disease by destabilizing PrP^C, which would predispose the molecule to aggregate. Alternatively a mutation could facilitate the interaction between PrP^C and PrP^{Sc} or affect the binding of a ligand or co-protein. In order to relate the folding stability of PrP^C to its propensity for forming PrP^{Sc}, several of the human mutations have been copied into the recombinant mouse protein (Liemann & Glockshuber 1999). Although this work broadly concluded that there is no absolute correlation between stability and disease, all of the fully penetrant pathogenic mutations show significant destabilization, while non-pathogenic polymorphisms have little effect.

Studies on transgenic animals have been widely used in the field and have provided several key insights. *Prnp*^{0/0} mice devoid of normal PrP^C are resistant to infection with prions and do not replicate the infectious agent (Bueler

et al. 1993). Introduction of prion genes from different species into these mice restores susceptibility to prions in a species-dependent manner. Thus there is an absolute requirement for PrP in disease pathogenesis.

Reconstitution of *Prnp*^{0/0} mice with transgenes expressing N-terminally truncated PrP have been performed to probe structure–function relationships in PrP. Expression of N-terminal deletion mutants to residue 106 was tolerated and allowed prion propagation. However, deletion beyond this led to severe ataxia and neuronal loss in the granular cell layer of the cerebellum (Shmerling *et al.* 1998), symptoms and pathology that were completely absent in the original *Prnp*^{0/0} mice. This observation has led to the hypothesis that PrP and a structural homologue compete for the same receptor or ligand. An additional gene (*Prn-d*) has recently been discovered within the *Prn-p* locus. It encodes a 179 residue protein with between 20% and 24% identity to PrP (depending upon the species) that is a plausible candidate for the putative competitor (Moore *et al.* 1999).

PrP in its entirety is unnecessary for prion propagation. Not only can the unstructured N-terminal 90 amino acids be deleted but also the first α -helix, the second β -strand and part of helix two. In transgenic animals a 106 amino-acid fragment of the protein comprising PrP Δ 23–88 Δ 141–176 was all that was required to confer susceptibility to, and propagation of, prions (Muramoto *et al.* 1996; Supattapone *et al.* 1999).

5. IN VITRO CONVERSION OF PrP^C TO OTHER CONFORMATIONS

It is now recognized that the adage ‘one sequence, one conformation’ is not strictly true. Depending on solvent conditions, probably any protein chain can adopt a variety of conformations in which there is a degree of periodic order, i.e. extensive regions of secondary structure. For instance, a recent systematic study of the conformations adopted by the glycolytic enzyme phosphoglycerate kinase shows that there are five distinct states that the chain can adopt in different media (Damaschun *et al.* 1999). However, such alternative states do not have precisely and tightly packed side-chains that are the hallmark of the native state of orthodox globular proteins.

Although, at physiological pH, recombinant fragments of PrP unfold via a two-state mechanism, an alternative folding pathway is observed at acidic pH. Studies on recombinant human PrP residues 90–231 (Swietnicki *et al.* 1997) and mouse PrP encompassing residues 121–231 identified a distinct, detergent-stabilized, equilibrium folding intermediate at pH 4.0. Circular dichroism spectroscopy indicated this intermediate was structured with predominantly β -sheet topology (Hornemann & Glockshuber 1998) and it has been proposed that this may be an intermediate on the pathway to PrP^{Sc} formation. Recent studies on a large fragment of the human prion protein (PrP^{91–231}) have shown that at acidic pH PrP can fold to a soluble monomer composed almost entirely of β -sheet in the absence of denaturants (Jackson *et al.* 1999). Reduction of the native disulphide bond was a prerequisite for β -sheet formation and these observations of alternative folding pathways dependent upon solvent

pH and redox potential could have important implications for the mechanism of conversion to PrP^{Sc}. Indeed this monomeric β -sheet state was prone to aggregation into fibrils with partial resistance to proteinase K digestion, both characteristic markers of PrP^{Sc}. Unusually for a protein with a predominantly helical fold, the majority of residues in PrP⁹¹⁻²³¹ have a preference for β -conformation (55% of non-glycine-proline residues). In view of this property, it is possible that the PrP molecule is delicately balanced between radically different folds with a high energy barrier between them—one dictated by local structural propensity (the β -conformation) and one requiring the precise docking of side-chains (the native α -conformation). Such a balance would be influenced by mutations causing inherited human prion diseases (Collinge 1997). It is also worthy of note that individuals homozygous for valine at polymorphic residue 129 of human PrP (where either methionine or valine can be encoded) are more susceptible to iatrogenic CJD (Collinge *et al.* 1991), and valine has a much higher β -propensity than does methionine.

The precise subcellular localization of PrP^{Sc} propagation remains controversial. However, there is considerable evidence implicating either late endosome-like organelles or lysosomes (Arnold *et al.* 1995; Mayer *et al.* 1992; Taraboulos *et al.* 1992; Laszlo *et al.* 1992). The environments of these organelles are evolved to facilitate protein unfolding at low pH prior to degradation by acid-activated proteases. It is possible that the α -PrP to β -PrP conversion, caused by reduction and mild acidification, is relevant to the conditions that PrP^C would encounter within the cell, following its internalization during recycling (Shyng *et al.* 1993). Such a mechanism could underlie prion propagation, and account for the transmitted, sporadic and inherited aetiologies of prion disease. Initiation of a pathogenic, self-propagating conversion reaction, with accumulation of aggregated β -PrP, may be induced by exposure to a 'seed' of aggregated β -PrP following prion inoculation, or as a rare stochastic conformational change, or as an inevitable consequence of expression of a pathogenic PrP^C mutant that is predisposed to form β -PrP. We are currently subjecting monomeric, oligomeric and fibrillar forms of β -PrP to bioassay in susceptible animals to determine whether such material is itself sufficient to constitute prion infectivity, or whether other cellular cofactors are also required. It remains to be demonstrated whether such alternative conformational states of the protein are sufficient to cause prion disease in an experimental host.

6. THE MOLECULAR BASIS OF PRION STRAIN DIVERSITY

A major problem for the 'protein-only' hypothesis of prion propagation has been how to explain the existence of multiple isolates, or strains, of prions. Such strains are distinguished by their biological properties: they produce distinct incubation periods and patterns of neuropathological targeting in inbred mouse lines. As they can be serially propagated in inbred mice with the same *Prnp* genotype, they cannot be encoded by differences in PrP primary structure. Furthermore, strains can be re-isolated in mice after passage in intermediate species with

different PrP primary structures (Bruce *et al.* 1994). Understanding how a protein-only infectious agent could encode such phenotypic information is clearly of considerable biological interest.

Support for the idea that strain specificity may be encoded by PrP itself was provided by study of two distinct strains of transmissible mink encephalopathy prions that can be serially propagated in hamsters—designated hyper (HY) and drowsy (DY). These strains can be distinguished by differing physicochemical properties of the accumulated PrP^{Sc} in the brains of affected hamsters (Bessen & Marsh 1992). Following limited proteolysis, strain-specific migration patterns of PrP^{Sc} on polyacrylamide gels were seen that related to different N-terminal ends of HY and DY PrP^{Sc} following protease treatment and implying differing conformations of HY and DY PrP^{Sc} (Bessen & Marsh 1994).

Recently, several human PrP^{Sc} types have been identified that are associated with different phenotypes of CJD (Parchi *et al.* 1996; Collinge *et al.* 1996b). The different fragment sizes seen on Western blots following treatment with proteinase K suggest that there are several different human PrP^{Sc} conformations. However, while such biochemical modifications of PrP are clearly candidates for the molecular substrate of prion strain diversity, it is necessary to be able to demonstrate that these properties fulfil the biological properties of strains—in particular that they are transmissible to the PrP in a host of both the same and different species. This property has been demonstrated in studies with CJD isolates, with both PrP^{Sc} fragment sizes and the ratios of the three PrP glycoforms (diglycosylated, monoglycosylated and unglycosylated PrP) maintained on passage in transgenic mice expressing human PrP (Collinge *et al.* 1996b). Furthermore, transmission of human prions and bovine prions to wild-type mice results in murine PrP^{Sc} with fragment sizes and glycoform ratios that correspond to the original inoculum (Collinge *et al.* 1996b). Variant CJD is associated with PrP^{Sc} glycoform ratios that are distinct from those seen in classical CJD. Similar ratios are seen in BSE in cattle and BSE when transmitted to several other species (Collinge *et al.* 1996b). These data strongly support the 'protein-only' hypothesis of infectivity and suggest that strain variation is encoded by a combination of PrP conformation and glycosylation. Furthermore, polymorphism in PrP sequence can influence the generation of particular PrP^{Sc} conformers (Collinge *et al.* 1996b). Transmission of PrP^{Sc} fragment sizes from two different subtypes of inherited prion disease to transgenic mice expressing a chimeric human mouse PrP has also been reported (Telling *et al.* 1996). As PrP glycosylation occurs before conversion to PrP^{Sc}, the different glycoform ratios may represent selection of particular PrP^C glycoforms by PrP^{Sc} of different conformations. According to such a hypothesis, PrP conformation would be the primary determinant of strain type with glycosylation being involved as a secondary process. However, since it is known that different cell types may glycosylate proteins differently, PrP^{Sc} glycosylation patterns may provide a substrate for the neuropathological targeting that distinguishes different prion strains (Collinge *et al.* 1996b). Particular PrP^{Sc} glycoforms may replicate most favourably in neuronal populations with a similar PrP glycoform

expressed on the cell surface. Such targeting could also explain the different incubation periods that also discriminate strains, targeting of more critical brain regions, or regions with higher levels of PrP expression, producing shorter incubation periods. Further supportive evidence for the involvement of PrP glycosylation in prion strain propagation has come from the study of transgenic mice expressing PrP with mutations interfering with N-linked glycosylation (DeArmond *et al.* 1997).

Recent work has shown strain-specific protein conformation to be influenced by metal binding to PrP^{Sc} (Wadsworth *et al.* 1999). Two different human PrP^{Sc} types, seen in clinically distinct subtypes of classical Creutzfeldt–Jakob disease, can be interconverted *in vitro* by altering the metal-ion occupancy. The dependence of PrP^{Sc} conformation on the binding of copper and zinc represents a novel mechanism for post-translational modification of PrP, and for the generation of multiple prion strains. This finding may also explain differences in molecular classification of classical CJD. An earlier study (Parchi *et al.* 1996) of PrP^{Sc} types in classical CJD described only two types of PrP^{Sc}, and these authors have argued that the types I and II we describe correspond to their type I, while our type III pattern corresponds to their type II (Parchi *et al.* 1997). However, these authors concede a degree of heterogeneity in their type I cases (Parchi *et al.* 1996). In a large-scale study of PrP^{Sc} types in CJD in conjunction with the UK National CJD Surveillance Unit, we demonstrated that patients classified as type I and type II using our criteria have quite distinct disease phenotypes, confirming the validity of our molecular classification. Type I human CJD is a distinct human prion disease with an aggressive clinical course and remarkably short clinical duration (Wadsworth *et al.* 1999).

Molecular strain typing of prion isolates can now be applied to molecular diagnosis of vCJD (Collinge *et al.* 1996b; Hill *et al.* 1997) and to produce a new classification of human prion diseases with implications for epidemiological studies investigating the aetiology of sporadic CJD. Such methods allow strain typing to be performed in days rather than the one to two years often required for classical biological strain typing. This technique may also be applicable to determining whether BSE has transmitted to other species (Collinge *et al.* 1996b) and thereby pose a threat to human health, for instance to sheep (Hill *et al.* 1998; Hope *et al.* 1999).

Such ability of a single polypeptide chain to encode information specifying distinct phenotypes of disease raises intriguing evolutionary questions. Do other proteins behave in this way? The novel pathogenic mechanisms involved in prion propagation may be of far wider significance and relevant to other neurological and non-neurological illnesses: indeed other prion-like mechanisms have now been described (Milner & Medcalf 1991) and the field of yeast and fungal prions has emerged (Wickner & Masison 1996; Wickner 1997; see Lindquist *et al.*, this issue).

Convincing evidence for the conformational change during conversion to PrP^{Sc} involving a region of the molecule that is unstructured in PrP^C has emerged. The epitope to a monoclonal antibody comprising residues 108–112 which is exposed in PrP^C has been shown to be buried in PrP^{Sc} (Peretz *et al.* 1997). Indeed in experiments assessing

the degree to which this epitope becomes exposed after partial denaturation of PrP^{Sc}, eight pathological distinct prion strains could be distinguished (Safar *et al.* 1998). Cell-free conversion reactions in which recombinant PrP^C can be recruited into a PK-resistant form when mixed with an excess of PrP^{Sc} have mimicked the species specificity seen in transmission studies. Although the *de novo* PrP^{RES} does not contain detectable prion infectivity (Hill *et al.* 1999a) cell-free conversion models are being used to identify potential inhibitors of conversion and to map interaction surfaces involved in the binding of PrP^C to PrP^{Sc} (Horiuchi & Caughey 1999; Caughey, this issue).

7. THE SPECIES BARRIER: BSE AND vCJD

Transmission of prion diseases between different mammalian species is restricted by a 'species barrier' (Pattison 1965). On primary passage of prions from species A to species B usually not all inoculated animals of species B develop disease and those that do have much longer and more variable incubation periods than those that are seen with transmission of prions within the same species, where typically all inoculated animals would succumb with a relatively short, and remarkably consistent, incubation period. On second passage of infectivity to further animals of the species B, transmission parameters resemble within-species transmissions with most, if not all, animals developing the disease with short and consistent incubation periods. Species barriers can therefore be quantitated by measuring the fall in mean incubation period on primary and second passage, or, perhaps more rigorously, by a comparative titration study. The latter involves inoculating serial dilutions of an inoculum in both the donor and host species and comparing the LD₅₀s obtained. The effect of a very substantial species barrier (for instance that between hamsters and mice) is that few, if any, animals succumb to disease at all on primary passage, and then at incubation periods approaching the natural life span of the species concerned.

In contrast, the species barrier limiting transmission of BSE from cattle to conventional mice is of much smaller effect. BSE can be readily transmitted to mice with most, if not all, inoculated animals succumbing to disease on primary passage (a high 'attack rate'). This relatively modest species barrier has been formally measured by comparative titration studies of the same BSE isolate by intracerebral inoculation into cattle and mice, indicating an approximately 1000-fold barrier (that is it takes 1000 times more BSE to kill a mouse than a cow) (Wells *et al.* 1998). The effect of this barrier on incubation periods is to increase mean incubation periods by around threefold and to increase dramatically the range of incubation periods seen.

Such experiments are usually performed using the most efficient, intracerebral, route of transmission. A formal titration of BSE in mice to determine an oral LD₅₀ has not been reported. However, oral challenge with *ca.* 10 g of BSE-affected cow brain killed the majority of exposed mice (Barlow & Middleton 1990). If the bovine-to-human species barrier were similar to that to mice this would suggest an oral LD₅₀ in humans of a similar order of magnitude as that for mice. Clearly, it is hoped that

the species barrier limiting transmission of BSE to humans will be of a far higher order. However, if we assume a pessimistic scenario that the barrier is similar (and it remains possible that it could be lower), extrapolation with the known incubation periods in the acquired human prion diseases, such as growth-hormone-related iatrogenic CJD or kuru, where transmission does not involve a species barrier (and mean incubation periods are around 10–15 years), would suggest mean incubation periods of BSE in humans of perhaps 30 years or more and a range extending from ten years towards or exceeding a normal human life span. Such estimations (Collinge 1999), based on very extensive experience of transmission studies across species from many research groups over several decades, suggest the need for caution with respect to optimistic assessments of likely human BSE epidemic size only three years after the recognition of vCJD.

Early studies on the molecular basis of the species barrier argued that it resided principally in differences in PrP primary structure between the species from which the inoculum was derived and the inoculated host. Transgenic mice expressing hamster PrP were, unlike wild-type mice, highly susceptible to infection with hamster prions (Prusiner *et al.* 1990). That most sporadic and acquired CJD occurred in individuals homozygous at *PRNP* polymorphic codon 129 supported the view that prion propagation proceeded most efficiently when the interacting PrP^{Sc} and PrP^C were of identical primary structure (Collinge *et al.* 1991; Palmer *et al.* 1991). However, it has long been recognized that prion strain type affects ease of transmission to another species. Interestingly, with BSE prions the strain component to the barrier seems to predominate, with BSE not only transmitting efficiently to a range of species, but maintaining its transmission characteristics even when passaged through an intermediate species with a distinct PrP gene (Bruce *et al.* 1994). For instance, transmission of CJD prions to conventional mice is difficult, with few if any inoculated mice succumbing after prolonged incubation periods, consistent with a substantial species barrier (Collinge *et al.* 1995b; Hill *et al.* 1997). In sharp contrast, transgenic mice expressing only human PrP are highly susceptible to CJD prions, with 100% attack rate and consistent short incubation periods which are unaltered by second passage, consistent with a complete lack of species barrier (Collinge *et al.* 1995b). However, vCJD prions (again comprising human PrP of identical primary structure) transmit much more readily to wild-type mice than do classical CJD prions, while transmission to transgenic mice is relatively less efficient than with classical CJD (Hill *et al.* 1997). The term 'species barrier' does not seem appropriate to describe such effects and 'species-strain barrier' or simply 'transmission barrier' may be preferable. The avidity of protein–protein interactions and the ease of conversion to a strain-specific PrP^{Sc} conformation are influenced both by the nature of the infecting conformation and the host PrP amino-acid sequence.

Mammalian PrP genes are highly conserved. Presumably only a restricted number of different PrP^{Sc} conformations (that are highly stable and can therefore be serially propagated) will be permissible thermodynamically, and

will constitute the range of prion strains seen. PrP glycosylation may be important in stabilizing particular PrP^{Sc} conformations. While a significant number of different PrP^{Sc} conformations may be possible among the range of mammalian PrP's, only a subset of these would be allowable for a given single mammalian PrP. Substantial overlap between the favoured conformations for PrP^{Sc} derived from species A and species B might therefore result in relatively easy transmission of prion diseases between these two species, while two species with no preferred PrP^{Sc} conformations in common would have a large barrier to transmission (and indeed transmission would necessitate a change of strain type). According to such a model of a prion transmission barrier, BSE may represent a thermodynamically highly favoured PrP^{Sc} conformation that is permissive for PrP expressed in a wide range of different species, accounting for the remarkable promiscuity of this strain in mammals.

Contribution of other components to the species barrier is possible and may involve interacting cofactors that mediate the efficiency of prion propagation, although no such factors have yet been identified.

A novel form of human prion disease, variant CJD (vCJD), was recognized in the UK in 1996 (Will *et al.* 1996) and implied the arrival of a new risk factor for CJD (Collinge & Rossor 1996). These epidemiological studies argued for a link with BSE and this idea was strongly supported by molecular strain typing studies (Collinge *et al.* 1996b). On the basis of molecular strain typing there is a close correlation between the physico-chemical properties of vCJD and BSE, both strains being associated with a high proportion of the diglycosylated form of PrP^{Sc}. When prions isolated from either bovine brain or human brain are transmitted to experimental mice, PrP^{Sc} isolated from the infected hosts is indistinguishable, either by site of proteinase K cleavage or glycoform ratio (Collinge *et al.* 1996b). Further evidence came from the biological as well as the molecular transmission characteristics (Hill *et al.* 1997): in particular, the incubation periods and neuropathology of the two strains produced in both transgenic mice and a variety of inbred strains of mice were identical (Hill *et al.* 1997; Bruce *et al.* 1997). That vCJD is human BSE is therefore supported by compelling experimental data. Moreover, it was recently found that vCJD can be further distinguished from other forms of CJD by the deposition of PrP^{Sc} in the lymphoreticular system (Hill *et al.* 1999), a tissue distribution that is specific to vCJD.

The species barrier between cattle BSE and humans cannot be directly measured but can be modelled in transgenic mice expressing human PrP^C, which produce human PrP^{Sc} when challenged with human prions (Collinge *et al.* 1995b). When such mice, expressing both human PrP valine 129 (at high levels) and mouse PrP, are challenged with BSE three possibilities could be envisaged: these mice could produce human prions, murine prions, or both. In fact, only mouse prion replication could be detected. Although there are caveats with respect to this model, particularly that human prion propagation in mouse cells may be less efficient than that of mouse prions, this result would be consistent with the bovine-to-human barrier being higher than the bovine-to-mouse barrier for this *PRNP* genotype. In the second

phase of these experiments, mice expressing only human PrP were challenged with BSE. While CJD isolates transmit efficiently to such mice at *ca.* 200 days, only infrequent transmissions at over 500 days were seen with BSE, consistent with a substantial species barrier for this human *PRNP* genotype (Hill *et al.* 1997). The *PRNP* valine 129 genotype was studied initially in attempts to produce an animal model of human prion disease, as this genotype was overrepresented amongst early cases of iatrogenic CJD (Collinge *et al.* 1991), suggesting increased susceptibility or shorter incubation periods in this genotype. However, it is important to repeat these studies in mice expressing only human PrP methionine 129 and in heterozygotes. So far, BSE appears to have transmitted only to humans of *PRNP* codon 129 methionine homozygous genotype (Collinge *et al.* 1996a; J. Collinge, unpublished data).

8. CELL DEATH AND PRION DISEASE

The precise molecular nature of the infectious agent and the cause of neuronal cell death remain unclear. Various hypotheses have been proposed to explain the mechanism of spongiform change and neuronal cell loss. These have included direct neurotoxic effects from a region of the prion protein encompassing residues 106–126 (Forloni *et al.* 1993) to increased oxidative stress in neurons as a result of PrP^C depletion that has been proposed to function as an antioxidant molecule (Brown *et al.* 1997b). It has also been suggested that PrP^C plays a role in regulating apoptosis, with disturbance of normal cellular levels of PrP during infection leading to cell death (Kurschner & Morgan 1995, 1996). Certainly there have been numerous recent reports of apoptotic cells being identified in the neuronal tissue of prion disease brains (Williams *et al.* 1997). Although PrP^C expression is required for susceptibility to the disease, a number of observations argue that PrP^{Sc} and indeed prions (whether or not they are identical) may not themselves be highly neurotoxic. Prion diseases in which PrP^{Sc} is barely or not detectable have been described (Medori *et al.* 1992a,b; Collinge *et al.* 1995a; Hsiao *et al.* 1990; Lasmézas *et al.* 1997). Mice with reduced levels of PrP^C expression have extremely high levels of PrP^{Sc} prions in the brain and yet remain well for several months after their wild-type counterparts succumb (Bueler *et al.* 1994). Conversely, Tg20 mice, with high levels of PrP^C, have short incubation periods and yet produce low levels of PrP^{Sc} after inoculation with mouse prions (Fischer *et al.* 1996). In addition, brain grafts producing high levels of PrP^{Sc} do not damage adjacent tissue in PrP knockout (*Prnp*^{0/0}) mice (Brandner *et al.* 1996).

The cause of neurodegeneration in prion diseases remains unclear. It remains possible that prion neurodegeneration is related, at least in part, to loss of function of PrP^C. That *Prnp*^{0/0} mice, other than those associated with overexpression of the *Prnp*-like gene *Dpl* (Moore *et al.* 1999), do not develop neurodegeneration could be due to compensatory adaptations during neurodevelopment. Complete or near complete ablation of PrP expression in an adult mouse using conditional gene expression methods has not yet been achieved. An alternative hypothesis is that a toxic, possibly infectious, intermediate

is produced in the process of conversion of PrP^C to PrP^{Sc}, with PrP^{Sc} present as highly aggregated material, being a relatively inert end-product. The steady-state level of such a toxic monomeric or oligomeric PrP (PrP^L) intermediate could then determine the rate of neurodegeneration.

9. CONCLUDING REMARKS

Prion diseases appear to be diseases of protein conformation and elucidating their precise molecular mechanisms may, in addition to allowing us to progress with tackling key public-health issues posed by vCJD, be of far wider pathobiological significance. It is of considerable interest that many of the commoner neurodegenerative diseases, such as Alzheimer's and Parkinson's disease and the polyglutamine repeat disorders (such as Huntington's disease), are also associated with abnormal protein aggregates. The ability of a single polypeptide chain to encode information is unprecedented and it seems likely that evolution will have used this mechanism in many other ways.

While the 'protein-only' hypothesis of prion propagation is supported by compelling experimental data, and now appears also able to encompass the phenomenon of prion strain diversity, the goal of the production of prions *in vitro* remains. PrP^{Sc}-like forms of PrP have now been produced from purified recombinant material, but none has yet been shown to be capable of producing disease in experimental animals that can be serially propagated. Success in such an endeavour would not only prove the 'protein-only' hypothesis—it would also serve as the essential model by which the mechanism can be understood in molecular detail.

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Discussion

M. B. Pepys (*Department of Medicine, Royal Free and University College Medical School, London, UK*). Have you investigated the effects of *in vitro* deglycosylation on the infectious material?

J. Collinge. We have not yet tried this type of experiment.

L. D. Barron (*Department of Chemistry, University of Glasgow, UK*). Your interesting results on the conversion of reduced human prion protein from α -helical to β -sheet structure, detected by UV CD, were obtained on fragment 91–231, which contains the structured C-terminal domain. How important do you think the ‘disordered’ N-terminal domain is in the mechanism of prion disease? The reason for my question is that we have some new results that suggest that hydrated polyproline II (PPII) helix may be an important fibrillogenic structural element in conformational disease (E. W. Blanch, L. A. Morozova-Roche, D. A. E. Cochran, A. J. Doig, L. Hecht and L. D. Barron, unpublished data). Recent spectroscopic studies (Pergamini *et al.* 1999) indicate that the complete prion protein is unusually plastic, which would be consistent with a large amount of hydrated PPII helix. In which case might the differences in glycosylation and metal binding in the prion proteins associated with different strains serve to modulate the fibrillogenic properties of the ‘disordered’ plastic domain in different ways?

J. Collinge. When we say it is unstructured, we mean that it is unstructured in solution under the conditions in which the NMR spectroscopy is carried out. The N-terminal region is highly conserved in mammals and it is hard to imagine that it does not adopt some sort of structure at some stage, and this may well involve metals. There is evidence that copper ions bind to this region, so this

region may well acquire some structure in its biologically functional form.

L. D. Barron. Polyproline II helix is very plastic, but is not detectable by NMR in solution since there is no inter-chain H-bonding.

J. Masel (*Department of Zoology, University of Oxford, UK*). There is evidence that very slowly replicating strains can interfere with faster replicating strains, so could it be that soluble β -prion protein is in fact infectious, but it replicates too slowly for its infectivity to be seen within the mouse lifespan?

J. Collinge. That is a good point because if we have produced infectivity in these experiments we do not know what strain it would be. Experiments are in progress to extend the lifespan artificially by serial passage, i.e. extracting the brains of injected mice at the end of their life and using homogenates to inject young mice.

A. Tahari-Alaoui (*University of Oxford, UK*). Have you tried to convert the full length PrP into the β -form?

J. Collinge. Yes, it is possible to convert the full length protein into the β -form under the same conditions we use for the truncated form. The CD spectrum is very similar but the product is even more protease-resistant—it will survive 500 $\mu\text{g ml}^{-1}$ of proteinase K.

P. Lansbury (*Department of Neurology, Harvard Medical School, USA*). Is there any additional evidence for the β -form being monomeric other than gel filtration?

J. Collinge. The NMR data indicate that this form is monomeric at high concentration.

C. M. Dobson (*Oxford Centre for Molecular Sciences, New Chemistry Laboratory, University of Oxford, UK*). We are used to thinking that this PrP protein is unusually susceptible to form the β -type of structure, but our experiments suggest that many other proteins are easier to convert to fibrils than PrP. So maybe this is in fact a difficult protein to convert, and perhaps it is transmissible because its sporadic conversion is so slow.

J. Collinge. That is an interesting perspective, but the conversions you are talking about are in the context of aggregation, not in conversion to a monomeric form.

C. M. Dobson. But the end result is conversion into fibrillar structures in both cases.

C. Exley (*Department of Chemistry, Keele University, UK*). Why did you try ATP on the conversion? I am surprised that some of your buffers apparently contain tens of micromolar concentrations of trace metals.

J. Collinge. The concentrations of metals we use are high compared with likely physiological concentrations, but we are not looking at the physiological state here. We are extracting the protein from the brains of humans or animals that have died from these diseases.

C. Exley. I am not referring to the physiological situation here. My point is that 20 micromolar copper ion concentration is a very high concentration to expect to contaminate the usual buffers.

J. Collinge. Our finding is that to reverse the effect of EDTA we have to add 20 micromolar copper ions. ATP was added as part of a large matrix of experiments with PrP to try to generate infectivity—in case there is an ATP-dependent cofactor that is required.

T. Feizi (*Imperial College School of Medicine, London, UK*). When studying the triplet patterns on SDS gels of protease-treated material, is this done with material extracted from whole brains or just from diseased parts of the brain? Could different cell types give different glycosylation patterns?

J. Collinge. We have not seen differences in pattern in extracts from different brain regions. I should emphasize that these patterns appear only after proteinase K treat-

ment. If you look at extracts before this treatment, most of the protein is diglycosylated.

T. Feizi. It would be interesting to know whether the product you observe is the product of a single cell type or many cell types.

J. Collinge. We have not yet begun to address this possibility.

Additional reference

Pergamini, P., Bramanti, E. & Ascoli, G. A. 1999 Structural dependence of the cellular isoform of prion protein on solvent: spectroscopic characterization of an intermediate conformation. *Biochem. Biophys. Res. Commun.* **264**, 972–978.