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Deadly Conformations—Protein Misfolding in Prion Disease

Review

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Novel infectious particles, termed prions, composed largely and perhaps solely of a single protein, are the likely causative agents of a group of transmissible spongiform encephalopathies that produce lethal decline of cognitive and motor function. As if the notion of a transmissible pathogenic protein is not jarring enough, evidence indicates that the responsible protein arrives at a pathogenic state by misfolding from a normal form that has ubiquitous tissue distribution. The remarkable nature of these diseases and the nature of the prion protein conversion process as we currently understand it are reviewed below.

Prion Diseases—Spongiform Encephalopathies Transmitted by Inoculation, Cannibalism, Genetic Inheritance

The first of these diseases to be recognized affected sheep, and its name, scrapie, derived from the observation that affected animals rubbed against the fences of their pens to stay upright, presumably reflecting the manifestation of ataxia. Transmissibility was accidentally but stunningly demonstrated in 1943 when a population of Scottish sheep was inoculated against a common virus with a formalin extract of lymphoid tissue unknowingly derived from an animal with scrapie—after two years, nearly 10% of the flock developed scrapie (Gordon, 1946). Some years later, a clinically and pathologically similar human disease, kuru, meaning ''trembling,'' was identified in highlanders of New Guinea (Gajdusek and Zigas, 1959; Hadlow, 1959). Here also, ataxia predominated, proceeding to death usually within 9 months. In the brains of these patients, characteristic ''plaque'' lesions, extracellular collections of proteinaceous material, were observed (see Figure 1). Transmissibility of kuru was first demonstrated following intracerebral inoculation of homogenate of kuru brain into chimpanzees (Gajdusek et al., 1966), but within the highlander population it was eventually surmised that transmission was occurring by ritual cannibalism (Gajdusek, 1977).

Three other clinically or pathologically similar neurodegenerative diseases have been recognized in humans, and for all of these, as with kuru, disease has been observed to be transmissible to experimental animals by intracerebral inoculation. In 1936, Gerstmann, Sträussler, and Scheinker described a condition with ataxia and progressive dementia, occurring after age 40, associated, as in kuru, with plaques in the brain of affected individuals (Gerstmann et al., 1936). Multiple affected family members were observed, in a pattern indicating autosomal-dominant inheritance. Similar genetic transmission has also been observed for a rare condition more recently described, familial fatal insomnia (FFI), exhibiting lethal insomnia and autonomic dysfunction associated with pathologic changes confined to nuclei in the thalamus (e.g., Manetto et al., 1992). By contrast, the more common condition, Creutzfeldt-Jakob disease (CJD), usually occurs sporadically and presents with dementia occurring after age 40, with pathology generally featuring spongiform degeneration (Figure 1 and see DeArmond and Prusiner, 1996, for review). While most CJD cases are sporadic in occurrence, autosomal-dominant transmission accounts for ~10% of cases. Horizontal transmission of CJD to chimpanzee was demonstrated early (Gibbs et al., 1968), but particularly notable have been cases of transmission between humans iatrogenically, through transplantation of infected corneas or injection of growth hormone derived from human pituitaries (see DeArmond and Prusiner, 1996). Even more striking have been a number of early-onset CJD cases with atypical pathology recently reported from Great Britain (Will et al., 1996 and see Figure 1), suggested to have been transmitted by consumption of meat from cows suffering from "mad cow" disease, a spongiform encephalopathy recently epidemic in British herds (see Anderson et al., 1996, concerning progression of the epidemic). The recent reports of production of a clinically and pathologically similar CJD in macaques by intracerebral injection of brain homogenate from afflicted cows (Lasmézas et al., 1996b), and of biochemical properties shared between the human cases and bovine spongiform encephalopathy (BSE) (Collinge et al., 1996), suggest that BSE is transmissible to man.

Involvement of a Protein

The nature of the transmissible spongiform encephalopathies has been addressed by testing the ability of infected brain homogenate to transmit disease to experimental animals. In initial studies transferring to chimpanzees and sheep, one or more years elapsed before onset of clinical disease, but, in subsequent studies with hamsters and mice, incubation periods as short as 70–150 days were achieved, greatly facilitating experimental work. Infectivity was found to be filterable, consistent with the behavior of a virus, but, differing from most viruses, formalin treatment did not completely abolish infectivity (e.g., Gordon, 1946). In 1966, Alper and coworkers made the additional surprising observation that the target size of the infectious material to UV inactivation at 254 nm, was relatively small (Alper et al., 1966, 1967). Rather, infectivity proved to be more sensitive to irradiation at 237 nm (Latarjet et al., 1970). This action spectrum suggested the possibility that a nucleic acid might not be involved. This led to a number of hypotheses about the nature of the infectious agent,

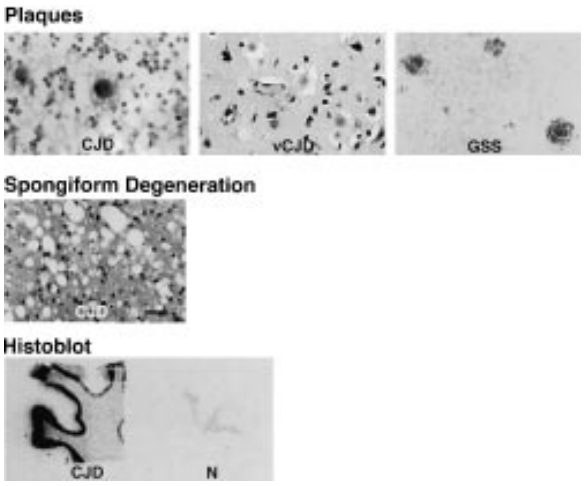

Figure 1. Neuopathological Findings in Transmissible Spongiform Encephalopathies

(Top Row) Plaque lesions in Creutzfeldt-Jacob disease (CJD) (first panel); the variant form of CJD (vCJD), recently shown to be transmissible to primates from BSE-infected cattle (second panel); and Gerstmann-Straüssler Scheinker disease (GSS) (third panel). In the first panel, the section is stained with PAS (periodic acid–Schiff reaction) and shows a centrally located plaque lesion exhibiting the "spiked ball" appearance typical of a kuru plaque (from DeArmond and Prusiner, 1995, with permission). In the second panel, the section is stained with PAS and shows a number of so-called "florid plaques" typical of vCJD, in each case with a central plaque lesion surrounded by a daisy-like pattern of vacuoles (photomicrograph kindly supplied by J. Ironside, University of Edinburgh; see also Will et al., 1996; and Lasmézas et al., 1996b). The third panel is a section immunostained after hydrolytic autoclaving (Muramoto et al., 1992) with anti-PrP antiserum, revealing the presence of PrP in the plaque lesions of GSS.

(Middle Row) Spongiform changes typical of CJD. Section obtained from an affected transgenic mouse, carrying a chimeric mouse-human-mouse transgene, that had been inoculated with brain homogenate from a human sporadic CJD case (from DeArmond and Prusiner, 1995, with permission).

(Bottom Row) Histoblot analysis (Taraboulos et al., 1992) of brain section from an individual with CJD and from an unaffected individual. Staining is with anti-PrP antibody, showing extensive staining of PrP in the cortical mantle of the affected individual (from DeArmond and Prusiner, 1995, with permission).

ranging from a replicating polysaccharide to a nucleoprotein complex (for review, see Prusiner, 1982). Among these models was a prescient speculation, in which Griffith suggested that "conversion" of a protein from a normal energetically favored conformation to another conformation, either spontaneously or by exogenous introduction of the altered conformation, could explain these diseases (Griffith, 1967).

The molecular nature of the infectious agent lay largely untested for 15 years until Stanley Prusiner and coworkers achieved the biochemical enrichment of infectious activity and showed its association with a specific protein. In early 1982, Prusiner and coworkers reported a 1000-fold enrichment of scrapie infectivity from homogenate of infected brain, achieved through a series of steps including polyethylene glycol precipitation, micrococcal nuclease digestion, limited proteinase K digestion, and sucrose density gradient centrifugation (Prusiner et al., 1982; Prusiner, 1982). The highest activity came from a fraction at the interface between 25% and 60% sucrose, where aggregates composed of amorphous material and flattened rods measuring 25 nm × 100–200 nm were observed. The enriched activity was inactivated by proteinase K, diethylpyrocarbonate, urea, chaotropes, phenol, and SDS, but was not abolished by nuclease treatments or UV irradiation. This behavior, typical of a protein, gave rise to the name attached by Prusiner and coworkers, "prion," for proteinaceous infectious particle (Prusiner, 1982; see also Prusiner et al., 1980).

The same workers identified a protein, designated PrP, resistant to limited proteinase K digestion, that was specifically present in infected hamster brain but not in normal brain and exhibited a relative migration in SDS–PAGE of 27–30 kDa (Bolton et al., 1982; Prusiner et al., 1982). Whether this species was a byproduct of infection, or was directly responsible, could not be immediately distinguished, although the copurification of proteinase K–resistant PrP 27–30 with infectivity offered circumstantial evidence that it was involved with causation. Similarly, rod structures, first observed by Merz et al. (1981), were observed in the proteinase K–treated extracts of infected brain and were found also to contain the PrP 27–30 core product. Preparations enriched for these rods were shown to be highly infectious (Prusiner et al., 1983; Diringer et al., 1983), although subsequent studies have shown that preparations devoid of visible structures can also be infectious. Coenrichment of PrP 27–30 and infectivity was observed in another setting, when immunoaffinity purification of detergent-lipid-solubilized infected brain extract was carried out, showing several 1000-fold enrichment of both PrP 27–30 and infectivity (Gabizon et al., 1988). This was consistent with the notion that there is tight linkage between infectivity and the presence of some form of the PrP protein. Nonetheless, despite years of effort, even in the purest samples, the ratio of PrP molecules to infectious units is $\sim 10^5$. At such low infectivity, it is impossible to exclude the possibility that other components, or covalent modifications, are required for infectivity. However, highly-purified infectious material has been shown to contain less than one molecule of nucleic acid larger than $\sim$100 nt for a particle-to-infectivity ratio near unity (Kellings et al., 1992). Thus, it seems likely that demonstration of the protein only hypothesis will require the production of infectious particles in vitro from purified PrP protein (that has a level of impurity of less than 1 part per infectious unit).

Prion Protein Is Host Encoded—A Conversion Process

Purification of PrP 27–30 made it possible to obtain NH_2-terminal amino acid sequence (Prusiner et al., 1984) and, ultimately, cDNA clones encoding PrP protein (Oesch et al., 1985; Chesebro et al., 1985). PrP mRNA proved to be the product of a single host cell nuclear gene. The primary structure of PrP encoded by the gene of a normal animal was found to be identical to that encoded by a cDNA from a scrapie-infected animal (Basler et al., 1986), and similar levels of mRNA were found in both settings (Chesebro et al., 1985; Oesch et al., 1985). Antibodies generated against PrP 27–30 identified the PrP

protein not only in the brain of uninfected animals but also in many visceral tissues as a 33–35 kDa glycosylated species, termed PrP^C. A protein of identical size was also observed in scrapie-infected brain extracts. Strikingly, when limited proteinase K digestion was carried out, PrP^C was completely degraded, whereas a fraction of the protein in infected brain, termed PrP^{Sc}, was only partially cleaved, removing 66 NH_2-terminal amino acids to produce the species PrP 27–30. Thus, the PrP protein appears to have at least two distinct conformational states: a protease-sensitive one found ubiquitously, and a protease-resistant one in the setting of infection. Perhaps linked to such protease-resistant behavior is the additional observation that, while PrP^C is a soluble protein, the PrP^{Sc} form is stubbornly insoluble, localizing in the amorphous aggregates in enriched fractions from infected brain (e.g., Meyer et al., 1986). In any case, the protease resistance of the PrP^{Sc} form has been relied on to allow the detection of PrP^{Sc} in situ in both experimental and clinical diagnosis. This is accomplished by pretreatment with proteinase K to remove PrP^C, followed by guanidine treatment to expose epitopes of PrP^{Sc} for immunolocalization (Taraboulos et al., 1992, and see Figure 1 histoblot).

Given the same primary structure of PrP^C and PrP^{Sc}, the process whereby the normal state of PrP protein is "converted" to the infection-associated form seemed likely to involve either posttranslational modification or a change in conformation (e.g., Hope et al., 1986). Extensive biochemical characterization has failed to find any covalent difference between the PrP^C and PrP^{Sc} proteins (e.g., Stahl et al., 1993). By contrast, physical measurements have demonstrated a dramatic conformational difference in the PrP forms. For example, Fourier transform infrared spectosocopy and circular dichroism indicate that the α-helical content of the PrP^C form is $\sim$40%, with little or no β sheet (Pan et al., 1993). By contrast, the PrP 27–30 form contains 50% β sheet and only $\sim$20% α helix (Caughey et al., 1991; Pan et al., 1993; Safar et al., 1993). The recently presented solution structure of a fragment of the mouse PrP^C has allowed a direct determination of secondary structure content of this portion of PrP^C (Riek et al., 1996). The agreement with the FTIR study is excellent: out of 109 resolvable residues in the PrP 121–232 species, 43 lie in α helix (40%), while only 8 residues lie in two short antiparallel β strands (7%) (see Figure 3B).

The Conversion Process—Endogenous PrP^C as the Target for "Infectious" PrP^{Sc}

The notion that endogenous PrP^C was involved with the development of infection was first supported by the observation that two strains of mice that had genetically determined long versus short incubation times in the face of prion exposure contained specific codon differences present at two positions in the PrP^C gene (Westaway et al., 1987). Subsequently, PrP genes in families with GSS, FFI, and CJD were found to encode either specific amino acid substitutions or particular polymorphisms (including insertion/deletion of members of an "octa repeat" motif in the NH_2-terminal portion) that could be linked to development of disease (see Prusiner, 1996, and Figure 3). For example, many GSS kindreds, including the original GSS family, harbor an amino acid substitution, P102L (Hsiao et al., 1989). In the case of sporadic CJD, most patients are homozygous for a polymorphism at residue 129 (encoding either methionine or valine), in the absence of any other mutation (Palmer et al., 1991). Similarly, the recent early-onset CJD cases potentially related to BSE exposure have affected individuals homozygous for M129 (Will et al., 1996). Interestingly, the corresponding mouse residue lies in the small, two-stranded β sheet region of the recently determined mouse PrP121–231 structure; this β sheet has been suggested to provide a nucleation point for conformational change during conversion (Riek et al., 1996; see Figure 3B).

Two different genetic studies with mice have provided perhaps the strongest evidence arguing that infectious particles are generated from the endogenous PrP^C protein. In one, spontaneous prion disease was observed in uninoculated transgenic mice expressing a mouse PrP with a substitution homologous to that in GSS patients (Hsiao et al., 1990; Hsiao et al., 1994; Telling et al., 1996a). Importantly, brain homogenates from these mice can transfer prion disease when inoculated into transgenic mice expressing low levels of the same mutant PrP protein, that would not otherwise develop disease (Hsiao et al., 1994; Telling et al., 1996a). Thus, all the components required to form infectious particles appear to be present endogenously in the mice. Moreover, it appears that removal of the endogenous PrP gene in the latter study led to earlier onset of disease and more severe pathology in the uninoculated transgenic strain, reflecting that the presence of wild-type PrP somehow interfered with disease production from the mutant transgene.

In a second avenue of study, a requirement for PrP^C protein in generating infectivity was demonstrated directly—mice with a disruption in the endogenous PrP gene (*Prnp*$^{0/0}$) were both resistant to prion disease and unable to generate new infectious particles (Büeler et al., 1993; Prusiner et al., 1993). A straightforward hypothesis suggested by these observations is that endogenous PrP^C is converted to PrP^{Sc} conformation by the action of an infectious form of the PrP molecule. However, given the low specific activity of even the purest PrP^{Sc} samples and the observation that under some circumstances it appears that there can be both disease and infectivity in the absence of protease-resistant material (e.g., the GSS mice), it remains possible that the infectious form of PrP is distinct from the protease-resistant PrP^{Sc} form.

Conversion Produces Host-Specific Prions and Is Restricted by Interspecies "Barriers"

The nature of the putative interaction between PrP^C and PrP^{Sc} that mediates conversion has been probed in vivo in transgenic mouse experiments. Studies of the "species barrier" have been particularly revealing. The species barrier is the phenomenon in which one species tends to be resistant to infection by prion particles generated in another. For example, higher primates were thought to be resistant to infection from ungulates, e.g.,

cows, until the recent cases of a new variant of CJD, termed vCJD, and the transmission of BSE to macaques. Moreover, mice are normally resistant to infection from the widely used 263K strain of hamster prion but become susceptible to this strain of hamster PrP^{Sc} (Scott et al., 1989) following transgenic introduction of the hamster PrP gene. Correspondingly, disruption of the endogenous mouse gene and introduction of a hamster transgene renders mice highly susceptible to hamster prions and resistant to mouse prions (Büeler et al., 1993; Prusiner et al., 1993). This data is most easily reconciled with a model in which there is a direct interaction between the infecting PrP^{Sc} and endogenous PrP^{C}, and in which that effective interaction is inhibited by differences in the PrP sequence. Between mouse and hamster, such a barrier must be mediated by one or more of the 16 differences (out of 254 residues) in the PrP sequence (Scott et al., 1993). As would be predicted for a homologous conversion reaction in which new PrP^{Sc} is generated from the endogenous PrP^{C}, when the species of PrP^{Sc} in the brains of transgenic mice expressing the hamster PrP^{C} was determined following challenge with hamster prions, the hamster PrP^{Sc} protein was observed (Prusiner et al., 1990). Correspondingly, if the transgenic animals were inoculated with mouse prions, mouse PrP^{Sc} was observed in the infected brain.

The location in the PrP^{C} structure of the homologous interaction with PrP^{Sc} was probed by producing transgenic mice bearing chimeric genes. When the midportion of the hamster sequence (codons 94–188), differing at 5 residues from mouse, was substituted for the corresponding region of mouse PrP, the transgenic mice were observed to become susceptible to hamster prions, producing, as expected, chimeric PrP^{Sc} (Scott et al., 1993 and see Figure 3A).

Conversion In Vitro

Despite considerable effort, it has not been possible to demonstrate the production of infectious particles in vitro. However, Caughey, Lansbury, and coworkers have demonstrated the ability of PrP^{Sc} to convert PrP^{C} in vitro to a form that has proteolytic resistance resembling that of PrP^{Sc} (Kocisko et al., 1994). In these studies, when metabolically-labeled PrP^{C} isolated by immunoprecipitation was treated with 3 M guanidine HCl and then diluted into a 50-fold excess of purified nonlabeled PrP^{Sc}, a portion was converted to a relatively protease-resistant form, which generated a species resembling PrP 27–30 upon proteinase K digestion. Because only a small amount of material was converted to a protease-resistant form and because this occurred in the presence of an excess of PrP^{Sc}, biological assay of the nascent protease-resistant material by measurement, for example, of infectivity was not possible. Interestingly, pretreatment of PrP^{Sc} with 3 M guanidine HCl, which produced reversible unfolding of PrP^{Sc}, increased the extent of conversion, suggesting that PrP^{Sc} itself may also need to undergo a conformational change for conversion to proceed, potentially accounting for the high particle:infectivity ratio. Supporting the proposal that the in vitro reaction faithfully reproduced that in vivo, species specificity in the conversion reaction between hamster, mouse, and the hamster-mouse chimeras was reproduced by the in vitro reaction (Kocisko et al., 1995). Moreover, there was preservation of "strain" specificity in vitro (discussed below), insofar as the distinct proteinase K resistance patterns of PrP^{Sc} from two mink prion strains, presumably reflecting different PrP^{Sc} conformations, were reproducible in the in vitro system (Bessen et al., 1995).

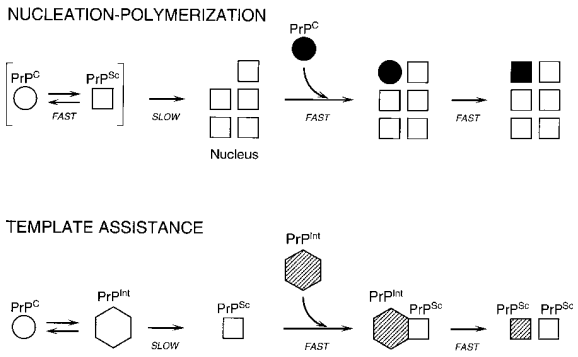

Figure 2. Schematic Model for the Conversion of PrP^{C} to PrP^{Sc}

In the nucleation-polymerization model, conversion between the PrP^{C} form (circles) and PrP^{Sc} form (squares) is inherently fast. However, in the absence of an aggregate large enough to act as a stable nucleus, designated by the collective of PrP^{Sc} squares, the PrP^{C} form is thermodynamically favored. In the template assistance model, the conversion of PrP^{C} or an altered conformation, PrP^{Int}, to PrP^{Sc} is extremely slow in the absence of PrP^{Sc}, but the conversion process is effectively irreversible. The PrP^{Sc} is able to propagate itself by catalyzing the conversion of other PrP^{Int} molecules to the PrP^{Sc} conformation. Additional unidentified factors, e.g., a molecular chaperone, might also be involved in the conversion process (see text).

Models for the Conversion of PrP^{C} to PrP^{Sc}

While the notion that a misfolded form of a protein could catalyze the refolding of native molecules into a distinct "misfolded" conformation might seem radical, such a process is by no means physically unreasonable. Two distinct mechanisms have been proposed to account for such behavior (Gajdusek, 1988; Prusiner, 1991; Jarrett and Lansbury, 1993; Cohen et al., 1994; Figure 2). In one model, formation of PrP^{Sc} is a nucleation-dependent polymerization process. In the absence of a preexistent aggregate, the conversion between PrP^{C} and PrP^{Sc} is reversible, but PrP^{Sc} monomer is less stable than PrP^{C}. PrP^{Sc} aggregates, however, promote the conversion of PrP^{C} by binding to and stabilizing the otherwise unfavored PrP^{Sc} conformation. The barrier to a stable conversion process thus lies at the level of the initial nucleation process, in which formation of low order aggregates is not favored, since the free energy gained from intermolecular interactions does not outweigh the entropic cost of binding until a minimum size nucleus is attained. The requirement that a nucleus be formed before conversion is stable predicts certain characteristics of the aggregation process, including dependence on exceeding a critical protein concentration for the initial formation of aggregates, and kinetics displaying a lag phase. The in vitro conversion process appears to show such features (Caughey et al., 1995), but whether a PrP^{Sc} nucleus is already present is unclear from the design of the study.

The relatively large size of the minimum stable nucleus would tend to make such a particle insoluble and could therefore account for the observation in the in vitro reaction that fractions containing higher-order PrP^{Sc} aggregates greater than 300 kDa in size could mediate the conversion to protease resistance while smaller-sized fractions could not. Infection would thus circumvent the slow step of nucleation by introducing a "seed" that initiates aggregation.

In a second proposed mechanism, the PrP^{Sc} form is inherently more stable than PrP^{C}, but kinetically inaccessible (Prusiner, 1991; Figure 2). In this case, PrP^{Sc} could promote conversion by catalyzing the rearrangement of a molecule of PrP^{C}, or of a partially destabilized intermediate, to the more stable PrP^{Sc} conformation (Figure 2). Infectivity would then rely on the ability of the PrP^{Sc} molecule to bind to and catalyze the conversion of existing intermediate molecules. By this template assistance model, the genetically inherited diseases result from mutations that increase the population of the unstable intermediate and/or enhance the rate at which this form spontaneously converts to PrP^{Sc}.

For both of the proposed mechanisms, there are physical precedents. In the case of nucleation-polymerization, there is a resemblance to tubulin polymerization, crystal growth, sickle hemoglobin formation, viral capsid assembly, and bacterial flagellar polymerization. Flagellar polymerization may be particularly instructive. The soluble monomer unit, flagellin, becomes incorporated into the growing end of a flagellum (Asakura et al., 1964, 1966). Monomers in solution, even at nearly millimolar concentration, occupy a conformation unable to spontaneously nucleate, but if a seed of fragmented flagellum is placed into the mixture, then polymerization rapidly ensues. Interestingly, the polymerizing monomers can assume the conformation of even heterologous seed material, reflecting a "templating" behavior. It should be pointed out that while the foregoing "aggregates" adopt a regular repeating structure, there is nothing in the physics underlying a nucleation process that requires that the aggregates formed must have long-range order.

There is also precedent for the template-assisted, catalyzed conversion mechanism, in which PrP^{C} is a metastable conformation that does not spontaneously form the more stable PrP^{Sc} at any appreciable rate. During the past few years, a number of proteins have been observed to occupy such conformations under kinetic control, i.e., they are separated from their true free energy minima by a large barrier. These include influenza hemagglutinin (Baker and Agard, 1994a), the serpin family of protease inhibitors (Sifers, 1995), and a number of proteases including subtilisin and α-lytic protease (Baker and Agard, 1994b). This last case of α-lytic protease is particularly revealing. Here, the interconversion between a molten globule–like intermediate, I, and the native state, N, is extremely slow, allowing little or no conversion over the course of a month (reflecting a barrier of $\sim$25 kcal/mole). Conversion, however, is dramatically accelerated by binding of the naturally-occurring propeptide region, in either *cis* or *trans*, allowing folding to N to occur within minutes (the propeptide lowers the barrier by $\sim$14 kcal/mole). This behavior raises the possibility that folding of PrP is also under kinetic control, with the PrP^{Sc} state thermodynamically favored but kinetically inaccessible. Infectious prion disease could then result if PrP^{Sc} were able to accelerate the conversion of PrP^{C} to PrP^{Sc} in a manner analogous to the catalyzed conversion between the I and N states of α-lytic protease.

It is important to note that the nucleation and catalyzed conversion mechanisms are not mutually exclusive. For example, there could be a hybrid mechanism by which the surface of an aggregate, which is initially formed by a nucleation process, catalyzes the conformational change of unconverted monomers. Indeed, in the case of flagella formation in vitro, kinetic studies show a lag between the initial, reversible, binding, and stable incorporation into the flagellum (Asakura, 1968). Moreover, NMR studies indicate that the NH_2 and COOH termini of flagellin, disordered in the monomers in solution, become ordered during the process of polymerization (Aizawa et al., 1990). By analogy, it seems attractive to consider that PrP^{C} could become converted in this manner, after an initial interaction with a PrP^{Sc} aggregate.

Other amyloid-forming diseases offer further opportunity for examining the mechanism of conformational rearrangement. There are at least 15 human diseases in which an accumulation of a specific protein can occur in characteristic insoluble fibers known as amyloid, which are typically 60–100 Å in diameter and exhibit characteristic birefringence when stained with the dye Congo Red (for review, see Kelly, 1996). These amyloid diseases result in a variety of different clinical presentations, dependent on the sites of amyloid deposition, and include Alzheimer's disease, where neurodegeneration occurs in association with deposition of the amyloid β protein. Despite distinct folds in the native state, all of the proteins involved in these diseases undergo conformational alteration to a common structure in the amyloid fibril, a "cross β" repeat structure in which β strands are aligned perpendicular to the axis of the amyloid fiber. A recent fiber diffraction study with synchrotron radiation suggests that, in fact, it is β sheets that are positioned perpendicular to the fiber axis and that they are arrayed as a continuous helix (Blake and Serpell, 1996). As with prion disease, the other amyloidoses can be initiated by inherited mutations in the respective coding sequences, which apparently destabilize the native state of these proteins, enabling them to rearrange to the common conformation in amyloid. Such destabilization has been elegantly demonstrated recently for two purified amyloidogenic lysozyme variants—while they were enzymatically active and crystallized in conformations nearly identical to wild-type, they exhibited little or no protection from deuterium exchange when incubated in solution at 37°C, unlike wild type (Booth et al., 1997). Lysozyme fibrils isolated from patient material, however, contained only the mutant lysozymes—the wild-type protein present in the heterozygous individuals was not recruited. This underscores the major difference that sets prion disease apart from other amyloidoses, namely that the aggregated form of PrP is also able to promote the rearrangement of unmutated protein, thereby allowing transmission of disease.

Recent studies with another amyloidosis, familial amyloidotic polyneuropathy, provide further insight into an

amyloidogenic conversion process (Kelly, 1996; Lai et al., 1996). The involved protein, transthyretin (TTR), is, in native form, a homotetramer whose subunits are eight-stranded β sheet sandwiches. In vitro, upon exposure to pH 4–5, TTR dissociates to monomers that undergo tertiary structural rearrangement, and amyloid formation ensues (Lai et al., 1996). As with prion conversion, control of TTR amyloid formation could lie either at the step of production of the amyloid aggregate or at the step of monomer rearrangement, invoking kinetic control. Both mechanisms have been observed with TTR in vitro. In support of a nucleation step, fibril formation was observed to exhibit a lag phase and to be accelerated after initiation by addition of amyloidogenic monomer. In support of kinetic control, a greater amount of TTR amyloid was formed at pH 4.4 during refolding from denaturant than was observed starting with native protein, reflecting a kinetic barrier between the amyloidogenic intermediate and the native tetramer.

Thus, for TTR, while both types of control have been observed in vitro, it remains unclear what step is rate limiting in vivo. How high is the kinetic barrier to formation of the amyloidogenic form at physiological temperature, pH, and ionic strength? In particular, without catalyzed formation of the amyloidogenic intermediate, how could there be enough accumulation of this intermediate to form a stable nucleus that would promote efficient polymerization? Alternatively, if the barrier to production of the intermediate is so high in vivo that a catalytic event is required, what mediates such an event in the absence of preexisting converted protein? Finally, given the observation of seeding phenomena in vitro, why is it that, unlike prions, TTR aggregates are apparently noninfectious? Is this a property of the greater stability of PrP^{Sc}? Or are the respective aggregates processed differently by the various organ systems involved? Concerning such potentially different physiology, two observations seem worth noting. In the case of TTR, a mechanism that clears TTR fibrils has recently been shown (Tan et al., 1995); and, in the case of prion disease, it has been observed that, even following intracerebral inoculation of mice with prions, there is early acquisition of infectivity in the spleen, long preceding any appearance of infectivity in the brain (Eklund et al., 1967; Kimberlin and Walker, 1979; Weissmann et al., 1997). Consistent with a primary replication step in the lymphoreticular system that favors neuroinvasion, SCID mice were relatively resistant to CNS disease following intraperitoneal inoculation (only 6 affected out of 18 animals), compared with immunocompetent littermates (13 of 14 animals) (Lasmézas et al., 1996a; see also Kitamoto et al., 1991). Presumably, those SCID animals that developed disease acquired CNS infection by direct neural spread, suggested in early studies to extend from peripheral nervous system to spinal cord to brain (e.g., Kimberlin and Walker, 1979).

Prion "Strains": Multiple Distinct PrP^{Sc} Conformations

Perhaps one of the most difficult phenomena to fathom in light of a protein only hypothesis is the existence of distinct prion "strains," reflecting observations that infectious material from different sources can produce distinct and reproducible patterns of incubation time, distribution of CNS involvement, and even pattern of proteolytic cleavage of PrP^{Sc}. These properties are retained even after repeated passage, as revealed by experiments in isogenic mice (e.g., Fraser and Dickinson, 1973; Bruce et al., 1976; Kimberlin et al., 1989; Carp and Callahan, 1991; DeArmond et al., 1993). Strikingly, there appear to be conformational differences in PrP^{Sc} that correlate with such behavior. A particularly good example of this is the observation in transmissible mink encephalopathy of two distinct presentations of faithfully transmissible disease, hyper (HY) and drowsy (DY), that describe the behavior of the affected animals, which are associated with characteristic incubation times and locations of neuropathology. The two presentations were found to be associated also with characteristic degrees of proteinase K susceptibility of PrP^{Sc}, with partial treatment producing different NH_2 termini (Bessen and Marsh, 1992, 1994). These different conformations could represent either different tertiary structures or, alternatively, different quaternary assemblies of the same fold. The latter case seems reminiscent of the ability of many proteins to pack their native forms into different crystal lattices, or of assembly of flagellar filaments, in which addition of different seeds results in the formation of distinct structures. Such diversity can be considerable, as in the case of the popular object of crystallization, hen egg lysozyme, shown to pack into at least five different crystallographic space groups. Alternatively, it remains possible that there are modifications such as N-linked glycosylation that confer strain-specific properties, although this modification does not appear to be necessary for acquisition of a proteinase K–resistant PrP^{Sc} in a cultured cell system (Taraboulos et al., 1990). Regardless of whether conformation or covalent differences are responsible, it seems possible that strain-specific properties of incubation time and brain localization may reflect targeting of different forms of PrP^{Sc} to specific CNS cells. These cells would then impart the same form to the newly-converted molecules (Hecker et al., 1992; Weissmann et al., 1997).

While primary structural differences are not necessary to produce different strains, an example of primary sequence origin of strain properties in human prion disease has recently been reported (Telling et al., 1996b). Human D178N FFI is associated with a proteinase K–resistant PrP^{Sc} of 19 kDa after deglycosylation, while both familial and sporadic CJD are associated with a 21 kDa species. Inoculation of the respective human brain homogenates into Prp-deleted mice containing a chimeric mouse–human mouse (MHuM) PrP transgene produced disease associated with the respectively sized PrP^{Sc}, indicating that the two distinct PrP^{Sc} species can template a single primary MHuM PrP structure into different conformations.

The importance of studying the origin and nature of strain differences has been emphasized recently by the reports of a number of cases of vCJD that appear to be linked to BSE epidemic in British cattle (Will et al., 1996). Despite the small number of cases, a number of observations suggests that vCJD represents a novel disease distinct from sporadic CJD. First, vCJD has a distinct pathology characterized by abundant "florid plaques," decorated by a daisy-like pattern of vacuolation (Figure 1). Second, there is a far younger age of onset than in

sporadic CJD. The notion that vCJD could be transmitted from cattle to primates was supported by the observation that intracerebral inoculation of BSE-infected brain extract into Macaque monkeys produced disease and pathology resembling that in the vCJD patients (Lasmézas et al., 1996b). This raised the possibility that vCJD was a newly-identified strain of prion that was less restricted by the species barrier. This was supported recently by studies examining the pattern of proteinase K–resistant PrP^{Sc} species from the vCJD patients, in particular comparing di-, mono-, and non-glycosylated species with those from brain homogenates of patients with sporadic or iatrogenic CJD, and homogenates from BSE-infected animals including cats and macaque (Collinge et al., 1996). vCJD was observed to share a common pattern with BSE-infected animals, distinct from that of sporadic or acquired CJD. The proteinase K–resistant diglycosylated species was particularly prominent, raising questions of whether this form of PrP^{C} is more susceptible to BSE-mediated conformational change or whether a population of cells preferentially producing diglycosylated PrP may be more readily targeted by BSE (Aguzzi and Weissmann, 1996).

Conversion Process In Vivo

The failure thus far to demonstrate conversion of PrP^{C} to an infectious form in vitro underscores the need for a better understanding of the factors present in vivo that facilitate this process. Studies of the biosynthesis and processing of the PrP proteins in cultured cells have provided important clues to where the conversion process might take place (Butler et al., 1988; Caughey et al., 1989). These studies indicate that the protein normally traverses the secretory pathway to reach the cell surface where it faces the external environment, anchored to the plasma membrane via a GPI anchor attached at its COOH terminus (Stahl et al, 1987). In reaching this destination, an NH_2-terminal secretory signal peptide of 22 amino acids is cleaved, and 23 COOH-terminal residues are also processed during addition of the anchor to S231. In scrapie-infected cultured cells, PrP^{C} was observed to undergo turnover with a $t_{1/2} = \sim 6$ hr (Caughey et al., 1989), but slow conversion of a small fraction (~5%) of newly synthesized, metabolically-labeled PrP^{C} was observed ($t_{1/2} = \sim 5$–15 hr), to a form that exhibited the same proteinase K resistance as PrP^{Sc} and that accumulated (Borchelt et al., 1990; Caughey and Raymond, 1991).

Additional studies in the cultured cell system showed that conversion to PrP^{Sc} could be blocked by addition of exogenous PI-specific phospholipase C or by proteases, suggesting that PrP^{C} undergoes conversion either at the cell surface or after internalization from the cell surface into the endocytic pathway (Caughey and Raymond, 1991; Borchelt et al., 1992). In support of a requirement for internalization, low temperature incubation (18°C), which retards endocytosis, also blocked production of PrP^{Sc} (Borchelt et al., 1992). Additional efforts to refine the localization have noted that GPI-anchored proteins localize at the cell surface in cholesterol-rich plasma membrane invaginations that are Triton X-100 insoluble, known as DIGS (detergent-insoluble glycosphingolipid-enriched membranes) (Brown and Rose, 1992; Smart et al., 1995). Supporting a role of such a compartment, treatment of the cultured cell system with the inhibitor of cholesterol biosynthesis, lovastatin, blocked the conversion process, but it was unclear whether this effect was mediated by failure of PrP^{C} to reach the cell surface or by disruption of the DIGS where conversion might take place (Taraboulos et al., 1995). Additional uncertainty is cast by the observation that absence of the GPI anchor from a truncated PrP inhibited but did not prevent production of the proteinase K–resistant PrP^{Sc} species in the cultured cells (Rogers et al., 1993).

Whatever the specific compartments involved, it seems clear that PrP^{C} reaches the cell surface and that this localization may make it an easily accessible target for exogenous PrP^{Sc}, although it seems equally clear that PrP^{Sc} presented from outside the cell could internalize down the same pathway as PrP^{C} and mediate conversion internally. Whichever the site, the notion that conversion could take place in a specific membranous compartment containing a specific subset of proteins has potential for reconstitution studies. If such a PrP^{C}-containing compartment is isolable as a low density Triton-insoluble membrane fraction, it should be possible to test for conversion with the isolated fraction, potentially allowing the delimiting of components that are critical to conversion.

Recent transgenic studies on the susceptibility of mice expressing chimeric human-mouse PrP^{C} suggest that at least one host factor other than PrP^{C}, tentatively termed factor X, might be involved in susceptibility to infection (Telling et al., 1995). Conceivably, factor X could be a molecular chaperone that binds to PrP^{C} and assists in altering its conformation. A precedent for chaperone involvement in a conversion process comes from recent studies in yeast, where the cytosolically localized product of the SUP35 gene, involved with translational termination, can be converted to a biologically inactive aggregated molecule, conferring a phenotype of nonsense suppression (PSI+) (Chernoff et al., 1995; Patino et al., 1996; Paushkin et al., 1996; see also Masison and Wickner, 1995). The SUP35 aggregates appear to act as a nucleus, promoting the aggregation of newly synthesized SUP35 protein, allowing propagation of the PSI+ state in a manner analogous to the PrP^{C}-to-PrP^{Sc} conversion process. Strikingly, maintenance of the PSI+ state was found to depend on the molecular chaperone, Hsp104, a large homohexameric single ring structure with two ATP-binding sites in each of its subunits, which has previously been shown to have a propensity to dissociate protein aggregates produced by heat shock (Parsell et al., 1994). Remarkably, either deletion of Hsp104 or its overexpression resulted in concordant disappearance of the SUP35 aggregates and loss of the PSI+ state. In the case of PrP conversion, a general chaperone component like Hsp104 has not so far been identified in the cellular locations where conversion appears to occur.

Structural Studies of PrP^{C}

Ultimately, an understanding of the conversion process will likely require knowledge of the three-dimensional structure of the different PrP conformations. A major

Figure 3. Structure of Prion Protein

(A) Primary structure of mature human PrP. The sequence of the mature protein, residues 23–231, after proteolytic processing of the NH_2-terminal secretory signal and the COOH-terminal region beyond the GPI anchor site, is shown. Mutations involved with inherited human disease are indicated in red, above the line, and naturally occurring polymorphisms are shown as numerator/denominator on the line, in purple. The asterisk indicates a mutation producing a stop codon at amino acid 145. Residues implicated in species barriers are also shown, between human and mouse (backlit in blue), and between mouse and hamster (backlit in yellow). Secondary structure of corresponding region 123–231 of mouse PrP, determined from the NMR structure (Riek et al., 1996), is shown above the primary sequence, with β strands represented by arrows and α helices by cylinders.

(B) Structure of mouse prion protein domain PrP121–231. Two opposite faces are shown of the mouse PrP121–231 structure recently determined by NMR (taken from Riek et al., 1996, with permission). Ribbon diagrams are shown in the two left panels, displaying the three α helices and the short antiparallel two-stranded β sheet. In the top left panel, green indicates well resolved loop structure, whereas purple indicates poorly resolved loop structure. A disulfide bond is shown in white. In the bottom left panel, side chains associated with inherited prion disease are shown in red, and residues that may be involved in the species barrier between mouse and human are shown in blue (Q168 side chain is not shown). (Note that side chain numbering corresponds to the human sequence shown in [A]). Two solvent-accessible glycosylation sites are also shown in green and the disulfide bond in yellow. Electrostatic potential plots are shown in the two right panels, displaying positive charge as blue and negative charge as red. The aspect of the molecule shown in the top panel has been suggested to possibly face a membrane, whereas the opposite, negatively-charged aspect (lower panels) may present a binding surface for an as yet unidentified ligand (see text).

advance in this direction is the recent NMR structure determination of a mouse PrP molecule containing residues 121–231 (Riek et al., 1996 and see Figure 3). This polypeptide was stable and soluble when programmed in E.coli for localization to the periplasm, and it comprises a significant portion of a minimal region of PrP, residues 80–231, that can mediate disease (Fischer et al., 1996; see also Muramoto et al., 1996). Given its solubility at millimolar concentration, and a high α helix/low β sheet composition, it seems probable that it occupies a conformation similar to that in intact PrP^C.

The secondary structure of PrP121–231 features three α helices and two short antiparallel β strands (Figure 3B). Glockshuber, Wuthrich, and coworkers speculate that this latter feature could be a "nucleation site" for a conformational transition to the β sheet–rich PrP^{Sc} form, that could presumably incorporate neighboring loops. Interestingly, the methionine/valine polymorphism affecting disposition to CJD maps into one of these strands. The observation that heterozygosity for Met/Val at this position is protective (Palmer et al., 1991) leaves one to wonder whether these strands might also be involved in intermolecular contacts involved in either the conversion process or in aggregation of PrP^{Sc}.

Analysis of the surface properties of the PrP121–231 molecule reveal two disparate faces (Figure 3B). One is overall electrostatically positive but contains intermingled hydrophobic patches, suggesting that it could face the cell membrane. The opposite face, by contrast, is electrostatically negative, containing the two sites of

glycosylation. Riek et al. suggest that it could be a site of binding of an as yet unidentified ligand. (Could this be PrP^C itself, on another cell, for example?) In addition, this surface bears at one edge containing the first α helix, a region suggested to act as an accessible binding site for PrP^{Sc}. This region contains 5 of 14 residues implicated by chimeric transgenic studies to be important for either the human-mouse or hamster-mouse species barrier (Figure 3A and 3B). Three of the remaining residues involved in the species barrier lie at the opposite edge of the molecule, located in a loop region between the second β strand and the second α helix (only 166 is shown in Figure 3B). The remaining five residues form a third putative PrP^{Sc} binding site located between residues 90 and 122, a region not present in the structure.

Interestingly, the sites of the species barrier and of disposing human mutations appear to be, so far, mutually exclusive. Whereas the region including α helix 1 appears to be a determinant of the species barrier, human mutations disposing to disease map to the region of the two other α helices, with three mapping into the hydrophobic core and three to the electrostatically negative surface. Such mutations could, correspondingly, either destabilize the structure or affect ligand binding.

With structural information of this sort now in hand, it will be possible to carry out a host of structure-function studies relating the regions of the species barrier and human mutations to the conversion process. For example, it should be possible to assess the relative importance of the three structural regions implicated in the species barrier. In addition, designed mutants with either decreased or increased PrP^C stability, measured in vitro with purified recombinant protein, will make it possible to test directly whether destabilization of the native PrP^C structure facilitates conversion in vivo. Finally, antibodies generated against peptides that are buried in the native PrP^C structure may potentially provide reagents for specifically detecting the PrP^{Sc} form. While PrP^C is at last yielding to structural analysis, by contrast, in the absence of protocols for solubilizing PrP^{Sc}, structural information on the converted form may require nonsolution techniques such as solid state NMR (e.g., Heller et al., 1996).

What Produces Prion Disease? Is It Deficiency of PrP^C or Production of PrP^{Sc}?

Mice develop normally despite complete absence of PrP^C (Büeler et al., 1992), but the issues of whether PrP^C has a function postnatally and what it may be have remained unresolved. In one report analyzing hippocampal slices from PrP^C null animals, GABA receptor–mediated fast inhibition was weakened (Collinge et al., 1994; see also Lledo et al., 1996), an effect that could be rescued by a transgene (Whittington et al., 1995), pointing to a possible postsynaptic function of PrP^C. In a more recent study, a strain of null mice deleted of the PrP coding sequence and part of the large intron developed ataxia by ~70 weeks of age, associated with loss of cerebellar Purkinje cells, further supporting the idea that PrP deficiency could produce neuronal cell death as a result of excitotoxic mechanisms (Sakaguchi et al., 1996). On the other hand, these clinical and pathologic features of PrP^C deficiency fail to recapitulate those of classic prion disease. Conversely, can production of PrP^{Sc} alone produce disease? An elegant study of Aguzzi, Weissmann, and coworkers has recently addressed this question (Brandner et al., 1996a). They engrafted brain tissue from a PrP^C-expressing normal mouse into a null animal and then inoculated with mouse prions. They observed the characteristic pathology of prion disease precisely within the engrafted tissue, but not within the null tissue. Nevertheless, PrP^{Sc} produced within the diseased tissue migrated into the neighboring null tissue. Thus, PrP^{Sc} by itself was insufficient to produce disease—i.e., simple exposure to exogenous PrP^{Sc} is insufficient for cytotoxicity. Rather, PrP^{Sc} may be toxic only when formed within the cell, or when presented from outside to a cell already expressing PrP^C, so that conversion ensues either at the cell surface or in an internalized compartment. Furthermore, Brandner and colleagues have also shown that propagation of infection across neural pathways in the CNS requires the presence of PrP^C along the pathway (Brandner et al., 1996b), insofar as grafts in null animals did not become infected following intraocular inoculation. Thus, in all cases, it appears that the presence of PrP^C is required for pathogenesis.

Perspective

An enormous body of data reveals the central role of the PrP protein in a group of related transmissible neurodegenerative diseases. These data demonstrate that PrP protein is required for the disease process and that the conformational conversion of the PrP protein from its normal soluble α-helical conformation to an insoluble β sheet state is intimately tied to the generation of disease and infectivity. Much about the conversion process remains unclear. In particular, is introduction of the PrP^{Sc} protein alone sufficient for infection in the presence of PrP^C? What is the nature of the conformational change? Given the ubiquitous distribution of PrP^C, why is prion pathology restricted to the central nervous system? What determines the species barrier? In addition to further resolving a fascinating biological process, the exposure of both cows and people to BSE underscores a need to answer these questions.

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