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Molecular Advances in Understanding Inherited Prion Diseases

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Abstract

The prion diseases are neurodegenerative disorders that have attracted great interest because of the possible link between bovine spongiform encephalopathy (BSE) and variant Creutzfeldt-Jakob disease (CTD) in humans. Possible transmission of these diseases has been linked to a single protein termed the prion protein. This protein is an abnormal isoform of a normal synaptic glycoprotein. The majority of prion diseases does not appear to be caused by transmission of an infectious agent but occur spontaneously with no known cause. The strongest supporting evidence that the prion protein is the causative agent in prion disease comes from specific inheritable forms of prion disease which are linked to single point mutations in the prion protein gene. Paradoxically, these point mutations, although autosomal dominant with 100% penetrance do not lead to disease until late in life. Molecular techniques are now being used extensively to determine how these point-mutations alter the prion protein's normal structure and activity. This review deals with the latest insights into how inherited mutations in the prion protein gene lead to neurodegenerative disease.

Index Entries: Prion; TSE; Gerstmann-Sträussler-Scheinker; Creutzfeldt-Jakob; Fatal Familial Insomnia; neurodegeneration; peptide.

Introduction

The prion protein (PrP^c) is a glycoprotein expressed at the cell surface as glycosyphosphatidylinositol (GPI)-anchored monomer. Recent crystolographic studies have suggested that the protein might form dimers by covalent linkage through a disulfide bond *(1)*. Although there is some evidence for dimerization in vitro *(2)*, there is currently little evidence for this in vivo and it is currently believed from nuclear magnetic resonance (NMR) studies *(3,4)* that the disulfide bond makes an intramolecular bond between two of the three helical regions of the C-terminus. PrP^c has been shown to be a cupro-protein binding between two and five atoms of copper *(5–8)*. The normal function of PrP^c is associated with its high concentration at the synapse *(9)*. Stud-

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ies have shown that a deficit of PrP^c-activity leads to a reduction in neuronal resistance to oxidative stress *(10)*. This manifests itself in the central nervous system (CNS) as altered synaptic activity such as reduced long-term potentiation *(11)* and may have the consequence of altering activities such as circadian rhythms *(12)*. Increased expression of PrP^c protects cells from oxidative assaults *(10)*. Analysis of isolated protein from both native and recombinant sources suggests that on binding copper PrP has an antioxidant activity like that of superoxide dismutase (SOD) *(13,14)*.

Prion diseases are a collection of human and animal diseases linked together by virtue of the fact that in those diseases PrP^c is converted to an abnormal isoform *(15)*. In sporadic forms of this disease this protein is designated PrP^{Sc} after the scrapie isoform isolated from the brains of mice infected experimentally with infectious isolates from the brains of sheep carrying the disease scrapie *(16)*. The abnormal form of PrP is characterized by structural conversion to a form rich in β-sheet as opposed to the helix rich PrP^c *(3)*. PrP^{Sc} is also greatly resistant to protease digestion and can form amyloid or fibrils *(17)*. A recent study has shown that PrP isolated from the brains of human patients with the sporadic prion disease, Creutzfeldt-Jakob disease (CJD) does not bind copper and has no anti-oxidant activity *(18)*. The implication is that the conversion process results in an aggregated inactive form of the protein.

Scrapie, bovine spongiform encephalopathy, CJD, variant CJD and the rare forms of prion diseases such as the inherited forms of human prion disease and forms spread by accidental transmission (Kuru and iatogenic CJD) are all fatal neurodegenerative diseases. They are characterized by long incubation periods with little or no symptoms and a rapid clinical phase characterized by dementia and motor disturbances on one hand and gliosis and vast neuronal loss on the other. As stated above PrP conversion is central to the pathology of these diseases. Most forms of these diseases can be transmitted to rodents or primates experimentally *(19–21)*. Prusiner *(22)* proposed that PrP^{Sc} can act as the infectious agent and this constitutes the "protein only" hypothesis of disease transmission. The central nature of PrP to disease transmission was shown by the work of Büeler et al. *(23,24)*. Mice in which expression of PrP^c is genetically ablated are resistant to infection with scrapie. Furthermore transplantation studies have shown that PrP^{Sc} generated in the brains of PrP-knockout mice has no deleterious effects on tissue devoid of PrP^c expression *(25)*. This shows that PrP^c expression is necessary for both disease transmission and the induction of neuronal death and gliosis. Models of neurotoxicity have focused on the neurotoxicity of a protein fragment of PrP^c in vitro *(26)*. This fragment is also toxic in vivo *(27)*. The mechanism of action suggests direct effects mediated by interaction with PrP^c *(14,28)* that modify neuronal resistance to oxidative stress *(29)*. Support for this has also come from studies with scrapie-infected cultures, which also demonstrate a reduced resistance to oxidative stress *(30)*. The toxic effect possibly comes from direct effects of the peptide or via toxic substances released from activated glia *(30–32)*.

Inherited Prion Diseases

Inherited forms of prion disease pose fundamental questions that both support the "protein only" hypothesis and also suggest that this model of disease cause is insufficient. This paradox has lead to intense research on inherited forms of these diseases despite the fact that they make up only a small percentage of a group of diseases that are rare in total numbers *(22)*. The inherited forms of prion disease have many similarities to the sporadic forms and more importantly deposition of abnormal PrP occurs in these inherited diseases. Point mutations in the coding domain of the single gene *(prnp)* for PrP are responsible for the majority of these inherited forms. These mutations are fully penetrant and are dominant implying that a single copy of the mutated gene is

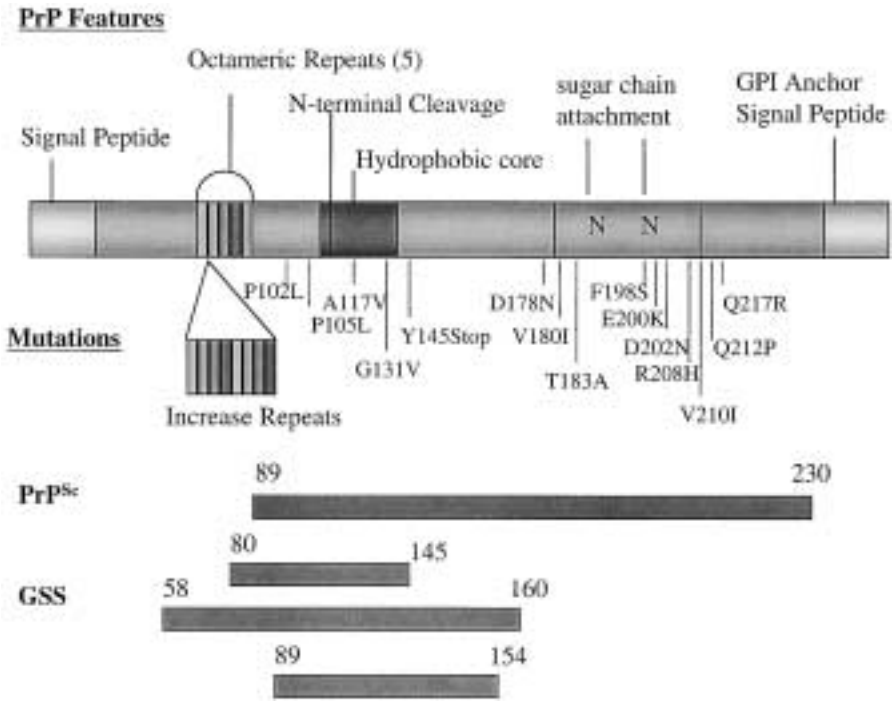

Fig. 1. Schematic representation of the primary structure of the prion protein. The location of many of the more frequently studied point mutations are shown. Also shown for comparison are the size of truncated PrP fragments found in CJD and GSS. Note that those found in GSS are truncated in the C-terminus as well as the N-terminus.

enough to cause disease in the carrier. However, the paradox lies in the fact that inherited forms of the disease do not occur at birth but when the carriers are, on average over the age of 50. This implies that during the majority of an individual's life some factors are suppressing onset of these diseases. Understanding what these factors might be has implications for both the cause and the possible prevention of all forms of prion disease including the more infamous variant CJD whose aetiology currently remains a matter of conjecture.

Currently there are more than 20 point mutations that cause inheritable prion diseases that are transmitted as autosomal dominant disorders. The three mains forms of inherited prion disease are familial Creutzfeldt-Jakob disease (fCJD), Fatal Familial Insomnia (FFI), and Gerstmann-Sträussler-Scheinker Syndrome (GSS).

GSS results from mutations causing changes in single amino-residues in human PrP. These include P102L, P105L, A117V, G131V, V180I, F198S, D202N, Q212P, and Q217R (Fig. 1). In GSS PrP deposits form but the depositions are not necessarily fibrillogenic. In GSS the PrP purified from brains can either be full-length or truncated at the N- or C-terminus or both. Some forms of GSS as well as showing deposition of abnormal PrP also show deposition of the tau protein in the form of paired helical filaments (tangles) normally associated with Alzheimer's disease (AD) or tauopathies. This is especially the case for the A117V form of GSS *(33)*. Another form of GSS (Y145stop) leads to a truncated form of PrP being expressed as a result of an amber stop codon being generated *(34)*. This is sometimes known as the vascular variant because of the large numbers of PrP deposits around cerebral blood vessels. Additionally this

form of GSS is also associated with tau-containing neurofibrillary tangles *(35)*.

Fatal Familial Insomnia is linked to different point mutations than GSS. Familial CJD is also linked to point mutations at D178N, T183A, E200K, V200I, R208H, and V210I. The methionine / valine polymorphism at codon 129 in prnp can influence the clinical phenotype produced by some of the point mutations. This is particularly marked in the D178N mutation. The D178N mutation will cause either FFI when a methionine is at codon 129 or fCJD when valine is at codon 129 *(36)*. The differences between these inherited forms of prion disease lies in the clinical signs that the diseases present with and also the average age of onset is different between GSS and fCJD. In particular FFI presents with a specific form of insomnia giving the disease its name.

As well as being linked to point mutations fCJD can also be caused by alterations to the octameric repeat region of the protein. The octameric repeats are the region at which copper binds to the protein *(37)*. Although it has been suggested that copper binds elsewhere *(7,8)*, there is little evidence that this occurs in vivo. In humans the number of complete repeats normally encoded by *prnp* is five whereas many other animals have only four. An increased number of octameric repeats of between 1 and 9 additional repeats are associated with inherited forms of disease *(38)*. Additionally, a new disease has recently been described which involves deletion of two of the octameric repeats *(39)*. In humans deletion of a single octameric repeat appears to have no pathological consequences. The mechanism by which the modifications of the octarepeats leads to disease is unknown at present but could possibly be related to altered binding of copper.

In the course of this review four different mechanisms will be discussed that might explain how PrP carrying inheritable mutations might cause the inherited forms of prion disease. These possible mechanisms involve: 1) structural changes due to amino acid substitution, 2) susceptibility to conformational conversion, 3) enhanced toxicity of the protein, and 4) disturbance in transport of the protein.

Secondary Structure

The most obvious place to look for an effect of amino-acid substitutions or insertions on the formation of abnormal protein is at the level of secondary structure. Folding dynamics of wild-type prion protein has been studies in detail (reviewed by Glockshuber; *40*). Computational models have suggested that because many of the mutants occur at key regions of the secondary structure that they might act as helix breakers. However there is scant evidence from structural studies to support this notion. Analysis of the NMR solution structure of PrP carrying the E200K mutation indicated that the secondary structures and backbone tertiary structure show very few variations from the wild-type protein *(41)*. The major change found was a redistribution of the surface electrostatic potential *(41)*. Such a change is more likely to effect the ability of the mutant PrP to interact with other proteins such as chaperones, which might be important in preventing the kind of conformational change that leads to PrP^{Sc} formation. Similarly studies of the thermodynamic stability of protein with the E200K mutant show that it is more susceptible to thermal denaturation than wild-type proteins *(42)*. However, this is not a common feature of proteins with inherited mutations as proteins with the D178N and P102L mutations were indistinguishable from wild-type proteins in terms of their thermodynamic stability. Another study looked at the thermodynamic stability of 8 mutants and found five that had a mild destabilizing effect *(43)*. These were V180 I, D178N, F198S, Q217R, and T183A, which was profoundly altered. In contrast E200K appeared to have the same stability as wild-type protein. Thus these findings are to some degree contradictory to those of the other investigators.

NMR and other structural studies have also found little differences between wild-type PrP

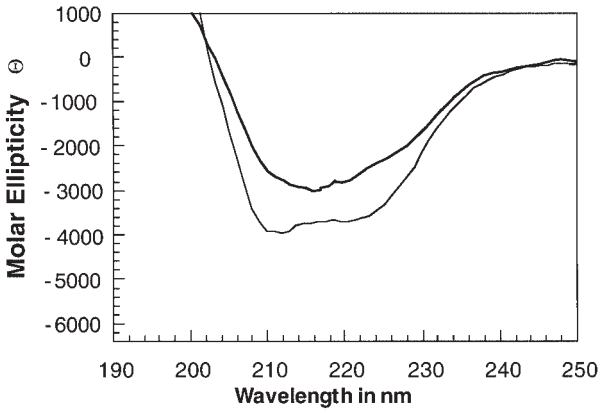

Fig. 2. Change in the circular dichroism (CD) spectrum of mutant PrP. The UV spectrum of wild-type and mutant human PrP was determined. The protein was purified from inclusion bodies as expressed in bacteria. The mutant protein contained the P102L mutation. The spectra were obtained at room temperature at pH.8 using 10 m*M* phosphate as a buffer. Thin line = wild-type PrP. Thick line = PrP with P102L mutation. Shown is the spectrum average of 5 sweeps measuring at 0.5 n*M* intervals for 2 s. Values are in molar ellipticity (θ).

and PrP carrying inheritable mutations *(44)*. Circular dichroism studies have picked up differences in protein with the P102L mutation as compared to wild-type (Fig. 2) but these changes are not associated with increased protease resistance and so appear not to alter conformational transition to the abnormal isoform directly *(45)*. Thus the general impression from structural studies at present is that any changes that the mutations cause are subtle and their effects are more likely to accelerate factors influencing conformation transitions rather than directly causing them.

Protein Conversion

It has been postulated that the fundamental metabolic change that links all prion diseases is that the normal isoform of the prion protein is converted into abnormal form such as PrP^{Sc} or another form. The abnormal forms differ from PrP^{c} in that they are altered confomationally, usually into a β-sheet rich, protease resistant aggregate that forms fibrils. These disease specific forms are then responsible for the ensuing pathological changes leading to neuronal loss, gliosis, spongiosis, and, through clinical complications, death of the patient. Therefore, if inherited mutations in the *prnp* gene are responsible for the inherited forms of prion disease, it would be expected that these mutations cause protein conversion to an abnormal form. However, the unexpected empirical finding is that most carriers of mutated *prnp* genes live without detectible neurological changes until late in life suggesting that PrP is either not converted to an abnormal isoform by these mutations or that this alone is insufficient to cause disease. However, the 100% penetrance of the mutations, even though late in life suggests that these mutations are sufficient. This paradox has lead investigators to determine if mutations in *prnp* do result in the formation of abnormal forms of PrP.

In studies of Libyian Jewish families carrying the E200K mutation analysis of the protein within the pathogenic PrP^{Sc} has been carried out. These studies reveal interesting information regarding participation of mutant and wild-type protein in the formation of abnormal protein deposits in fCJD. fCJD among these families is unusual as there are some reported cases of individuals who are homozygous for the E200K mutation where as almost all other cases of inherited prion diseases worldwide are heterozygous with one allele of *prnp* coding for wild-type protein *(46)*.

However, in PrP deposits in the brains of heterozygous patients both wild-type and E200K mutant PrP can be identified *(47)*. However, it is unclear at present if the wild-type protein is converted to PrP^{Sc} or not and it has been suggested that the wild-type protein does not contribute to the disease process. Mutant proteins expressed in CHO cells in culture show properties like PrP^{Sc} although their infectious nature has not been demostrated *(48,49)*. However, some studies using other cell types suggest that protein with the E200K mutation can still behave like PrP^{c} suggesting that

expression of the mutation does not automatically confer protease resistance of like that of PrP^{Sc} as would be expected from the studies of patients with the mutation *(50)*.

Another change that results in abnormal PrP is the production of abnormal fragments of the mutant PrPs. These fragments which are generated may or may not be resistant to protease digestion. The amber mutation at Y145 causes truncation of PrP. This protein fragment has a number of peculiarities in the diseases. A proportion of the protein retains its N-terminal signal peptide suggesting failure to be exported from cells *(34)*. Most of the mutant protein is rapidly degraded. When degradation is impaired this protein accumulates in the cell and shows protease resistance *(34)*. The protein is often degraded to a fragment of ~7.5 kD in size. In the brain it is this fragment that is deposited in aggregates around blood vessels *(35)*. Similar sized fragments are also found in deposits in other forms of GSS *(51,52)*.

In GSS with the A117V mutation a 7 kD fragment can be isolated from the brains of patients with an N-terminus starting at either residue 89 or 90 *(53)* and a number of different C-termini *(54)*. In addition, small N and C-terminal fragments could also be detected but in vitro experiments suggest that only the 7 kD fragment would be involved in fibril formation *(54)*. In GSS with the F198S mutation a fragment of 8 kD can be isolated with an N-terminus starting at residue 74 *(53)*. In other forms PrP can be detected as an 11 kD form starting at amino residue 58 *(55)*. As these fragments are the result of proteolytic degradation, it is likely that GSS mutants alter the conformation of the protein protecting specific sites from digestion. In CJD PrP is in general full-length or N-terminally truncated. It appears that only in the inherited forms of the disease C-terminally truncated fragments accumulate *(52,56,57)*. This again suggests that the mutations themselves might alter the conformation of the protein to increase protease sensitivity in these regions. This at first might appear contradictory as it would be expected that the mutations might increase resistance. However, if one considers that a disordering of the pattern of degradation of the protein might generate an abnormal fragment that could more rapidly generate protease resistance before the degradation process is complete, then it is possible that this abnormal degradation could trigger the conformational conversion of the protein.

Analysis of PrP-peptides based on the hydrophobic core of the protein has suggested that hydration effects the kind of structures that these peptides will form *(58)*. Dehydration of the peptides leads to increased β-sheet formation in the form of hydrogen-bonded slabs. The presence of GSS like mutations such as P102L increased β-sheet formation and enhanced folding of the peptide into compact units, which would significantly enhance the formation of β-sheet fibrils in intact protein *(58)*.

Evidence that peptides bearing GSS mutants can cause disease in vivo come from studies with transgenic mice *(59)*. The trangenic mice carrying the P102L mutation and were injected with 55 amino-residue peptides also carrying the P102L mutation. Two forms of peptide were injected. Either the peptide was injected in β-sheet rich form or one that lacked β-sheet. The β-sheet rich form only caused a neurodegenerative disease in the mice that resembled scrapie. These results suggest it is structural conversion induced by the β-sheet peptide that initiates the disease in these mice.

Mice overexpressing PrP carrying the P102L mutation of GSS develop a spontaneous disease *(60)*. These results at first suggested that this was all that was sufficient for the development of disease. However, a repetition of the experiments in which mice expressed this mutation but at a level comparable to normal expression showed that this was not the case. Such transgenic mice carrying the GSS P102L mutation did not develop disease *(61)*. However, the mice showed an increased and altered susceptibility to infection with experimental scrapie. Again these experiments suggest that GSS mutations may not necessarily cause conversion of the protein to an abnormal isoform but PrP carrying the mutations is

more susceptible to influences that may induce such conversion.

Inherited prion diseases can be transmitted experimentally from the brain tissue of afflicted patients to mice carrying a chimeric PrP construct with part of the human gene *(62)*. Mutant PrP would be expressed in the brains of patients all their life even if the expression level of the abnormal protein was somehow suppressed. There is no evidence concerning whether disease could be transmitted to mice from the brain of an asymptomatic carrier of a PrP mutant. However, if such transmission cannot occur then transmission of mutant PrP alone would not be sufficient for the disease suggesting some other factor must come into play to modify PrP to make it infectious. However, as stated earlier, this is also suggested by the fact that individuals carrying mutant PrPs do not develop disease until late in life. Discovery of an age-related onset factor for prion diseases would have huge ramification for both diagnosis and possibly treatment of all prion diseases.

Toxicology

Studies of the toxicity of abnormal forms of PrP have concentrated either on PrP^{Sc} *(63)* derived from rodents or on peptides such as PrP106-126 *(26)* based on the human sequence. PrP106-126 (identical to amino-residues 106-126 of the human sequence of PrP) has been demonstrated to be toxic in the retina proving its toxicity in vivo *(27)*. A variety of findings have suggested a number of mechanism of action of this peptide based on in vitro studies. One or a combination of these mechanisms may play a part in the action of PrP^{Sc} in vivo. In particular it has been suggested that PrP106-126 has two effects. One involves direct interactions with neurones either binding to PrP^{c} and causing reduction in neuronal resistance to oxidative stress or by directly interacting with the membrane *(64)* causing changes in membrane fluidity or making channels into the membrane, which undermine the electrochemical gradient *(65)*. The toxic mechanism also appears to require indirect effects either mediated by glutamate accumulating as a result of failed astrocytic clearance *(31,66)* or superoxide and other toxic substances generated by microglia *(29,32)*. Some in vitro studies have suggested that these indirect effects are unnecessary. However, it was not clearly demonstrated in these studies that these indirect effects were not participating in the toxic mechanism. Studies showing involvement of indirect effects do not exclude direct interaction of PrP106-126 with neurones that are killed. Without reduction in neuronal resistance to oxidative stress the degree of microglia / astrocyte activation is insufficient for the indirect effects to trigger toxicity. What is clear from comparing in vivo *(25)* and in vitro *(29,67)* findings is that expression of PrP^{c} is essential for the toxicity of both Prp106-126 and PrP^{Sc}. This shows that PrP106-126 is an effective mimic of PrP^{Sc} and that all studies of PrP106-126 toxicity must use PrP-deficient cells as control for nonspecific effects not related to the kind of neurotoxicity seen in prion disease. Thus even in vitro both PrP106-126 and PrP^{Sc} require specifically neuronal expression of PrP^{c} to mediate direct effects on neurones and the indirect effects of toxic substances release by glia in order to initiate apoptosis in neurones *(63)*. The indirect effects do not require microglia to express PrP^{c}. The execution of apoptotic death initiated by PrP106-126 involves calcium influx through L-type calcium channels and NMDA receptors *(68,69)*, mitochondrial damage *(70)*, and activation of caspases *(71)*.

Toxicity mediated by PrP^{Sc} or PrP106-126 is caused by protein or peptides with the wild-type sequence of the prion protein. However, the in vitro systems developed to study PrP106-126 have been used to analyse how mutations alter this toxicity. Two studies have shown that mutations associated with inherited prion diseases modify the toxicity of PrP peptides *(72,73)*. Forloni et al. *(72)* studied a peptide PrP89-106 (amino-residues 89-106 of the human PrP sequence) and

showed no toxicity to cultured cortical neurones. However, a modified peptide carrying the P102L substitution of GSS showed significant toxicity but without converting the peptide to a fibrillar form. In contrast the mutation P105L had no effect. Other peptides studied in this report include PrP169-185, PrP195-213 and PrP201-220 (numbers correspond to human PrP amino residue sequence). These peptides showed mild neurotoxicity. Modified forms of these peptides were used to test the neurotoxicity of the GSS mutations D178N, F198S, E200K, V201I, and Q217R. Of these mutations only D178N increased the toxicity of the corresponding peptide. This mutation also enhanced the fibrillar nature of the peptide as determined by turbidity and electron microscopy. Additionally the D178N mutation also enhanced the ability of the peptide to enhance astrocyte proliferation. Thus only mutations in the vicinity of the hydrophobic domain of the protein appear to alter its neurotoxicity when studied with peptides.

The only mutation to lie in the palendromic region of the protein (also in the hydrophobic core) is A117V. In contrast to Forloni et al. *(72)* who found no effect of the mutation on the toxicity of the PrP106-126 peptide Brown *(73)* showed that PrP106-126 carrying this point mutation was far more toxic to wild-type neurones. This more detailed study examined the effect of this mutation in greater detail. In contrast to wild-type peptide (wt-PrP106-126) which is not toxic to neurones that do not express PrP^c, A117V-PrP106-126 showed significant toxicity to neurones from PrP-knockout mice. Similarly, the mutant peptide also showed an increased β-sheet content as determined by circular dichroism spectroscopy. The toxicity of A117V-PrP106-126 also did not require microglia and its toxicity is not increased by increasing the number of microglia present in the culture. These results suggest that the mechanism of action of the mutant peptide was quite different to the wild-type peptide. This implies that in inherited forms of prion disease abnormal PrP might have toxic effects quite different to that or in sporadic CJD or other forms of prion disease.

PrP106-126 has been shown to bind tubulin *(74)* and cells taking up labeled PrP106-126 show that the peptide is associated with microtubules inside cells *(75)*. The effect of PrP106-126 on the rate of polymerization of tubulin showed that it had no effect on the rate of polymerization in the absence of tau *(73)*. However, tau rapidly accelerates the rate of tubulin polymerization. PrP106-126 inhibits this effect. When PrP106-126 carries the A117V mutation inhibition of tau stimulated tubulin polymerization is greatly enhanced. The relation of this effect to the altered mechanism of toxicity of A117V-PrP106-126 was shown by taxol, which inhibited the toxicity of A117V-PrP106-126 but had no effect on the toxicity of wt-PrP106-126. A117V-PrP106-126 also greatly enhances calcium entry into neurones *(73)*. Furthermore toxicity of A117V-PrP106-126 is inhibited by blockers of L-type calcium channels. Studies have shown that destabilization of the cytoskeleton can cause neuronal death by calcium entry through L-type channels *(76)*. Therefore, it is possible that the mechanism of action of the A117V mutant form of PrP could have its neurotoxic mechanism through this activity. However, such a possible mechanism has not been validated in vivo.

In another recent article, a second neurotoxic domain in PrP has been identified with toxicity more potent than that of PrP106-126 *(77)*. The findings suggest that PrP contains two domains that are potentially toxic. The first is the hydrophobic domain, which is approximately equivalent to PrP112-136 in the unstructured part of the protein. The second is contained within the structured C-terminus approximately equivalent to PrP121-231. Peptide dissection of this toxic domain suggests that it lies within PrP163-220. However, this toxic domain is probably conformationally sensitive and might correspond to the hydrophobic pocket in the globular domain of the protein. Interaction between PrP121-231 and PrP106-126 abolished the toxicity of either domain. Although PrP121-231 applied to cultures of cerebellar neurones is

toxic, PrP112-231 is not. Circular dichroism analyses of these two protein fragments show a moderately different structure suggesting that possibly PrP112-231 folds differently to PrP121-231. The NMR structures of PrP121-231 and full-length prion protein have been determined *(3,4)*. These structures show little difference but the NMR structure of PrP112-231 has not been determined.

The interaction of a peptide fragment of the PrP and PrP121-231 have allowed analysis of the possible effects of GSS mutations on PrP structure *(77)*. PrP121-231 recombinant proteins were generated carrying GSS mutations. Some mutations enhance the toxicity of PrP121-231 directly (Fig. 3). E200K was found to increase the toxicity of PrP121-231 to cultured neurones. The F198S mutation also has a similar effect. More interestingly some GSS point mutations inhibit the interaction of PrP121-231 and the inhibitory peptide PrP113-125. Where as the peptide completely inhibited the toxicity of PrP121-231 to neurones it had no effect on PrP121-231 carry the D178N and only a small effect on that carrying the F198S mutation (Fig. 4). Circular dichroism showed that the inhibitor peptide directly altered the structural characteristics of wild-type PrP121-231 but had no effect on D178N mutant protein. Similarly, the spectra of the E200K and F198S mutants were also less altered than the wild-type protein. This suggests that GSS mutations might have their effect by altering hydrostatic or other noncovalent interactions within the intact prion protein. These alterations might result in more rapid conversion of PrP to an altered isoform or lead to a toxic protein fragment following metabolic degradation.

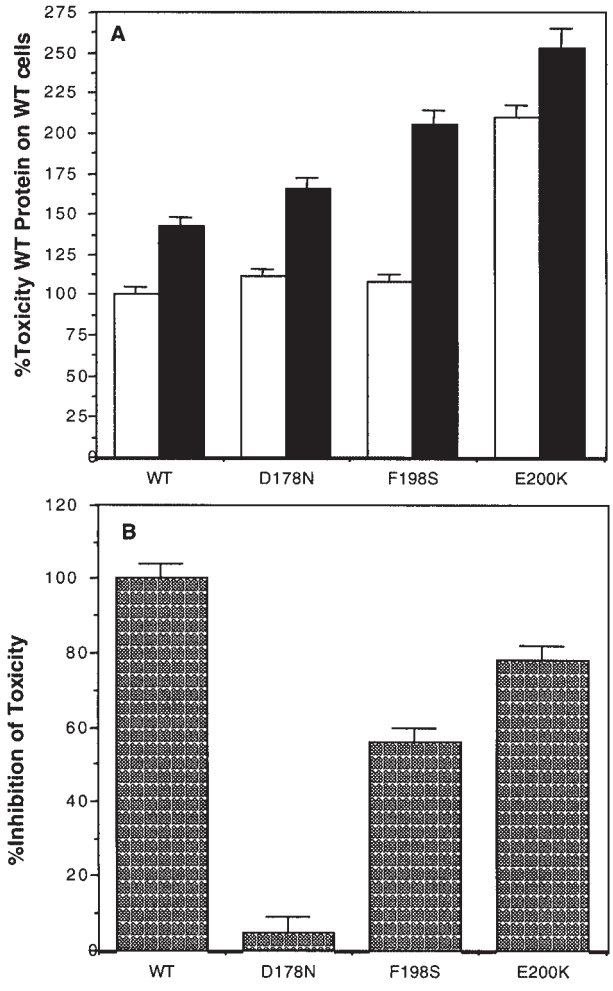

Fig. 3. Toxicity of peptides carrying inherited mutations. **(A)** The toxicity of PrP121-231 either with the wild-type sequence (WT) or with one of the mutations E200K, F198S, or D178N was determined. The peptides were applied at 50 μ*M* to cerebellar neurones in serum-free medium. The cerebellar cells were either derived from wild-type mice (light bars) or mice knocked-out for prion protein expression (dark bars). Survival was determined using an MTT assay. **(B)** 50 μ*M* of a peptide PrP106-126 was applied in parallel with the 50 μ*M* of the proteins shown in (A) above. An MTT assay was used to measure the toxicity of the treatment. The ability of the peptides to inhibit the toxicity of the PrP121-231 proteins was measured.

Cellular Transport

Most studies of topology in relation to the plasma membrane have indicated that the prion protein is linked to the membrane by a glycosylphosphatidylinositol (GPl) anchor *(78–81)*. However, some studies have suggested that in in vitro translation systems PrP

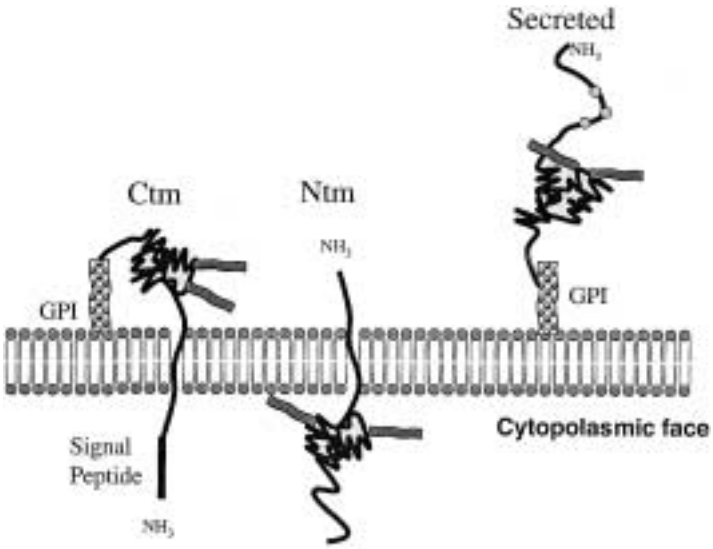

Fig. 4. Schematic of transmembrane forms of PrP. Shown are the orientations of the three theoretical forms of PrP in terms of orientation to the plasma membrane. The three forms are the secreted form anchored to the outside of the cell by a GPI anchor, and the Ctm and Ntm form that are both transmembrane and differ in their orientation of insertion into the membrane.

can be generated both as a secreted form and a transmembrane form (Fig. 4). A stop transfer element (STE) within the protein sequence has been proposed to regulate this activity. Analysis of protein incorporated in mycelles mixed with in vitro translation systems has suggested that the protein can end up in two orientations in the membrane either with the N-terminus in the cytsolic compartment (Ctm) or the C-terminus in the cytosolic compartment (Ntm) of the cell *(82)*. In normal cells the percentage of total PrP that would be in either of these forms would be negligible. Analysis of mutant forms of the PrP have suggested that point mutations increase the percentage of Ctm-PrP expressed by cells. In particular the A117V mutation appears to induce Ctm-PrP. The A117V mutation is one of the rarer forms of GSS and in contrast to other forms there appears to be no PrP^{Sc} deposition and the disease cannot be transmitted to rodents *(20,21)*. When this point mutation is transgenically expressed in mice a percentage of the protein is expressed in the Ctm form. Another double point mutation termed KH→II converts all PrP to Ctm-PrP. Mice expressing PrP with mutation develop a spontaneous disease that does not show signs of PrP deposition *(82)*. It has been suggested that Ctm-PrP also develops in noninherited forms of prion disease but this finding is controversial and the suggestion that Ctm-PrP is the cause of neurodegeneration in prion diseases *(83)* in general is interesting but is unsubstantiated at present. Therefore although transmembrane forms of PrP exist, they do not appear to play a major role in disease.

Investigation of the possible involvement of transmembrane forms of PrP in prion disease have moved to studies of protein expression in transfected cells. The general finding of these studies is that mutation in or near the proposed transmembrane domain induce formation of Ctm-PrP, but other point mutations elsewhere in the protein do not induce an increase in transmembrane forms of PrP *(84)*.

The use of transfected cells expressing fusion proteins composed of GFP linked to PrP has allowed analysis of cellular localization of PrP^{c} (Fig. 5). Although studies show that GFP-

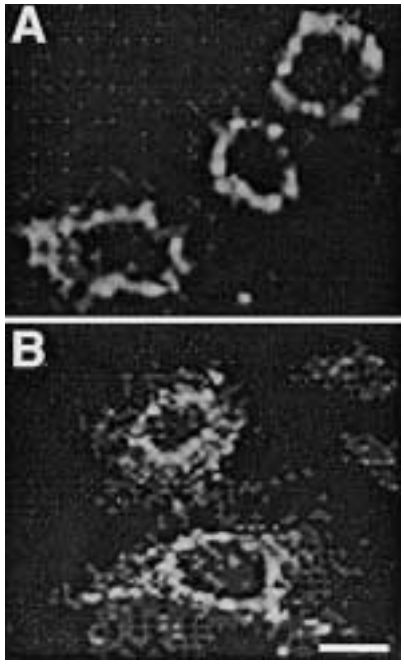

Fig. 5. Inherited mutations alter the cellular localization of PrP. Neuroblastoma cells were transfected with a construct to express the prion protein as a fusion with the green fluorescent protein. Cells were also transfected with a mutagenized construct carrying the KH→II mutation (*see* text). Stable transfected cells were analyzed via confocal microscopy. The wild-type protein is expressed predominantly at the cell surface **(A).** However, the KH→II mutant was detected predominatly within the cell, suggesting that the mutant protein remains trapped within the endoplasmic reticulum and golgi and does not reach the cell surface. Scale Bar = 10 μm.

PrP^c is localized on the cell surface, golgi, or endoiplamic reticulum (ER), some labeled protein can be detected in the cytoplasm *(85)*. GSS or fCJD associated mutations appear to cause a redistribution of labeled protein away from the cell surface. There appears to be increased retention of protein in the ER especially with the D178N, F198S, and a nine octameric-repeat expansion. This does not appear to be the case for protein with the equivalent of the E200K mutation, although there is some dispute about this and it may depend on the cell type used *(86)*. These results suggest that mutations in PrP could have their effect by altering protein trafficking of PrP *(85)*.

The rate of turnover of PrP^c at the membrane of cells is regulated by the extracellular copper concentration, possibly by interaction of the copper with the copper-binding domain in the N-terminus of the protein *(87)*. Expansion of the octameric-repeat region to mimic the expansion mutation that is found in fCJD inhibits the ability of copper to enhance cellular turnover of PrP^c *(88)*. Similarly deletion of the copper-binding domain entirely also inhibits this copper-enhanced turnover *(89)*. This suggests that the expansion mutation might inhibit the normal copper-dependent activity of PrP^c.

Investigation of membrane attachment of PrP in cultured cells has shown that when converted to PrP^{Sc} the protein cannot be released from the cells by cleaving the GPI anchor *(90)*. This effect is not due to the protein taking up a transmembrane of sequestrated orientation with regards to the membrane as it can be labeled and degraded with proteases *(91)*. Denaturation of PrP^{Sc} leads to a cleavable GPI anchor, suggesting this altered membrane association has something to do with the altered structure of the protein rather than modification of the anchor. Similarly, point mutations also effect membrane anchoring of PrP. In particular, P102L, D178N, and F198S alter protein association with membrane *(48,49)*. The E200K seems to only have a minor effect in this regard *(50)*, but then again it has also been suggested that the E200K mutant shows different trafficking and the majority of protein does not reach the cell surface *(86)*. Similarly, the expansion mutation of the octameric repeats also causes abnormal membrane association *(92)*.

Future Prospects

The advance in understanding the molecular nature of inherited prion diseases has focused on changes in the prion protein either in terms of conformation, location in cells, molecular

breakdown, or toxicity. Unfortunately at present none of these findings can explain why it takes so long for patients to show clinical signs of the disease if they have the mutant gene from birth. The best explanation for this is that the mutant forms of the protein are somehow less efficient at interacting with chaperones that might help prevent conformational changes leading to abnormal structure. Alternatively, as has been proposed for sporadic forms of the disease, there is a kinetic barrier that normally prevents protein conversion. Following a probabilistic scenario, eventually, sufficient free energy is trapped by a molecule of PrP for the barrier to be overcome and this leads to conformational change. The altered molecule can then interact with more PrP molecules, creating the domino effect of protein conversion that has now been reproduced in vitro *(93)* resulting in large amounts of abnormal protein. The effects of the mutations may act to reduce the amount of free energy required to overcome this kinetic barrier. Nevertheless the barrier is sufficiently high that the probability is still low that conversion will occur. The one problem with this is that if that were the case the number of people with GSS or CJD would increase with age instead of being tightly clustered in the 50 and 60s age groups. It is probably more likely that another factor still to be identified that is age-dependent and initiates conversion.

The current understanding of the mechanism of conversion of PrP to an abnormal form, induced by inherited mutations, is summarized in Table 1. As can be seen, there is currently no single explanation for the effects of all the mutations. It is quite possible that different mutations will have different mechanisms of action or that single mutations will have their effects through multiple pathways.

Understanding the mechanisms by which mutations cause protein conversion is important. Clues to the destabilizing nature of such mutations might point to a general mechanism that would also be valid for other forms of prion disease. A technique that could inhibit or reverse such conversion would be of value in treating all forms of these diseases. The tools and techniques that are currently being used to investigate these techniques are likely to provide such valuable advances in the near future.

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