Role of PrP in prion diseases

C Weissmann, H Büeler, A Sailer,
M Fischer, M Aguet,
Institut für Molekularbiologie I, Universität Zürich, Zürich, Switzerland
A Aguzzi
Institut für Neuropathologie, Universitätsspital, Zürich, Switzerland

Scrapie, the prototype of a group of diseases designated as transmissible spongiform encephalopathies (TSEs) or prion diseases, is a naturally occurring affliction of sheep which was recognized more than 250 years ago. Characteristically, affected animals present with twitching, excitability, intense itching and finally paralysis and death. Transmission of the disease by inoculation of healthy sheep and goats with lumbar cord of diseased animals was first demonstrated in 1936[1] and soon confirmed by Gordon.[2] This led to the recognition of the unusual properties of the pathogenic agent (later designated as 'prion'[3], such as the extremely long incubation periods, exceeding 1 year, and resistance to high temperatures, formaldehyde treatment and UV irradiation.[2,4,5]

In a separate development, Creutzfeld-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS) and kuru were characterized as slow degenerative diseases of the central nervous system. The suggestion by Hadlow[6] that these diseases might be the counterpart of scrapie in humans was followed by long-term inoculation studies by Gajdusek and his colleagues which resulted in the transmission, first of kuru[7], then of CJD[8] to chimpanzees. Later, GSS was also transmitted to the chimpanzee[9] and to the mouse.[10] Recently we have witnessed the emergence of a new form of a prion disease, namely bovine spongiform encephalopathy (BSE), which is attributed to the feeding of cattle with meat and bone meal supplements derived from scrapie-contaminated sheep and then cattle offal.[11,12]

The overall properties of the prion differ from those of any known virus or viroid[2–4,13] and early on gave rise to speculations that it might be devoid of both nucleic acid and protein,[14] consist of protein only[15–17] or be a polysaccharide[17a] or a membrane fragment.[18] In this article we review the current hypotheses regarding the pathogenesis of

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Note added in proof: Unheated brain extracts of PrP[0/0] mice 25, 33 and 48 weeks after inoculation with scrapie prions show no scrapie infectivity. Thus, the positive result for the 20-week PrP[0/0] mouse (Table) is likely accidental.

this class of diseases (for recent reviews, *see* Refs 19–24), in particular the so-called 'protein only' hypothesis and the major lines of evidence in its support, and describe experiments carried out in our laboratory demonstrating the essential role of the host gene *Prn-p* in the pathogenesis of scrapie.[25]

THE 'PROTEIN ONLY' HYPOTHESIS

Prusiner has suggested that the prion is devoid of nucleic acid and identical with PrP[Sc], a modified form of PrP[C] (the 'protein only' hypothesis).[26] PrP[C] is a normal host protein[27–29] encoded within a single exon of a single copy gene[30] and is found predominantly on the outer surface of neurons, attached by a glycosyl phosphatidyl inositol anchor[19,26,31,32] but also in a variety of other tissues, both in the embryonic and the adult mouse.[33,34]

PrP[Sc] is defined as a largely protease-resistant form of PrP[C] which readily forms aggregates after treatment with detergents and protease.[27, 35–37] It accumulates intracellularly, in cytoplasmic vesicles[38,39] and is the major component of the extracellular amyloid plaques characteristic for some forms of prion diseases. No chemical differences have so far been detected between PrP[Sc] and PrP[C][19,40,41], however, it must be stressed that the ratio of infectious units to PrP[Sc] molecules is in the order of 1:100 000,[42] so that if the infectious entity were a subspecies of PrP[Sc] or a different modification of PrP altogether a chemical difference between PrP[C] and the infectious subspecies could be analytically undetectable.

Prusiner proposed that PrP[Sc], when introduced into a normal cell, causes the conversion of Prp[C] or its precursor into PrP[Sc][19,27,43,45–46] (Fig. 1A). The nature of the conversion is unknown and could be due to a chemical or conformational modification, during or after its synthesis. However, the existence of many different strains of scrapie which can be propagated in one and the same inbred mouse line, and the apparent mutability of the agent[21–23] are cited in support of the virino hypothesis (Fig. 1B), which holds that the infectious agent consists of a nucleic acid genome and the host-derived PrP, which is recruited as some sort of coat.[27,48] No credible evidence for such a nucleic acid has yet been forthcoming.[22,43,44,48] Finally, the possibility that the infectious agent is a virus with unusual properties is still upheld by some.[24,49]
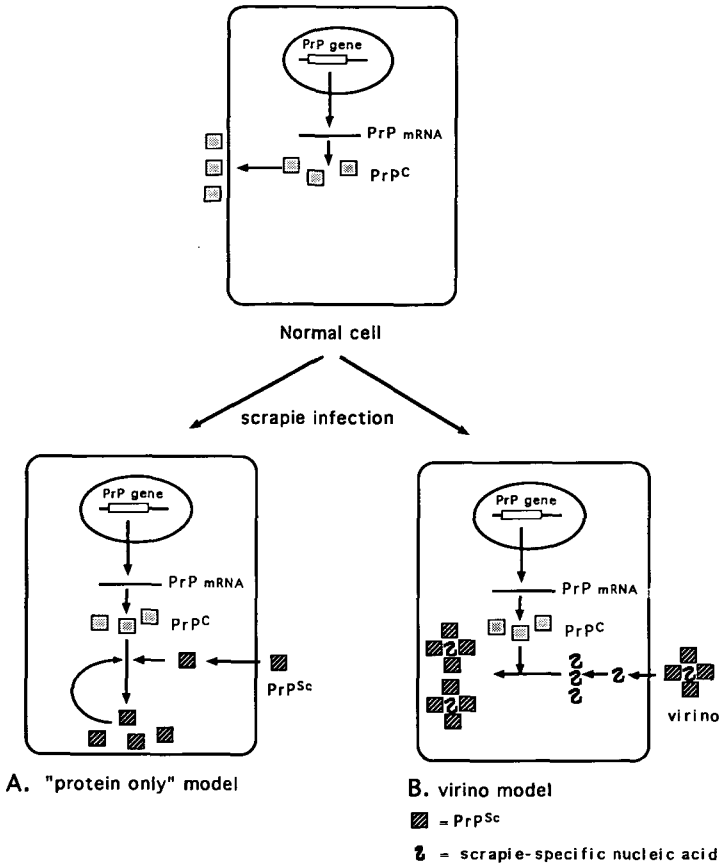
Fig. 1 Models for the propagation of the scrapie agent (prion). (A) The 'protein only' model assumes that the prion is identical with PrP^{Sc}. Exogenous PrP^{Sc} causes the conversion of the normal cellular protein PrP^C into PrP^{Sc}. (B) The 'virino hypothesis' assumes that the infectious agent consists of a scrapie-specific nucleic acid associated with or packaged in PrP^{Sc}. The 'scrapie specific nucleic acid' (to date never found) is replicated in the cell and recruits PrP^C into association with it. Strain specificity would be mediated by the nucleic acid.

PREVIOUS EVIDENCE BEARING ON THE RELATIONSHIP OF PrP TO TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

Scrapie infectivity is associated with PrP^{Sc}

Purification of scrapie infectivity results in a preparation highly enriched with regard to PrP^{Sc}.[35,50,51] Conversely, purification of PrP^{Sc} by affinity chromatography on an anti-PrP antibody column leads to enrichment of infectivity.[52]

A nucleic acid larger than about 100 nucleotides is not essential for infectivity of scrapie prion preparations

This claim is based on: (a) the unusually small target size of scrapie infectivity for UV and ionizing radiation[16,53,54]; (b) the low ratio of nucleic acids to infectious units in highly purified prion preparations[44] and the failure to find scrapie-specific nucleic acid in prion preparations or scrapie-infected brain tissue[22,43,55]; (c) resistance of infectivity to treatment with agents modifying or damaging nucleic acids.[3] Collectively, these data suggest that a nucleic acid of more than 50–100 nucleotides is not required for infectivity (but *see* Ref. 56 for a different conclusion).

The susceptibility of a host to scrapie infection is co-determined by the prion inoculum and the PrP gene

The significance of the host PrP genotype for the susceptibility to scrapie infection and the course of the disease is revealed by two sets of findings. First, the incubation time for one and the same prion isolate may be different in distinct mouse strains, and is determined predominantly by *Sinc*[57,58] or *Prn*-i[59,60] which is very closely linked to or coincident with *Prn-p*, the gene encoding PrP.[59–63] Second, when prions are transmitted from one animal species to another, disease often develops only after a very long incubation period, if at all. However, upon serial passaging in the new species, the incubation time may decrease dramatically and then stabilize. At least in the case of hamster and mouse, this so-called species barrier[64] can be overcome by introducing into the recipient host the PrP transgene from the prion donor.[45,65] Moreover, prion preparations from mice carrying hamster PrP-transgenes and inoculated with hamster scrapie prions are highly infectious to the hamster but not to the mouse. The same transgenic mouse strain, infected with mouse-derived prions, yields preparations highly infectious for mice but not for hamsters.[45] Within the framework of the 'protein only' hypothesis this means that hamster PrP[C], but not murine PrP[C], is a suitable substrate for conversion to hamster PrP[Sc] by hamster prions and vice versa.

Hereditary forms of spongiform encephalopathies are linked to mutations of the PrP gene

The human prion diseases, Creutzfeldt-Jakob (CJD) and Gerstmann-Sträussler-Scheinker disease (GSS), are very rare in the overall population, but also occur as a familial form.[8,9,66,67] Hsiao et al[68] found that in two apparently unrelated GSS families the disease is tightly linked to a proline-to-leucine change in codon 102 of one of the *Prn-p* alleles. Subsequently other GSS and CJD families were identified, carrying the

102 mutation or one of a number of other mutations in the PrP gene (for a review see Ref. 69). Prusiner[26] proposed that the mutations allow spontaneous conversion of PrP[C] into PrP[Sc] with a frequency sufficient to allow the disease to be expressed within the lifetime of the individual. Sporadic CJD would be attributable to a somatic mutation in the *Prn-p* gene or to rare instances of spontaneous conversion of PrP[C] into PrP[Sc] (Fig. 2A).

Hsiao et al.[70] showed that mice carrying a murine PrP transgene with the pro→leu mutation corresponding to the human GSS mutation at position 102 spontaneously come down with a lethal scrapie-like disease. However, it has not yet been reported whether or not the brains of these animals contain prions.

SUSCEPTIBILITY TO SCRAPIE OF MICE DEVOID OF PrP[C]

The 'protein only' hypothesis predicts that in the absence of PrP[C], mice should be resistant to scrapie infection, both with regard to symptoms and to propagation of the infectious agent.

Generation and properties of *Prn-p*[0/0] mice

Mice devoid of PrP[71] were generated by the general approach described by others.[72–75] In short, one *Prn-p* allele of murine embryonic stem (ES) cells was disrupted (Fig. 2A) by homologous recombination with a 4.8 kb DNA fragment in which codons 4–187 of the 254-codon open reading frame, which is located within a single exon, were replaced by a neomycin phosphotransferase (*neo*) gene under the control of the HSV TK promoter (Fig. 2B). In the resulting construction (Fig. 2C) the first 3 *Prn-p* codons, the *neo* encoding sequence and the residual 67 *Prn-p* codons were fused in frame, with one nonsense codon interposed between the initial *Prn-p* codons and the *neo* sequence and two nonsense codons between the latter and the residual *Prn-p* sequence (Fig. 2D). Thus, the synthesis of PrP or any fragment thereof is precluded. Blastocysts from black mice were injected with *agouti* ES cells carrying the disrupted *Prn-p* gene and implanted into foster mothers. Chimeric males (showing *agouti* patches) were mated with wild type black mice and *agouti* offspring carrying the disrupted gene were identified by PCR analysis. *Prn-p*[0/+] heterozygotes were mated and 176 superficially indistinguishable offspring analyzed by PCR. Of these, 24% were homozygous for the disrupted *Prn-p* gene.

As shown by Northern analysis, normal PrP mRNA was not detectable in brain from *Prn-p*[0/0] homozygotes, however substantial quantities of a fused mRNA containing the *neo* and the residual *Prn-p* sequence were present. Western analysis of brain proteins showed that
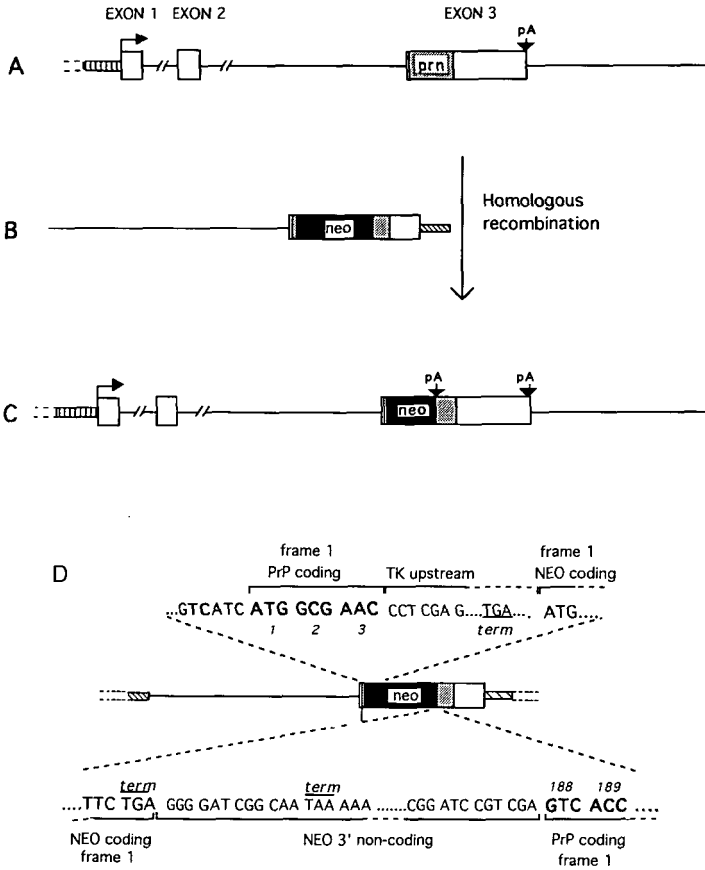
Fig. 2 Construction of the PrP targeting vector. (A) Map of the murine PrP gene.[88] (B) The targeting vector was constructed by replacing 552 bp of the *Prn-p* coding region (extending from position 10–562) by a 1.1 kb cassette containing the HSV thymidine kinase promoter followed by the *neo* gene. (C) The disrupted PrP gene. (D) The first 3 *Prn-p* codons, the *neo* coding sequence and the residual 67 *Prn-p* codons were fused in frame, with one nonsense codon interposed between the initial *Prn-p* codons and the *neo* sequence and two nonsense codons between the latter and the residual *Prn-p* sequence.

PrP was undetectable in *Prn-p[0/0]* samples and present at about half the normal level in *Prn-p[0/+]* samples.

No gross abnormalities were noted in *Prn-p[0/0]* mice at the macroscopic or microscopic level, in particular of the brain, skeletal muscle and visceral organs.[71,76] Disruption of the *Prn-p* gene had no detectable effect on the normal maturation of the lymphocyte subsets. No significant difference in the response of splenocytes from *Prn-p[0/0]* and *Prn-p^{+/+}* mice to activation by concanavalin A was detected.[71]

Because PrP^C is a predominantly neuronal protein and present in a high proportion of hippocampal neurons, the learning ability of Prn-$p^{0/0}$, Prn-$p^{+/+}$ and Prn-$p^{0/+}$ mice, all derived from the mating of the first generation of heterozygotes, was compared. Three tests, the **swimming navigation test**,[77] the **Y-maze discrimination** test[78] and the **two-way avoidance (shuttlebox)** test[79] revealed no differences between the three groups of mice.

Homozygous Prn-$p^{0/0}$ mice are fertile and normal progeny result from homozygous Prn-$p^{0/0}$ breeding pairs. No abnormalities of homozygotes were recorded during 18 months of observation. It is surprising that a protein expressed in many areas of the brain and in other tissues and whose gene has been found in mammals, birds and fish can be ablated without apparent detrimental effects. It is possible that its function is too subtle to be detected under laboratory conditions, or that it can be replaced by some other gene product(s), particularly if adaptive processes are facilitated during development.

Challenge of Prn-$p^{0/0}$ and Prn-$p^{0/+}$ mice with scrapie prions

Mice devoid of PrP were challenged with mouse prions and proved to be completely protected against scrapie disease, at least up to 14 months after inoculation (Fig. 3). Moreover, even heterozygous Prn-$p^{0/+}$ mice proved to be partially protected, inasmuch as scrapie-inoculated animals showed signs of scrapie only **253-337** days after inoculation but are still alive after 380 days, while all Prn-$p^{+/+}$ controls died within about 180 days. Moreover, disease progression in Prn-$p^{0/+}$ mice is distinctly slower than in Prn-$p^{+/+}$ mice, the interval between first symptoms and death being 13 days in the case of Prn-$p^{+/+}$ mice while no Prn-$p^{0/+}$ mice have died to date, about 3 months after the appearance of scrapie symptoms (Büeler and Weissmann, unpublished results).

We conclude that development of scrapie symptoms and pathology is strictly dependent on the presence of PrP and that incubation time and disease progression are inversely related to the level of PrP. It has previously been found that the length of the scrapie incubation time for hamster-derived prions in mice expressing SHaPrP genes was inversely related to the level of SHaPrP.[45]

Are prions propagated in Prn-$p^{0/0}$ mice?

It was shown that if infectious agent is propagated in Prn-$p^{0/0}$ mice, this would be at a level about five orders of magnitude lower than that in Prn-$p^{+/+}$ animals.[25] Scrapie prion titers were determined by preparing tissue extracts at various times after inoculation, heating them for 20 min at 80°C to destroy any adventitious conventional pathogens and inoculating them into wild type indicator mice. In scrapie-
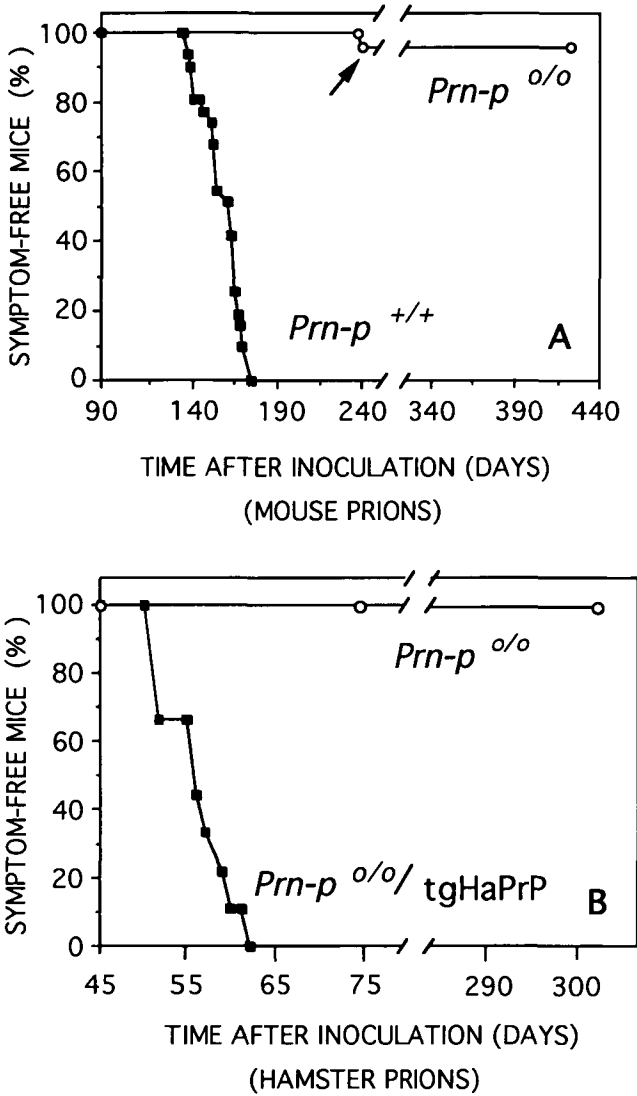
Fig. 3 Susceptibility of mice with various PrP genotypes to inoculation with scrapie prions. Mice were inoculated intracerebrally and the percentage of mice remaining free of scrapie symptoms was plotted as a function of time. (A) *Prn-p⁰ᐟ⁰* and *Prn-p⁺ᐟ⁺* mice were inoculated with mouse prions. One *Prn-p⁰ᐟ⁰* mouse (arrow) showed ataxic gait and was sacrificed at 240 days: it showed no histopathological changes typical for scrapie and brain homogenate from this mouse did not transmit scrapie to CD-1 indicator mice after more than 170 days. (B) *Prn-p⁰ᐟ⁰* and *Prn-p⁰ᐟ⁰*/tgHaPrP mice (*Prn-p⁰ᐟ⁰* mice carrying Syrian hamster transgenes) were inoculated with hamster scrapie prions.

inoculated *Prn-p⁺ᐟ⁺* animals, infectious agent was detected in the brain

at 8 weeks and increased to about 8.6 log LD_{50} units/ml by 20 weeks after inoculation (Table). No infectious agent was detected in $Prn-p^{0/0}$ animals 25 weeks after inoculation, the latest time point for which data are presently available. However, in a parallel assay using unheated samples, a low level of infectivity, about 3.2 log LD_{50} units/ml, was found in the 20 week sample from $Prn-p^{0/0}$ animals. Because only one $Prn-p^{0/0}$ brain sample in the entire experiment was positive, accidental contamination of the homogenate or infectivity due to residual traces of inoculum in the occasional mouse cannot be excluded. However, the possibility must be considered that the infectious agent is something other than PrP^{Sc}, and that in the absence of PrP^{Sc} in the host it is much less infectious than in its presence. This question can only be resolved by further experiments, which are now underway.

Table   Prion titers in brain and spleen of $Prn-p^{+/+}$ and $Prn-p^{0/0}$ mice[a]

Time after inoculation	Brain (heated)		Brain (not heated)		Spleen (heated)	
	$PrP^{+/+}$	$PrP^{0/0}$	$PrP^{+/+}$	$PrP^{0/0}$	$PrP^{+/+}$	$PrP^{0/0}$
4 days	< 1.5	2.0[b]	n.d.	n.d.	5.7 ± 0.9	2.3[c]
2 weeks	< 1.5	< 1.5	n.d.	n.d.	6.2 ± 0.8	< 1.5
8 weeks	5.4	< 1.5	7.7 ± 0.6	< 1.5	6.9 ± 1.0	< 1.5
12 weeks	6.8	< 1.5	7.1 ± 1.6	< 1.5	5.9 ± 0.6	< 1.5
20 weeks	8.6	< 1.5	7.7 ± 1.1	3.2 ± 1.4[d]	6.9 ± 0.6	< 1.5
23/25 weeks	8.1 ± 0.8	< 1.5	n.d.	n.d.	n.d.	< 1.5

[a]The samples designated 'heated' were kept 20 min at 80°C prior to inoculation. The titers of brain homogenates recovered at 8, 12 and 20 weeks after inoculation and of the Chandler-derived mouse prion inoculum 'RML' (8.5 log LD_{50} units/ml) were determined by end point dilution. The titers of the other samples were determined by the incubation time assay (Ref. 25) [b]1/6 mice died after inoculation with 10^{-1} diluted homogenate. [c]2/6 mice died after inoculation with 10^{-1} diluted homogenate [d]6/6 mice died after inoculation with 10^{-1} diluted homogenate

Restoration of susceptibility to scrapie by introduction of PrP transgenes.

When ablation of a gene product gives rise to a specific phenotype, in this case resistance to scrapie, it is desirable to demonstrate that reinstatement of the gene restores the original phenotype. Indeed, insertion of mouse PrP genes rendered $Prn-p^{0/0}$ mice susceptible to mouse prions (Fischer and Weissmann, unpublished data). More interestingly, introduction of hamster PrP transgenes into $Prn-p^{0/0}$ mice rendered them very susceptible to hamster-derived prions (56 ± 3 days incubation time) but much less so (303 ± 19 days) to mouse-derived prions, demonstrating the requirement of a homotypic relationship between incoming

prion and resident PrP protein for prion propagation and development of pathology, as foreshadowed by the results of Prusiner et al.[45]

IMPLICATIONS AND OUTLOOK

Both the biochemical and genetic data discussed in this review support the proposal that the prion is composed partly or entirely of a PrP isoform (either PrP^{Sc} or a subfraction of it), and that protein-encoding nucleic acid is not an essential component. Other explanations of the data, such as that PrP^{C} is or contributes to a receptor for the scrapie agent, while less likely, are not yet formally excluded.

How is PrP^{C} converted to PrP^{Sc}?

No chemical differences between PrP^{C} and PrP^{Sc} have been detected, however, as pointed out above, the ratio of infectious units to PrP^{Sc} molecules is about $1:10^5$. Therefore, chemical analyses may not bear on the structure of a conjectural minor component responsible for the infectivity. Nonetheless, this minor component is likely PrP^{C}-derived, as indicated by the genetic evidence, and is as resistant to proteinase digestion as PrP^{Sc}.[80]

Prusiner postulated that the difference between PrP^{C} and PrP^{Sc} is conformational. In Figure 4A it is suggested that a molecule of PrP^{Sc} binds to PrP^{C} and thereby imposes its conformation upon it. The species barrier is explained by the assumption that heterologous PrP species interact poorly and/or that the conversion only occurs rarely. Mutations of the Gerstmann-Sträussler and CJD type would allow spontaneous, albeit very rare conversion events, yielding PrP^{Sc} that can then act catalytically (Fig. 4B). Sporadic cases of CJD could be attributed to even rarer cases of spontaneous conversion of wild type PrP^{C} or of a somatically mutated PrP^{C}.

It has so far not been possible to denature infectious preparations containing PrP^{Sc} and renature them to regain infectivity and/or protease resistance[81] contrary to previous reports,[82,83] nor has it been possible to convert PrP^{C} into PrP^{Sc} in a cell free system.[84] In addition, PrP^{C} does not, or only in the rarest of cases, spontaneously convert to PrP^{Sc} in vivo, despite the inordinate stability of PrP^{Sc}. To explain these findings within the framework of the 'protein only' model, Figure 4C suggests that the PrP^{Sc} state is separated from that of PrP^{C} and of a random coil by very high activation energy barriers, while the PrP state is separated from the random coil state by a far lower barrier. Thus, the only way PrP^{C} can convert to PrP^{Sc} is by 'tunnelling' through the activation energy barrier by a catalyzed, probably energy-dependent process which might involve chaperonins.

THE "PROTEIN ONLY" HYPOTHESIS"
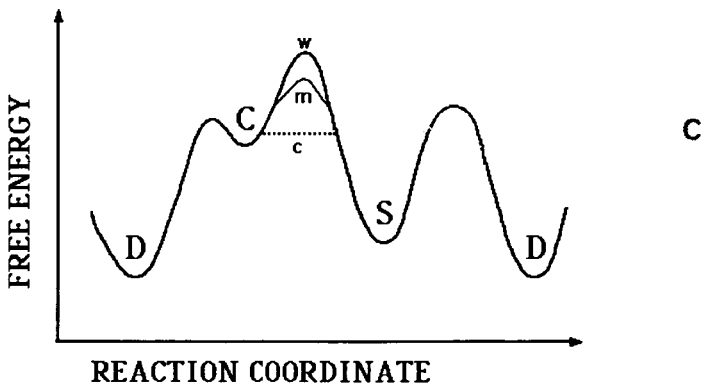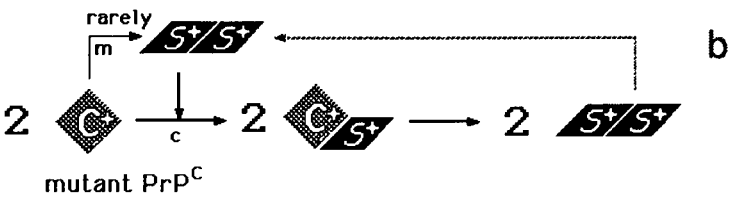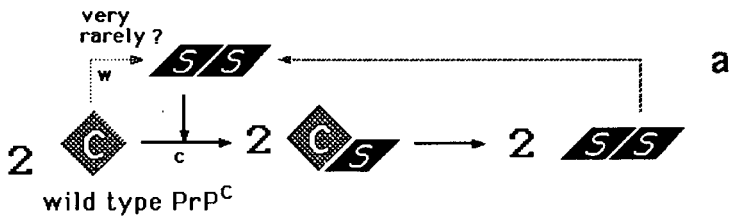
Fig. 4 Model for the catalyzed conformational conversion of PrP^C to an infectious PrP form. (A) Following a proposal by Prusiner[45] PrP^C (C) is converted to the conformer PrP^{Sc} (S) via dimers formed with exogenously introduced PrP^{Sc}. This results in an exponential cascade of conversion. Sporadic forms of prion disease (such as sporadic CJD) may come about when an extremely rare event (w) leads to spontaneous conversion of PrP^C to PrP^{Sc} and gives rise to a conversion cascade. (B) In the case of certain mutations in PrP^C (C+) spontaneous conversion (m) to PrP^{Sc} may occur about a million times more frequently than in the case of the wild type protein, but still remains a rare event, explaining why familial forms of prion diseases arise only late in life. (C) Postulated energy diagram for different forms of PrP. The PrP^C conformation (C) is separated from the PrP^{Sc} conformation (S) by a high activation energy barrier, and from the denatured state (D) by a low activation energy barrier. Thus a spontaneous conformational change from PrP^C to PrP^{Sc} (pathway w) will be extremely rare. Interaction of PrP^C with PrP^{Sc} facilitates the conversion (pathway c) by lowering the activation energy for the transition. In the case of certain mutations in PrP^C the activation energy for the conversion (pathway m) is lowered, increasing the probability of the spontaneous conformational change to PrP^{Sc}.

Scrapie strains

As mentioned above, the finding that there are many distinct strains of scrapie prions which can be propagated in one and the same mouse strain (homozygous with regard to its PrP gene) is not readily explained by the 'protein only' hypothesis (for a review, see Ref 23) because it implies that an incoming PrP^{Sc} strain can convert one and the same PrP precursor into a likeness of itself, and that this can happen for several if not many different strains. Two subsidiary hypotheses have been suggested to circumvent this difficulty. The 'unified theory'[85] proposes that PrP^{Sc} is associated with a small host-derived nucleic acid which is not required for infectivity but determines the characteristic phenotype of the strain. This nucleic acid would be replicated by host cell enzymes and then associate with newly formed PrP^{Sc} leading to preservation of the prion's phenotype. The 'targeting theory'[86] (and KH Meyer, personal communication 1991) proposes that PrP^{Sc} carries a variable modification, for example carbohydrate residues, which target it to a specific subset of cells. These cells would impart the same modification to the newly formed PrP^{Sc} molecules. Different strains would thus be targeted to different subsets of cells and retain their specific modification (Fig. 5). This hypothesis is supported by the observation that different hamster prion strains[86] or mouse prion strains[87] give rise to different patterns of PrP^{Sc} deposition in the brain. It predicts that if two different prion strains are propagated through a singular cellular species they should emerge with identical properties.

PRACTICAL IMPLICATIONS OF PRION RESEARCH

The results of Büeler et al[25,71] show that it is possible to generate normal mice which are resistant to scrapie by knocking out their PrP genes.
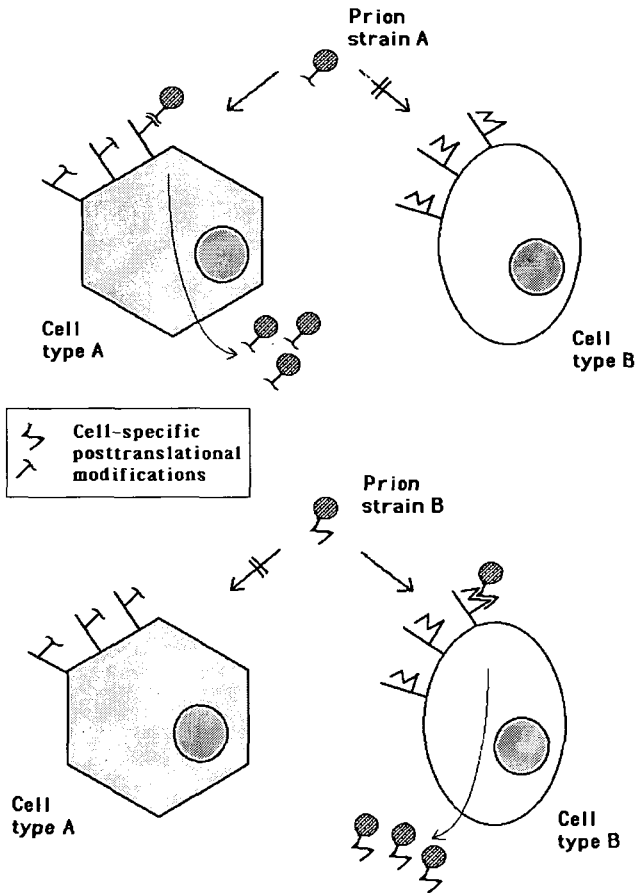
Fig. 5 The 'targeting model' to explain prion strain specificity. It is assumed that strain specificity of the prion is imparted by a cell specific post-translational modification which targets the prion to the same type of cell as the one in which it was synthesized. A possible modification would be glycosylation.

In principle it should thus be possible to breed sheep or cattle resistant to this disease, either by PrP gene disruption or by the introduction of transgenes expressing PrP antisense RNA. Moreover, the fact that *Prn-p^{0/+}* heterozygous mice show much longer scrapie incubation times than their wild type counterparts argues that disease progression may be rate-limited by the PrP^C concentration. This conclusion is consistent with the observation that in several mouse lines containing hamster PrP transgenes the incubation time for hamster prion-induced scrapie is a function of the hamster PrP expression level.[45] A practical implication of this conclusion is that a moderate reduction of PrP^C synthesis, such

as might eventually be achieved by antisense oligonucleotide therapy, could substantially mitigate disease progression in incipient cases of spongiform encephalopathies.

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