Transmissible and genetic prion diseases share a common pathway of neurodegeneration

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Prion diseases can be infectious, sporadic and genetic[1–4]. The infectious forms of these diseases, including bovine spongiform encephalopathy and Creutzfeldt-Jakob disease, are usually characterized by the accumulation in the brain of the transmissible pathogen, an abnormally folded isoform of the prion protein (PrP) termed PrP^{Sc}. However, certain inherited PrP mutations appear to cause neurodegeneration in the absence of PrP^{Sc} (refs 5–8), working instead by favoured synthesis of ^{Ctm}PrP, a transmembrane form of PrP (ref. 9). The relationship between the neurodegeneration seen in transmissible prion diseases involving PrP^{Sc} and that associated with ^{Ctm}PrP has remained unclear. Here we find that the effectiveness of accumulated PrP^{Sc} in causing neurodegenerative disease depends upon the predilection of host-encoded PrP to be made in the ^{Ctm}PrP form. Furthermore, the time course of PrP^{Sc} accumulation in transmissible prion disease is followed closely by increased generation of ^{Ctm}PrP. Thus, the accumulation of PrP^{Sc} appears to modulate *in trans* the events involved in generating or metabolising ^{Ctm}PrP. Together, these data suggest that the events of ^{Ctm}PrP-mediated neurodegeneration may represent a common step in the pathogenesis of genetic and infectious prion diseases.

We have previously shown that certain mutations in PrP (including the A117V mutation associated with human prion disease) alter its biogenesis at the endoplasmic reticulum, causing a higher percentage of PrP molecules to be synthesized in a transmembrane form that we termed ^{Ctm}PrP (ref. 9). Expression of ^{Ctm}PrP-favouring mutations in transgenic mice resulted in neurodegenerative changes similar to those observed in prion disease[9]. The detection of ^{Ctm}PrP (but not PrP^{Sc}) in the brains of human patients and in transgenic mice carrying ^{Ctm}PrP-favouring mutations suggested that elevated ^{Ctm}PrP causes neurodegeneration[9], but whether this mechanism of neurodegeneration is also involved in the pathogenesis of transmissible prion disease has been unclear. To explore this question we first generated mice with mutant PrP transgenes differing in their propensity to form ^{Ctm}PrP, and subsequently assessed their susceptibility to PrP^{Sc}-induced neurodegeneration.

Figure 1a shows the *in vitro* translocation products of four mutants of Syrian hamster prion protein (SHaPrP) that alter the amount of ^{Ctm}PrP synthesized at the endoplasmic reticulum. Transgenic (Tg) mice expressing each of these mutant PrPs in the FVB/$Pmp^{0/0}$ background (null for the endogenous PrP gene) were generated and characterized (see Table 1 and Fig. 1b). The Tg[SHaPrP(KH → II)$_H$], Tg[SHaPrP(KH → II)$_M$], Tg[SHaPrP(A117V)$_H$] and Tg[SHaPrP(N108I)$_H$] mice were observed to develop signs and symptoms of neurodegenerative disease at about 60, 472, 572 and 312 days, respectively (Fig. 1c and Table 1). By contrast, neither the Tg[SHaPrP(ΔSTE)] mice nor mice expressing lower levels of the disease-associated transgenes, Tg[SHaPrP(KH → II)$_L$], Tg[SHaPrP(A117V)$_L$] and Tg[SHaPrP(N108I)$_L$], developed spontaneous disease (Table 1). Biochemical

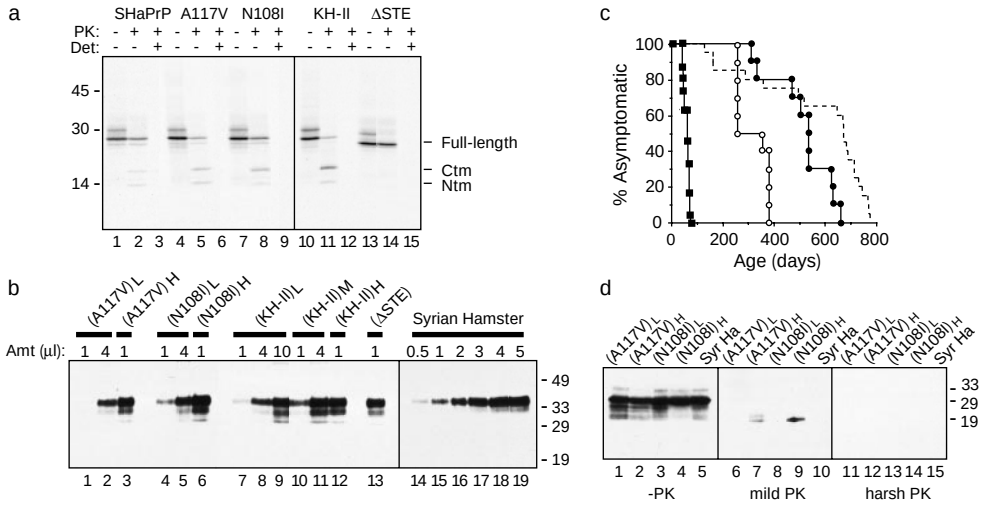

Figure 1 Dose response of ^{Ctm}PrP-induced neurodegeneration. **a**, Topology of wild-type and mutant PrP molecules at the endoplasmic reticulum. *In vitro* synthesized transcript coding for each PrP construct (indicated above the gels) was used to programme a rabbit reticulocyte lysate cell-free translation reaction containing microsomal membranes and a competitive peptide inhibitor of glycosylation. Following translation, samples were either left untreated or digested with PK in the absence or presence of 0.5% Triton X-100 ('Det') as indicated above the gel. The positions of the full-length PrP molecule, the amino-terminal fragment (indicative of ^{Ntm}PrP) and the carboxy-terminal fragment (indicative of ^{Ctm}PrP) generated by PK digestion are marked on the right. **b**, Level of expression of various transgenic mouse lines. Varying amounts (in microlitres, indicated above the lanes) of 10% brain homogenate from each transgenic mouse were immunoblotted for PrP with the 13A5 monoclonal antibody and compared to a titration of Syrian hamster brain homogenate. **c**, Time course of development of symptoms in Tg[SHaPrP(A117V)$_H$] mice (closed circles) and Tg[SHaPrP(N108I)$_H$] mice (open circles). Non-transgenic control animals (dashed line) and Tg[SHaPrP(KH → II)$_H$] mice (closed squares; data from ref. 9) are shown for comparison. **d**, Analysis of various transgenic mice and Syrian hamsters (as indicated above the gel) for ^{Ctm}PrP and PrP^{Sc} as described in Methods. Tg[SHaPrP(A117V)$_H$] and Tg[SHaPrP(N108I)$_H$] samples were from clinically ill mice, and Tg[SHaPrP(A117V)$_L$] and Tg[SHaPrP(N108I)$_L$] samples were from mice (which showed no signs of disease) aged at least 600 days. The fragment of PrP resistant to PK under the 'mild' conditions, indicative of ^{Ctm}PrP, is only seen in the Tg[SHaPrP(A117V)$_H$] and Tg[SHaPrP(N108I)$_H$] samples. No PK resistant PrP^{Sc} was observed in any of the samples.

analyses of brain tissue from each of these lines of transgenic mice revealed elevated ^{Ctm}PrP, but no PrP^{Sc}, in the lines which developed disease (Fig. 1d). Together, the data in Fig. 1 recapitulate the point that increased synthesis of the ^{Ctm}PrP form of PrP is associated with the development of neurodegenerative diseases.

More remarkable is the apparent dose response relationship, seen in two ways, between ^{Ctm}PrP and disease. First, the more strongly ^{Ctm}PrP synthesis is favoured at the endoplasmic reticulum (KH → II > N108I > A117V), the earlier is the onset of spontaneous disease (Table 1). Second, lowering the level of expression of each of these mutations below an apparent threshold stops both the generation of ^{Ctm}PrP (Fig. 1d and ref. 9) and development of disease (Table 1). Furthermore, the three transgenic lines expressing the KH → II mutation develop disease at times inversely correlated with their respective levels of expression (Table 1). These observations demonstrate that both the ^{Ctm}PrP-favouring quality of a mutation and its level of expression contribute to the development of neurodegeneration.

The transgenic mice with differing propensities to make ^{Ctm}PrP were used to establish the relationship between ^{Ctm}PrP and PrP^{Sc}. We first examined the susceptibility to PrP^{Sc} of transgenic mice with similar levels of transgene expression but differing propensities to make ^{Ctm}PrP: these mice were Tg[SHaPrP(ΔSTE)] and Tg[SHaPrP(A117V)$_H$]. Upon inoculation with Sc237 hamster prions, we found that the Tg[SHaPrP(ΔSTE)] and Tg[SHaPrP(A117V)$_H$] mice developed illness at approximately 323 and 54 days, respectively (Fig. 2a, Table 1). Biochemical analysis of representative mice at the time of disease onset revealed that the Tg[SHaPrP(ΔSTE)] mice contained substantially more PrP^{Sc} than the Tg[SHaPrP(A117V)$_H$] mice (Fig. 2b). Thus, the transgenic line that generates higher ^{Ctm}PrP is more susceptible to PrP^{Sc}, developing disease at a lower level of overall PrP^{Sc} accumulation.

We next compared the susceptibility to Sc237 of Tg[SHaPrP(KH → II)$_L$] versus Tg[SHaPrP(KH → II)$_M$] mice (Fig. 2c), and Tg[SHaPrP(A117V)$_L$] versus Tg[SHaPrP(A117V)$_H$] mice (Fig. 2e). By holding the mutation constant, issues regarding a potential barrier to propagation are avoided, while still changing the propensity to generate ^{Ctm}PrP by adjusting the level of expression. As expected, lowering the level of expression increased the incubation time to disease following Sc237 inoculation. More remarkably, however, we found that with both the KH → II and A117V mutants, the lower-level expressors contained more PrP^{Sc} at the time of disease onset (Fig. 2d and f). A similar inverse relationship between level of expression and amount of PrP^{Sc} at disease onset has been observed in mice expressing different levels of wild-type PrP (ref. 10 and unpublished observations). Thus, as above, the mice with a diminished propensity to form ^{Ctm}PrP had accumulated higher levels of PrP^{Sc} at the time of disease onset.

To integrate the above inoculation data into a single plot, we ranked the different lines of transgenic mice by their relative propensities to generate ^{Ctm}PrP using a measure that we term the Ctm-index (see Table 1). This index is derived by multiplying the percentage of chains synthesized in the ^{Ctm}PrP topology by the level of transgene expression, thus combining the two parameters known to influence ^{Ctm}PrP generation. Figure 2g shows that a relationship exists between the Ctm-index and the amount of PrP^{Sc} that had accumulated at disease onset. The ability of PrP^{Sc} to cause disease seems to be a function of the propensity of the host to generate ^{Ctm}PrP.

The data in Fig. 2 demonstrate two important points. First, very different levels of PrP^{Sc} accumulation are observed at the time of onset of neurologic dysfunction upon inoculation of the various lines of transgenic mice. Given that the strain of the mice is identical in each case, with the only differences being in the nature and level of expression of the PrP transgene, we conclude that accumulation of protease-resistant PrP^{Sc} is not likely to be the most proximate cause of disease; subsequent events (apparently involving ^{Ctm}PrP) are likely to be involved. Second, there is a relationship between the amount of accumulated PrP^{Sc} and the factors that modulate ^{Ctm}PrP generation. Such a relationship argues that ^{Ctm}PrP and PrP^{Sc} are part of a pathway in which each can potentially influence, either directly or indirectly, the metabolism of the other.

One way to explain the data in Fig. 2g is if accumulation of PrP^{Sc} causes increased generation of ^{Ctm}PrP, which then elicits neurodegeneration. Thus, transgenic mice that have a greater propensity to generate ^{Ctm}PrP (that is, a high Ctm-index) would require less PrP^{Sc} accumulation before ^{Ctm}PrP generation is increased beyond the threshold needed to produce detectable neurologic disease. This model would explain the inverse relationship observed in Fig. 2g, and makes two additional predictions. First, as transgenic mice that substantially favour synthesis of PrP in the ^{Ctm}PrP form can entirely circumvent the requirement for PrP^{Sc} in the development of neurodegenerative disease, tissue from such mice should not be infectious. Second, ^{Ctm}PrP levels should increase during the course of PrP^{Sc} accumulation in infectious prion disease. These predictions were tested.

To assess the transmissibility of ^{Ctm}PrP-associated disease, brain homogenate from clinically ill Tg[SHaPrP(KH → II)$_H$] mice was

Table 1 Characteristics of transgenic mouse lines used in this study

Transgenic line name	Transgenic line number	% Ctm *in vitro**	Level of PrP expression (relative to SHa)†	Ctm-index‡	Age of onset of disease (days)§	^{Ctm}PrP *in vivo*‖	Sc237 inoculation time (days)¶
TgSHaPrP(ΔSTE)$_H$	F1788	6	4	24			323 ± 14 (9/9)
TgSHaPrP(A117V)$_L$	E15781	31	0.4	12			70 ± 2 (6/6)
TgSHaPrP(A117V)$_H$	E15727	31	4	124	572 ± 35 (5/5)	+	55 ± 1 (6/6)
TgSHaPrP(N108I)$_L$	E15786	35	1	35			311 ± 3 (3/3)
TgSHaPrP(N108I)$_H$	E15790	35	5	175	312 ± 24 (7/7)	+	233 ± 5 (9/9)
TgSHaPrP(KH → II)$_L$	E12485	48	0.4	19			257 ± 2 (9/9)
TgSHaPrP(KH → II)$_M$	F1220	48	1	48	472 ± 13 (6/6)	+	181 ± 5 (10/10)
TgSHaPrP(KH → II)$_H$	F1198	48	4	192	58 ± 11 (24/24)#	+	ND☆

* Values were derived from quantification of Fig. 1a.
† Levels of PrP expression were determined by quantitative western blotting with the 13A5 monoclonal antibody and are expressed relative to PrP expression in Syrian hamster (SHa; see Fig. 1b for a representative experiment).
‡ The Ctm-index for each transgenic line is derived by multiplying the values in the preceding two columns.
§ The age of onset of clinical symptoms [average ± s.e.m. (n/n_0)].
‖ Biochemical assay for determining the presence of ^{Ctm}PrP *in vivo* was as previously described[9], and carried out on either clinically ill animals (in the case of transgenic lines developing illness) or mice over 600 days of age (in the case of transgenic lines that do not develop neurodegeneration).
¶ Time from inoculation with Sc237 SHa prions to development of neurologic signs of dysfunction [average ± s.e.m. (n/n_0)].
# Data from ref. 9.
☆ ND, not determined.
H, higher; M, medium; L, lower.
TgSHaPrP, transgenic Syrian hamster prion protein.

inoculated intracerebrally into four hosts: Tg[SHaPrP(KH → II)$_L$] mice expressing the KH → II mutation at low levels; Tg[SHaPrP] mice overexpressing wild-type SHaPrP; FVB/Prnp$^{0/0}$ mice with a homozygous disruption of the PrP gene; and Syrian hamsters. As shown in Fig. 3, homogenate from terminally sick Tg[SHaPrP(KH → II)$_H$] mice did not induce neurological illness at rates that were different from those inoculated with control Tg[SHaPrP] homogenate when directly compared in three independent hosts. Furthermore, biochemical and pathological examination of representative brain tissue from animals depicted in Fig. 3 at up to 625 days after inoculation did not show any evidence of PrP^{Sc} or neurologic disease in either experimental or control animals (data not shown). However, inoculation of Tg[SHaPrP(KH → II)$_L$] mice with Sc237 prions readily generated PrP^{Sc} in brain tissue (Fig. 2d), which re-transmitted disease to Tg[SHaPrP(KH → II)$_L$] and Tg[SHaPrP] mice (data not shown). Although PrP(KH → II) is capable of being converted into PrP^{Sc}, the ^{Ctm}PrP-associated disease in Tg[SHaPrP(KH → II)$_H$] mice does not generate detectable PrP^{Sc} and is therefore not infectious. Lack of transmission provides further support for the hypothesis that neurodegeneration in these genetic prion diseases is caused by ^{Ctm}PrP directly.

The second prediction made on the basis of the data in Fig. 2g was that accumulation of PrP^{Sc} in infectious prion disease should induce increased generation of ^{Ctm}PrP, which would subsequently lead to neurodegeneration. Unfortunately, testing this prediction directly is hampered by the biochemical properties of the accumulating PrP^{Sc} (ref. 11). Being highly protease-resistant and heterogeneous in its fractionation, it tends to contaminate substantially all subcellular fractions. In addition, it interferes with the assays for ^{Ctm}PrP detection, which are also based on protection from proteases. Because PrP^{Sc} is not readily degraded by the cell and accumulates to very high levels[12], even very small amounts of contamination of subcellular fractions are sufficient to make detection of slight increases in ^{Ctm}PrP difficult. Thus, an alternate method is required to monitor the effect of accumulating PrP^{Sc} on the topology of newly synthesized PrP (see Fig. 4a).

To design such an experiment, we took advantage of three observations. First, a species barrier to PrP^{Sc} conversion exists between mouse and Syrian hamster[13,14]. Second, in contrast to the species barrier for PrP^{Sc} formation, no species-specific differences in the synthesis, translocation or topology are observed between mouse prion protein (MoPrP) and SHaPrP (ref. 9 and data not shown). And, finally, monoclonal antibodies highly specific to SHaPrP (which do not cross-react with MoPrP) are available to distinguish between expression of these two PrP transgenes[15]. Thus, in such double-transgenic animals we can use hamster-^{Ctm}PrP formation as a 'reporter' of signalling in *trans* during the course of accumulation of mouse PrP^{Sc}. For this experiment, the double-transgenic mice which synthesize both MoPrP and SHaPrP (see Methods) are inoculated with mouse prions (of the RML strain). Then, at various intervals during the time course of accumulation of PrP^{Sc} and development of disease, individual mice are killed and

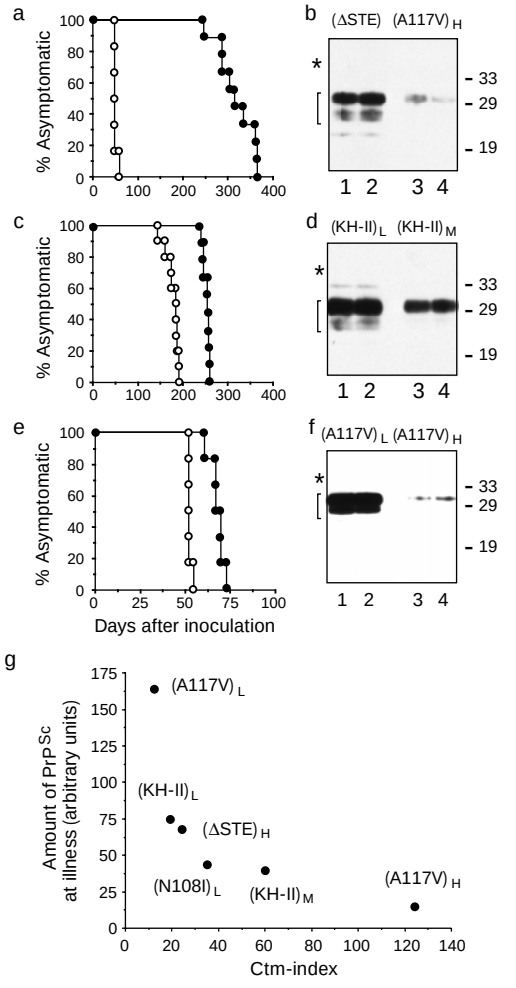

Figure 2 Relationship between ^{Ctm}PrP and PrP^{Sc}. **a**, **c**, **e**, Time course of development of illness in various transgenic lines following inoculation with Sc237 hamster prions. **b**, **d**, **f**, Relative levels of protease-resistant PrP^{Sc} at time of illness in corresponding transgenic lines. Duplicate samples of each line were digested using 'harsh PK' conditions as described in Methods and equivalent amounts of each sample separated by SDS-PAGE. The C-terminal PrP27-30 fragment characteristic of PrP^{Sc} (indicated with a bracket on the left) was detected by immunoblotting with the 13A5 monoclonal antibody. The position of undigested, full-length PrP is indicated with an asterisk on the left. **g**, The Ctm-index for each transgenic line (see Table 1) was plotted against the amount of PrP^{Sc} accumulated at the time of illness following inoculation with Sc237 prions. **a**, Open circles: Tg[SHaPrP(A117V)$_H$]; closed circles: Tg[SHaPrP(ΔSTE)]. **c**, Open circles: Tg[SHaPrP(KH→II)$_M$]; closed circles: Tg[SHaPrP(KH→II)$_L$]. **e**, Open circles: Tg[SHaPrP(A117V)$_H$]; closed circles: Tg[SHaPrP(A117V)$_L$].

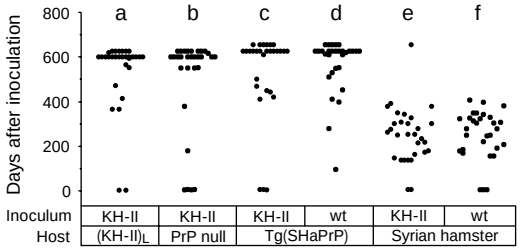

Figure 3 Lack of transmission of ^{Ctm}PrP induced neurodegenerative disease. Terminally ill Tg[SHaPrP(KH → II)$_H$] mice ('KH → II'; **a**–**c**, **e**) and clinically normal Tg(SHaPrP] mice ('wt'; **d**, **f**) were sacrificed and homogenates of the brain tissue inoculated intracerebrally into various hosts as indicated below the graph. The host animals used were Tg[SHaPrP(KH → II)$_L$]['(KH → II)$'_L$], FVB/Prnp$^{0/0}$ ('Prp null'), Tg[SHaPrP] and Syrian hamsters. The time (in days) at which individual animals died following inoculation is plotted. Deaths by all causes, including those related to the inoculation itself, are plotted. The experiments were terminated after ~600 days with none of the remaining animals showing any signs or symptoms of neurologic disease. Each experiment represents three sets of 10 host animals injected with inoculi prepared from three separate animals to be tested. It should be noted that in our animal care facility, hamsters routinely live about 200–400 days, while mice live longer than 600 days (data not shown).

examined for total PrP^{Sc} accumulation and for the presence of hamster ^{Ctm}PrP (see Fig. 4a). The principle is that, following inoculation, only MoPrP will be a substrate for prion replication and PrP^{Sc} formation[13]. The effect of this PrP^{Sc} accumulation on the ability of cells to generate (or not generate) ^{Ctm}PrP can be assessed by examining SHaPrP.

Clinical disease developed in these animals about 9 weeks after inoculation (data not shown). We found that PrP^{Sc} accumulated in these mice during this 9-week time course, with the earliest times at which it was detectable being approximately 5–6 weeks (Fig. 4b). As expected, the SHaPrP was not found to have formed any PrP^{Sc} by both biochemical criteria in this study (Fig. 4b) and infectivity criteria in previous studies[13]. However, a significant increase in the amount of ^{Ctm}PrP was noted upon examination of the SHaPrP (Fig. 4c). Such an increase was not observed in a parallel set of mice that did not receive the inoculum (data not shown). These findings, coupled with the observation that ^{Ctm}PrP can cause neurodegeneration in the absence of a transmissible forms of PrP (ref. 9, Figs 1 and 3), suggest that PrP^{Sc} accumulation may cause disease by inducing the synthesis of ^{Ctm}PrP *de novo*.

These findings suggest causal relationships between PrP^{Sc} accumulation, the events of ^{Ctm}PrP formation and metabolism, and the development of neurodegenerative disease. Three complementary and independent lines of evidence argue for this conclusion. First, increasing the generation of ^{Ctm}PrP beyond a certain threshold (by modulating a combination of PrP mutation and level of expression) results in neurodegeneration in the absence of PrP^{Sc} formation (Figs 1 and 3 and ref. 9). Second, the amount of accumulated PrP^{Sc} needed to cause neurodegenerative disease is influenced by the propensity of the host to generate ^{Ctm}PrP (Fig. 2). And third, the brain appears to contain increasing levels of ^{Ctm}PrP during the course of accumulation of PrP^{Sc} (Fig. 4). Taken together, the data are suggestive of three successive stages in the pathogenesis of prion diseases (Fig. 5).

Infectious prion diseases are proposed to work by initiating the steps of Stage I, the accumulation of PrP^{Sc}. Genetic prion diseases could in principle work at either Stage I or II. If the PrP mutation in question results in the spontaneous formation of PrP^{Sc}, Stage I would be initiated, PrP^{Sc} would replicate and accumulate, and subsequently cause increased elevation of ^{Ctm}PrP (Stage II). Such a mechanism seems plausible for the E200K mutation which causes familial Creutzfeldt-Jakob disease[16]. Thus, PrP^{Sc} is seen in these patients[17], and the disease is readily transmissible to experimental animals[7]. Alternatively, certain other PrP mutations could bypass

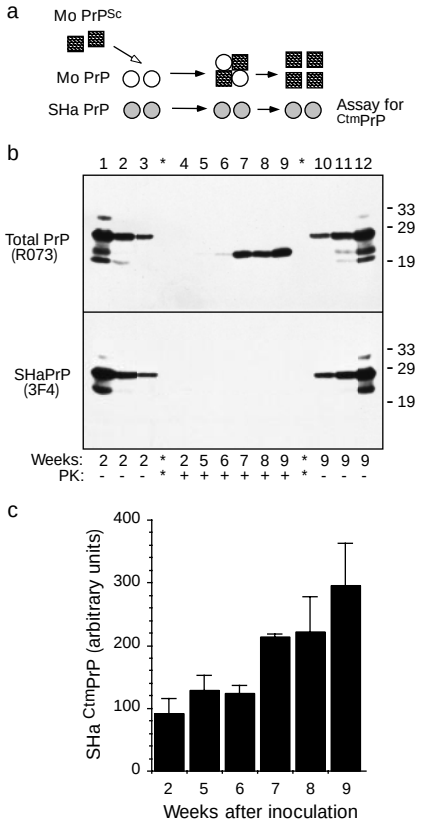

Figure 4 ^{Ctm}PrP generation during time course of PrP^{Sc} accumulation. **a**, Diagram of experimental design. Double transgenic mice (see Methods) expressing both SHaPrP (shaded circles) and MoPrP (open circles) are inoculated with RML mouse prions (crosshatched squares). Over time, host MoPrP is converted to $MoPrP^{Sc}$ and accumulates. During this time course, the SHaPrP is not converted to $SHaPrP^{Sc}$ owing to the species barrier and may therefore be assayed for ^{Ctm}PrP. **b**, Relative amounts of total PrP^{Sc} and $SHaPrP^{Sc}$ in mice at various times (in weeks) after inoculation with RML. Homogenate was digested using the 'harsh PK' conditions (see Methods), treated with PNGase, and analysed by SDS-PAGE and immunoblotting with either the R073 polyclonal antibody (to detect total PrP) or the 3F4 monoclonal antibody (to selectively probe for SHaPrP). An equivalent amount of homogenate is analysed in each lane except lanes 2 and 11 (which contain one-fourth as much) and lanes 3 and 10 (which contain one-tenth as much). **c**, Relative amounts of Syrian hamster ^{Ctm}PrP (detected selectively using the 3F4 monoclonal antibody) at various times after inoculation with RML. Each bar represents the average ± s.e.m. of three determinations.

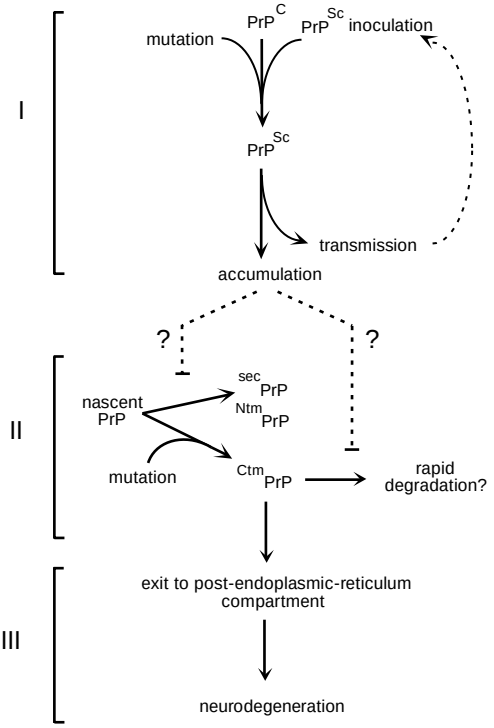

Figure 5 Three-stage model of prion disease pathogenesis. Stage I is the formation and accumulation of PrP^{Sc}. This could be initiated by either inoculation or spontaneous conversion of a mutated PrP^{C} to PrP^{Sc}. Stage II comprises the events involved in generating ^{Ctm}PrP. These events could be affected *in trans* at a currently unknown step (dashed lines with question marks) by accumulated PrP^{Sc} or *in cis* by certain mutations within PrP. Stage III represents the events (currently unknown) involved in ^{Ctm}PrP-mediated neurodegeneration. This is likely to involve exit of ^{Ctm}PrP to a post endoplasmic-reticulum compartment as a first step. In this model, PrP^{Sc} can cause disease (via ^{Ctm}PrP), but is not absolutely necessary. By contrast, we suggest, based on the data, that ^{Ctm}PrP production is necessary and sufficient for the development of disease.

Stage I altogether by directly causing an increase in ^{Ctm}PrP generation. The A117V mutation resulting in human Gerstmann-Sträussler-Scheinker[18] disease seems likely to work by such a mechanism. This would explain why this disease has not been transmissible[6–8], and why PrP^{Sc} has not been detected in the brain tissue of these patients[6,9].

The final stage in prion disease pathogenesis includes the mechanisms by which ^{Ctm}PrP, once generated, leads to neurodegenerative disease. The mechanism by which this occurs and the intracellular pathways that are involved remain entirely unclear. However, it does not appear to be the case that ^{Ctm}PrP is simply misfolded, retained or accumulated in the endoplasmic reticulum, or that it elicits an unfolded protein response. This is suggested by the observation that essentially all of the ^{Ctm}PrP has been trafficked beyond the endoplasmic reticulum (ref. 9), which is the site of the presently known quality-control machinery for protein folding in the secretory pathway[19,20]. In addition, disease can be elicited by transgenes expressed at close to physiologic levels, as is the case with Tg[SHaPrP(KH → II)$_M$] animals or human cases of Gerstmann-Sträussler-Scheinker disease containing the A117V mutation. Thus, a more selective means by which ^{Ctm}PrP induces neurodegeneration is suggested by the available data.

The framework described in Fig. 5 proposes several new areas for future studies. First, the regulated events in ^{Ctm}PrP biogenesis and trafficking remain to be elucidated. The reconstitution of the early events of PrP translocation and topology determination in a cell-free system amenable to fractionation appears promising for the identification of *trans*-acting factors regulating ^{Ctm}PrP synthesis[21,22]. Also, the availability of several mutants influencing topology should facilitate studies aimed at defining later events in the trafficking of ^{Ctm}PrP. Second, studying the metabolism of ^{Ctm}PrP will allow a better understanding of how these events are modulated *in trans* by PrP^{Sc} accumulation. Such studies are likely to clarify the relationship between the events of PrP^{Sc} accumulation and ^{Ctm}PrP-mediated neurodegeneration. Given that PrP^{Sc} accumulation may affect several metabolic functions of the cell[23–25], it is plausible that one or more of these influence ^{Ctm}PrP generation to elicit disease. The availability of PrP mutants that act at a step beyond PrP^{Sc} accumulation to directly cause ^{Ctm}PrP mediated neurodegeneration (ref. 9 and Fig. 1) should help to establish the downstream events in prion disease pathogenesis. □

Methods

Cell-free translation and translocation

Transcription of the relevant coding regions using SP6 polymerase, translation in rabbit reticulocyte lysate containing microsomal membranes from dog pancreas, and proteolysis were as described[9]. Translation reactions were carried out at 32 °C for 40 min, and proteolysis reactions at 0 °C for 60 min using 0.5 mg ml^{-1} proteinase K. Products were immunoprecipitated with the R073 antibody[26], separated by SDS-PAGE on 15% acrylamide gels and visualized by autoradiography.

Transgenic mouse production and characterization

Transgenic mice expressing mutants (see Table 1) were generated as described (ref. 10 and references therein). PrP expression was assessed by immunoblotting of brain tissue homogenate with 13A5 mAb (ref. 27) and comparing to serial dilutions of normal Syrian hamster brain tissue (Fig. 1b and Table 1). Observation of these mice for development of spontaneous illness was as described[13]. The double transgenic mice expressing both SHaPrP and MoPrP (see Fig. 4) were generated by crossing Tg[SHaPrP]/Prnp$^{0/0}$ (line A3922, ref. 9) to Tg[MoPrP]/Prnp$^{0/0}$ (line B4053, ref. 28). Transmissibility (see Fig. 3) was assessed by intracerebral inoculation of 1% brain homogenate (w/v) into mice (30 μl per animal) or hamsters (50 μl per animal) as described[13]. The Sc237 strain of hamster prions (used in Fig. 2) and RML (Rocky Mountain Laboratories) strain of mouse prions (used in Fig. 4) have been described previously[29,30].

Assessment of brain for ^{Ctm}PrP and PrP^{Sc}

Brain tissue (either freshly removed or stored frozen at −80 °C following flash-freezing in liquid nitrogen) was homogenized in PBS (at 5% w/v or 10% w/v) by successive passage through 16, 18 and 20 gauge needles. For ^{Ctm}PrP detection ('mild' proteolysis conditions), 17-μl aliquots of the sample (at a concentration of 25 μg μl^{-1}) were adjusted (in a final volume of 20 μl) to 1.0% NP-40, 0.25 mg ml^{-1} PK and incubated for 60 min on ice. For PrP^{Sc} detection ('harsh' proteolysis conditions), 17 μl samples (at a concentration of 25 μg ml^{-1}) were adjusted (in a final volume of 20 μl) to 0.5% NP-40, 0.5% deoxycholate, 0.1 mg ml^{-1} PK and incubated for 60 min at 37 °C. Note that the difference between mild versus harsh digestion conditions, while operational, is not subtle, as it involves a 37° change in temperature of incubation, and the presence of non-ionic detergent versus mixed micelles of non-ionic and ionic detergents. The proteolysis reactions were terminated by the addition of PMSF to 5 mM, incubating for an additional 5 min, and transferring the sample to 5 volumes of boiling 1% SDS, 0.1M Tris, pH 8.9. Samples were then digested with PNGase as directed by the manufacturer, resolved by 10% tricine-SDS-PAGE, transferred to nitrocellulose, and probed with either the 3F4 or 13A5 monoclonal antibody[15,27] or the RO73 polyclonal antibody[26].

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