De novo generation of a transmissible spongiform encephalopathy by mouse transgenesis

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Communicated by Charles Weissmann, Scripps Florida, Jupiter, FL, October 23, 2008 (received for review August 4, 2008)

Most transmissible spongiform encephalopathies arise either spontaneously or by infection. Mutations of *PRNP*, which encodes the prion protein, PrP, segregate with phenotypically similar diseases. Here we report that moderate overexpression in transgenic mice of mPrP(170N,174T), a mouse PrP with two point mutations that subtly affect the structure of its globular domain, causes a fully penetrant lethal spongiform encephalopathy with cerebral PrP plaques. This genetic disease was reproduced with 100% attack rate by intracerebral inoculation of brain homogenate to *tga20* mice overexpressing WT PrP, and from the latter to WT mice, but not to PrP-deficient mice. Upon successive transmissions, the incubation periods decreased and PrP became more protease-resistant, indicating the presence of a strain barrier that was gradually overcome by repeated passaging. This shows that expression of a subtly altered prion protein, with known 3D structure, efficiently generates a prion disease.

amyloid | neurodegeneration | prion | species barrier | transgenic mice

Prions cause fatal neurodegenerative disorders termed transmissible spongiform encephalopathies (TSEs). The prion contains a misfolded and aggregated form, PrPSc, of the host cellular prion protein, PrPC (1). Accordingly, *Prnp*o/o mice that lack the gene encoding PrPC resist prion infection (2).

Human familial TSEs co-segregate with more than 20 mutations in the prion gene, PRNP (3–5). Some of these genetic diseases have been transmitted to primates or mice (6, 7). De novo production of infectious prions from PRNP with point mutations would represent a useful tool for elucidating the origin of prion infectivity in genetic TSEs. Transgenic mice expressing Prnp with point mutations, insertions, or deletions develop a spectrum of neurological diseases with clinical or histological features reminiscent of TSEs (8–11). However, de novo infectivity was claimed only for mice expressing PrnpP101L, which mimics the Gerstmann-Sträussler-Scheinkerassociated P102L point mutation of human PRNP. PrnpP101L mice were reported to transmit disease to nine of 148 (6%) hamsters (10) but not to mice expressing WT PrP (10, 12). Transgenic mice expressing high amounts of *Prnp*^{P101L} were thought to transmit spongiform encephalopathy to mice expressing lower amounts of PrnpF101L (10, 12), yet further studies led to the re-interpretation of this phenomenon as disease acceleration rather than bona fide transmission (13). A further set of transgenic mice expressing Prnp^{P101L} produced by targeted gene substitution did not develop a neurological disease (6).

Mammalian PrP^C consists of a flexibly disordered N-terminal segment and a globular C-terminal domain containing three α-helices and two short antiparallel β -strands (14). However, the loop linking the β 2 strand with the α 2 helix displays remarkable species-specific variations. The β 2— α 2 loop region of human, cattle, mouse, dog, and cat is disordered, whereas that of elk is precisely defined (15–18). This feature is caused by S170N/N174T substitutions (numbering according to ref. 19), as documented by studies of variant mouse PrP containing the two residues 170N and 174T (16).

To probe the pathogenic impact of the well structured loop observed by NMR (16), we expressed in vivo a murine *Prnp* mini-gene with the S170N and N174T exchanges. This mouse-elk PrP chimeric molecule, henceforth termed RL-PrP for "rigid loop PrP," accumulated in detergent-resistant membranes similarly to WT PrP. Surprisingly, RL-PrP transgenic mice developed a transmissible spongiform encephalopathy. The novel variety of prions produced by RL-PrP transgenic mice was distinct from other commonly used laboratory prion strains.

Result

mPrP(170N,174T) Expression in Transgenic Mice. Two point mutations, $G \rightarrow A$ and $A \rightarrow C$, were introduced at nucleotide positions 506 and 518 of the *Prnp* ORF within the pHGPrP vector (20), causing it to encode RL-PrP [supporting information (SI) Fig. S1 A, B, E and F embedded in a "half-genomic" Prnp minigene. Linearized constructs were microinjected into B6;129S5-Prnp^{+/o} zygotic pronuclei and founders were bred to B6;129S5-Prnp^{o/o} mice. Transgene copy numbers and brain protein expression levels were determined by quantitative Southern and Western blotting of hemizygous mice. Line tg1020 mice had ≈ 45 copies of the transgene (Fig. S1C) and expressed approximately two- to threefold WT PrP levels in brain (Fig. S1D). PrP expression in tg1020 mice was higher than in most corresponding WT tissues (Fig. S2). In the tg1020 mouse line, RL-PrP^C glycosylation patterns, presence in detergentresistant membranes, and functionality as shown by rescue of Shmerling disease (Fig. S2) (11) were indistinguishable from those of PrPC (Figs. S2 and S3).

Spontaneous Neurodegenerative Disease in tg1020 Mice. Clinical monitoring of aging tg1020 mice revealed a progressive neurological illness with ataxia, hind limb paresis, weight loss, lethargy, kyphosis, and occasionally pruritus, which slowly progressed in severity over 1 to 3 months (Fig. 1A). Onset occurred at 145 to 637 days of age (50% incidence by 364 days; n=47; Fig. 1B). Footprint tracks demonstrated circling movements of tg1020 mice indicative of ataxia (Fig. S4).

Brains of clinically diseased tg1020 mice contained multicentric

Author contributions: C.J.S., M.H., K.W., and A.A. designed research; C.J.S., K.P.R.N., S.H., M.H., G.M., P.S., D.O., T.R., P.P.L., C.J., J.F., and L.S. performed research; K.P.R.N. contributed new reagents/analytic tools; C.J.S., K.P.R.N., S.H., M.H., P.P.L., C.J., J.F., K.W., and A.A. analyzed data; and C.J.S., S.H., K.W., and A.A. wrote the paper.

Conflict of interest statement: K.P.R.N. has interest in BioChromix AB (Linköping, Sweden), which owns some intellectual property in the amyloid binding luminescent conjugated polymers, PTAA and PTMI, described. The other authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/ 0810680105/DCSupplemental.

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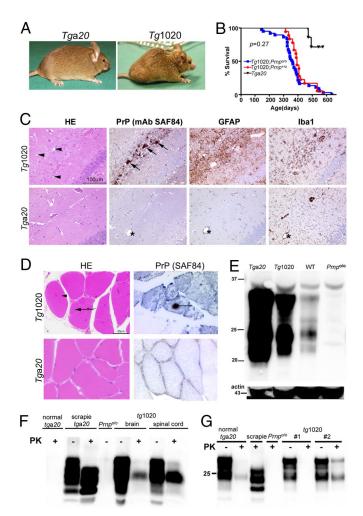


Fig. 1. RL-PrP mice (tg1020) develop a lethal neurologic disease with lesions in brain and muscle. (A) Clinically healthy tga20 mouse (Left) and tg1020 mouse showing kyphosis and paraparesis, with hind legs tucked under the body. (B) Survival plots of tg1020;Prnpo/o, tg1020;Prnp+/o, and tga20 mice. Tga20 mice showed the typical life expectancy of C57BL/6 mice (41) held in specified pathogen-free environments, whereas tg1020;Prnpo/o and tg1020;Prnp+/o mice displayed median survival times of 363 and 394 days, respectively. (C) Hippocampi of tg1020 and tga20 mice. Arrows indicate spongiform change. Linear arrays of PrP aggregates were accompanied by severe gliosis and microglial activation, as detected by antibodies to PrP, GFAP, and Iba1, respectively. Asterisk indicates a small vessel in a section of a tga20 brain. (D) Myopathy in tg1020 but not tga20 mice, with centralized nuclei (arrowhead), split fibers (arrow), and hypervariable fiber calibers. PrP immunostains show deposits (arrow) in affected tg1020 muscles. (E) Muscle PrP expression in tg1020 mice is higher than in WT but lower than in tga20 mice. (F) PK-resistant PrP in tg1020 brains revealed by immunoblotting. There is no shift in the electrophoretic mobility, indicating lack of amino terminal cleavage of PrP. (G) Some young tg1020 mice (60 days old) also show PK-resistant PrP (mouse 2). Mouse 1 does not have PK-resistant PrP.

PrP deposits in the stratum lacunosum-moleculare of the hippocampus, within the corpus callosum, and in the cingulum. Deposits were sometimes surrounded by vacuoles as in florid plaques of variant Creutzfeldt-Jakob disease (Fig. 1C and Fig. S4). Individual PrP deposits were 1 to 3 μ m in diameter and often arranged in clusters of 25 to 50 particles. The density of PrP deposits varied from isolated clusters to widespread zones of diffuse aggregates (Fig. 1C). Spongiform change, astrogliosis, and microgliosis were also evident (Fig. 1C).

Peripheral nerves and skeletal muscle of RL-PrP mice developed pathologic changes similar to those reported in mice overexpressing WT or mutant PrP (12, 21). Sciatic nerves displayed "onion bulbs" indicative of cycles of active demyelination and re-myelination, as well as macrophages and Schwann cells with myelin-filled vacuoles (Fig. S5). Myofibers displayed degeneration and regeneration, varied markedly in size, and contained angular and split fibers as well as centralized nuclei (Fig. 1D). PrP immunohistochemistry revealed large, coarse PrP aggregates in tg1020 myofibers, whereas very few aggregates (≤2 per muscle section) were observed in myofibers of tga20 mice, which over-express WT Prnp (20) (Fig. 1D). PrP expression in muscle of tg1020 mice was higher than in WT mice, but lower than in tga20 mice (Fig. 1E).

Although expression in tga20 brain was consistently twofold higher than in tg1020 mice, and the PrP distribution in tg1020 brains was similar to that of tga20 mice (Fig. S2), tga20 mice did not spontaneously develop neurological disease, spongiform encephalopathy, or brain plaques during lifetimes up to 900 days.

Certain pathologies induced by PrP mutants are partially or fully suppressed by co-expression of WT-PrP (11, 22), whereas others are unaffected (23). To investigate the influence of WT-PrP on the RL-PrP disease, we bred tg1020 to WT mice. The clinical course of spontaneous disease and the median survival time were unaffected by WT PrP co-expression (P = 0.27, log-rank test; Fig. 1B). We also did not identify any influence of WT-PrP co-expression on the severity of spongiosis and the occurrence of PrP-immunoreactive plaques (data not shown). We then assessed the resistance of RL-PrP to proteolysis. After stringent digestion (50 or 100 μ g/ml proteinase K [PK], 37 °C, 30 min), we detected PrP with a mobility corresponding to ≈25 kDa in a clinically sick 145-day-old tg1020 mouse and in a clinically healthy 60-day-old tg1020 mouse, whereas some young tg1020 mice had PK-sensitive PrP. PK digestion did not induce the typical electrophoretic mobility shift to PrP²⁷⁻³⁰, suggesting that the amino terminus of RL-PrP was not cleaved (Fig. 1 F-G), similarly to patients with Gerstmann-Sträussler-Scheinker syndrome (24).

Prion Strain Barriers Between Mice Expressing RL-PrP or WT PrP. Local structural variations (16) imposed by the RL-PrP mutations may create transmission barriers to prion infection. We tested this possibility by intracerebrally inoculating groups (n = 4) of tg1020and tga20 mice with 10⁴ logLD₅₀ of mouse-adapted Rocky Mountain Laboratory (RML) prions. Whereas tga20 mice developed scrapie after 74 \pm 6 days, tg1020 mice developed scrapie only after 323 \pm 92 days, which decreased to 171 \pm 11 days upon second passage to tg1020 mice (Fig. 2A, panel vii). The lower PrP^C expression levels in tg1020 mice does not account for the prolonged and broadened incubation time, as 10³ logLD₅₀ of the same inoculum produced disease within 170 ± 12 days in WT mice, which express even lower levels of PrP^C. Therefore, expression of RL-PrP created a transmission barrier for RML prions.

Transmissibility of RL-PrP-Induced Disease. We then asked whether tg1020 mice spontaneously generate prions. To minimize all risks of laboratory prion contamination, tg1020 mice were housed in a room that had never hosted prion-infected animals, factory-new instruments were used for tissue collection, and all working surfaces for harvesting and processing tissues were protected with disposable covers. We inoculated tg1020 brain homogenate into tga20 mice, as RL-PrP displays a prion conversion barrier for WT-adapted inoculum and tga20 mice are permissive for xenogeneic strains such as chronic wasting disease (CWD) (25). After inoculation with brain homogenates derived from six terminally sick tg1020 mice, all 28 inoculated tga20 mice (n = 4-6 per group, 100% attack rate) developed neurologic signs (e.g., kyphosis, hind leg clasp, paraparesis, ataxia, and pruritus) by 481 ± 59 days after inoculation (Fig. 2A, panels i-vi; Table S1). When inoculated into further tga20 mice, brain homogenates from tg1020-infected tga20 mice caused similar neurologic signs in all recipients (n = 35), but with a shorter incubation period (343 \pm 122 days) suggestive of strain adaptation

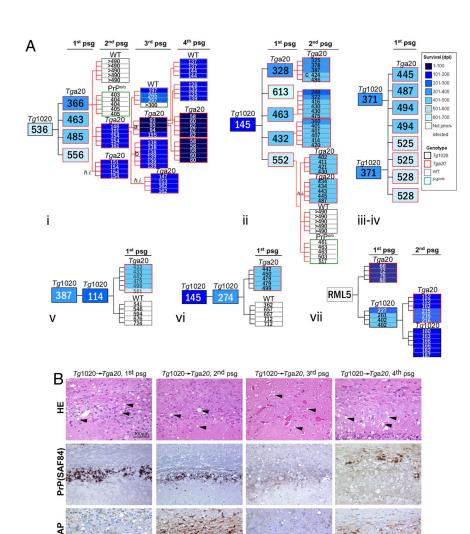


Fig. 2. Ta1020 prion infection transmits to taa20 mice, but not to Prnpolo mice. (A) Survival of mice inoculated with tg1020 brain homogenate (groups i-vi). Box outline colors denote mouse genotypes and fill colors indicate survival times as in the legend. For the first passage, only mice with incubation times more than 300 days are shown. (h.i. = heat inactivated.) Group vii shows RML5 passaged into tga20 and tg1020 mice, and the second passage from RML-infected tg1020 mice. (B) Histopathology from transverse brain sections at the level of the hippocampus and the corpus callosum, all from group i (A). Sections from first to fourth passages of tg1020 to tga20 mice reveal consistent spongiform changes (arrowheads). PrP deposits and astrocyte reactivity, as indicated by SAF84 and GFAP antibody staining, were apparent in all passages in tga20 mice. PrP immunostain reveals that PrP deposit sizes decreased progressively, with granular aggregates in the fourth passage. Mock-inoculated mice did not show spongiform change or PrP staining, and gliosis was minimal (data not shown).

(Fig. 2A, panels *i-vi*; Table S2). Further serial passages to *tga20* led to even shorter incubation periods, and *tga20*-adapted prions were also transmissible to WT mice (Fig. 2 A and B and Fig. S6; Table S2 and Movie S1).

Brain homogenates recovered from tg1020-inoculated tga20 mice were heat-inactivated at $60\,^{\circ}\text{C}$ for 20 min and inoculated into further tga20 mice. The recipient tga20 mice developed neurologic disease, spongiform encephalopathy, and plaques with the same incubation period (153 ± 2 days) as in the corresponding experiment without heat inactivation (153 ± 5 days). In a subsequent passage, brain homogenate was heat-inactivated at $80\,^{\circ}\text{C}$ for $25\,^{\circ}\text{min}$ and was again infectious, showing that the infectious agent was highly heat-resistant (Fig. 2A, panel i).

Because *tga20* mice over-express PrP, and PrP nucleation may be concentration-dependent, we systematically tested whether serial inoculation of brain homogenate among *tga20* mice may uncover any spontaneous transmissible pathology. Brain homogenates from 30 young and old *tga20* mice (including four mice 450–900 days old) were inoculated into 16 mice. After 250 days, the latter mice were killed, and four groups of pooled brain homogenates were inoculated into 17 further *tga20* mice. After 250 more days, pooled brain homogenates from the latter mice were further inoculated into eight *tga20* mice (Fig. S7). In a further series, brain homogenate from healthy *tga20* and WT mice was inoculated into five *tga20* mice that were killed at 563 days after inoculation (Fig. S7). None of these transmission attempts resulted in neurologic disease of re-

cipients, even after the third serial passage. We never detected spontaneous neurologic disease or PrP plaques in *tga20* mice aged up to 30 months.

Because nucleation may be time-dependent, extremely aged tga20 mice might develop subclinical levels of prion infectivity even if clinically healthy at 900 days or older. Although no infectivity was detectable in the experiments described earlier, we again attempted transmission from brain homogenates of two 600- and 900-day-old tga20 mice. Three of six tga20 recipient mice developed neurologic disease and brain PrP aggregates at 422 ± 50 days post-inoculation. This suggests that low levels (≈ 1 ID₅₀/3 mg tissue) of prion infectivity may sporadically arise in very old tga20 mice. The apparent occurrence of prion infectivity in some tga20 mice is different from the prion generation in RL-PrP mice, as the former were four to six times older than the youngest tg1020 mice containing infectivity, and approached the natural murine lifespan, whereas the latter elicited 100% attack rates, even when inocula were prepared from 145-day-old tg1020 mice.

RL-PrP Prion Strain Properties. Brain homogenates from tga20 mice infected with RL-PrP (RL $\rightarrow tga20$) were subjected to PK digestion and Western blotting. PrP from first- and second-passage RL $\rightarrow tga20$ mice was mostly PK-sensitive (Fig. 3*A*). Weak residual PrP bands of unchanged electrophoretic mobility were identified after PK digestion, similarly to the signal observed in tg1020 mice. However, by the third (n = 3/4) and fourth (n = 5/5) passage

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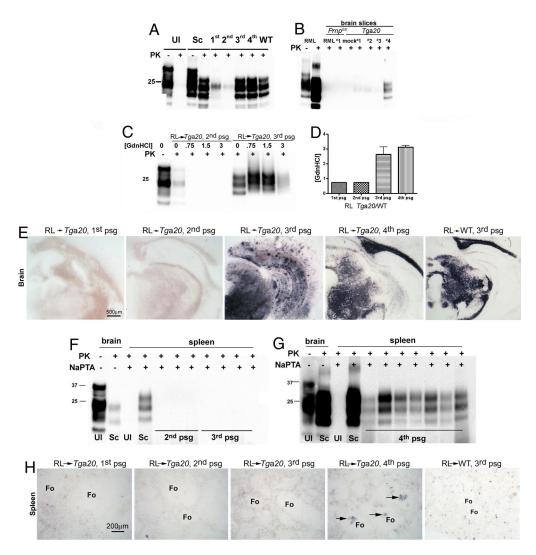


Fig. 3. Evolution of RL-PrP features over four passages of $tg1020 \rightarrow tga20$ mice. (A) Western blot from brain shows that the PrP switched from mostly PK-sensitive in the first and second passages to PK-resistant, with cleavage of the amino terminus in the third and fourth passages. (UI, uninfected, Sc. scrapie-infected.) (B) Western blot from Prnpolo and tga20 organotypic brain slices exposed to brain homogenate from second passage of $tg1020 \rightarrow tga20$ (n = 4 mice) shows a switch to a PK-resistant PrP in mouse 4. (C) Conformation stability assay indicates that PrP stability also changes between the second and third passages. (D) The change in the [GdnHCl], in which <25% of the original PrP signal remains, is shown for all four passages of $tg1020 \rightarrow tga20$ (n = 4-5 mice per group). No differences were identified in the first two passages. (E) Histoblot of brain treated with PK and stained for PrP maps the location of PK-resistant PrP. (F) No PK-resistant PrP was evident in the spleens in the second and third passages of tg1020 \rightarrow tga20. (G) PrPSc was detected in all spleens tested after the fourth passage of $tg1020 \rightarrow tga20$. (H) Histoblots of spleen show PrPSc deposits (arrows) only in the fourth passage of $tg1020 \rightarrow$ tga20. (Fo, follicles.)

derived from mouse "a" (Fig. 2A), RL \rightarrow tga20 brain homogenates displayed abundant PK-resistant PrP^{27–30} (Fig. 3A).

We then subjected tg1020 homogenates to the prion organotypic slice culture assay (26). We exposed brain slices to brain homogenates from four second-passage $RL \rightarrow tga20$ mice (Fig. 2, panel ii, from group including "c"), all of which had predominantly PK-sensitive PrPSc. Homogenates from one of four mice induced PK-resistant PrP formation in the brain slices (Fig. 3B), suggesting either that the switch to PK resistance is a stochastic event, or that a PK resistant strain had been progressively selected.

We quantified the resilience of PrPSc from each passage to guanidinium hydrochloride (GdnHCl) using a modified conformational stability assay (27). PrPSc from third- and fourth-passage RL \rightarrow tga20 brains displayed enhanced stability with >25% of the signal resisting exposure to 3 M GdnHCl (Fig. 3 C and D). Accordingly, PK-treated histoblots of frozen brain sections were strongly positive upon the third and fourth passages of RL \rightarrow tga20 mice and the third passage to WT mice (Fig. 3E).

PrPsc accumulation in lymphoid organs is a distinctive feature of some, but not all, prion strains (28). First, second, and third passage of RL \rightarrow tga20 mice did not induce PrPsc in the spleen (Figs. 3 F and H). However, fourth-passage RL \rightarrow tga20 mice accumulated splenic PrPsc, as revealed by both Western blots and histoblots (Fig. 3 G and H). The absence of lymphotropism in the third passage suggests that the strain continued to evolve between the third and the fourth passage.

Differential Staining of RL-PrP with Luminescent Conjugated Polymers

(LCPs). Prion strains often differ in the affinity of PrP deposits for amyloidotropic dyes (29). This property can differentiate strains even when propagated in congenic hosts. The pathological deposits of tg1020 mice, $RL \rightarrow tga20$ mice (all four passages), and $RL \rightarrow WT$ mice were consistently negative for thioflavin T and Congo red, yet were stained by the amyloid-binding LCPs polythiophene acetic acid (PTAA; anionic) and polythiophene methyl imidazole (PTMI; cationic) (30, 31) (Fig. 44). These pathological deposits were confirmed to consist of PrP by immunohistochemical staining of consecutive sections with antibody SAF84 to PrP (data not shown).

To distinguish RL-PrP from other PTAA-positive deposits, we acquired fluorescence spectra. The averaged ratios (R) of emission intensities at wavelengths $R_{532:641}$ and $R_{532:Emax}$ (E_{max} , emission maximum) recorded for individual animals allowed for unequivocal discrimination between RL \rightarrow tga20 and aggregates of other strains (Fig. 4B). The $R_{532:641}$ values of PTAA-stained tg1020 and RL \rightarrow tga20 plaques were nearly identical (analysis of variance [ANOVA], P < 0.05), supporting the notion that tga20 aggregates originated from the RL prion strain. In contrast, PTAA spectra from the RL-PrP aggregates differed significantly from those of other mouse-adapted prion strains, and PTAA did not detect bovine spongiform encephalopathy or RML aggregates (Fig. 4 C and D; ANOVA, P > 0.05). Therefore, the morphological characteristics, the tinctorial properties of RL-PrP aggregates, and the emission spectra are similar in tg1020 mice and across four serial passages to

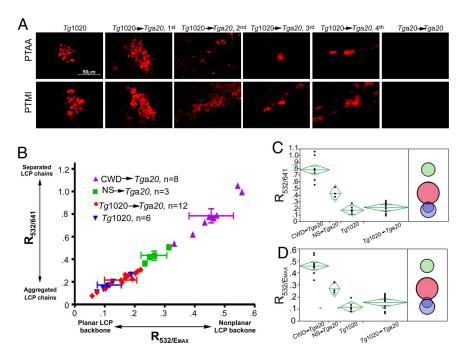


Fig. 4. LCP fluoroscopy distinguishes RL-PrP from other mouse prion strains. (A) Tg1020 and $Tg1020 \rightarrow$ Tga20 PrP aggregates in brain tissue were stained using anionic and cationic conjugated poly-electrolyte probes (PTAA and PTMI, respectively). Spectral intensity measurements were recorded from PTAA-stained sections at 10-nm intervals between 500 and 750 nm. Spectral un-mixing visualizes the pixels with similar spectral profiles. (B) Intensity ratios of fluorescence, $R_{532/641}, \ and \ R_{532/Emax}, \ from \ PTAA-stained \ plaques \ in$ individual CWD ightarrow tga20-, NS ightarrow tga20-, and tg1020 ightarrowtga20-infected mice, as well as un-inoculated tg1020 mice. (NS, natural sheep scrapie.) (C and D) Statistical analysis was performed using ANOVA followed by Tukey-Kramer honestly significantly different (HSD) test for the spectral ratios, R_{532/641}, and R_{532/Emax} (general α level of 0.05). The green diamonds illustrate the sample means and the corresponding 95% CIs (one black dot per animal). Lines across each diamond represent group means. The vertical span of each diamond represents the 95% CI for each group. Overlap marks are drawn above and below the group mean. Comparison circle plots are shown for Tukey-Kramer HSD test (Right). Circles for means that are significantly different do not intersect, or intersect so slightly that the outside angle of intersection is <90°.

tga20 and WT mice, and profoundly differ from all other murine PrP deposits in our laboratory, including those induced in mice by CWD, bovine spongiform encephalopathy, natural sheep scrapie, and RML prions.

Discussion

We report that the alteration of two codons in the PrP reading frame, whose conformational consequences on the encoded protein are understood in atomic detail (16), induces a progressive neurologic disease in transgenic mice. Although clinical signs arose stochastically and often late in life, the penetrance of the disease was 100%. The RL-PrP disease, unlike the syndromes associated with overexpression of WT PrP (21), was caused by the loop mutations, given that tga20 mice that expressed >2-fold higher PrP protein levels than RL-PrP mice did not develop spontaneous PrP plaques, histological signs, or clinical prion disease.

Several internally deleted PrP variants cause neurodegeneration, which is suppressed by co-expression of WT PrP (11, 22, 32). In contrast, the concomitant presence of PrP did not influence any of the clinical and histological parameters of the RL-PrP disease. Therefore, the RL-PrP disease arises by fundamentally different mechanisms than the aforementioned syndromes.

Conversely, the spongiform degeneration, the conspicuous PrP plaques, and the deposition of partially PK-resistant PrP in the brain of tg1020 mice were suggestive of a bona fide prion disease. This conjecture was substantiated by the transmissibility of the RL-PrP disease to young tga20 and, after adaptation, to WT mice, but not to $Prnp^{0/0}$ mice. Disease transmission occurred even after extensive heat-treatment of tg1020 brain homogenate, virtually ruling out any conventional infectious agent.

The failure to directly transmit disease from RL-PrP to WT mice, and the highly variable incubation periods of first-passage RL-PrP in tga20 mice, may point to a transmission barrier imposed by the 170N and 174T substitutions in mPrP. This barrier appeared to be bidirectional, as tg1020 mice experienced long, highly variable incubation periods upon inoculation with RML mouse prions. The incubation time and its variability decreased by $\approx 50\%$ on second passage in tg1020 mice, as would be expected from strain adaptation (25, 33, 34).

Progressive alterations in the disease phenotype occurred over the course of four RL-PrP passages in tga20 mice, such as shortening of incubation periods, a shift from plaque-like to synapticdiffuse cerebral PrP deposits, progressively increased resistance of PrP to PK, and, by the fourth passage, acquisition of lymphotropism. These changes suggest that the conformation and/or the size of the PrP aggregates underwent gradual changes. Alternatively, the original seeds in RL-PrP mice may be heterogeneous and propagate at different rates, with the fittest sub-strains being gradually selected and eventually becoming dominant. However, the evolution of biochemical properties and pathological features was not accompanied by shifts in PTAA emission spectra, indicating that certain core properties of RL-PrP prions were stable over at least four passages. PTAA interacts anisotropically with amyloid, and its emission spectrum reports on the structure of PrPSc aggregates (29). The stability of PTAA spectra suggests that an aspect of the structure of RL-PrP aggregates is faithfully propagated onto WT PrP aggregates over many generations. Conversely, the strain adaptation phenomena described may result from interaction with ancillary, non-PrP constituents.

After several passages into tga20 mice, RL-PrP acquired PK resistance and proteolysis patterns similar to those of the murine laboratory prion strain, RML. These phenomena are unlikely to stem from contamination or cross-infection with RML prions. First, RML-infected brains are PTAA-negative (29), whereas RL-PrP aggregates of tg1020 mice and all passages therefrom were consistently PTAA-positive. Second, RL $\rightarrow tga20$ PrP was PK-resistant by the third passage, yet was not lymphotropic, whereas RML is heavily lymphotropic (35). Third, the clinical disease and incubation period varied vastly within and between groups of mice inoculated with two second-passage RL $\rightarrow tga20$ mice, whereas the incubation period of RML is highly consistent, with <5 days variation within a group. Fourth, brain histoblots of third-passage RL $\rightarrow tga20$ revealed large, patchy aggregates, unlike the diffuse stains of RML.

What is the mechanism initiating RL-PrP prion formation? The loop region of PrP contains several glutamine and asparagine residues, which are common in amyloidogenic proteins and may act as "hot spots" in protein aggregation (36). In amyloid aggregates of short fibril-forming polypeptides, glutamines and asparagines can form multiple intermolecular hydrogen bonds (i.e., "steric zippers") (37). NMR spectroscopy and molecular dynamics simulations indicate that the N170 residue protrudes highly directionally from PrPC, whereas S170 is disordered (16,

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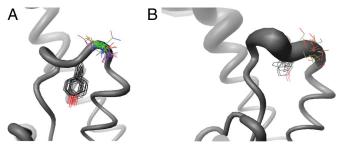


Fig. 5. The RL-PrP and WT-mPrP loop regions. (A) The 169Y and 170N side chains are quite precisely defined in the NMR structure of RL-PrP (16), and the side chain of 170N (green) protrudes from the core of the molecule. (B) The 169Y and 170S side chains in the NMR structure of WT-mPrP are disordered. The different features of the side chains of residues 169 and 170 in the two proteins are also predicted by molecular dynamic simulations (38).

38). Hence the N170 asparagine side chain might engage in long-range intermolecular hydrogen bonds with complementary sequences and initiate steric zippers (Fig. 5). Alternative mechanisms of amyloidogenesis are equally conceivable, including the disruption of interactions between the loop region and hypothetical anti-amyloidogenic chaperones.

If the RL substitutions are pathogenic for mice, why was no spontaneous prion disease observed in transgenic mice overexpressing cervid PrP^C (39, 40)? In addition to the two loop residues, cervid and mouse PrP^C differ at 11 further positions within their globular domains, seven of which are located near the C-terminal end of the α 3 helix. This site is thought to be important for species-specific molecular interactions in TSE transmission. Therefore, some of these substitutions may even have evolved to counteract enhanced aggregation tendencies. A comparison of the three-dimensional structures of elk PrP with murine PrP shows that the orientation of the α 3 helix is closely similar in the two proteins (16). However, a change in the polarity and/or charge of specific amino acid side chains in this area might nonetheless modify the specificity of this surface area for intermolecular interactions. In view of these results, it will be interesting to assess the impact wrought by the structural features of the rigid loop onto the enigmatic pathogenesis and transmission barriers of cervid CWD. Further studies may help clarify the significance of the $\beta 2$ — $\alpha 2$ loop region on prion disease development and species barriers.

Materials and Methods

Refer to SI Text for detailed materials and methods and for a description of the transgene construct and mouse characterization.

Prion Inoculations. For detailed methods, refer to SI Text. Briefly, mice were intracerebrally inoculated into the left parietal cortex with 30 μ l of a 5% brain homogenate from an un-inoculated tg1020 mouse, an un-inoculated tga20 mouse, and subsequently from tga20 mice infected with RL-PrP prions. Brains from terminal mice were collected using new instruments on new surface covers.

Histopathology and Immunohistochemical Stains. For detailed methods, refer to SI Text. Briefly, 2- μ m-thick sections were cut onto positively charged silanized glass slides and stained with hematoxylin and eosin, or immunostained using antibodies for PrP (SAF84), astrocytes (GFAP), or microglia (Iba1).

ACKNOWLEDGMENTS. We thank Frank Baumann, Jens Pahnke, Gino Miele, Sei-ichi Yusa, Kirsten Mertz, Amedeo Caflisch, and Pietro Alfarano for discussions; Petri Vilkman, Marianne König, and the animal care staff for technical support; and Michel Mittelbronn and Burkhard Seifert for help with statistics. This study was supported by the European Union TSEUR collaborative research project (A.A.) and Understanding Protein Misfolding and Aggregation by NMR program (K.W.), the Swiss National Science Foundation (A.A.), the National Competence Centers for Research on Neural Plasticity and Repair (A.A.) and on Structural Biology (K.W.), National Institutes of Health grants K08-AI01802 and 5R21NS055116 (to C.J.S.), the Foundation for Research at the University of Zürich (C.J.S.), the US National Prion Research Program (C.J.S. and A.A.), the Knut and Alice Wallenberg Foundation (K.P.R.N.), the Max-Clöetta and Bonizzi-Theler Foundations (M.H.), Stammbach Foundation (A.A.), and ETH Zürich (K.W.).

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