MATERIALS AND METHODS

Construction of transgenic mice. The construction and breeding of Tg(MoPrP-A)4053 and Tg(MoPrP,Δ23-88)9949/Prnp0/0 mice have been described previously (S1, S2). FVB and CD1 mice as well as Syrian hamsters were obtained from Charles River Laboratories (Wilmington, MA). C57B6 mice are from Jackson Laboratories (Bar Harbor, ME).

Production of recMoPrP(89–230). We used the pPho 41 secretion vector employing the alkaline-phosphatase promoter and the periplasmic bacterial signal peptide from Shigella toxin II, for expression of MoPrP(89–230) in E. coli (S3). High levels of expression were obtained for MoPrP(89–230) that corresponds to the sequence of MoPrP 27-30. The transfected bacteria were grown in a 10 L fermenter and harvested 36 h after inoculating the fermentation media. The recombinant protein was released by
disruption of the bacteria in 8 M GdnHCl/100 mM DTT, pH 8.0, and purified by size-exclusion chromatography (26 mm × 600 mm, Pharmacia, Uppsala, Sweden) and reverse-phase chromatography. Using a C-4 preparative column (25 mm × 250 mm; Vydac, Hesperia, CA), the desired recMoPrP(89–230) was eluted at approximately 34% acetonitrile using a linear gradient of 0–34% acetonitrile. The molecular weight of recMoPrP(89–230) was determined by laser desorption and electrospray mass spectrometry.

Production of amyloid fibrils. Amyloid fibrils were formed on incubation of recMoPrP(89–230) (0.6 mg/ml) at 37°C in 3 M urea, 0.2 M NaCl, 50 mM sodium acetate buffer, pH 5.0, as previously described (S4). The kinetics of fibril formation were monitored using a thioflavin T binding assay (S5).

Preparation of inocula. Inocula were prepared by dialysis of 2 ml of recMoPrP(89–230) fibrils in 2 L of sterile phosphate-buffered saline (PBS) without calcium or magnesium, pH 7.2, that was changed 3 times over 2 days. The concentration of recMoPrP(89–230) in the inocula was ~0.5 mg/ml.

Ten percent brain homogenates in sterile PBS without calcium or magnesium were prepared by repeated extrusion through syringe needles of successively smaller size, from 18- to 22-gauge. New, sterile, individually packaged needles, syringes, and tubes were used. All work was carried out in laminar flow hoods to avoid cross-contamination.

Determination of prion incubation periods. Mice of either sex, aged 7 to 10 weeks, were inoculated intracerebrally with 30 µl of recMoPrP(89–230) amyloid fibrils, control
PBS, or 1% brain homogenate in calcium- and magnesium-free PBS plus 5 mg of bovine serum albumin per ml. Inoculation was carried out with a 27-gauge disposable hypodermic needle inserted into the right parietal lobe. After inoculation, mice were examined daily for neurologic dysfunction. Standard diagnostic criteria were used to identify animals exhibiting signs of prion disease (S6, S7). In each group, some animals whose deaths were imminent were sacrificed, and their brains were removed for histologic and biochemical analysis. The RML prion strain propagated in Swiss mice was originally provided by W. Hadlow (Rocky Mountain Laboratory, Hamilton, MT) and was passaged in Swiss CD1 mice obtained from Charles River Laboratories (S8). Me7 and 139H inocula were obtained from R. Kimberlin and the C506 inoculum was provided by C. J. Gibbs (S9-S11). The DY inoculum was a gift from R. Marsh and the 301V inoculum was obtained from H. Fraser (S12, S13).

**Neuropathology.** Brains were removed rapidly at the time of sacrifice, immersion-fixed in 10% buffered formalin, and embedded in paraffin. Eight-micrometer-thick sections were stained with hematoxylin and eosin for evaluation of prion disease. Peroxidase immunohistochemistry with antibodies to glial fibrillary acidic protein was used to evaluate the degree of reactive astrocytic gliosis. Immunohistochemistry of PrPSc was performed by the hydrated autoclaving method using the PrP-specific HuM-R2 recombinant monoclonal antibody fragment (Fab) (S14).

**Protease digestion and PrP immunostaining.** Nuclei and debris were removed from brain homogenates by centrifugation at 1,000 × g for 10 min. Homogenates were adjusted to 1 mg of protein per ml in 100 mM NaCl–1 mM EDTA–2% Sarkosyl–50 mM Tris-HCl (pH 7.5). Twenty micrograms of proteinase K (PK; Boehringer Mannheim) per
ml was added to 0.5 ml of adjusted homogenate to achieve a ratio of total protein to enzyme of 50:1. After incubation at 37°C for 1 h, proteolytic digestion was terminated by the addition of 8 ml of 0.5 M phenylmethylsulfonyl fluoride in absolute ethanol. Both PK-digested and undigested samples were prepared for sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis by mixing equal volumes of adjusted homogenate and 2× sample buffer.

Following electrophoresis, Western blotting was performed as previously described (S6). Membranes were blocked with 5% nonfat milk protein in calcium-and magnesium-free PBS plus 0.1% Tween 20 (PBST) for 1 h at room temperature. Blocked membranes were incubated with primary PrP-specific recFab HuM-D13 at 1 µg/mL in PBST for 1 h at 4°C (S14). After incubation with primary Fab, membranes were washed 3× for 10 min in PBST, incubated with horseradish peroxidase–labeled anti-human Fab secondary antibody (ICN), diluted 1:5,000 in PBST for 25 min at room temperature, and washed again 3× for 10 min in PBST. After chemiluminescent development with an ECL reagent (Amersham) for 1 to 5 min, blots were sealed in plastic covers and exposed to ECL Hypermax film (Amersham). Films were processed automatically in a Konica film processor.

**Human-mouse chimera (HuM) Fab preparation.** Sequences obtained from a phage display library (S15) were utilized for the D13 and R2 clones, and the variable sequences were inserted into an expression plasmid containing human immunoglobulin G (IgG) Fab framework. *Escherichia coli* 33B6 competent cells were transformed with plasmid containing HuM Fab sequences, and a single bacterial colony from a Luria-
Bertani medium agar plate containing 100 mg of ampicillin was grown in 500 ml of Luria-Bertani media containing 100 mg of ampicillin per ml at 30°C overnight. A Biostat B controller (B. Braun, Melsungen, Germany) was used together with a 10 L vessel for all fermentation procedures. Fermentation was carried out in medium containing (per liter) MT-8 salts (0.26 g of potassium phosphate dibasic, 0.13 g of sodium phosphate monobasic dihydrate, 0.5 g of ammonium sulfate, 0.1 g of sodium citrate dihydrate, and 0.15 g of potassium chloride per liter); 0.5 g of isoleucine; 20% NZ amines; 20% yeast extract; 1 mM magnesium sulfate; 50% glucose; trace metals; and 100 mg of ampicillin per mg and was completed in 40 to 48 h. The fermented culture was pelleted at 8,000 rpm for 1 h at 4°C in an Avanti J-20 centrifuge (Beckman), and the pellet was stored at 20°C. The frozen paste was resuspended in five volumes of 2 mM imidazole–20 mM sodium phosphate–250 mM sodium chloride (pH 7.0), homogenized in a tissue homogenizer at 9,600 rpm, and was processed twice in a Microfluidizer M-110 EH (Microfluidics Co., Newton, MA). The cell paste was titrated to 0.1% polyethylenimine (PEI) (5% stock solution at pH 8.0) and was stirred at 4°C for 30 min. The processed sample was then spun down with a Sorval J-10 (Beckman) at 10,000 rpm for 30 min at 4°C; the pellet was discarded and the supernatant was used for the purification procedure.

For purification, the sample was diluted in an equal volume of 20 mM imidazole (pH 7.0). The solution was loaded onto a Sepharose Fast Flow (Amersham Pharmacia, Uppsala, Sweden) column, and the recombinant HuM Fab was eluted with a linear gradient of five column volumes (cv) of 0 to 100% 20 mM imidazole–500 mM sodium acetate (pH 7.0). The resulting peak was directly applied onto an IMAC column, and it
was eluted with 5 cv of 200 mM imidazole (pH 7.0). The peak was dialyzed with three changes of 100 volumes of 10 mM Tris-HCl (pH 7.2) at 4°C overnight. The dialyzed recombinant material was further purified through a Sepharose Fast Flow utilizing a 0–100% linear gradient of 10 mM Tris-HCl–500 mM sodium chloride (pH 7.2). The final purified HuM Fab peak was filtered through a 0.45-mm pore-size sterile filter and stored at 4°C.
Fig. S2

A

Vacuolation Scores

Unseeded
Seeded

LC FC DG CA LT MT Cd MS LS Cm Cg Cw Bs

B

Vacuolation Scores

RML

LC FC DG CA LT MT Cd MS LS Cm Cg Cw Bs

Legname et al.
Legname et al.

Fig. S3

A

Vacuolation Scores

FVB
Tg4053

LC FC DG CA LT MT Cd MS LS Cm Cg Cw Bs

B

Vacuolation Scores

FVB
Tg4053

LC FC DG CA LT MT Cd MS LS Cm Cg Cw Bs
SUPPLEMENTARY FIGURE LEGENDS

Fig. S1. Expression and refolding of recMoPrP(89–230). (A) Expressed and purified recombinant PrPs (S3) were separated in 16% Tris-glycine SDS-PAGE gel (Invitrogen) and silver-stained. M: protein molecular weight markers; lane 1: wt recMoPrP(89–230); lane 2: wt recMoPrP(23–230) is shown for comparison. Molecular weight markers are expressed in kilodaltons (kDa). Mass spectrometry measurements for full-length recMoPrP(23–230) and the N-terminally truncated recMoPrP(89–230) were made and compared to the theoretical mass. (B) recMoPrP(89–230) (0.5 mg/ml) in 0.6 ml was incubated at 37°C in 3 M urea, 0.2 M NaCl, 50 mM sodium acetate buffer, pH 5.0, using a conical shaker oscillating at 600 rpm (S4). Seeded PrP amyloid fibrils were prepared using the same conditions as those used for the unseeded fibrils except 1% (w/w) of freshly prepared, preformed fibrils composed of recMoPrP(89–230) was added to the reaction mixture. Kinetics of amyloid formation for unseeded recMoPrP(89–230) (filled circles) and seeded (open squares) were monitored using the thioflavin T binding assay (S5). Inocula (0.5 mg/ml) for bioassays were prepared by dialysis of 2 ml of recPrP fibrils using 2 L of stirred PBS buffer, pH 7.2, that was changed 3 times over 2 days. (C) Electron micrograph of amyloid fibrils formed from recMoPrP(89–230) negatively stained with ammonium molybdate. Scale bar, 50 nm.

Fig. S2. Vacuolation score histograms from Tg9949 mouse brains indicate that vacuolation phenotype is different for the three inoculates: unseeded and seeded recMoPrP(89–230) amyloid (A) and RML prions (B). The vacuolation histogram is a semiquantitative estimate of the area of a brain region occupied by vacuoles. Bs, brainstem (pons); CA, cornu ammonis of the hippocampus; Cd, caudate nucleus; Cg,
cerebellar granule cell layer; Cm, cerebellar molecular layer; Cw, cerebellar white matter; DG, dentate gyrus of the hippocampus; FC, frontal cortex; LC, limbic cortex (cingulate gyrus); LS, lateral septal nuclei; LT, lateral thalamic nuclei; MS, medial septal nuclei; MT, medial thalamic nuclei.

**Fig. S3.** Vacuolation score histograms from FVB and Tg4053 mice inoculated with seeded recMoPrP(89–230) amyloid (A) or with RML prions (B). See Fig. S2 for legends.
Table S1. Transmission of synthetic and natural prion strains to Tg9949 mice.

<table>
<thead>
<tr>
<th>Mouse strain (expression level)</th>
<th>Inoculum (^b)</th>
<th>Incubation time (Days ± SEM)</th>
<th>n/n₀ (^d)</th>
<th>Terminated at (days)</th>
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<tbody>
<tr>
<td>Tg9949(^a) (16×)</td>
<td>Seeded recMoPrP</td>
<td>516 ± 27</td>
<td>7/7</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>MoSP1</td>
<td>258 ± 25</td>
<td>7/7</td>
<td>n/a</td>
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<tr>
<td></td>
<td>Unseeded recMoPrP</td>
<td>590 ± 46</td>
<td>4/4</td>
<td>n/a</td>
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<tr>
<td>PBS(^c)</td>
<td></td>
<td>0/7</td>
<td>620</td>
<td></td>
</tr>
<tr>
<td>RML(CD1)</td>
<td></td>
<td>160 ± 4</td>
<td>11/11</td>
<td>n/a</td>
</tr>
<tr>
<td>RML(Tg9949)</td>
<td></td>
<td>143 ± 7</td>
<td>10/10</td>
<td>n/a</td>
</tr>
<tr>
<td>Me7(C57B6)</td>
<td></td>
<td>220 ± 4</td>
<td>7/7</td>
<td>n/a</td>
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<tr>
<td>301V(CD1)</td>
<td></td>
<td>433 ± 25</td>
<td>4/8</td>
<td>512</td>
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<tr>
<td>C506(C57B6)</td>
<td></td>
<td>253 ± 30</td>
<td>10/10</td>
<td>n/a</td>
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<tr>
<td>Sc237(SHa)</td>
<td></td>
<td>479 ± 14</td>
<td>3/8</td>
<td>525</td>
</tr>
<tr>
<td>139H(SHa)</td>
<td></td>
<td>491 ± 19</td>
<td>3/5</td>
<td>533</td>
</tr>
<tr>
<td>DY(SHa)</td>
<td></td>
<td>491 ± 19</td>
<td>3/5</td>
<td>533</td>
</tr>
<tr>
<td>DY(CD1)</td>
<td></td>
<td>143 ± 5</td>
<td>8/9</td>
<td>201</td>
</tr>
<tr>
<td>FVB (1×)</td>
<td>MoSP1</td>
<td>154 ± 4</td>
<td>9/9</td>
<td>n/a</td>
</tr>
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<td>RML(CD1)</td>
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<td>117 ± 3</td>
<td>10/10</td>
<td>n/a</td>
</tr>
<tr>
<td>Tg4053(^a) (8×)</td>
<td>MoSP1</td>
<td>90 ± 1</td>
<td>10/10</td>
<td>n/a</td>
</tr>
<tr>
<td>RML(CD1)</td>
<td></td>
<td>59 ± 2</td>
<td>10/10</td>
<td>n/a</td>
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</tbody>
</table>

\(^a\) Transgenes were expressed in FVB/Prnp\(^0/0\) mice. Expression levels of PrP transgene product given in parentheses are relative to SHaPrPC\(^0\) levels in Syrian hamster brain. PrP levels were determined by immunoblots of serially diluted brain homogenates.

\(^b\) Text in parentheses indicates the host in which the inoculum was last passaged. MoSP1, prions derived from Tg9949 mice inoculated with seeded recPrP amyloid.

\(^c\) Tg9949 mice were inoculated with PBS at ~50 days of age and did not show any signs of neurologic dysfunction when sacrificed at 620 days after inoculation. One additional Tg9949 mouse was sacrificed at 530 days after inoculation and evaluated for neuropathologic changes and protease-resistant PrP on Western blotting.

\(^d\) Number of mice developing prion disease/number inoculated and still alive when the experiment was terminated as indicated in the last column.
REFERENCES


