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N-terminally tagged prion protein supports prion propagation in transgenic mice

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Abstract

The eight amino acid sequence, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, representing the FLAG peptide, was inserted after codons 22 or 88 of the mouse (Mo) prion protein (PrP) gene. Inclusion of the FLAG sequence at these locations interfered neither with the cellular processing of PrP^C nor its conversion into PrP^{Sc}. Inclusion of the FLAG epitope at residue 22 but not at residue 88 facilitated immunodetection of tagged PrP by anti-FLAG monoclonal antibodies (mAbs). Inoculation of transgenic (Tg) mice expressing N-terminally tagged MoPrP with Mo prions resulted in abbreviated incubation times, indicating that the FLAG sequence was not deleterious to prion propagation. Immunopurification of FLAG-tagged $MoPrP^C$ in the brains of Tg mice was achieved using the calcium-dependent anti-FLAG M1 mAb and non-denaturing procedures. Although the function of PrP^C remains unknown, our studies demonstrate that some modifications of PrP^C do not inhibit the one biological activity that can be measured, i.e., conversion into PrP^{Sc}. Tagged PrP molecules may prove useful in the development of improved assays for prions as well as structural studies of the PrP isoforms.

Keywords: epitope tagging; prion protein; transgenic mice

Prions are infectious pathogens afflicting humans and animals which cause fatal degenerative diseases of the central nervous system (CNS) (Prusiner, 1994). They are composed largely, if not entirely, of the scrapie isoform of the prion protein (PrP) designated PrP^{Sc} (Prusiner, 1982), which is derived from the host-encoded cellular prion protein (PrP^C) (Basler et al., 1986) through a post-translational process (Borchelt et al., 1990). A fundamental event in the propagation of prions is the conformational transition of α-helices in PrP^C into β-sheets in PrP^{Sc} (Pan et al., 1993). Evidence from transgenic (Tg) mouse studies suggests a requirement for an additional component(s) referred to as protein X in this conversion process (Telling et al., 1995).

Purification of PrP^{Sc} was facilitated by virtue of its relative resistance to proteolytic degradation and insolubility in non-denaturing detergents (Bolton et al., 1982; Prusiner et al., 1982). Purification of PrP^C has been more problematic. Immunoaffinity chromatography of PrP^C yielded only small amounts of purified protein (Turk et al., 1988). Improved purification of PrP^C was accomplished by a multi-step purification procedure involving detergent extraction and separation by immobilized Cu^{2+} ion affinity chromatography, followed by cation-exchange chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Pan et al., 1992).

The production of monoclonal antibodies (mAbs) against PrP, in particular against MoPrP, has been frustrating. The generation of mouse anti-MoPrP mAbs has been hindered by immune tolerance to inoculated MoPrP antigens, presumably because of the recognition of MoPrP as self in immunized animals (Prusiner et al., 1993; Williamson et al., 1996). Recently, a variety of artificial epitope sequences have been described that were shown to be useful for tagging and detecting recombinant proteins. These include the "Strep tag" (Schmidt & Skerra, 1994), a nine–amino acid peptide sequence, consisting of Ala-Trp-Arg-His-Pro-Gln-Phe-Gly-Gly; the c-myc tag, an eleven–amino acid sequence from human c-myc consisting of Glu-Gln-Lys-Leu-Leu-Ser-Glu-Glu-Asp-Leu-Asn, which is recognized by mAb 9E10 (Manstein et al., 1995); an epitope derived from an influenza virus hemagglutinin (HA) subtype represented by the thirteen–amino acid sequence Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ile-Glu-Gly-Arg recognized by mAb 12CA5 (Murray et al., 1995); and the Glu-Glu-Phe sequence recognized by the mAb YL1/2 to α-tubulin (Stammers et al., 1991). Inclusion of artificial epitopes in recombinant proteins avoids the

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necessity for development of a specialized scheme or functional assay for protein purification and circumvents the need to raise mAbs against the protein.

One such tag, the eight amino acid FLAG marker peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, has a number of features which make it effective not only for immunodetection but also for immunoaffinity purification of recombinant proteins (Kunz et al., 1992). A particularly useful feature is the calcium-dependent binding of the anti-FLAG M1 mAb to recombinant proteins containing the FLAG peptide at the N-terminus (Prickett et al., 1989). Removal of calcium ions by chelation with EDTA permits efficient immunoaffinity purification of FLAG fusion proteins without denaturation. A further advantage of the FLAG system is that it allows cleavage of the FLAG peptide from purified protein because the tag contains the rare five amino acid recognition sequence for enterokinase.

In order to overcome the lack of anti-MoPrP mAbs, we created novel recombinant FLAG-tagged PrP constructs by engineering the FLAG peptide into MoPrP. PrP contains a 22 amino acid signal peptide that is proteolytically processed during maturation. We engineered the sequence for the FLAG peptide after codon 22 of the MoPrP gene; amino acid 23 of the recombinant FLAG-MoPrP is therefore Asp, the first amino acid of the FLAG peptide. Because detection with the anti-FLAG M1 mAb requires that the FLAG sequence occurs at the N-terminus of recombinant proteins (Prickett et al., 1989), our ability to detect tagged PrP^C with the anti-FLAG M1 mAb demonstrates that inclusion of the FLAG sequence at this position does not prevent proteolytic cleavage of the signal peptide. In contrast, a second construct tagged PrP^C containing the FLAG sequence at an internal location after codon 88 was not detected by the anti-FLAG M1 mAb. Both FLAG-tagged PrP molecules were efficiently expressed and processed in cultured cells and we were able to immunopurify N-terminally FLAG-tagged PrP on an anti-FLAG M1 mAb affinity column. More importantly, biological activity was conserved in FLAG-tagged PrP: inclusion of the FLAG epitope at either location did not inhibit formation of PrP^{Sc} and the recombinant N-terminally FLAG-tagged MoPrP fusion protein retained the ability to support prion propagation in Tg(FLAG–MoPrP) mice inoculated with mouse prions. These studies open the possibility of obtaining large quantities of highly purified, biologically active FLAG-tagged PrP from Tg mice for biochemical and structural studies which promises to facilitate research into mechanisms of prion replication.

Results

Construction of epitope-tagged PrP expression cassettes

The FLAG system has the advantage of efficient, one-step immunoaffinity purification without denaturation using the anti-FLAG M1 mAb, which binds to proteins containing the FLAG peptide in a calcium-dependent system. Recognition of FLAG fusion proteins by the anti-FLAG M1 mAb relies on the location of the FLAG epitope at the N-terminus of the protein (Prickett et al., 1989). Since PrP is processed in cells by the removal of a 22 amino acid N-terminal signal peptide, we engineered the FLAG sequence immediately distal to the signal peptidase cleavage site at amino acid residue 22 of PrP (Fig. 1). A second construct in which the FLAG sequence was inserted at an internal location of MoPrP between codons 88 and 89 was also produced (Fig. 1B).

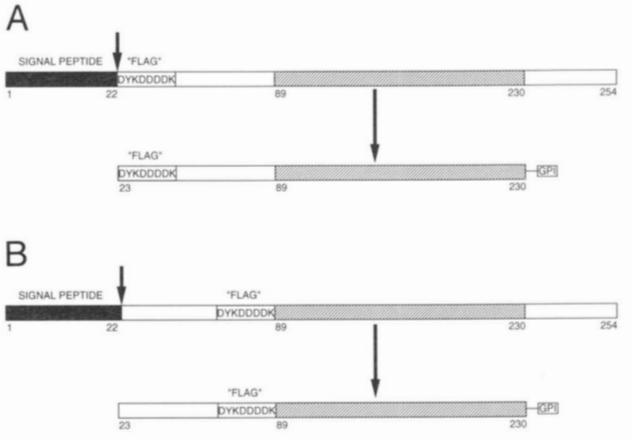

Fig. 1. Structure of FLAG-tagged PrP constructs. **A:** N-terminally FLAG-tagged mouse PrP. The N-terminal 22–amino acid signal peptide, shown shaded, is removed from the primary PrP translation product by cleavage with signal peptidase after amino acid residue 22. In the N-terminally tagged FLAG–PrP construct, amino acid 23, the N-terminal amino acid of mature PrP^C is aspartate, the first amino acid residue of the eight amino acid FLAG peptide. Proteolytic cleavage also occurs at C-terminus where 24 amino acids are removed and a glycosylphosphatidylinositol anchor is attached. **B:** PrP with a FLAG tag at amino acid 88. In both A and B, the protease-resistant core of PrP27-30, consisting predominantly of amino acid residues 89–230, is shown hatched. In both FLAG-tagged constructs, proteinase K treatment results in the loss of the FLAG epitope. For convenience, the numbering of amino acid residues refers to the locations in untagged wild-type MoPrP.

Conversion of FLAG-tagged PrP^C into PrP^{Sc} in ScN2a cells

The two FLAG-tagged PrP sequences were engineered into an MHM2PrP expression cassette that has been described previously (Scott et al., 1992). This variant consists of the MoPrP gene containing the two–amino acid epitope for monoclonal antibody 3F4, derived from SHaPrP. The resulting FLAG-tagged MHM2PrP expression cassettes were cloned into the SPOX.II neo expression vector (Scott et al., 1992), which allows selection of cells expressing recombinant PrP to be selected in the presence of G418. Chronically infected ScN2a cells, which continuously produce $MoPrP^{Sc}$ (Butler et al., 1988), were transfected with SPOX.II neo-based FLAG-tagged MHM2PrP and control MHM2PrP constructs, and neomycin-resistant cells were selected in medium containing G418. MoPrP is not recognized by anti-PrP 3F4 mAb, so the inclusion of the 3F4 epitope allows discrimination between ectopically expressed recombinant PrP and endogenous MoPrP.

Western blots of cell lysates from stable transformants of ScN2a cells transfected with the FLAG-tagged MHM2PrP constructs show that inclusion of the FLAG epitope after amino acid residues 22 or 88 does not prevent expression or processing of PrP (Fig. 2A, B, lane 3). Treatment of cells expressing N-terminally FLAG-tagged MHM2PrP with phosphatidylinositol phospholipase C (PIPLC) resulted in release of the protein into the medium showing that the tagged protein was attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor (data not shown).

The placement of a foreign hydrophilic sequence at these two locations raised concerns that the recombinant FLAG-PrP would be unable to support prion propagation, perhaps by affecting the ability of PrP^C to adopt a conformation essential for PrP^{Sc} conversion and the propagation of infectious prions. Hallmarks of

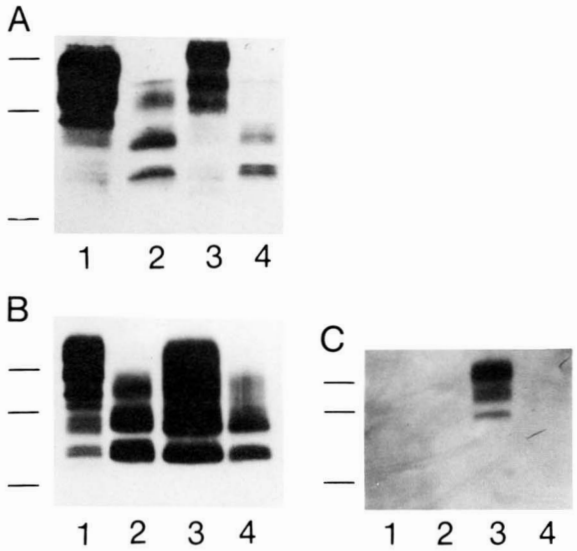

Fig. 2. Expression of FLAG-tagged PrP and conversion to PrP^{Sc} in ScN2a cells. Aliquots of NP-40 detergent lysates from ScN2a cell lines expressing FLAG-tagged MHM2PrP or control cells expressing untagged MHM2PrP were either untreated or digested with 20 μg proteinase K for 60 min at 37 °C. Samples were resolved by SDS-PAGE and analyzed by Western blotting. The blots were exposed to either anti-PrP 3F4 mAb or anti-FLAG M1 mAb in the presence of 1mM $CaCl_2$, developed using enhanced chemiluminescence (ECL) (Amersham Corporation) and exposed to X-ray film. **A:** Immunodetection of N-terminally FLAG-tagged PrP^{C} and PrP^{Sc} with anti-PrP 3F4 mAb. Lane 1: Detergent lysate from ScN2a cells expressing MHM2PrP not treated with proteinase K; lane 2: detergent lysate from ScN2a cells expressing MHM2PrP treated with proteinase K; lane 3: detergent lysate from ScN2a cells expressing N-terminally FLAG-tagged MHM2PrP not treated with proteinase K; lane 4: detergent lysate from ScN2a cells expressing N-terminally FLAG-tagged MHM2PrP treated with proteinase K. **B:** Immunodetection of $MHM2PrP^{C}$ and PrP^{Sc} with FLAG inserted at amino acid residue 88 with anti-PrP 3F4 mAb. Lane 1: detergent lysate from ScN2a cells expressing MHM2PrP not treated with proteinase K; lane 2: detergent lysate from ScN2a cells expressing MHM2PrP treated with proteinase K; lane 3: detergent lysate from ScN2a cells expressing MHM2PrP with FLAG inserted at amino acid residue 88 not treated with proteinase K; lane 4: detergent lysate from ScN2a cells expressing MHM2PrP with FLAG inserted at amino acid residue 88 treated with proteinase K. **C:** The calcium dependent anti-FLAG M1 monoclonal antibody detects N-terminally FLAG-tagged PrP^{C} but not PrP tagged at an internal location. Lane 1: detergent lysate from ScN2a cells expressing MHM2PrP with FLAG inserted at amino acid residue 88 not treated with proteinase K; lane 2: detergent lysate from ScN2a cells expressing MHM2PrP with FLAG inserted at amino acid residue 88 treated with proteinase K; lane 3: 10% brain homogenate from a $Tg(FLAG–MoPrP)7755/Prnp^{0/0}$ mouse not treated with proteinase K; lane 4: 10% brain homogenate from a $Tg(FLAG–MoPrP)7755/Prnp^{0/0}$ mouse treated with proteinase K. The positions of protein molecular weight markers are shown to the left of the blot and correspond to molecular masses of (from top to bottom) 35.5, 29.1, and 14.5 kDa.

PrP^{Sc} are its insolubility in detergents and relative protease resistance. Limited proteinase K digestion of PrP^{Sc} in infected ScN2a cells produces PrP 27–30 consisting predominantly of residues 89 to 230 (Fig. 1). Using the anti-PrP 3F4 mAb to detect proteinase K-resistant FLAG-tagged MHM2PrP, we found that, like ScN2a cells expressing untagged MHM2PrP (Fig. 2A, B, lane 2), ScN2a cells efficiently produced PrP^{Sc} from MHM2PrP tagged with the FLAG sequence at either location (Fig. 2A, B, lane 4). Thus, addition of the FLAG-tag at positions 22 or 88 interferes neither with the expression of PrP^{C} nor its conversion to PrP^{Sc}.

Detection of FLAG-tagged PrP by anti-FLAG M1 mAb

The calcium-dependent anti-FLAG M1 mAb antibody reacts only with fusion proteins in which the FLAG epitope is exposed at the N-terminus (Prickett et al., 1989). Calcium-dependent anti-FLAG M1 mAb immunoreactivity was only observed with $MoPrP^{C}$ containing the FLAG sequence after amino acid residue 22 (Fig. 2C, lane 3) and not in $MoPrP^{C}$ containing the FLAG sequence positioned internally after amino acid 88 (Fig. 2C, lane 1). Since the protease-resistant core of PrP^{Sc} extends from amino acid residues 89 to 230, proteinase K treatment of FLAG-PrP results in the loss of the FLAG epitope at residues 22 and 88. Thus, only N-terminally FLAG-tagged $MoPrP^{C}$ is detected with this antibody (Fig. 2C, lane 3).

Removal of Asn-linked oligosaccharides from MoPrP by digestion of denatured PrP^{C} with peptide N-glycosidase F (PNGase F) resulted in the disappearance of the highly glycosylated 33–35 kDa PrP^{C} band (Fig. 3, lane 1) and the persistence of two unglycosylated PrP species (Fig. 3, lane 3) when immunoblots were probed with the anti-PrP RO73 polyclonal antiserum. These bands represent unglycosylated full-length PrP^{C} of ~27 kDa corresponding to GPI-containing deglycosylated full-length PrP^{C} (Haraguchi et al., 1989) and an N-terminally processed ~17 kDa intermediate of PrP^{C}, which is generated by cleavage of PrP^{C} (Pan et al., 1992; Taraboulos et al., 1995). This truncated form has also been detected in human brain where cleavage occurs at either residue His 111 or Met 112 (Chen et al., 1995). The anti-PrP RO73 polyclonal antiserum also detected glycosylated N-terminally FLAG-tagged $MoPrP^{C}$ (Fig. 3, lane 2). PNGase F treatment of N-terminally FLAG-tagged $MoPrP^{C}$ also resulted in the appearance of two unglycosylated PrP species (Fig. 3, lane 4). Untagged wild-type (wt) MoPrP was not recognized by the anti-FLAG M1 mAb (Fig. 3, lanes 5 and 7), whereas glycosylated FLAG-$MoPrP^{C}$ 33–35 kDa and full-length deglycosylated FLAG-MoPrP were detected with the anti-FLAG M1 mAb (Fig. 3, lanes 6 and 8). Immunoreactivity of these species with the the anti-FLAG M1 mAb was highly dependent upon the presence of calcium ions (Fig. 3, lanes 9–12). The 17 kDa degradation product of FLAG–$MoPrP^{C}$ was not detected with the anti-FLAG M1 mAb since the FLAG epitope is cleaved upon formation of this product (Fig. 3, lane 8).

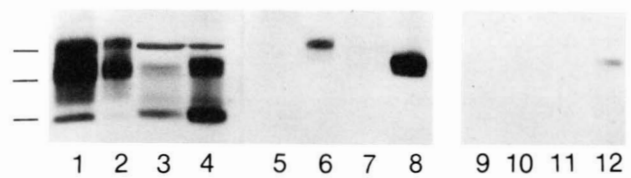

Fig. 3. Inclusion of the FLAG tag after amino acid residue 22 does not interfere with N-terminal processing of PrP. Aliquots of 10% brain homogenates from CD-1 or $Tg(FLAG–MoPrP)7755/Prnp^{0/0}$ mice were either treated or untreated with peptide N-glycosidase F (PNGase F) and analyzed by SDS-PAGE and Western blotting. Immunoblots were exposed to anti-PrP RO73 polyclonal antiserum (lanes 1–4) or anti-FLAG M1 mAb in the presence of 1mM $CaCl_2$ (lanes 5–8), or 2mM EDTA (lanes 9–12). Western blots were developed using enhanced chemiluminescence (ECL) (Amersham Corporation) and exposed to X-ray film. Lanes 1, 5 and 9: CD-1 mouse brain homogenate; lanes 2, 6 and 10: $Tg(FLAG–MoPrP)7755/Prnp^{0/0}$ mouse brain homogenate; lanes 3, 7 and 11: CD-1 mouse brain homogenate treated with PNGase F; lanes 4, 8 and 12: $Tg(FLAG–MoPrP)7755/Prnp^{0/0}$ mouse brain homogenate treated with PNGase F. The positions of protein molecular weight markers are shown to the left of the blot and correspond to molecular masses of (from top to bottom) 31, 21.5, and 14.5 kDa.

Since calcium dependent detection with the anti-FLAG M1 mAb requires that the FLAG sequence occur at the N-terminus of recombinant proteins (Prickett et al., 1989), we conclude that cleavage of the signal peptide after amino acid residue 22 is not inhibited by inclusion of the FLAG peptide at this location, and that aspartic acid, the first amino acid of the inserted FLAG peptide, is therefore the first residue at the N-terminus of mature FLAG–PrP.

Transgenic mice expressing FLAG-tagged MoPrP

Since the acquisition of protease resistance indicated that N-terminally FLAG-tagged MHM2PrP^{C} could be converted into PrP^{Sc} in cultured cells, we were encouraged to produce Tg mice to determine whether FLAG-tagged MoPrP could support mouse prion infectivity in vivo. The FLAG sequence at codon 22 was engineered into a MoPrP expression cassette, generating FLAG–MoPrP, which was cloned into the cosSHa.Tet cosmid expression vector for Tg mouse production. Five Tg founders were produced: three derived from microinjection of FVB mouse embryos and two from FVB/Prnp$^{0/0}$ mouse embryos. To simplify analysis, high copy number Tg(FLAG–MoPrP) mice derived only from the FVB/Prnp$^{0/0}$ background were used for mouse prion transmission studies because only transgene-expressed FLAG–MoPrP and not endogenous wt MoPrP is expressed in this case. Interference of transgene directed prion propagation by endogenous wt MoPrP has been observed in other situations (Telling et al., 1994, 1995, 1996).

Using the anti-PrP RO73 polyclonal antiserum, we estimated by serial dilution and immuno-dot blotting that the level of FLAG–MoPrP expression in brain extracts from one line designated Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ was ~100-fold higher than the level of wt MoPrP expression in CD-1 mice (Fig. 4A).

Propagation of prions in Tg(FLAG–MoPrP)Prnp$^{0/0}$ mice

High copy number Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice were inoculated intracerebrally with mouse RML prions. Inoculated mice developed clinical signs of scrapie with an average incubation time of ~52 days (Fig. 4B). This is similar to mouse RML prion incubation times observed in high copy number Tg(MoPrP-A)4053 mice, which overexpress MoPrP about eight-fold higher than wt mice (Carlson et al., 1994; Telling et al., 1996) and is considerably shorter than non-Tg Prnp^{a} mice, which have average incubation times of ~120 days (Fig. 4B).

As expected, the brains of clinically sick Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice inoculated with mouse RML prions contained proteinase K-resistant PrP 27–30, detectable with the anti-PrP RO73 polyclonal antiserum (Fig. 5). Because the inoculated Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice were derived from the fertilized embryos of FVB/Prnp$^{0/0}$ mice, which express no endogenous wt MoPrP, the RO73-reactive PrP^{Sc} in the brains of these mice is derived exclusively from FLAG–MoPrP^{C}.

Inclusion of the FLAG epitope in FLAG–MoPrP facilitated *in situ* detection of FLAG–MoPrP by the histoblot technique (Taraboulos et al., 1992). Using the anti-FLAG M1 mAb and the anti-PrP RO73 polyclonal antiserum, the distribution of FLAG–MoPrP^{C} and FLAG–MoPrP^{Sc} was analyzed in brain sections of Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice inoculated with mouse RML prions (Fig. 6B). The distribution of FLAG–MoPrP^{C} was found to be the same with both antibodies (Fig. 6A, B). Histoblot detection of FLAG–MoPrP^{Sc} in the brains of Tg(FLAG–MoPrP) mice inoculated with mouse RML prions was achieved with only the anti-PrP

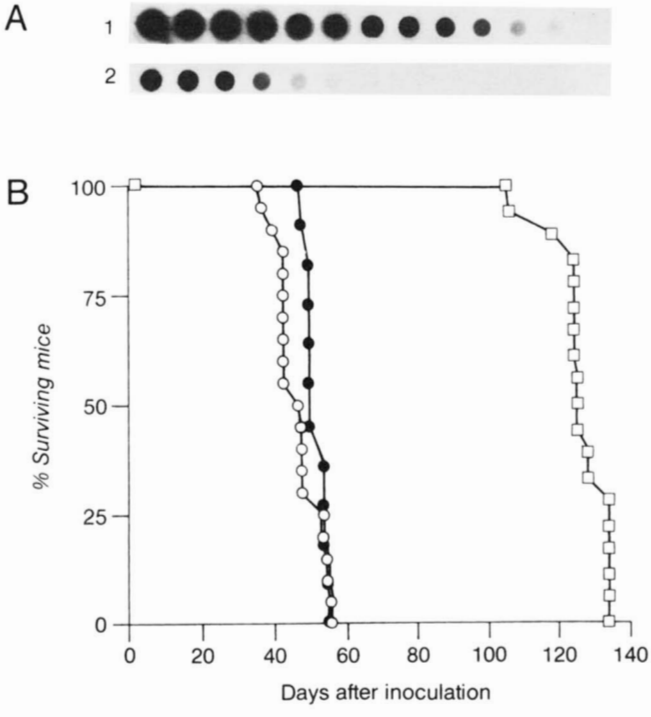

Fig. 4. Transgenic mice overexpressing FLAG-tagged MoPrP support the propagation of mouse prions. **A:** Overexpression of FLAG-tagged MoPrP in Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice. The total concentrations of protein in 10% brain homogenates from Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice and CD-1 mice were determined by bicinchoninic acid assay. Aliquots of brain homogenates containing equal amounts of total protein were subjected to two-fold serial dilutions which were applied to immobilized nitrocellulose filters. Panel 1: Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mouse brain homogenate; panel 2: CD-1 mouse brain homogenate. The blot was probed with anti-PrP RO73 polyclonal antiserum, developed using enhanced chemiluminescence (ECL) (Amersham Corporation) and exposed to X-ray film. **B:** Transgenic mice overexpressing FLAG-tagged MoPrP support mouse prion propagation. Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice (closed circles), Tg(MoPrP-A)4053 mice (open circles) and non-Tg PrP-A mice (squares) were inoculated intracerebrally with ~10^{6} ID_{50} units of RML prions. The data were plotted as a percentage of total number of mice with normal CNS function.

RO73 polyclonal antiserum (Fig. 6C) and not the anti-FLAG M1 mAb (Fig. 6D), because the FLAG epitope is lost upon proteinase K digestion of FLAG–MoPrP (see Fig. 2). The pattern of FLAG–MoPrP^{Sc} distribution was similar to that of MoPrP^{Sc} observed in brains of non-Tg mice inoculated with RML prions but with pronounced punctate staining in the cortex.

Neuropathological features of the brains of clinically sick Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice included generally less severe white matter vacuolation compared to non-Tg mice. Reduced severity of neuropathic changes is a feature that has previously been observed in the brains of ill Tg(MoPrP-A)4053/FVB mice. Tg(MoPrP-A)4053/FVB mice overexpress MoPrP and have shortened mouse RML prion incubation times (Telling et al., 1996). Hydrolytic autoclaving using anti-PrP RO73 polyclonal antiserum revealed the presence of diffuse FLAG–PrP-containing plaques (Fig. 7A) accompanied by intense focal glial fibrillary acidic protein (GFAP) staining, indicative of reactive astrocytic gliosis (Fig. 7B), which appeared at the same locations as the punctate FLAG–MoPrP^{Sc} staining detected by histoblotting.

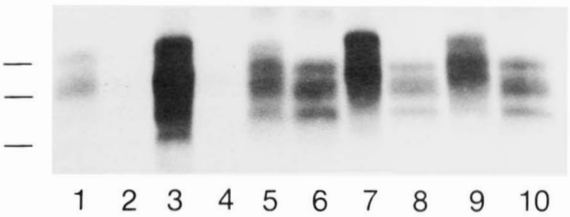

Fig. 5. The brains of Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice inoculated with mouse prions contain protease-resistant FLAG–MoPrP^{Sc}. The total concentrations of protein in 10% brain homogenates were determined by bicinchoninic acid assay. Aliquots of brain homogenates containing equal amounts of total protein in each case were either treated with 20 μg proteinase K for 60 min at 37 °C (even numbered lanes) or untreated (odd numbered lanes). Samples were resolved by SDS-PAGE and analyzed by Western blotting. The blot was exposed to the anti-PrP RO73 polyclonal antiserum, developed using enhanced chemiluminescence (ECL) and exposed to X-ray film. Lanes 1 and 2: uninoculated CD-1 mouse; lanes 3 and 4: uninoculated Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mouse; lanes 5 and 6: CD-1 mouse inoculated with mouse RML prions; lanes 7 and 8: Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mouse inoculated with mouse RML prions; lanes 9 and 10: Tg(MoPrP-A)4053/FVB mouse inoculated with mouse RML prions. The positions of protein molecular weight markers are shown to the left of the blot and correspond to molecular masses of (from top to bottom) 35.5, 29.1, and 14.5 kDa.

Immunopurification of FLAG–MoPrP from Tg mice

Since the anti-FLAG M1 mAb efficiently recognizes tagged MoPrP^{C} in Tg(FLAG–MoPrP) mice and the levels of FLAG–MoPrP expression are ~100-fold higher than wt MoPrP expression in non-Tg mice, we expected that Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice would be an excellent source for large amounts of FLAG–MoPrP^{C} obtainable in high purity under non-denaturing conditions by immunoaffinity chromatography. Brain tissue from uninoculated Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice was homogenized in TBS/10 mM $CaCl_2$ and the homogenate applied to an antibody column composed of anti-FLAG M1 mAb coupled to agarose beads. After applying the homogenate, the column was washed extensively and then bound FLAG–MoPrP was eluted with TBS containing 2 mM EDTA. FLAG–MoPrP was analyzed in samples from each stage by SDS-PAGE and Western blotting using anti-PrP RO73 polyclonal antiserum (Fig. 8). FLAG–MoPrP was bound to the anti-FLAG M1 mAb column in the presence of 10 mM $CaCl_2$ but the appearance of FLAG–MoPrP in the flow-through and the first wash suggests that binding was not complete. The bound FLAG–MoPrP was, however, efficiently eluted from the column in the presence of 2 mM EDTA. A silver-stained gel of the immuno-

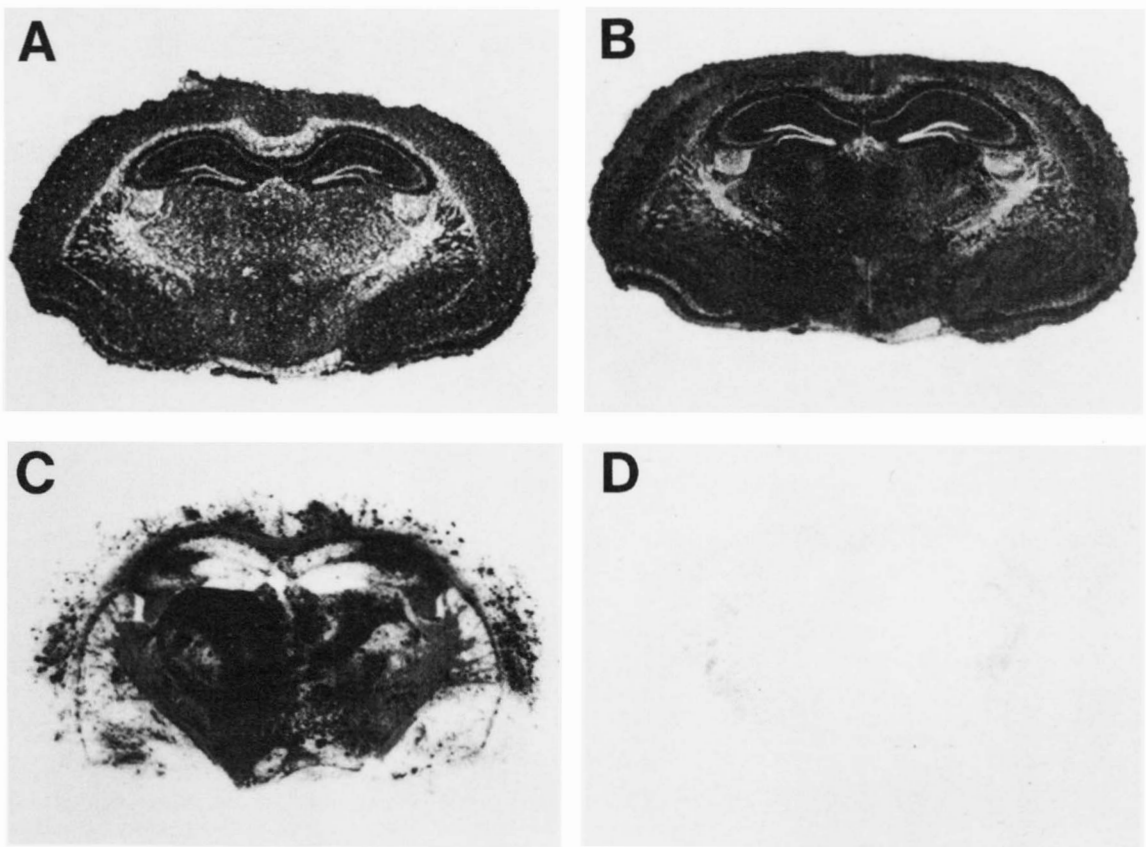

Fig. 6. In situ detection of PrP in Tg(FLAG–MoPrP) mice with the anti-FLAG M1 mAb. Serial coronal sections were made at the level of the hippocampus and thalamus in Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice inoculated with mouse RML prions. **A:** Histoblot not treated with proteinase K; developed using the anti-PrP RO73 polyclonal antiserum. **B:** Histoblot not treated with proteinase K; developed using the anti-FLAG M1 mAb. **C:** Histoblot treated with proteinase K; developed using the anti-PrP RO73 polyclonal antiserum. **D:** Histoblot treated with proteinase K; developed using the anti-FLAG M1 mAb.

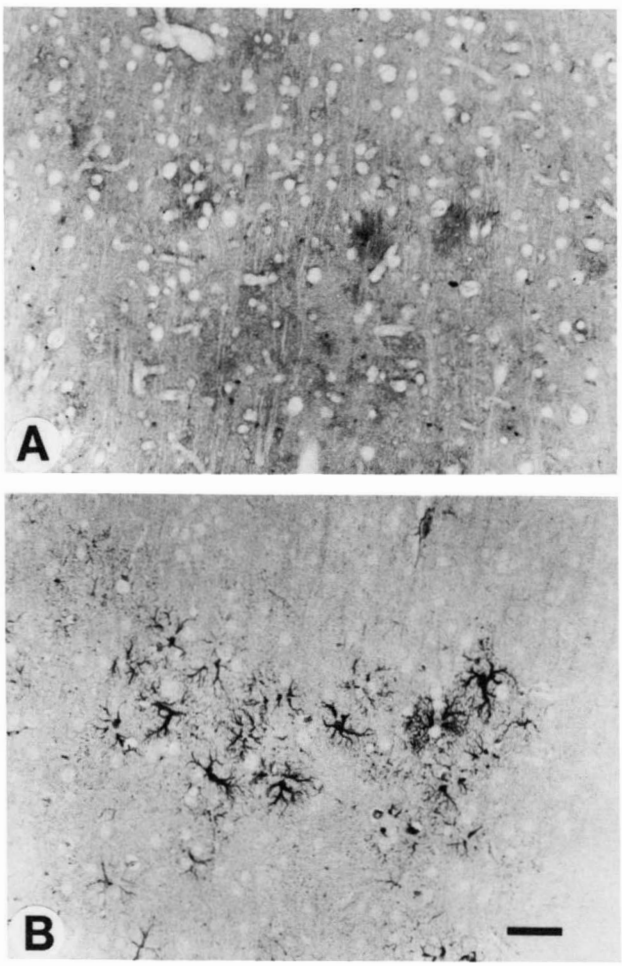

Fig. 7. Neuropathology in the brains of clinically sick Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice inoculated with mouse RML prions. Sections of the cerebral cortex were stained with immunoperoxidase using anti-PrP RO73 antiserum in panel A and anti-GFAP antibody in panel B. Bar = 50 μ.

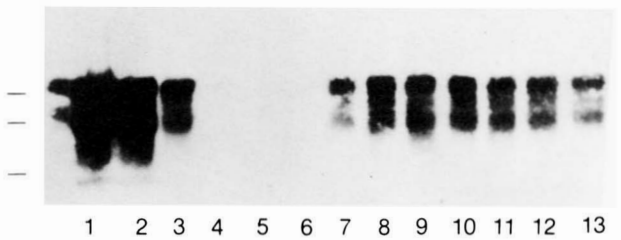

Fig. 8. Immunopurification of FLAG–MoPrP from Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice. The brain of an uninoculated Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mouse was homogenized in TBS containing 10 mM $CaCl_2$ and 0.1% NP-40 detergent. The preparation was applied to a 5 mL column bed composed of anti-FLAG M1 mAb coupled to agarose beads. The flow-through was reapplied repeatedly to ensure maximal binding. The column bed was washed three times with 15 mL TBS/10 mM $CaCl_2$. Bound FLAG–MoPrP was eluted with TBS containing 2 mM EDTA which was applied in eight 1-mL aliquots for 10 min each. Samples from each fraction were resolved by SDS-PAGE and analyzed by Western blot. The blot was exposed to anti-PrP RO73 polyclonal antiserum, developed using enhanced chemiluminescence (ECL) (Amersham Corporation) and exposed to X-ray film. Lane 1: 10% brain homogenate from Tg(FLAG–MoPrP) mouse; lane 2: flow-through; lanes 3–5 consecutive 15 mL TBS/10 mM $CaCl_2$ washes; lanes 6–13: 1 mL elutions in TBS/2 mM EDTA. The positions of protein molecular weight markers are shown to the left of the blot and correspond to molecular masses of (from top to bottom) 35.5, 29.1, and 14.5 kDa.

purified samples revealed, in addition to protein bands with the molecular weight of FLAG–MoPrP, protein bands at other locations, indicating that immunopurification of FLAG–MoPrP by anti-FLAG M1 mAb is not a one-step procedure (data not shown).

Recently, an improved FLAG tag has been generated in which the fifth amino acid of the FLAG sequence was changed from an aspartate residue to a glutamate residue. The binding affinity of the anti-FLAG M1 mAb was increased six-fold in Western blots over the original FLAG sequence (Knappik & Pluckthun, 1994). This raises the possibility that Tg mice expressing PrP with the improved FLAG tag may be an even better source of recombinant PrP in the future.

Discussion

In our initial studies involving epitope tagging of PrP, we inserted Syrian hamster (SHa) PrP epitopes into MoPrP (Taraboulos et al., 1990; Scott et al., 1992). Until recently, the only mAbs available for detection of PrP were those that reacted with SHaPrP but not MoPrP (Barry & Prusiner, 1986; Kascsak et al., 1987). The problem of generating anti-MoPrP antibodies has recently been overcome by first immunizing mice in which the PrP gene has been ablated (Prusiner et al., 1993) and then producing libraries of recombinant Fabs from the RNA of these Prnp$^{0/0}$ mice (Williamson et al., 1996).

The studies reported here were initiated because Ca^{2+} dependent mAbs to the FLAG epitope were available (Prickett et al., 1989). Such mAbs offered the possibility of eluting PrP from affinity columns by addition of low levels of EDTA to chelate the Ca^{2+} and release the bound PrP; under such conditions, the conformation of PrP^C might remain "native." Since the function of PrP^C remains elusive, assessing whether a particular preparation of PrP^C possesses a native conformation is difficult. We faced this problem in spectroscopic studies of the secondary structure of PrP^C where only procedures known not to denature most proteins were used in the purification of PrP^C (Pan et al., 1993). To determine whether or not inclusion of the FLAG epitope affected the native state of PrP^C, we measured the ability of FLAG–PrP^C to be converted into PrP^{Sc} in ScN2a cells and to support the propagation of scrapie prions *in vivo*. As reported here, we found that two different FLAG-tagged PrP^C constructs supported PrP^{Sc} formation (Fig. 2, 5, 6) while N-terminally FLAG-tagged PrP^C supported prion propagation in Tg mice (Fig. 4) and could be immunopurified using a Ca^{2+}-dependent anti-FLAG antibody (Fig. 8).

On the conversion of PrP^C into PrP^{Sc}

The results of our studies provide a blueprint for assessing whether any modified PrP^C molecule remains functional, at least insofar as it continues to be eligible for conversion into PrP^{Sc}. Earlier studies showed that the N-terminus of PrP^{Sc} could be truncated without loss of scrapie infectivity (Prusiner et al., 1982, 1984) and correspondingly, the truncation of the N-terminus of PrP^C still allowed its conversion into PrP^{Sc} (Rogers et al., 1993) and supported prion propagation (Fischer et al., 1996). Our results demonstrate that

modification of the N-terminus of PrP^C with a foreign sequence is not deleterious to either PrP^{Sc} formation or prion propagation.

Alternative epitope tags

While FLAG may be a suitable epitope for PrP^C isolation, other foreign epitopes may prove superior for PrP^{Sc} isolation. It may be preferable to produce constructs with tags other than the FLAG epitope, for instance a polyhistidine tag, the Strep tag (Schmidt & Skerra, 1994), the human c-Myc epitope, which is recognized by the monoclonal antibody 9E10 (Manstein et al., 1995), the influenza virus hemagglutinin (HA) epitope, recognized by the monoclonal antibody 12CA5 (Murray et al., 1995), or the Glu-Glu-Phe sequence recognized by the monoclonal antibody YL1/2 to α-tubulin (Stammers et al., 1991).

We have also constructed transgenic mice expressing recombinant MoPrP with a nine amino acid peptide sequence, referred to as the "Strep tag" at the same N-terminal PrP location (N. Heye, G. Telling, & S. B. Prusiner, unpubl. results). The Strep tag peptide consists of the sequence Ala-Trp-Arg-His-Pro-Gln-Phe-Gly-Gly, which has been demonstrated to have intrinsic streptavidin-binding activity when fused to the C-terminus of polypeptides (Schmidt & Skerra, 1994). Recombinant tagged protein can be directly purified by affinity chromatography from the host cell extract on immobilized streptavidin, the bound protein being eluted with 1 mM biotin. Because elution of bound recombinant Strep-tagged PrP does not rely on the use of metal ions for binding and elution, it is likely that this approach may be more feasible for the isolation of PrP^{Sc} from the brains of scrapie-infected Tg mice. These mice are currently under study to determine whether they will also support prion replication.

Mapping the structure of PrP^{Sc}

The placement of epitope tags at various positions in PrP might have the advantage of allowing us to assess which epitopes are exposed and which are buried if certain tagged constructs can be converted into PrP^{Sc} while others cannot. Using the four-helix bundle model of PrP^C (Huang et al., 1994), epitopes can be engineered either at regions of proposed α-helical structure, which we presume change in conformation during prion replication, or in the loop regions connecting these α-helices. Such information could elucidate the unfolding and refolding of PrP^C into PrP^{Sc}. A comparison of exposed and buried FLAG epitopes in both isoforms of PrP might also be useful in exploring the hypothesis that prion diversity represents different conformers of PrP. Although placement of FLAG at amino acids 22 and 88 does not interfere with PrP^{Sc} formation, it seems likely that placement of FLAG at some other internal positions within PrP is likely to prevent PrP^{Sc} formation.

New immunoassays for PrP^{Sc}

If an epitope tag can be engineered into a region of PrP^C where it is hidden from detection by Abs but still allows conversion of the molecule into PrP^{Sc}, then such recombinant PrP molecules might be ideally suited for a PrP^{Sc} immunoassay. Because the conformational transition is so large during the conversion of PrP^C into PrP^{Sc}, it is likely that an epitope buried in PrP^C might become exposed in PrP^{Sc}. If such an epitope tag can be designed that is detected in PrP^{Sc} but not PrP^C, then transgenes carrying such epitope-tagged PrP molecules might form the basis for a rapid assay for prions in humans, cattle, and sheep. The report of possible prion transmission from cattle to humans makes the need for rapid prion assays all the more pressing (Will et al., 1996).

Materials and methods

Construction of epitope-tagged PrP expression cassettes

To construct a PrP gene in which the FLAG tag is inserted after codon 22, a new recognition sequence for the restriction enzyme StuI was created upstream of the MoPrP signal peptidase cleavage site. The StuI site was created by changing a T for an A at nucleotide position 57 of the MoPrP gene. This was achieved by PCR-mediated mutagenesis in which one of the primers for the reaction contained the T–A mismatch. Complementary synthetic 42 and 47 oligonucleotide sequences were annealed and used to replace the DNA sequence between the newly created StuI site and a pre-existing PflMI site, downstream of the signal peptidase cleavage site at nucleotide position 76. The replacement reconstituted the distal portion of the signal peptide and the proximal sequence of mature MoPrP and resulted in the insertion of the eight-amino acid FLAG sequence immediately distal to the signal peptidase cleavage site. In this way, aspartate, the first amino acid of the FLAG sequence, becomes amino acid 23, the first amino acid of the mature PrP (Fig. 1A).

A second construct in which the FLAG sequence was inserted at an internal location of MoPrP between codons 88 and 89 was produced by replacing sequences between the Pvu II site at nucleotide position 210 and the Asp718 site at nucleotide position 277 with complementary synthetic oligonucleotides containing the FLAG sequence (Fig. 1B).

After verification of the predicted nucleotide sequences, the FLAG-tagged sequences were subcloned into the MHM2 expression cassette and then cloned into the SPOX.II neo vector (Scott et al., 1992), allowing direct selection of neomycin-resistant transfected cells in medium containing G418. The FLAG sequence was also cloned into MoPrP to produce FLAG–MoPrP, which was cloned into the cos.SHaTet expression vector for Tg mouse production.

Cultured cells expressing FLAG-tagged PrP

Mouse neuroblastoma N2a cells chronically infected with mouse prions (ScN2a cells) (Butler et al., 1988) were grown in six-well plates and transformed to neomycin resistance by DOTAP-mediated transfection (Boehringer Mannheim Biochemicals) with SPOX.II neo constructs containing either FLAG-tagged MHM2PrP or untagged MHM2PrP. Neomycin-resistant colonies became apparent ~2 weeks after transfection and growth in medium containing G418. Colonies from individual plates were pooled and passaged.

When stable cultures were established, NP-40 detergent lysates were isolated from either FLAG-tagged MHM2PrP or control MHM2PrP-expressing cells for expression studies. In some experiments, PrP attached to the cell surface released by treatment with PIPLC as described (Harris et al., 1993).

Analysis of protease-resistant FLAG-tagged MHM2PrP or untagged MHM2PrP in stable cell lines was accomplished by digesting a 1 mL aliquot of cell lysate derived from a confluent 100 mM

dish with proteinase K at a concentration of 20 μg/mL for 1 h at 37 °C. Detergent-resistant, proteinase K-resistant proteins were purified by centrifugation at 40,000 × *g* for 1 h and the pellets were suspended in 20 μL lysis buffer. Digested or undigested cell lysates were added to an equal volume of 2X SDS-PAGE sample buffer and processed for SDS-PAGE analysis. Immunoblotting was performed using anti-PrP 3F4 mAb directed against SHaPrP (Kascsak et al., 1987).

The production of transgenic mice expressing FLAG-tagged PrP

The N-terminally FLAG-tagged MoPrP open reading frame (ORF) expression cassette was cloned into the cos.SHaTet expression vector (Scott et al., 1992) to produce the cosSHa.Tet FLAG–MoPrP clone. The isolation and screening of recombinant cosmid cos.SHaTet clones has been described (Scott et al., 1993). The NotI cosmid fragment, recovered from large scale DNA preparations, was used for microinjection of the pronuclei of fertilized FVB/N or FVB/Prnp$^{0/0}$ mouse embryos as described (Scott et al., 1992). FVB/Prnp$^{0/0}$ are mice in which both copies of the prion protein gene (Prnp) are disrupted and therefore do not produce endogenous MoPrP.

Five Tg founders were produced: three were derived from FVB embryos and two from FVB/Prnp$^{0/0}$ embryos. Genomic DNA isolated from tail tissue of weanling animals was screened for the presence of incorporated transgenes using probes that hybridize to the 3′-untranslated region of the SHaPrP gene contained in the cosSHa.Tet vector (Scott et al., 1992). By comparing the hybridization signals of DNA from weanling mice with standardized DNA samples we estimated that one line, Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$, had a transgene copy number in excess of ~60 copies. Using the anti-PrP RO73 polyclonal antiserum, we estimated by serial dilution and immuno-dot blotting that the level of FLAG–MoPrP expression in brain extracts from the Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ line was ~100-fold higher than wt MoPrP expression in normal CD-1 mice.

Preparation of brain homogenates

A 10% (w/v) homogenate of a sample of thawed brain tissue was prepared in phosphate-buffered saline lacking calcium and magnesium ions by repeated extrusion through an 18 gauge syringe needle followed by a 22 gauge needle. For immunopurification studies, Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mouse brain tissue was homogenized in Tris Buffered Saline (TBS) containing 10 mM $CaCl_2$. The mouse RML prion isolate (Chandler, 1961) was provided by Dr. W. Hadlow and was passaged in Swiss mice from a closed colony at the Rocky Mountain Laboratory (Hamilton, Montana) or in Swiss CD-1 mice obtained from Charles River Laboratories (Wilmington, Massachusetts).

Determination of incubation periods

Mice were inoculated intracerebrally with 30 μL of brain extract using a 27 gauge needle inserted into the right parietal lobe. The preparation of inocula and criteria for diagnosis of scrapie in mice have been described (Carlson et al., 1986). Beginning 30 days after inoculation, mice were examined for neurologic dysfunction every 3 days. When clinical signs of CNS dysfunction appeared, the mice were examined daily. To confirm the clinical diagnosis, the brains of some animals whose death was obviously imminent were taken for histopathological and biochemical studies.

Immunoblot analysis

For determination of the relative levels of PrP expression in Tg and non-Tg mouse brains, total protein concentrations were determined by bicinchoninic acid assay and immuno-dot blots as described previously (Scott et al., 1993). Analysis of protease-resistant PrP in brain homogenates of inoculated Tg(FLAG–MoPrP)FVB/Prnp$^{0/0}$ 7755 and normal mice was accomplished by digesting a 50 μL aliquot of a 10% brain homogenate in TNE-Sarkosyl (Tris, pH 7.4, 10 mM NaCl, 1 mM EDTA, 2% Sarkosyl) with proteinase K at a concentration of 20 μg/mL for 1 h at 37 °C. Digested or undigested samples were added to an equal volume of 2X SDS-PAGE sample buffer and processed for SDS-PAGE analysis. For deglycosylation, aliquots of brain homogenates from CD-1 or Tg(FLAG–MoPrP)FVB/Prnp$^{0/0}$ 7755 mice were digested overnight with recombinant PNGase F (New England Biolabs) as specified by the supplier. Western blots were performed as described previously (Towbin et al., 1979; Barry & Prusiner, 1986), using the enhanced chemiluminescent (ECL) detection method (Amersham, Arlington Heights, Illinois). The blot was exposed to X-ray film for 5–60 s. Anti-PrP RO73 polyclonal antiserum and anti-PrP 3F4 mAb were used at a final dilution of 1:5000 and anti-FLAG M1 mAb was used according to the manufacturer's recommendations (IBI-Kodak, New Haven, Connecticut).

Neuropathology

Brains were dissected rapidly after sacrifice of the animal and immersion fixed in 10% buffered formalin. The tissue was embedded in paraffin and 8 μM thick histological sections were prepared for GFAP and PrP immunohistochemistry as described previously (Scott et al., 1989; Telling et al., 1996).

Histoblot analysis

Histoblots for localization of PrP^C or protease-resistant PrP were made by pressing 16 μL thick unfixed cryostat sections of brain to nitrocellulose paper as previously described (Taraboulos et al., 1992). To localize PrP^{Sc}, the histoblot was exposed to 400 μg/mL proteinase K for 18 h at 37 °C to eliminate PrP^C, exposed to 3 M guanidinium thiocyanate to denature the remaining PrP^{Sc}, followed by immunostaining with anti-PrP RO73 polyclonal antiserum or anti-FLAG M1 mAb.

Immunopurification of FLAG–MoPrP from Tg(FLAG–MoPrP) mouse brain

Brain material from uninoculated Tg(FLAG–MoPrP)7755/Prnp$^{0/0}$ mice was homogenized in TBS/10 mM $CaCl_2$ and NP-40 was added to a final concentration of 0.1%. The preparation was applied to a 5 mL column bed comprising anti-FLAG M1 mAb coupled to agarose beads. The flow-through was reapplied several times to ensure optimal binding. The column bed was then washed three times with 15 mL TBS/10 mM $CaCl_2$. FLAG–MoPrP bound to the column was eluted with TBS/2 mM EDTA which was applied in eight 1-mL aliquots for 10 min each. Samples from each fraction were resolved by SDS-PAGE and analyzed by Western blotting. FLAG–MoPrP was detected using anti-PrP RO73 polyclonal antiserum.

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