TABLE 2

Comparison of titer of infectivity and PrP-res level

PrP-res levels, quantified relative to recombinant PrP from digital immunoblot images, and infectivity titer, measured by ID_{50} bioassay. Detection limit of the immunoblot system was estimated to be equivalent to 25 μg of PrP-res/g wet weight brain.

Model	PrP-res	PrP-res	Titer[a]
	μg/g tissue[b]	*% of ME7*	*IU/g tissue*
Wt/ME7	1994	100	$10^{8.5}$
101LL/ME7	1040	52	$10^{7.8}$
101LL/263K(a)	498	25	$10^{8.7}$
101LL/263K(b)	<25	<1.3	$10^{7.3}$
101LL/263K(c)	<25	<1.3	$10^{7.5}$
101LL/GSS(d)	<25	<1.3	$10^{9.8}$
101LL/GSS(e)	<25	<1.3	$10^{7.2}$

[a] Titer of infectivity per gram of brain tissue as calculated from ID_{50} bioassay in mice using the Karber calculation.

[b] The actual amount of PrP-res quantified from the blots (0.5–2 mg/g) is higher than would be predicted for mouse tissue and may reflect the use of recombinant PrP for calibration, because this does not possess any post-translational modifications and may therefore display altered antibody affinity. However, this internal control acts to normalize each blot and, therefore, ensures that the relative proportions of PrP-res between each model are real, despite possible errors in the absolute quantification.

res in the 101LL transgenic mice compared with wild-type mice, although this was associated with a 0.7 log drop in titer (Table 2). In 101LL/263K(a) the limit of PrP-res detection was 62.5 μg/ml brain homogenate, which was approximately half the level in 101LL/ME7 and one quarter the level in Wt/ME7. For all other samples, no PrP-res was detectable in even the most concentrated (1 mg/ml) sample examined (Table 2, Fig. 2, and supplemental Fig. S1*A*). Digital imaging of immunoblots and quantitation of PrP-res relative to recombinant PrP control allowed the calculation of PrP^{Sc} concentration (mean PrP-res grams per gram wet weight of tissue) in each sample (Table 2). The level of sensitivity for the immunoblot, determined using recombinant PrP, was 5–10 ng, therefore the level of PrP-res in samples that showed no PK-resistant material must be below this threshold. Previous studies have shown that PrP-res from other well characterized rodent scrapie strains with titers ranging from $10^{5.5}$ to 10^{9} can be easily identified on immunoblot of 1% brain homogenate following PK treatment (supplemental Fig. S1*B*). These data would suggest that tissue containing titers of 10^{7} to 10^{9} IU/g should contain levels of PrP-res, which can be easily identified by immunoblot. However, for 101LL/GSS- and 101LL/263K-infected tissue this was clearly not the case. Although we cannot eliminate the possibility that PrP-res was indeed present below the threshold level of the immunoblot, a poor correlation between the level of infectivity and the amount of PrP-res in the brain is nevertheless clearly established. To confirm that the failure to detect PrP-res on these immunoblots was not simply a consequence of the loss of the monoclonal antibody epitope (8H4) duplicate blots were also probed with a second monoclonal antibody (7A12) and a polyclonal antibody (1B3), which detects multiple epitopes in PrP. These results confirmed the low PrP-res levels in 101LL/GSS and 101LL/263K tissues (data not shown). Although the combination of monoclonal and polyclonal antibodies used to examine these tissues makes it unlikely that a form of PrP-res exists that has not been detected in our immunoassays, this possibility has not been totally excluded and we continue to investigate these tissues with new antibodies.

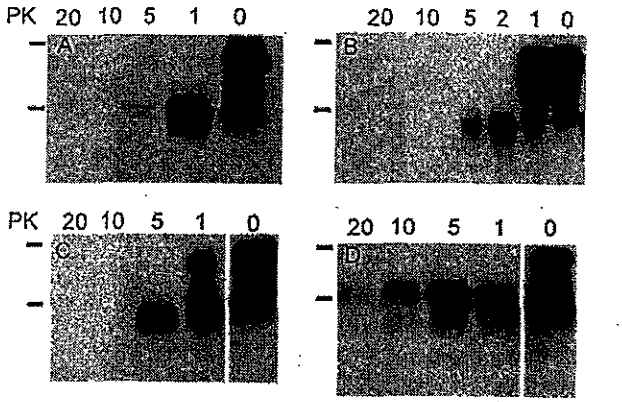

FIGURE 3. **PK resistance of PrP in 101LL/GSS and 101LL/263K brain tissue.** Brain homogenates in Nonidet P-40 lysis buffer were digested with varying concentrations of proteinase K at 37 °C for 1 h. Samples were subjected to SDS-PAGE and immunoblotting to determine the PK sensitivity of the PrP present in 101LL/GSS and 101LL/263K tissue. Representative images show: *A*, uninfected 101LL control mouse brain; *B*, uninfected Wt 129/Ola control mouse brain; *C*, 101LL/263K(b) mouse brain; and *D*, 101LL/263K(a) mouse brain. The PK concentration used for digestion is shown *above* each *lane* (micrograms/ml). Blots were probed with mAb 8H4. *Bars* indicate molecular mass markers of 36 and 30 kDa.

Are Alternative Forms of PrP Associated with Infectivity?—Although PrP-res was present at low or undetectable levels in tissues from 101LL/GSS- and 101LL/263K-infected mice, it is possible that forms of PrP other than PrP-res may be infectious (28). Alternative forms of PrP such as transmembrane PrP (29, 30), cytoplasmic PrP (31, 32), and PrP with amino acid insertions or deletions (33–36) have been linked with disease. In addition, a PK-sensitive variant of PrP^{Sc}, $sPrP^{Sc}$, has been recently described (20–22) that may represent an intermediate in the refolding of PrP^{C} to PrP^{Sc} during the disease process and could therefore be associated with infectivity. To test whether $sPrP^{Sc}$ may account for the dissociation between PrP-res and infectivity in 101LL/263K and 101LL/GSS tissues we examined the protease resistance of PrP in such brains by digesting with a range of PK concentrations from 1 μg/ml to 20 μg/ml. Homogenates from Wt/ME7, 101LL/ME7, and uninfected 101LL and 129/Ola mice were also treated with varying PK concentrations as controls. Digestion was stopped by the addition of phenylmethylsulfonyl fluoride to 1 mM, and samples were analyzed by immunoblot (Fig. 3). In the positive controls (Wt/ME7 and 101LL/ME7) PrP-res was evident in all dilutions, with the PK-resistant core still visible after treatment with 20 μg/ml PK (data not shown). PrP in the uninfected controls was found to be sensitive to PK concentrations >5 μg/ml, and produced mildly PK-resistant fragments at PK concentrations of 2–5 μg/ml under the digestion conditions used here (Fig. 3). PrP in the 263K-infected 101LL brains showed variable PK resistance, in agreement with the level of PrP-res detectable in each homogenate. Thus, 101LL/263K(a) showed PrP-res at 20 μg/ml, but 101LL/263K(b) and -(c) showed a similar pattern of PK resistance to uninfected mice (Fig. 3). In addition, samples from both 101LL/GSS(d) and 101LL/GSS(e) showed a PK-sensitivity pattern identical to that of uninfected 101LL mice (data not shown).

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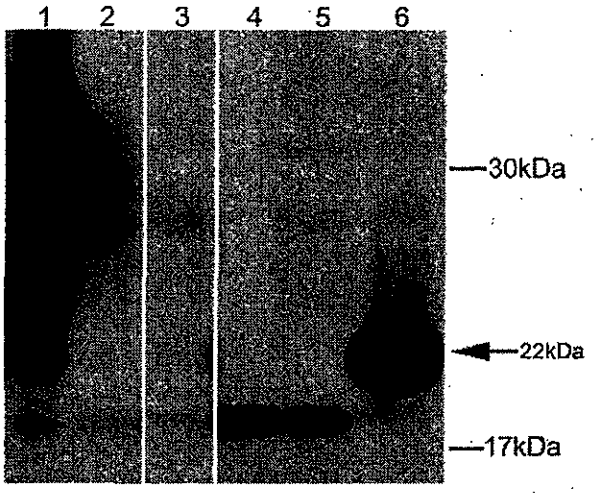

FIGURE 4. **Cold PK treatment of tissues from high titer/low PrP-res models.** 101LL/GSS and 101LL/263K tissues taken from the same transmission experiments as those shown in Table 1 were subjected to cold PK digestion on ice. Uninfected and Wt/ME7-infected brains were also digested as controls. *Lane 1*, undigested 101LL/GSS brain homogenate; *lane 2*, 101LL/263K(g); *lane 3*, 101LL/GSS(f); *lane 4*, 101LL uninfected control; *lane 5*, Wt129/Ola uninfected control; *lane 6*, Wt/ME7 infected control. *Lanes 2–6* were treated with 250 μg/ml PK on ice for 1 h and de-glycosylated with peptide *N*-glycosidase F. ME7 control was loaded at ~25% of the concentration of *lanes 2–5* to allow comparison. The blot was probed with mAb 7A12. The image has been cropped from a single blot to remove lanes with samples that are not relevant to this figure.

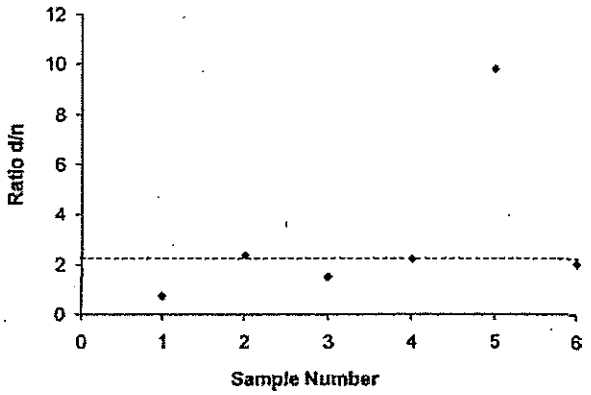

FIGURE 5. **CDI analysis of 101LL/GSS and 101LL/263K brain homogenate.** Samples of 101LL/GSS brain homogenate, 101LL/263K homogenate, and uninfected or ME7-infected controls were analyzed for the presence of PrP^{Sc} using a CDI. Samples were precipitated with sodium phosphotungstate, and pellets were resuspended in either distilled water or 4 M guanidinium-HCl to provide native and denatured samples. These were analyzed in a sandwich ELISA using mAb FH11 as capture and mAb 7A12 as detector. Ratio of denatured to native (*d/n*) signal plotted to show presence of PrP^{Sc}. *Sample 1*, 101LL/GSS(j); *sample 2*, 101LL/GSS(k); *sample 3*, 101LL/263K(m); *sample 4*, 101LL/263K(n); *sample 5*, 101LL/ME7; and *sample 6*, uninfected 101LL mouse. All samples were assayed in duplicate. *Dotted line* indicates cut-off value, which was calculated as the d/n ratio of the uninfected 101LL plus 10%.

The presence of $sPrP^{Sc}$ in brain tissue has also been demonstrated by performing cold PK digestion, *i.e.* PK digestion on ice (21, 22). $sPrP^{Sc}$ has been previously identified in samples that showed no PrP-res (using standard digestion conditions of 20 μg/ml for 1 h at 37 °C) by the presence of a 22-kDa band on immunoblot after digestion with PK on ice and subsequent de-glycosylation with peptide *N*-glycosidase F (21, 22). Although we aimed to perform all procedures on each individual mouse brain, the limited tissue size meant this was not possible for the cold PK analyses carried out here. However, cold PK digestion was performed on brain tissue taken from mice showing positive clinical and vacuolar signs of TSE, but low levels of PrP deposition in the same primary transmission experiments as those listed in Table 2 (details in supplemental Fig. S2 and Table S1). These tissues failed to demonstrate any marked increase in the 22-kDa PK-resistant PrP band after cold PK digestion (Fig. 4, *lanes 2* and *3*). When compared with the ME7 control (Fig. 4, *lane 6*, loaded at 25% concentration of *lanes 2–5*), the low levels of PrP apparent in *lanes 2* and *3* after digestion with PK on ice demonstrate that $sPrP^{Sc}$ cannot account for the high titer of infectivity in the 101LL/263K and 101LL/GSS models.

Although PrP^{Sc} is generally defined by its partial resistance to PK digestion, it can also be identified using immunoassays that exploit the differential binding of anti-PrP antibodies to PrP^{Sc} in the native and denatured state. Epitopes that are hidden in the native PrP^{Sc} conformation become exposed on denaturation in increasing concentrations of guanidine hydrochloride, leading to an increase in antibody binding. This observation is the basis of the CDI, where levels of PrP^{Sc} are calculated by measuring the ratio of the denatured to native signal (d/n ratio) in a sandwich ELISA (20, 37, 38). An increase in d/n ratio indicates the presence of PrP^{Sc}, which produces the increased signal obtained on denaturation of the sample. Because this assay does not use PK digestion to identify abnormal PrP, it can also be used to identify $sPrP^{Sc}$. To confirm the absence of large amounts of PrP-res or $sPrP^{Sc}$ in the models described here, CDI analysis was performed on tissues from 101LL/GSS- and 101LL/263K-infected mice. Tissue from animals detailed in Table 1 was not analyzed due to limited sample availability, but analysis was performed on tissues from 101LL/GSS- and 101LL/263K-infected mice with confirmed clinical and pathological TSE disease, but little or no PrP^{Sc} in the brain (supplemental Fig. S3 and Table S1). The d/n ratios obtained for all four infected animals ranged from 0.73 to 2.39, which were similar to or lower than the uninfected 101LL control (d/n ratio of 2.01). The 101LL/ME7 control gave a d/n ratio of 9.8 (Fig. 5). These data confirm the limited PK digestion studies, proving that no PrP^{Sc}-like conformers are present in 101LL/GSS- and 101LL/263K-infected tissues that could account for the observed titers of infectivity.

Immunoprecipitation Using PrP^{Sc}-specific Monoclonal Antibodies—Several mAbs have been generated that specifically bind PrP^{Sc} isoforms, but not PrP^{C}. These antibodies can therefore isolate PrP^{Sc} from non-PK-treated tissue homogenates by immunoprecipitation, ensuring that all abnormal PrP isoforms are identified. This technique has been used by others to demonstrate the presence of $sPrP^{Sc}$ in the brains of mice overexpressing 101L-PrP (22). Here, PrP^{Sc}-specific motif grafted mAbs 89–112 and 136–158 (25) were used to immunoprecipitate PrP from brain tissue homogenates of 101LL/GSS- and 101LL/263K-infected mice. Tissues analyzed were taken from mice showing positive clinical and vacuolar signs of TSE but low levels of PrP deposition in the same primary transmission experiments as those used to determine titer of infectivity in each model (details in supplemental Fig. S2 and Table S1). Positive control mAb D13 (which precipitates only the cellular form of PrP) and negative control mAb b12 were also

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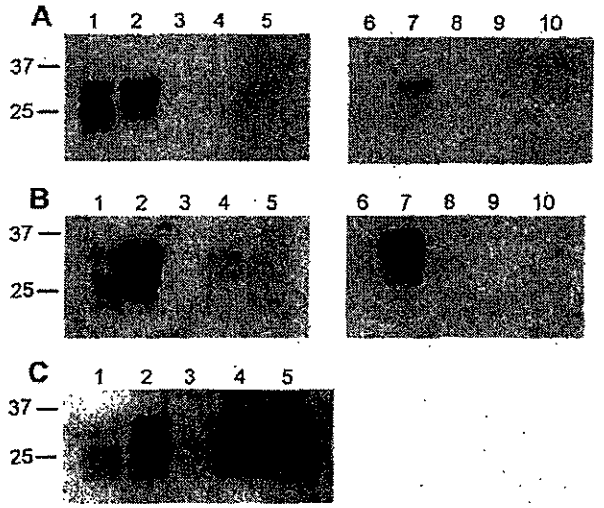

FIGURE 6. **Immunoprecipitation using PrP^{Sc}-specific monoclonal antibodies.** 101LL/GSS and 101LL/263K tissues taken from the same transmission experiments as those shown in Table 1 were subjected to IP using PrP^{Sc}-specific mAbs 89–112 and 136–158 to determine whether forms of PrP^{Sc}, which were sensitive to PK, were present in these tissues. mAb D13, which precipitates only cellular PrP, and mAb b12, which recognizes the HIV gp120 antigen, were used as IP controls. In *A*: *lanes 1–5*, 101LL/GSS(h); *lanes 6–10*, uninfected 101LL; in *B*: *lanes 1–5*, 101LL/263K(i); *lanes 6–10*, uninfected 101LL; in *C*: RML scrapie Wt control. *Lanes 1* and *6*, crude brain homogenate; *lanes 2* and *7*, IP with mAb D13 (positive control antibody); *lanes 3* and *8*, IP with mAb b12 (negative control antibody); *lanes 4* and *9*, IP with mAb 89–112; *lanes 5* and *10*, IP with mAb 136–158.

included in all experiments. For all 101LL/GSS and 101LL/263K tissues examined, extremely low levels of PrP^{Sc} were immunoprecipitated by both PrP^{Sc}-specific antibodies (Fig. 6). These levels were estimated by immunoblot to be 100- to 1000-fold less than those precipitated from control RML-infected mouse brain. Results from these immunoprecipitations therefore support our previous biochemical data, which show no evidence of PK-sensitive forms of PrP^{Sc} in brain tissue from 101LL/GSS- and 101LL/263K-infected mice.

DISCUSSION

PrP^{Sc} is thought to be the sole component of the prion, or TSE infectious agent. For this reason it has become the main target for TSE diagnostic assays, where identification of PrP^{Sc} in post-mortem brain tissue indicates a TSE-positive animal. However the relationship between PrP^{Sc} and TSE infectivity has not been definitively demonstrated, and concerns have been raised by earlier reports of disease transmission in the apparent absence of PrP-res (16, 18). In particular, 101LL gene-targeted transgenic mice inoculated with GSS P102L or 263K succumb to a disease, which is highly transmissible to both 101LL and wild-type mice but shows extremely low levels of PrP-res in the brain. Extended analyses of this model (described here) have now used quantitative assays to unequivocally demonstrate that titers of 10^7 to 10^9 IU/g can be present in brain tissue, which shows little or no abnormal PrP accumulation by standard immunoblot analysis, IHC, CDI, or immunoprecipitation. These titers are similar to or higher than those observed in our well characterized, high titer control strain ME7, but for 4 of 5 brains analyzed, PrP-res levels were below the limit of detection of our immunoblot assay (<1.3% of the amount of PrP-res in wild-type ME7 tissue). Previous studies have shown that PrP-res from other well characterized rodent scrapie strains with titers ranging from $10^{5.5}$ to 10^9 can be easily identified on immunoblot of 1% brain homogenate following PK treatment. Based on these previous data, it would be predicted that the tissues studied here should contain titers far below 10^5 IU/g tissue. However the transmission data clearly show that 101LL/GSS- and 101LL/263K-infected tissues contained high titers of infectivity, which exceed those measured in both 79V- and 22A-infected tissue (supplemental Fig. S1*B*). These data suggest that current diagnostic assay systems that rely on PrP^{Sc} detection might fail to identify some highly infectious tissues. To this end, tissues from 101LL/GSS- and 101LL/263K-infected mice are currently being assessed in several of these assay systems in our laboratory.

Several independent studies have previously shown that one TSE infectious unit is composed of ~10^5 PrP^{Sc} molecules (2, 14, 15). In contrast to these studies the data obtained from 101LL/GSS- and 101LL/263K-infected tissues indicate that the number of PrP^{Sc} molecules per unit of infectivity must display a wide range, with 101LL/GSS and 101LL/263K tissues showing between 10 to 1000 times fewer PrP-res molecules per unit infectivity than Wt/ME7. Alternatively, these data could indicate that only a very small proportion of PrP^{Sc} present in TSE-infected tissue is actually infectious. This lack of correlation between levels of PrP-res and infectivity does not support PrP-res as the infectious agent of TSE.

Because PrP-res does not appear to be a major component of infectivity in this study, it is possible that another form of PrP is responsible for disease in these mice. We have shown previously that 101LL mice can form PrP-res when inoculated with other rodent TSE strains (39); therefore, the lack of PrP^{Sc} in these models is not due to an inherent inability of 101L-PrP to convert to a protease-resistant isoform. In contrast to the gene-targeted transgenic 101LL mice described here, transgenic mice, which overexpress 101L-PrP at levels 8- to 16-fold higher than endogenous PrP, develop a spontaneous neurological disease that appears to be associated with a PK-sensitive form of PrP^{Sc} (21, 22). We have found no evidence of $sPrP^{Sc}$ in 101LL/GSS or 101LL/263K brain tissue by either limited PK digestion studies or CDI analysis. Additionally, motif-grafted mAbs 89–112 and 136–158, which specifically bind PrP^{Sc}, did not immunoprecipitate PK-sensitive forms of PrP^{Sc} from 101LL/GSS or 101LL/263K brain tissue, even though these mAbs have been shown to immunoprecipitate abnormal PK-sensitive PrP^{Sc} from 101L-overexpressing transgenic mice.[6] One possible reason for this discrepancy between models is that disease in 101LL/GSS and 101LL/263K mice is due to a TSE infection, which has been transmitted from a known infected source, and can be further passaged to both 101LL and wild-type 129/Ola mice (18, 19). In contrast the disease observed in transgenic mice overexpressing 101L PrP does not transmit to wild-type mice and only appears to accelerate the phenotype already present in mice expressing lower levels of the transgene (17, 22). This

[6] A. Bellon and R. A. Williamson, unpublished data.

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