Brief Communications

Prion Transmission Prevented by Modifying the β2-α2 Loop Structure of Host PrP^C

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Zoonotic prion transmission was reported after the bovine spongiform encephalopathy (BSE) epidemic, when >200 cases of prion disease in humans were diagnosed as variant Creutzfeldt-Jakob disease. Assessing the risk of cross-species prion transmission remains challenging. We and others have studied how specific amino acid residue differences between species impact prion conversion and have found that the β2-α2 loop region of the mouse prion protein (residues 165–175) markedly influences infection by sheep scrapie, BSE, mouse-adapted scrapie, deer chronic wasting disease, and hamster-adapted scrapie prions. The tyrosine residue at position 169 is strictly conserved among mammals and an aromatic side chain in this position is essential to maintain a 3_{10}-helical turn in the β2-α2 loop. Here we examined the impact of the Y169G substitution together with the previously described S170N, N174T "rigid loop" substitutions on cross-species prion transmission *in vivo* and *in vitro*. We found that transgenic mice expressing mouse PrP containing the triple-amino acid substitution completely resisted infection with two strains of mouse prions and with deer chronic wasting disease prions. These studies indicate that Y169 is important for prion formation, and they provide a strong indication that variation of the β2-α2 loop structure can modulate interspecies prion transmission.

Key words: **conversion; transmission; amyloid; TSE; prions**

Introduction

Transmission of prion diseases between individuals can be remarkably efficient and has led to epidemics, as seen with bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD) (Anderson et al., 1996; Miller and Williams, 2003). Disease occurs when β-sheet-rich prion protein aggregates, known as PrP^{Sc}, template the conversion of the normal cellular prion protein, PrP^C, in an autocatalytic process (Prusiner, 1982). Although the mechanism of PrP conversion is not yet clear, single-residue differences between PrP^{Sc} and host PrP^C have been reported to inhibit prion replication (Priola and Chesebro, 1995; Kaneko et al., 1997; Perrier et al., 2002).

The cellular mouse prion protein consists of a flexibly disordered 100-residue amino-terminal domain and a globular C-terminal domain of similar size, which includes three α-helices and a short β-sheet (Riek et al., 1996, 1997). Although the overall architecture of the globular domain is highly conserved, NMR studies revealed structural variations in the β2-α2 loop region (residues 165–175) among mammalian PrPs. Specifically, at 20°C, the β2-α2 loop is disordered in the NMR structures of PrP^C from mouse, cattle, and humans (Riek et al., 1996; López Garcia et al., 2000; Zahn et al., 2000). Elk, bank vole, and horse PrP^Cs display a structurally well-defined β2-α2 loop at 20°C (Gossert et al., 2005; Christen et al., 2008; Pérez et al., 2010) and have been referred to as "rigid loop PrP^Cs" (RL-PrP^C). Inserting the β2-α2 loop sequences of elk, bank vole, or horse PrP into mouse PrP results in hybrid mouse prion proteins showing RL-PrP^C behavior (Gossert et al., 2005; Christen et al., 2008; Pérez et al., 2010).

We have previously found that the β2-α2 loop structure profoundly impacts interspecies prion transmission. Transgenic mice expressing a mouse PrP variant with the elk substitutions

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S170N and N174T [*Tg(MoPrP*^{170,174}*)*] showed a prolonged incubation period when exposed to mouse-adapted prions, indicating a transmission barrier (Sigurdson et al., 2010). Wild-type (WT) mice are relatively resistant to infection with CWD or hamster prions (Race et al., 2002; Raymond et al., 2007) yet are susceptible to infection with sheep scrapie and BSE (Hill et al., 1997; Bruce et al., 2002). *Tg(MoPrP*^{170,174}*)* mice showed a complete switch in species barriers, in that the mice were susceptible to prions from deer and hamsters, which contain N170, but resisted infection with prions from sheep and cattle, which contain S170 (Sigurdson et al., 2010). In contrast, the D167S substitution in mouse PrP (Pérez et al., 2010), which also results in RL-PrP^{C} behavior, had no effect on prion species barriers in transgenic mice (Bett et al., 2012), suggesting that in this case a primary sequence variation determines the ease of interspecies prion transmission.

Recent NMR studies revealed the reason for the poor structural definition of the β2-α2 loop in PrP^{C}s of many mammals, showing that there is a conformational equilibrium between two different loop structures (i.e., a 3_{10}-helix turn and a type I β-turn) (Damberger et al., 2011; Christen et al., 2012, 2013). In prion proteins showing a structurally poorly defined loop, the exchange between the two forms at 20°C is sufficiently slow to broaden the NMR signals beyond detection, whereas in RL-PrP^{C}s, the exchange is sufficiently rapid to observe the dominant 3_{10}-helix form. Replacement of Y169 with glycine results in a PrP^{C} containing only the type I β-turn loop structure (Damberger et al., 2011). These observations on PrP^{C} structures provided the basis for the present investigations of the effect from this rather dramatic local conformational change on the ease of interspecies transmission of TSEs. To this end, we investigated the conversion of variant mouse PrP containing the amino acid substitutions Y169G, S170N, and N174T by a variety of infectious prions from different species.

Materials and Methods

Generation of transgenic mice expressing MoPrP^{169,170,174}. Single-point mutations that alter the amino acid sequence of the mouse to 169G, 170N, and 174T were generated within a pMECA subclone, based on pHGPrP (Fischer et al., 1996), using the Stratagene point mutagenesis kit (primers: forward, 5′-GGCCA GTG GAT CAG GGC AAC AAC CAG AAC ACC TTC GTG CAC GAC-3′ and rc 5′-GTC GTG CAC GAA GGT GTT CTG GTT GTT GCC CTG ATC CAC TGGCC-3′). The PmeI/NheI pMECA subclone was then cloned into the PmeI/NheI sites of the pHG-PrP plasmid, and the entire ORF was sequenced (Rosenberg et al., 1977). The *Prnp* minigene sequence was excised with NotI/SalI, and constructs were microinjected into the pronucleus of fertilized B6;129S5-*Prnp*$^{+/o}$ oocytes (*Prnp*-KO Zurich I) using conventional methods (Rülicke, 2004). Founder lines were identified by PCR for the transgene as previously described (Sigurdson et al., 2011), and founders were bred to *Prnp*$^{o/o}$ mice. Nine transgene-carrying founder mice were identified that transmitted the transgene to their progeny; *Tg(Prp*^{169,170,174}*)70–91Biat*. Lines were maintained by crossing with *Prnp*$^{o/o}$ mice. Mice were maintained under specific pathogen-free conditions.

Prion inoculations. WT (C57BL/6), *Tg(MoPrP*^{169,170,174}*)*, or *Tga20* transgenic mice (groups of $n = 4–6$ mice) of either sex were intracerebrally inoculated into the left parietal cortex with 30 μl of brain homogenate containing RML or 22L mouse scrapie prions, or CWD prions from a naturally infected mule deer previously shown to contain infectious prions (Sigurdson et al., 2006). Uninfected brain homogenate was inoculated into the same mouse genotypes as a negative control. Mice were monitored three times weekly, and TSE was diagnosed according to clinical criteria, including ataxia, kyphosis, stiff tail, hind leg clasp, and hind leg paresis. Mice were killed at the onset of terminal disease when showing signs including weight loss, tremors, slow movements, and severe kyphosis, or by ~600 d after inoculation. Incubation period was calculated from the day of inoculation to the day of terminal clinical disease.

Table 1. Incubation period of *Tg(MoPrP*^{169,170,174}*)* and WT mice inoculated with mouse or cervid CWD prions

Inoculum	Genotype	Incubation period (mean ± SE days)[a]	Attack rate
RML	WT	163 ± 4	5/5
	Tg(MoPrP^{169,170,174}*)*	384 ± 43	0/5
22L	WT	131 ± 2	5/5
	Tg(MoPrP^{169,170,174}*)*	365 ± 29	0/5
CWD	WT	638 ± 3	0/4
	Tg(MoPrP^{169,170,174}*)*	515 ± 35	0/5

[a]All but one of the *Tg(MoPrP*^{169,170,174}*)* mice were more than 500 d of age when they were euthanized because of concurrent disease. No brain lesions or PrP^{Sc} was evident in these animals.

Sodium phosphotungstic acid (NaPTA) precipitation and Western blotting. A total of 10% brain homogenates from all prion-inoculated mice were prepared in PBS using a Beadbeater tissue homogenizer. Samples were subjected to NaPTA precipitation as previously described (Wadsworth et al., 2001).

PrP peptide ELISA. The peptide ELISA was performed as described by Lau et al. (2007) with minor modifications. PrP was measured by standard sandwich ELISA on a 96-well plate precoated with 2.5 μg/ml POM-2 antibody. A biotinylated POM-1 antibody (50 ng/ml) followed by streptavidin HRP and an Ultra TMB-ELISA substrate (Thermo Scientific) was used for detection. RML-infected and uninfected control brain samples were included in every experiment. Samples were run in triplicate.

Histopathology and immunohistochemical stains. Two-micron-thick sections were stained with hematoxylin and eosin or immunostained using antibodies for PrP as previously described (Bett et al., 2012).

Protein misfolding cyclic amplification (PMCA). The *in vitro* prion replication and PrP^{Sc} detection of amplified samples was performed as previously described (Castilla et al., 2008). Briefly, 50 μl of brain homogenate was seeded with 5 μl of each of prion seed and subjected to sonication in a sonicator water bath at 37°C-38°C. The sonicator settings were as follows: 20 s at power setting 70%–80% followed by 30 min of incubation (model S-700MPX, QSonica). Three serial rounds of PMCA were performed, and all sonicated samples were digested with 50–100 μg/ml of PK for 1 h at 42°C. To test for PrP^{Sc} in L87 mice, each brain homogenate was subjected to 4 experimental repetitions, each performed with four replicates. Blots were probed with monoclonal antibodies 6D11 or 6H4.

Results

Mouse characterization

We developed transgenic mice that express variant murine PrP^{C} with the Y169G, S170N, and N174T substitutions under the prion promoter. Nine lines of *Tg(MoPrP*^{169,170,174}*)* were bred on a PrP knock-out background and found to have onefold to twofold PrP^{C} expression levels in the brain compared with WT mice. Because PrP function can be assessed only indirectly, we tested whether mutant PrP functions similarly to WT PrP in rescuing early death in mice expressing amino-terminally truncated PrP (F35) (Shmerling et al., 1998). MoPrP^{169,170,174} rescued the early death of the F35 mice, similar to WT MoPrP (*F35*: 92 ± 6 d; *F35/WT*: 259 ± 15 d; *F35/MoPrP*^{169,170,174}: 207 ± 25 d; mean ± SE; $n = 5–9$/group).

Line 87 (L87) mice, which expressed PrP at slightly less than WT levels (data not shown), were selected for prion inoculation experiments. MoPrP^{170,174} is aggregation-prone and leads to a spontaneous transmissible spongiform encephalopathy, with prion plaques in the brain and skeletal muscle of aged mice (Sigurdson et al., 2009). Here, we did not observe spontaneous prion disease or plaques in the brain of aged L87 mice.

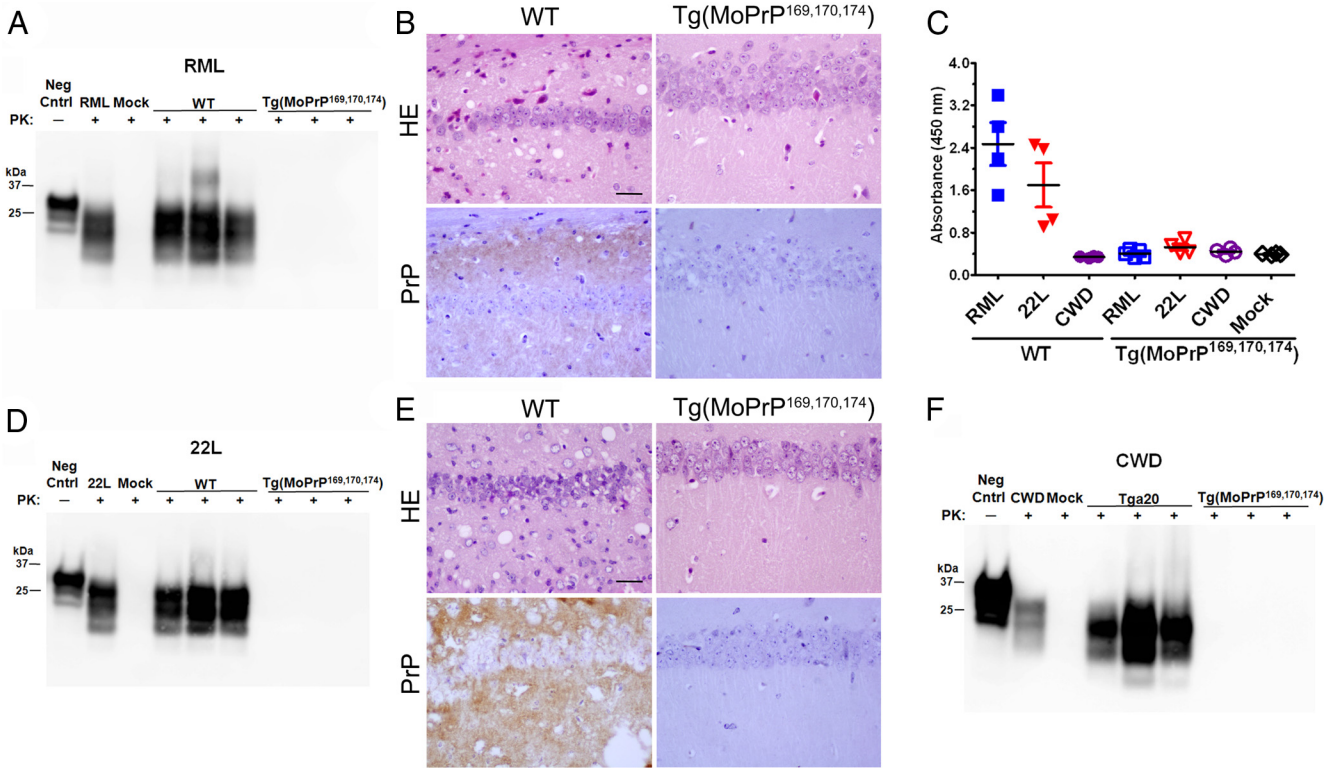

Figure 1. *Tg(MoPrP^{169,170,174})* mice inoculated with mouse or deer prion-infected brain show no PrP^{Sc} accumulation in the brain by Western blot, ELISA, or PrP immunohistochemistry. Brain homogenates from *Tg(MoPrP^{169,170,174})*, WT, or Tga20 mice, which had been inoculated with mouse prions (RML, 22L), deer prions (CWD), or uninfected brain (mock), were subjected to NaPTA precipitation and Western blotting. PrP^{Sc} is seen only in WT and Tga20 mice expressing mouse PrP (***A***, ***D***, ***F***). ***A***, Western blot of RML-inoculated *Tg(MoPrP^{169,170,174})* mice at 384–466 d after inoculation. ***B***, Brain at hippocampus shows no PrP^{Sc} deposits in the *Tg(MoPrP^{169,170,174})* mice inoculated with RML. ***C***, Peptide ELISA reveals no PK-sensitive PrP^{Sc} in *Tg(MoPrP^{169,170,174})* mice inoculated with RML, 22L, or CWD, whereas PrP^{Sc} was present in WT mice inoculated with RML or 22L prions. WT mice resisted infection with deer CWD. ***D***, Western blot of 22L-inoculated *Tg(MoPrP^{169,170,174})* mice at 378–529 d after inoculation. ***E***, Brain at hippocampus shows no PrP^{Sc} deposits in the *Tg(MoPrP^{169,170,174})* mice inoculated with 22L. Western blot of CWD-inoculated *Tg(MoPrP^{169,170,174})* mice at 493–618 d after inoculation. ***A***, ***D***, ***F***, The negative control shows the PrP signal from the brain of a *Tg(MoPrP^{169,170,174})* mouse.

Species barriers to mouse prions

We assessed the impact of the Y169G, S170N, and N174T residue exchanges on prion conversion *in vivo*. *Tg(MoPrP^{169,170,174})* and WT mice were intracerebrally inoculated with RML mouse prions on the same day. WT mice developed prion disease by 173 d, whereas none of the RML-exposed *Tg(MoPrP^{169,170,174})* mice developed clinical signs of prion disease, after up to 466 d after inoculation (Table 1), or showed any evidence of prion infection in the brain by Western blotting using sodium phosphotungstic acid precipitation or PrP immunohistochemistry (Fig. 1*A*,*B*). Mild vacuolation was apparent in brains of some *Tg(MoPrP^{169,170,174})* mice. We considered the possibility that these mice had developed PK-sensitive multimers of PrP. We therefore tested for PrP aggregates using a PrP-peptide ELISA assay (Lau et al., 2007), but no PK-sensitive PrP aggregates were revealed in the *Tg(MoPrP^{169,170,174})* mice (Fig. 1*C*). PrP^{Sc} is readily detected in the brains of mice expressing 50% of WT PrP^{c} levels at 200 d after inoculation with RML (C.J.S., unpublished data), indicating that the absence of PrP^{Sc} in *Tg(MoPrP^{169,170,174})* mice was not due to the slightly lower PrP^{c} expression levels.

Because the PrP^{Sc} conformation impacts the conversion efficiency of PrP^{C} (Telling et al., 1996; Atarashi et al., 2006), we tested a second mouse-adapted prion strain known as 22L. Whereas the WT mice were highly susceptible to 22L mouse prions, the *Tg(MoPrP^{169,170,174})* mice resisted infection after up to 529 d post inoculation, and they did not reveal any evidence of PrP^{Sc} by peptide ELISA, Western blot, or PrP immunostaining of brain sections (Fig. 1*C*–*E*; Table 1).

Species barriers to deer prions

Transgenic mice that express mouse PrP with a β2-α2 loop homologous to deer and elk *[Tg(MoPrP^{170,174})]* are susceptible to deer CWD prions (Sigurdson et al., 2010). To determine whether the additional Y169G substitution would affect CWD susceptibility, we inoculated *Tg(MoPrP^{169,170,174})* and WT mice, as well as Tga20 mice as previously reported, with deer CWD (Sigurdson et al., 2006, 2010). However, unlike *Tg(MoPrP^{170,174})* and Tga20 mice, the *Tg(MoPrP^{169,170,174})* mice completely resisted infection with the same CWD inoculum, suggesting that the Y169G substitution contributed to a species barrier to CWD (Fig. 1*F*; Table 1). Thus, MoPrP^{169,170,174} was not converted by either two strains of mouse prions or by deer prions.

Confirming absence of prion propagation in *Tg(MoPrP^{169,170,174})* mice

To assess whether low levels of PrP^{Sc} exist in the brains of the *Tg(MoPrP^{169,170,174})* mice inoculated with mouse or deer prions, we performed serial PMCA to detect PrP^{Sc} that may have been present at low levels (Saá et al., 2006). PrP^{C} substrate from uninfected *Tg(MoPrP^{169,170,174})* or healthy WT mouse brain was seeded with prion-exposed *Tg(MoPrP^{169,170,174})* mouse brain homogenate, and the samples were subjected to three serial rounds (24 h per round) of PMCA. None of the brain samples from the prion-exposed *Tg(MoPrP^{169,170,174})* mice showed any detectable PK-resistant PrP with either of the substrates used, whereas RML- and 22L-infected WT brain samples showed efficient PrP^{Sc} amplification in WT substrate after one round of PMCA (Fig. 2).

Substrate		RML control	22L control	Samples inoculated in Tg(MoPrP^{169,170,174}) mice: Mock 1	Mock 2	22L 1	22L 2	22L 3	CWD 1	CWD 2	CWD 3	RML 1	RML 2	RML 3	Unseeded
	PMCA round														
Tg(MoPrP^{169,170,174})	R1	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	R2	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	R3	1/4	1/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
C57BL/6	R1	4/4	4/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	R2	4/4	4/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	R3	4/4	4/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

Figure 2. No PrP^{Sc} is detected in the brains of *Tg(MoPrP^{169,170,174})* mice by serial PMCA. Brain homogenates from inoculated *Tg(MoPrP^{169,170,174})* mice (378 – 618 dpi) were used in an attempt to seed WT or *Tg(MoPrP^{169,170,174})* normal brain homogenate over 3 rounds of serial PMCA. Four technical replicates were performed, and the proportion of positives noted. RML- and 22L-infected WT brain samples were used to seed WT mouse brain homogenate as a positive control (C57BL/6), and to seed *Tg(MoPrP^{169,170,174})*. Unseeded samples were used as negative controls.

In one of four experiments, there was a very low level of conversion in one of four replicate samples of *Tg(MoPrP^{169,170,174})* substrate directly seeded with RML or 22L after three rounds of serial PMCA.

Species barriers to cattle, sheep, or hamster prions

Because neither mouse nor deer prions could detectably convert MoPrP^{169,170,174} *in vivo*, suggesting a strong species barrier, we further tested whether MoPrP^{169,170,174} could be converted by prions from cattle, sheep, or hamsters using the PMCA assay. As a source of PrP^{C}, we used brain homogenate from *Tg(MoPrP^{169,170,174})* or WT mice. To determine whether any PrP^{Sc} could be amplified in the *Tg(MoPrP^{169,170,174})* substrate, we performed three serial rounds of PMCA amplification. The WT mouse substrate amplified PrP^{Sc} from 263K hamster scrapie after 2 rounds, and from sheep scrapie and cattle BSE after 3 amplification rounds. Remarkably, neither prions from sheep, cattle, or hamster, nor from deer, converted MoPrP^{169,170,174}, even after 3 rounds of PMCA (Fig. 3).

Discussion

Here we found that transgenic mice expressing a variant mouse PrP containing the three amino acid substitutions S170N, N174T, and Y169G in the β2-α2 loop completely resisted infection with two different strains of mouse prions. The previously generated "rigid loop" mice, which express mouse PrP with the S170N and N174T substitutions, were susceptible to mouse prions, whereby all mice developed prion disease with prolonged and variable incubation periods indicative of a transmission barrier (Sigurdson et al., 2010). The additional replacement of the strictly conserved tyrosine in the β2-α2 loop with glycine now abolished all conversion in the *Tg(MoPrP^{169,170,174})* mice. There was no detectable PrP^{Sc} in the brain of any of these mice, even after incubation periods of up to 466 d, whereas the WT mice developed disease after 173 d. Together, these results suggest that the added substitution of Y169G had a profound inhibitory effect on TSE transmission.

The *Tg(MoPrP^{170,174})* mice were 100% susceptible to CWD; however, the *Tg(MoPrP^{169,170,174})* mice showed no detectable CWD prion infection *in vivo* or *in vitro*. The *Tg(MoPrP^{170,174})* mice express higher PrP^{C} levels than the *Tg(MoPrP^{169,170,174})* mice, so there was the possibility that differences in CWD susceptibility were due to different PrP^{C} expression levels. However, this is highly unlikely, as three rounds of PMCA also failed to show PrP conversion. We also tested whether the MoPrP^{169,170,174} could be converted by hamster, cattle, or sheep prions and found no conversion of the MoPrP^{169,170,174} seeded by any of these prions in three rounds of the PMCA assay. Therefore, none of the prions from four of detectable conversion of MoPrP^{169,170,174}. Mouse prions successfully converted one of four replicates of MoPrP^{169,170,174}, suggesting that the barrier to mouse prions is weaker than the barrier to prions from the other four species. Three rounds of PMCA were performed to robustly compare the *Tg(MoPrP^{169,170,174})* with the WT substrate yet avoid false positive signals from spontaneous conversion (three PMCA rounds have previously replicated known species barriers) (Kurt et al., 2009). It remains possible that MoPrP^{169,170,174} might be converted by prions through further PMCA rounds.

Few single-residue substitutions in PrP^{C} have been reported to completely abolish prion infection. The E219K substitution may delay or prevent prion infection in humans and mice, depending on the prion strain and the genetic background of the mice (Perrier et al., 2002; Hizume et al., 2009). G96S inhibits CWD infection in a transgenic mouse model (Meade-White et al., 2007), yet there are CWD-infected deer expressing PrP with serine in position 96 (Johnson et al., 2006). Transgenic mice expressing mouse PrP with the single Q168R β2-α2 loop substitution show a complete barrier to infection with RML mouse prions (Perrier et al., 2002). The Q172R substitution, which also lies within the β2-α2 loop, potently inhibits *in vitro* conversion by mouse or hamster prions (Geoghegan et al., 2009).

Because the substitution of Y169 in MoPrP with glycine results in a quite dramatic local change of the molecular conformation, where the β2-α2 loop forms a type I β-turn and there is no evidence for a dynamic admixture of other significantly populated structures (Damberger et al., 2011; Christen et al., 2013), the present data show that, in addition to the primary structure effects indicated by studies of different variant PrPs (see above), the conformation of the β2-α2 loop in PrP^{C} may also impact the ease of TSE transmission between different species. We await with interest the results of *in vivo* experiments (in progress in one of our laboratories) with transgenic mice expressing MoPrP with different single-amino acid substitutions in position 169. Because these substitutions result either in an apparently "pure" type I β-sheet turn structure or in maintaining the dynamic equilibrium between two loop structures (Damberger et al., 2011; Christen et al., 2012; Christen et al., 2013), as observed in natural PrP^{C}s of mammalian species, these experiments should provide additional information on the apparently intricate interplay between the amino acid sequence of the β2-α2 loop and its conformation in their effects on TSE transmission.

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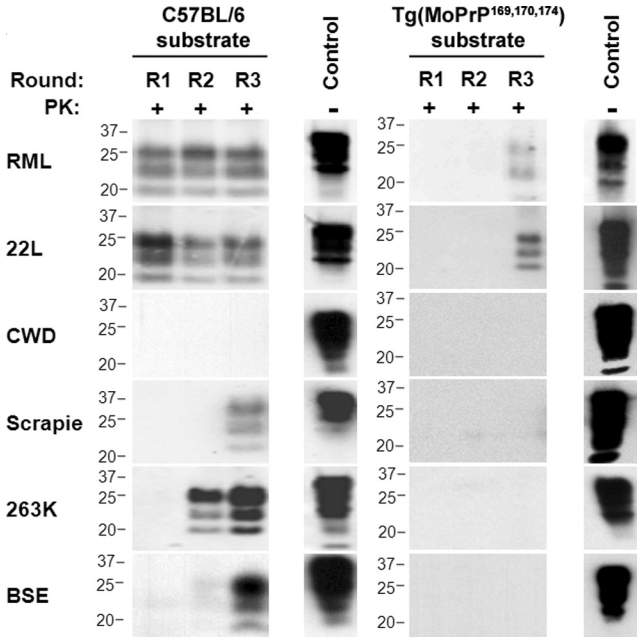

Figure 3. PMCA assay reveals either poor or no conversion in the *Tg(MoPrP^{169,170,174})* substrate by prions originating from different species. Mouse-scrapie strains RML and 22L, deer CWD, sheep scrapie, hamster scrapie 263K, and cattle BSE were subjected to three serial rounds of PMCA using WT mouse (C57BL/6) or *Tg(MoPrP^{169,170,174})* brain homogenates as the substrate. Although none of the prions from deer, hamster, cattle, or sheep were amplified using *Tg(MoPrP^{169,170,174})* mouse brains, all of the prions, with the exception of CWD, were amplified using WT mouse brains. CWD has previously been amplified in transgenic mice expressing cervid PrP (Green et al., 2008). Low level conversion was noted in one of four *Tg(MoPrP^{169,170,174})* samples seeded with RML or 22L prions.

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