TITLE: Role of the goat K_{222}-PrP^{C} polymorphic variant in prion infection resistance.

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The prion protein encoding gene (prnp) strongly influences the susceptibility of small ruminants to Transmissible Spongiform Encephalopathies (TSEs). Hence, selective breeding programs have been implemented to increase sheep resistance to scrapie. In goats, epidemiological and experimental studies provided some association between certain polymorphisms of the cellular prion protein (PrP\text{C}) and resistance to TSEs. Among them, the Q/K polymorphism at the PrP\text{C} codon 222 (Q/K\text{222}) yielded the most promising results. In this work, we investigated the individual effect of K\text{222}-PrP\text{C} variant on the resistance/susceptibility of goats to TSEs. For that purpose, we generated two transgenic mouse lines expressing either Q\text{222} (wild type) or K\text{222} variant of the goat PrP\text{C}. Both mouse lines were intracerebrally challenged with a panel of TSE isolates. Transgenic mice expressing the wild type (Q\text{222}) allele were fully susceptible to the infection with all tested isolates, whereas transgenic mice expressing similar levels of the K\text{222} allele were resistant to all goat-scrapie and cattle-BSE isolates but not to goat-BSE isolates. Finally, the heterozygous K/Q\text{222} mice displayed a reduced susceptibility to the tested panel of scrapie isolate. These results demonstrate a highly protective effect of K\text{222} variant against a broad panel of different prion isolates and further reinforce the argument supporting the use of this variant in breeding programs to control TSEs in goat herds.
The objective of this study was to determine the role of the K222 variant of the prion protein (PrP) on the susceptibility/resistance of goats to Transmissible Spongiform Encephalopathies (TSEs). Results showed that transgenic mice expressing the goat K222-PrP polymorphic variant are resistant to scrapie and Bovine Spongiform Encephalopathies (BSE) agents. This protective effect was also observed in heterozygous Q/K222 animals. Therefore, the single aminoacid exchange from Q to K at codon 222 of the cellular prion protein provides resistance against TSEs. All the results presented here support the view that the K222 polymorphic variant is a good candidate for selective breeding programs to control and eradicate scrapie in goat herds.
INTRODUCTION

Scrapie is an infectious neurodegenerative disease naturally affecting sheep and goats. It belongs to the group of Transmissible Spongiform Encephalopathies (TSEs) or prion diseases, such as Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease in cervids. TSEs are characterized by the accumulation in the central nervous system (CNS) of a pathological aberrant form (PrPSc) of the host cellular prion protein (PrPC) (1).

Scrapie occurrence is mainly determined by the host prion protein encoding gene (prnp) (2, 3) and the prion strain (4), thus resulting in distinct disease phenotypes (showing differences in the PrPSc deposition pattern, incubation period, pathogenesis or clinical signs). Sheep and goats share the same PrPC amino acid sequence but with a variety of polymorphisms. Sheep prnp genotypes V136R154Q171 and A136R154Q171 are associated with high susceptibility to classical scrapie while the A136R154R171 genotype is linked to resistance (5-8). This information was used in some European Union member states to implement selective breeding programs in order to promote the A136R154R171 haplotype within the ovine herd (9).

In goats, half a hundred polymorphisms in the open reading frame (ORF) of the prnp gene have been described (10-12), including some silent mutations, a three octapeptide repeats variant (13) and a nonsense mutation at codon 32 (14). Although several polymorphisms are shared by sheep and goats, to date, only some correlations between some variants of the goat PrPC and susceptibility to TSE have been published. The I/M142 aminoacid substitution was associated with a slightly decreased risk of developing scrapie (15-17), and prolonged incubation times after challenge with scrapie and BSE prions (18). Same effect on the incubation period was attributed to the three-repeat/glycine 102 genotype (13, 18). In natural outbreaks, goats carrying the H/R143...
polymorphism showed a partial decrease in their susceptibility to scrapie prions (15, 19). Likewise, a low susceptibility to scrapie was also linked to the R/H_{154} and R/Q_{211} variants of the goat PrP\(^C\) (15, 20); albeit, the R/H_{154} polymorphism arose as a risk factor for goat atypical scrapie too (21). Besides, other PrP\(^C\) variants were linked to resistance to scrapie, such as the N/D_{146} and N/S_{146} polymorphisms (22). However, the most encouraging results are those related to the Q/K_{222} polymorphism. The absence or marked under-representation of scrapie positive goats expressing the K_{222} variant in numerous herds from different countries (11, 12, 15, 20, 22-25) suggests that K_{222} PrP\(^C\) variant strongly influences goat susceptibility to TSE agents. Recently, heterozygous Q/K_{222} goats were reported to show a strong resistance to classical scrapie after intracerebral inoculation (26). These evidences should be reinforced by experimental data, but the wide number of genetic variants harbored in the goat prnp hinders the \textit{in vivo} determination of the individual effect of the K_{222}PrP\(^C\) variant on this matter.

In this work, we analyze the role of the K_{222} variant of the goat PrP\(^C\) on the susceptibility to prion infection. For that purpose, two transgenic mouse lines, one expressing the wild type goat PrP\(^C\) sequence and another expressing the same PrP\(^C\) sequence but with a single aminoacid exchange from Q to K at codon 222, were generated. Both mouse lines were intracerebrally (IC) challenged with a panel of TSE isolates and their susceptibilities were assessed and then compared.

**MATERIALS AND METHODS**

**Ethics statement**

Animal experiments were carried out in strict accordance with the recommendations in the guidelines of the Code for Methods and Welfare Considerations in Behavioral Research with Animals (Directive 86/609EC) and all efforts were made to minimize suffering. Experiments were approved by the Committee
on the Ethics of Animal Experiments of the author’s institution (INIA); Permit Number: CEEA2009/003.

Transgenic mice

The procedures used to generate transgene constructs have been described elsewhere (27). Briefly, the open reading frame (ORF) of goat wt-prnp was isolated by PCR amplification and sequenced, being identical to goat prnp previously reported (GenBank accession number SY200301). The primers used created a XhoI restriction enzyme site adjacent to the translation start and stop sites of the goat PrP ORF (5’-CTCGAGATCATGGTGAAAAGCCACATAGGC-3’ and 3’-CTCGAGCTACTATGAGAAAAATGAG-5’ respectively). The PCR fragment was sub cloned into the pGEM-T Easy Vector System (Promega) following the manufacturer’s instructions. The expression vector MoPrP.Xho (28) was used for the production of transgenic mice. This vector contains the murine PrP promoter, exon 1, intron 1, exon 2 and 3’ untranslated sequences. The PrP ORF fragment was excised from the pGEM-T vector using the restriction enzyme XhoI and inserted into the MoPrP.Xho expression vector (28), also digested with the XhoI enzyme, resulting in the plasmid pMo-GoPrP.Xho. This plasmid was mutated to generate a K222-PrP plasmid (pMo-GoK222-PrP.Xho) using the kit QuikChange® II XL (Stratagene, California, USA) with specific oligonucleotides (5’-GTGCATCACCCAGTACAAGAGAGAATCCCAGGC-3’ and 3’-GCCTGGGATTCTCTCTTGTACTGGGTGATGCAC-5’) following the procedures described by the manufacturer. MoPrP.Xho expression vector contains the wt murine prnp gene, including the murine prnp promoter (exon-1, intron-1, exon-2 and 3’ untranslated sequences; but not intron-2 and murine PrP ORF).

Transgenic mouse lines were generated as previously described (27). Briefly, transgenes were excised from their expression vectors (pMo-GoPrP.Xho or pMo-
GoK222-PrP.Xho) by the restriction endonuclease Not I leading to DNA fragments of approximately 12 Kb. DNAs were then purified using sodium chloride gradients as previously described (29) and resuspended in TE (10 mM Tris, pH 7.4, 0.1 mM EDTA) at a final concentration of 2 to 6 ng/ml. Finally, DNAs were microinjected into pronuclear stage embryos collected from super-ovulated B6CBAF1 females mated with 129/Ola males carrying a null mutation in their endogenous PrP (30).

DNA was extracted from founders’ tail biopsies using an Extract-N-Amp Tissue PCR kit (Sigma-Aldrich) following the manufacturer’s instructions. The presence of the goat transgene in these founders was identified by PCR amplification using specific primers for the mouse PrP exon 2 and goat PrP ORF. The primers used were 5´-CATTCTGCCTCCTAGGTACCC-3´ and 5´-GCTTGTTTCCACGTGACTGCGG-3´.

MuPrP+/- goPrP+/- founders were backcrossed with homozygous PrP null animals (muPrP-/-) to obtain homozygosis for the null mutation (muPrP-/-, goPrP+/+). The absence of the murine PrP ORF in the transgenic mice generated was confirmed by PCR amplification using the primers: 5´-ATGGCGAACCTTGGCTACTGGC-3´ and 5´-GATTATGGGTACCCCTCCTTGG.

**Analysis of PrP expression in transgenic mice.** Whole brains from either mice or goat were homogenized in extraction buffer (0.5% NP40, 1% sodium deoxycholate, 10mM EDTA in PBS pH 7.4, and the Complete™ cocktail of protease inhibitors from Roche). Samples were pre-cleared by centrifugation at 2,000 g for 5 minutes, after which an equal volume of 2x SDS reducing sample loading buffer was added to all samples and each one was boiled for 5 minutes before loading on a SDS/12% polyacrylamide gel. For the immunoblotting experiments, the monoclonal antibodies (mAbs) FH11 (31) or 12B2 (32) were used at a concentration of 1 μg/mL. FH11 recognizes the goat PrP amino-terminal region (amino acids 23 to 85) and 12B2
recognizes 93WGQGG97 epitope of the goat PrP sequence. Immunocomplexes were
detected using horseradish peroxidase conjugated anti mouse IgG. Immunoblots were
developed with enhanced chemiluminescence.

**Transmission studies**

Transgenic mice were challenged with a panel of TSEs including cattle-BSE
goat-BSE and different goat-scrapie isolates (see Table 1 for isolates information).
Inocula were prepared from infected brain tissues as 10% (w/v) homogenates in 5%
glucose.

Groups of 6-9 individual identified animals (6-7 weeks-old) were anesthetized
and intracerebrally inoculated with 20 ul of 10% brain homogenate in the right parietal
lobe using a 25-gauge disposable hypodermic needle. As a control, 6-7 animals of each
line were inoculated with healthy goat brain to discard the appearance of a spontaneous
prion disease. Mice were observed daily and their neurological status assessed twice a
week. When the progression of the disease was evident, or at the end of their lifespan
(=650 days), mice were euthanized for ethical reasons. During necropsy, brain was
harvested at -20ºC for determining the presence of PrP\textsuperscript{res} by Western blot (WB).
Survival time was expressed as the mean of the survival days post inoculation (dpi) of
all the PrP\textsuperscript{res} positive mice, standard error included. Attack rate was determined as the
proportion of PrP\textsuperscript{res} positive mice from all the mice inoculated.

**Western blot**

175 mg of brain tissue were homogenized in 5% glucose in distilled water in
grinding tubes (Bio-Rad) adjusted to 10% (w/v) using a TeSeETM Precess 48TM
homogenizer (Bio-Rad) following the manufacturer instructions. To determine the
presence of PrP\textsuperscript{res} in transgenic mice brains, 100 μl of 10% brain homogenate were
analysed by Western blots as previously described (33). For immunoblotting,
membranes were incubated with Sha31 mAb (34) that recognizes 148YEDRYYRE155 epitope of the goat-PrP sequence. Immunocomplexes were detected with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech) after incubating the membranes for 1 hour, and immunoreactivity was visualized by chemiluminescence with ECL Plus (GE Healthcare Amersham Biosciences).

### Histopathological analysis

All procedures concerning the histopathological analysis of mice infected brains were performed as previously described (35). Brain samples were immediately fixed in neutral-buffered 10% formalin (4% 2-formaldehyde) during mice necropsy and paraffin embedded later. After deparaffinization, 2 µm-thick tissue slices were stained with haematoxylin and eosin. Lesion profiles of the brains were established following the standard method by Fraser & Dickinson (36). Paraffin Embedded Tissue (PET) blots and Immunohistochemistry (IHC) were conducted as described by Andreoletti et al. (37-38).
RESULTS

**Goat PrP^C expression in transgenic mice**

Several mouse lines (founders) were obtained for each transgene (Q\textsubscript{222}-PrP\textsuperscript{C} or K\textsubscript{222}-PrP\textsuperscript{C}). Founder animals also expressing the endogenous murine prnp gene (prnp\textsuperscript{mu+/go+/}) were crossed with prnp null mice (prnp\textsuperscript{mu-/-}) to obtain transgene-hemizygous lines in a murine prnp null background (prnp\textsuperscript{mu-/-go+/}). The absence of the murine prnp was determined by PCR using specific primers (data not shown). Then, PrP\textsuperscript{C} expression level was determined in brain homogenates by serial dilution in Western blotting using the 12B2 mAb in comparison with the PrP\textsuperscript{C} levels found in goat brain homogenates. Q\textsubscript{222}-Tg501 and K\textsubscript{222}-Tg516 lines were selected based on the fact that they have PrP\textsuperscript{C} expression levels in brain similar to those in goat (Fig. 1). In addition, both Q\textsubscript{222}-PrP\textsuperscript{C} and K\textsubscript{222}-PrP\textsuperscript{C}, expressed in brain of transgenic mice, showed an electrophoretic profile similar to that observed in the PrP\textsuperscript{C} of goat brain (Fig. 1).

Finally, hemizygous Q\textsubscript{222}-Tg501 and K\textsubscript{222}-Tg516 mouse lines were used for transmission studies.

**Transgenic mice expressing K\textsubscript{222}-PrP variant are resistant to a broad panel of goat-scrapie isolates**

The Q\textsubscript{222}-Tg501 mouse line expressing the wild type goat PrP\textsuperscript{C} was susceptible to all inoculated scrapie isolates. Intracerebrally challenged animals displayed 100% attack rate and mean survival times ranging from 250 to 650 days post inoculation (dpi) (Table 2). No differences were observed between the electrophoretic profiles of the PrP\textsuperscript{res} in the inoculated isolates and those of the PrP\textsuperscript{res} present in the brain of challenged Q\textsubscript{222}-Tg501 mice (Figure 2).

In contrast, none of the K\textsubscript{222}-Tg516 mice succumbed to the inoculation with any of these scrapie isolates (Table 2). These animals were sacrificed at 650 dpi without
clinical signs and were scored PrP<sup>res</sup> negative in their brains by western blot. The absence of PrP<sup>res</sup> in their brains was confirmed by either immunohistochemistry (IHC) or PET-blot. These animals exhibited some vacuolation mainly at the level of the dorsal medulla, and mesencephalic tegmentum. However, this vacuolation was also observed in the same areas in old Q<sub>222</sub>-Tg501 and K<sub>222</sub>-Tg516 mice inoculated with healthy goat brain. Contrary, all the infected Q<sub>222</sub>-Tg501 mice displayed PrP<sup>res</sup> deposits in their brains which were accompanied of histopathological alterations characteristic of prion infection (data not shown).

It is important to note that Q<sub>222</sub>-Tg501 and K<sub>222</sub>-Tg516 mouse lines, both expressing similar amounts of PrP<sup>C</sup> in their brain, only differ among their PrP<sup>C</sup> sequence on the Q/K<sub>222</sub> substitution, thus demonstrating that this single aminoacid exchange is the only responsible for the differential susceptibility/resistance to scrapie observed between these mouse lines.

None of the Q<sub>222</sub>-Tg501 or K<sub>222</sub>-Tg516 mice inoculated with the healthy brain sample succumbed to disease (Table 2). They were euthanized at the end of their life span (650 dpi) without neither clinical signs nor PrP<sup>res</sup> in their brains.

Transgenic mice expressing the K<sub>222</sub>-PrP variant are resistant to cattle-BSE but not to goat- or sheep-BSE.

Both Q<sub>222</sub>-Tg501 and K<sub>222</sub>-Tg516 mouse lines were fully susceptible (100% attack rates) to goat and sheep-BSE isolates, displaying similar mean survival times ranging from 400 to 500 dpi (Table 2). However, the inoculation of the cattle-BSE isolate in Q<sub>222</sub>-Tg501 and K<sub>222</sub>-Tg516 mice produced markedly different results. While Q<sub>222</sub>-Tg501 mice showed a 100% attack rate and a survival time of 583±57 dpi, K<sub>222</sub>-Tg516 mice were sacrificed at the end of their lifespan (650 dpi) without neither clinical signs nor PrP<sup>res</sup> in their brains (Table 2). Moreover, K<sub>222</sub>-Tg516 mice were...
totally susceptible to cattle-BSE passaged in Q222-Tg501 mice with a mean survival time of 434±30 dpi.

Western blot analysis of brain-PrP<sup>res</sup> from goat-BSE isolates revealed a typical PrP<sup>res</sup> banding pattern, characterized by low size fragments (19kDa fragment for the aglycosyl-band) and prominent diglycosylated species. This pattern was indistinguishable from that observed in both Q222-Tg501 and K222-Tg516 mice infected with goat or sheep-BSE isolates. Moreover, an identical PrP<sup>res</sup> banding pattern was observed in Q222-Tg501 mice inoculated with cattle-BSE (Fig. 3).

Histopathological analysis confirmed the absence of both PrP<sup>res</sup> deposits and spongiform changes in the brain of all K222-Tg516 mice inoculated with cattle-BSE. However, when these mice were infected with goat-BSE isolates, they exhibited PrP<sup>res</sup> deposition patterns and lesion profiles similar to those observed in Q222-Tg501 mice infected with either cattle or goat-BSE (data not shown).

Altogether these results suggest a low transmission barrier to cattle-BSE in transgenic mice expressing the wild type (Q222) goat PrP<sup>C</sup>. Conversely, the K222 amino acid substitution seemed to give rise to a transmission barrier that limits the propagation of cattle-BSE prions. Nevertheless, this transmission barrier is abolished once the isolate is previously passaged in a wt-goat PrP<sup>C</sup> expressing host (sheep, goat and Q222-Tg501 mouse) (Table 2).

**Heterozygous Q/K222 mice showed lower attack rates and/or longer survival times depending on the inoculated isolate**

To accurately determine the role of K222-PrP variant on goat resistance/susceptibility to TSEs, K222-Tg516 mice were crossbred with Q222-Tg501 mice to generate heterozygous (Q/K222) mice. These animals were intracerebrally inoculated with the same panel of TSE isolates previously used (Table 2).
Heterozygous Q/K_{222} animals were resistant to Goat-Sc I3 and Goat-Sc S2 isolates. These animals were sacrificed at 650 dpi without having shown clinical signs and no detectable PrP^{res} was found in their brains by WB, IHC or PET-blot. However, Q/K_{222} mice succumbed to the infection with Goat-Sc F2 isolate displaying reduced attack rates (83.3\%) and longer survival times than those observed in Q_{222}-Tg501 mice. Moreover, those Q/K_{222} mice challenged with Goat-Sc F10 isolate were sacrificed at 650 dpi also without clinical signs, but PrP^{res} was detected in their brains (Table 2). Taken together these results suggest that in heterozygous Q/K_{222} mice the K_{222}-PrP^{C} variant interferes with the replication of all tested scrapie agents.

When inoculated with cattle-BSE, heterozygous Q/K_{222} animals showed prolonged survival times than Q_{222}-Tg501 mice (Table 2). However, goat-BSE isolates displayed similar transmission features in all transgenic mouse models challenged (Q_{222}-Tg501, K_{222}-Tg516 and Q/K_{222} mice) independently of the expressed PrP^{C} variant; thus indicating low or non-existent interference of K_{222}-PrP^{C} variant with BSE agent replication.

Histopathological studies confirmed the presence of PrP^{res} and spongiform changes in the brains of heterozygous Q/K_{222} mice infected with Goat-Sc F2, Goat-Sc F10 and goat and cattle-BSE isolates. PrP^{res} deposits and vacuolation profile in these mice were similar but not identical to those determined in Q222-Tg501 mice infected with the same isolates (data not shown). However, we cannot exclude that these slight differences are linked to the old age of Q/K222 mice since they displayed longer survival times than Q222-Tg501. Further passages are necessary to conclude at this point.
DISCUSSION

In this study, we use a transgenic mice model to assess the role of the K222-PrPC variant in the resistance/susceptibility of goats to TSEs. For that purpose, we generated two transgenic mouse lines expressing either the wt (Q222-Tg501) or the K222 variant (K222-Tg516) of the goat PrPC. Both mouse lines expressing similar amounts of PrPC were intracerebrally challenged with a panel of prion isolates.

When TSEs transmission efficiency was assessed in Q222-Tg501 mice, these animals showed high susceptibility (100% attack rates) to all the tested scrapie and BSE isolates. PrPres profiles in western blot of all scrapie and BSE isolates were not altered after passage in these Q222-Tg501 mice (Figure 2 and 3). Goat-BSE displayed a weak decrease in survival times when compared with cattle-BSE, confirming the low transmission barrier of goat to cattle-BSE previously reported in experimentally challenged goats (39). According to these data, we propose transgenic mice Q222-Tg501 expressing the wild type goat PrPC as a valuable model for both studying the susceptibility/resistance of small ruminants to TSEs and characterizing TSE strains affecting these species.

In contrast to the high susceptibility of Q222-Tg501 mice, K222-Tg516 mice were fairly resistant to all the classical scrapie isolates inoculated independently of their origin or PrPres signature. Since Q222-Tg501 mice and K222-Tg516 mice express exactly the same PrPC sequence except for the Q/K222 aminoacid substitution and with similar expression levels, we can assume that the lack of transmission efficiency of classical scrapie is linked to the K222 aminoacid. This conclusion is consistent with previous epidemiological studies carried out in different European goat scrapie outbreaks where the K222-PrPC variant was only detected in healthy goats (12, 15, 23, 24).
together these results suggest that the K222-variant of the goat PrP<sup>C</sup> is strongly resistant to a wide diversity of classical scrapie strains.

On the other hand, heterozygous Q/K222 mice were clearly more resistant to the different scrapie isolates than Q<sub>222</sub>-Tg501 mice. These results support the contention that K<sub>222</sub>-PrP<sup>C</sup> variant provides a dominant negative effect over the wild type PrP<sup>C</sup> sequence. This phenomenon would be consistent with the stone fence model (40) which predicts that, for a given TSE agent, the incorporation of a conversion incompetent PrP<sup>C</sup> variant would interfere with the wild type PrP<sup>Sc</sup> replication, resulting in a lower efficacy of prion propagation. This decreased propagation efficacy would result in reduced attack rates and/or prolonged survival times, as shown in our study; and could explain the few epidemiological evidence linking scrapie with K222 allele (11, 15, 22).

BSE transmission experiments showed that mice expressing the K<sub>222</sub>-PrP<sup>C</sup> variant (K<sub>222</sub>-Tg516 mice) were resistant to cattle-BSE but not to goat-BSE or sheep-BSE isolates (Table 2). The failure of the cattle-BSE isolate to be transmitted in K<sub>222</sub>-PrP<sup>C</sup> mice can be explained by the dual influence of both the PrP<sup>C</sup> sequence differences in the prion cross-species transmission (2, 3) and the effect of the Q/K<sub>222</sub> aminoacid substitution. This way, the effect of the PrP<sup>C</sup> primary sequence of the donor (bovine) is not strong enough to avoid cattle-BSE replication in goat wt Q<sub>222</sub>-Tg501 mice. However, after the Q/K<sub>222</sub> substitution in K<sub>222</sub>-Tg516 mice, cattle-BSE is unable to replicate. This view is fully consistent with the fact that K<sub>222</sub>-Tg516 mice were susceptible to BSE only after passage in goat or Q<sub>222</sub>-Tg501 mice (Table 2). Therefore, transmission barrier towards BSE agent is complex being modulated not only by K<sub>222</sub> allele but also by other determinants as the PrP amino acid differences between host and donor (goat versus cattle). Interestingly, this transmission barrier was abolished when K<sub>222</sub>-PrP<sup>C</sup> variant was present in heterozygosis, indicating that, for BSE, K<sub>222</sub>
aminoacid substitution does not interfere with the conversion of wild type Q222-PrPC into PrPSc.

An inhibitory effect on prion replication of equivalent K222 variants has also been described in other species. Human K219-PrPC allele (homologous to codon 222 in goat PrPC) was reported to protect humans against Creutzfeldt-Jakob-Disease (41) while the equivalent K218 variant of the mouse PrPC significantly reduced the fibrils aggregation kinetics and generated non proteinase K-resistant PrP (42). A plausible explanation for this inhibitory effect could be the insertion of an additional positive charge at codon 222, provided by the lysine aminoacid (K), thus interfering with the PrPC/PrPSc interaction and resulting in abolished or low conversion rates of PrPSc (43). All these considerations suggest that codon 222 must play an important role on PrPC conformation and points it out as a target for future TSEs studies.

The use of transgenic mice in our transmission studies allowed us to determine the Q/K222 aminoacid substitution as the unique responsible for the observed resistance to TSEs, excluding other genetic factors. In addition, the intracerebral inoculation route provided the best scenario for prion replication as the inoculum is directly placed in the target tissue. Therefore, although our procedure mimics neither the natural route of scrapie infection nor the complex pathogenesis involving prion replication in peripheral tissues, our results offer solid arguments supporting that the K222 variant of the goat PrPC provides a strong resistance against a wide diversity of classical scrapie isolates and reinforce the view that K222 allele is a good candidate for the development of breeding programs for resistance against scrapie in commercial goat population.
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REFERENCES


Figure legends

Figure 1. Brain PrP<sup>C</sup> expression in hemizygous Q<sub>222</sub>-Tg501 (A) and K<sub>222</sub>-Tg516 (B) mouse lines in comparison with goat brain. Immunoblotting of PrP<sup>C</sup> detected with 12B2 mAb. Direct sample (10% brain homogenates) and ½ serial dilutions were loaded on 12% Bis-Tris gels. The figure illustrates a representative set of three independent experiments. Relative molecular mass in kilodaltons at the left side.

Figure 2. PrP<sup>res</sup> of goat scrapie isolates both before and after transmission in Q<sub>222</sub>-Tg501 mice. Immunoblots of brain PrP<sup>res</sup> detected with Sha31 mAb. Similar quantities of PrP<sup>res</sup> were loaded for adequate comparison. Molecular weights (in kDa) are shown at the right side of the blot.

Figure 3. PrP<sup>res</sup> of BSE isolates before and after transmission in either Q<sub>222</sub>-Tg501 or K<sub>222</sub>-Tg516 mice. Immunoblots of brain PrP<sup>res</sup> detected with Sha31 mAb. Similar quantities of PrP<sup>res</sup> were loaded for adequate comparison. Molecular weights (in kDa) are shown at the right side of the blot.
**TABLE 1.** Description of the different isolates used in this work.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin (local code)</th>
<th>Goat PrP Genotype</th>
<th>Description and references</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat-BSE1</td>
<td>Fr (CH0064)</td>
<td>I_{136}Q_{154}S_{240}/I_{136}R_{154}S_{240}</td>
<td>Terminally ill goat intracerebrally inoculated with Ca-BSE isolate</td>
<td>INRA¹</td>
</tr>
<tr>
<td>Goat-BSE2</td>
<td>Fr (CH1075)</td>
<td>I_{136}R_{154}S_{240}/I_{136}R_{154}P_{240}</td>
<td>Terminally ill goat intracerebrally inoculated with Ca-BSE isolate</td>
<td>INRA¹</td>
</tr>
<tr>
<td>Sheep-BSE</td>
<td>Fr (ARQ0)</td>
<td>wt*</td>
<td>Pool of terminally ill ARQ/ARQ sheep inoculated with Ca-BSE isolate (44)</td>
<td>INRA¹</td>
</tr>
<tr>
<td>Ca-BSE</td>
<td>Fr (139)</td>
<td></td>
<td>BSE from a naturally infected cow (44)</td>
<td>INRA¹</td>
</tr>
<tr>
<td>Goat-Sc F2</td>
<td>Fr (CP40)</td>
<td>wt*; S/P_{240}</td>
<td>Classical scrapie from an experimentally infected goat</td>
<td>INRA¹</td>
</tr>
<tr>
<td>Goat-Sc F10</td>
<td>Fr (2143)</td>
<td>wt*; S/P_{240}</td>
<td>Classical scrapie from a naturally infected goat</td>
<td>INRA¹</td>
</tr>
<tr>
<td>Goat-Sc I3</td>
<td>It (121429/1/1)</td>
<td>wt*</td>
<td>Classical scrapie from a naturally infected goat</td>
<td>IZSTO²</td>
</tr>
<tr>
<td>Goat-Sc I9</td>
<td>It (85792/1/1)</td>
<td>wt*; S/P_{240}</td>
<td>Classical scrapie from a naturally infected goat</td>
<td>IZSTO²</td>
</tr>
<tr>
<td>Goat-Sc S2</td>
<td>Sp (C-163)</td>
<td>wt*; S/P_{240}</td>
<td>Classical scrapie from a naturally infected goat</td>
<td>UNIZAR³</td>
</tr>
<tr>
<td>Goat-Sc S3</td>
<td>Sp (C-645)</td>
<td>wt*</td>
<td>Classical scrapie from a naturally infected goat</td>
<td>UNIZAR³</td>
</tr>
<tr>
<td>Healthy goat brain</td>
<td></td>
<td></td>
<td>Brain from a not infected goat</td>
<td>INRA¹</td>
</tr>
</tbody>
</table>

*Wild type (wt) goat prion protein genotype: A_{136}R_{154}P_{240}/A_{136}R_{154}P_{240}.

1French National Institute for Agricultural Research (INRA), Nouzilly, France.
2Istituto Zooprofilattico Sperimentale del Piemont, Italy.
3Universidad de Zaragoza, Spain.
TABLE 2. Transmission of a panel of prion isolates to Q222-Tg501, K222-Tg516 and heterozygous Q/K222 mice.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mean survival time in days ± SEM (n/n₀)³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q₂₂₂⁻Tg₅₀₁ (Q₂₂₂⁻/-)</td>
</tr>
<tr>
<td>Goat-BSE1</td>
<td>497±31 (5/5)</td>
</tr>
<tr>
<td>Goat-BSE2</td>
<td>484±34 (7/7)</td>
</tr>
<tr>
<td>Sheep-BSE</td>
<td>485±62 (7/7)</td>
</tr>
<tr>
<td>Ca-BSE</td>
<td>583±57 (6/6)</td>
</tr>
<tr>
<td>Ca-BSE/Tg501ᵇ</td>
<td>326±26 (6/6)</td>
</tr>
<tr>
<td>Goat-Sc F10</td>
<td>465±19 (7/7)</td>
</tr>
<tr>
<td>Goat-Sc F2</td>
<td>250±36 (4/4)</td>
</tr>
<tr>
<td>Goat-Sc I3</td>
<td>659±10 (5/5)</td>
</tr>
<tr>
<td>Goat-Sc I9</td>
<td>600±43 (5/5)</td>
</tr>
<tr>
<td>Goat-Sc S2</td>
<td>449±62 (9/9)</td>
</tr>
<tr>
<td>Goat-Sc S3</td>
<td>298±22 (6/6)</td>
</tr>
<tr>
<td>Healthy goat brain</td>
<td>&gt;650 (0/6)</td>
</tr>
</tbody>
</table>

³n/n₀: diseased, PrP<sup>Sc</sup>: positive/inoculated animals.

ᵇPool of terminally ill wt-Tg501 mice inoculated with Ca-BSE isolate.

ND: No data