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Mutated but Not Deleted Ovine PrP^C N-Terminal Polybasic Region Strongly Interferes with Prion Propagation in Transgenic Mice

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ABSTRACT

Mammalian prions are proteinaceous infectious agents composed of misfolded assemblies of the host-encoded, cellular prion protein (PrP). Physiologically, the N-terminal polybasic region of residues 23 to 31 of PrP has been shown to be involved in its endocytic trafficking and interactions with glycosaminoglycans or putative ectodomains of membrane-associated proteins. Several recent reports also describe this PrP region as important for the toxicity of mutant prion proteins and the efficiency of prion propagation, both *in vitro* and *in vivo*. The question remains as to whether the latter observations made with mouse PrP and mouse prions would be relevant to other PrP species/prion strain combinations given the dramatic impact on prion susceptibility of minimal amino acid substitutions and structural variations in PrP. Here, we report that transgenic mouse lines expressing ovine PrP with a deletion of residues 23 to 26 (KKRP) or mutated in this N-terminal region (KQHPH instead of KKRPK) exhibited a variable, strain-dependent susceptibility to prion infection with regard to the proportion of affected mice and disease tempo relative to findings in their wild-type counterparts. Deletion has no major effect on 127S scrapie prion pathogenesis, whereas mutation increased by almost 3-fold the survival time of the mice. Deletion marginally affected the incubation time of scrapie LA19K and ovine bovine spongiform encephalopathy (BSE) prions, whereas mutation caused apparent resistance to disease.

IMPORTANCE

Recent reports suggested that the N-terminal polybasic region of the prion protein could be a therapeutic target to prevent prion propagation or toxic signaling associated with more common neurodegenerative diseases such as Alzheimer's disease. Mutating or deleting this region in ovine PrP completes the data previously obtained with the mouse protein by identifying the key amino acid residues involved.

Transmissible spongiform encephalopathies (TSE), or prion diseases, form a class of fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle (1, 2). TSE are caused by proteinaceous infectious agents termed prions, which are mainly, if not solely, composed of misfolded, aggregated conformers of the ubiquitously expressed, host-encoded normal cellular glycosylphosphatidylinositol-anchored glycoprotein PrP^C (where C indicates cellular). Biochemically, scrapie prion protein (PrP^{Sc}) conformers most often show pronounced resistance to protease digestion and insolubility in nonionic detergents. The PrP^{Sc} pathogenic conformer triggers, with the help of potential additional cellular cofactors, the conversion of PrP^C into PrP^{Sc} by a poorly understood (autocatalytic) process involving structural interactions between PrP^{Sc} and PrP^C (3). Distinct prion strains can be isolated within defined host species, causing disease with the specific tempo and pattern of (neuro)pathology. Prion strains are thought to differ in their PrP^{Sc} conformations at the level of the tertiary and/or quaternary structure (1, 4).

Prions can propagate between species, as exemplified by the emergence of a variant Creutzfeldt-Jakob disease through dietary exposure to BSE prions. Generally, cross-species transmissions are less efficient than intraspecies ones, as evidenced by incomplete attack rate and extended incubation times in recipient animals. Transgenic modeling (5) and studies of scrapie in sheep and goat flocks (6) indicate that minimal amino acid divergences in the PrP protein between species can profoundly affect prion susceptibility. At the molecular level, structural compatibility between infecting PrP^{Sc} strain types and host PrP^C is thought to dictate prion capacity to propagate and to establish disease within and between species (1, 4).

Native PrP^C has an unstructured N-terminal domain and a globular C-terminal domain composed of three α-helices and a short antiparallel β-sheet. Investigating the PrP^C regions involved in the conformational interaction with PrP^{Sc} during intra- and cross-species transmission events mostly relies on PrP^C mutagen-

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esis and assessment of convertibility in cell-free systems, cells, and transgenic mice. As could be expected, some of the identified regions, including a polybasic region (residues 98 to 110), lie within the PrP globular domain known to be remodeled conformationally in the process of PrP^{Sc} formation. Outside the PrP^{Sc} amyloid core, a second, highly conserved, polybasic region ($_{23}$KKRPKPG GW$_{31}$) at the extreme N terminus has received special interest. Studies using motif-grafted antibodies have suggested that this region might dock PrP^{Sc} during prion propagation (7–9). Mice overexpressing mouse PrP with a deletion of this short region (residues 23 to 31; [PrPΔ23–31]) displayed dramatically delayed disease and reduced accumulation of brain PrP^{Sc} upon mouse RML (Rocky Mountain Laboratory scrapie) prion infection despite the correct expression pattern of the mutant PrP^{C} protein (10). Transgenic mice expressing physiological levels of the mutant protein were resistant to RML infection, accumulating subclinical levels of PrP^{Sc} in their brains (10). Similar results were obtained with mouse PrP with a deletion of residues 23 to 88 (11). The presence of the N-terminal polybasic region, while unnecessary to convert PrP^{C} into PrP^{Sc}, was key to generating bona fide infectious prions in a cell-free conversion assay (12). Independently of a prion infectious context, the N-terminal polybasic region has been implicated in a number of PrP functions, including Cu^{2+}-dependent PrP endocytosis (13, 14), tubulin interactions (15), glycosaminoglycan binding (16) leading to regulation of β-secretase cleavage of the Alzheimer's amyloid precursor protein (17), stimulation of DNA repair (18), and PrP neuroprotection (19–22). The direct involvement of this region in the interaction with the Aβ_{42} oligomers responsible for Alzheimer's disease and potentially with other β-conformers suggests a broader implication in mediating neurotoxic signaling (23, 24).

The N-terminal polybasic region is strictly conserved among prion-susceptible mammalian species. Yet the question remains as to whether this domain is a promiscuous docking site relative to other prion strains and PrP species. A second important question is whether some amino acids are more important than others in the region of residues 23 to 31. We therefore generated transgenic mouse lines expressing ovine PrP(136V154R171Q) (the VRQ allele [PrP^{VRQ}]) deleted or mutated in the N-terminal polybasic region and assessed their susceptibility to scrapie and BSE prions relative to that of transgenic mice expressing wild-type ovine PrP.

MATERIALS AND METHODS

Ethics statement. All animal experiments were approved by the local ethics committee of the Institut National de la Recherche Agronomique (Comethea permit number 12/034).

Constructs. The N-terminally deleted or mutated ovine PrP-expressing vectors were derived from the previously described ovine PrP^{VRQ} (136V154R171Q allele) phgPrP (25)-based tg1 construct (26). The tg1 plasmid DNA was digested by BspEI and SmaI to isolate a short DNA fragment encompassing the start of the transcription unit of the gene. The region encoding amino acids (aa) 23 to 28 (KKRPKP) was mutated into a partially deleted (KP, i.e., KKRP deletion) or mutated (KQHPHP) sequence by PCR amplification using the forward oligonucleotide 5′-AAC AGATACAAGCATTTAAGCCAGTCCGGAGCGGTGACTCAT-3′ that includes the BspEI restriction site and the reverse oligonucleotide 5′-TG CCCGGGATACCGGCTTCCACCGGTGTTCCACCCTCCAGGCTTGC AGAGGCCGACATCAGTCCAC-3′ or 5′-TGCCCGGGATACCGGCT TCCACCGGTGTTCCACCCTCCAGGCGATGGGTGTCGTTTGCA GAGGCCG-3′ for the deleted or the mutated sequence, respectively. Both reverse oligonucleotides include an SmaI restriction site. Following

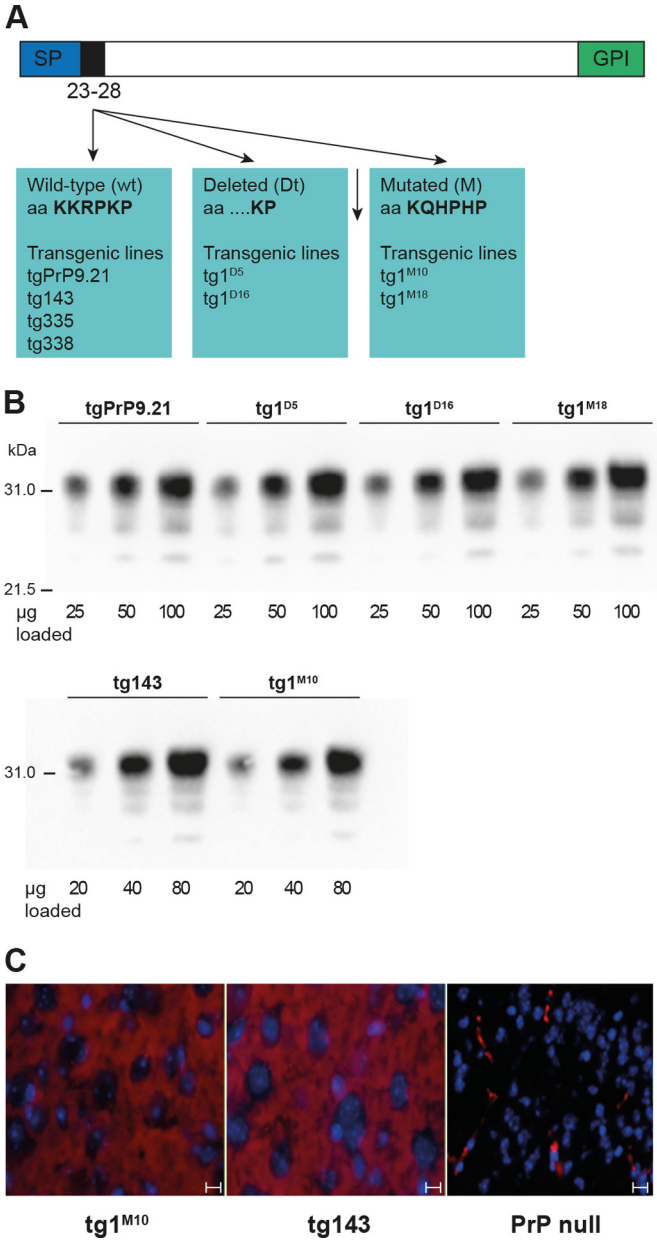

FIG 1 Schematic of the transgenic lines generated in the study and PrP^{C} expression levels in these lines. (A) Schematic of the transgenic lines generated in the study. Location and mutated sequences within the ovine PrP protein associated with the different transgenic lines used in this experiment are indicated. (B) Immunoblot (Pc248 antibody) showing PrP^{C} expression levels in the brain of the mutant lines compared to levels in wild-type lines. (C) Distribution of PrP^{C} in the brain of the PrP transgenic lines. Brain sections of tg1^{M18} and tg143 mice (at the level of the cerebral cortex) were stained with anti-PrP Sha31 antibody and the nuclear marker 4′,6-diamidino-2-phenylindole. Brain sections from FVB/N PrP$^{0/0}$ (PrP null) mice were used as a negative control. Scale bar, 10 μm.

PCR amplification, cloning into pGEM-T, and sequence analysis, both mutated sequences were excised from pGEM-T by BstEI and SmaI restriction digests and cloned into the corresponding sites of the tg1 vector, leading to the tg1^{D} (D for deleted) and tg1^{M} (M for mutated) constructs (Fig. 1).

Generation of transgenic mice and expression analysis. The NotI/SalI tg1^{D} and tg1^{M} vector-free gel-purified inserts were injected into

FVB/N *Prnp* knockout mouse eggs, as previously described (26, 27). Identification of the transgenic founders and transgenic offspring was performed by PCR analysis of genomic DNA extracted from tail biopsy specimens using primers 5′-AACCGCTATCCACCTCAGG-3′ and 5′-AAAG AGGATCACACTTGC-3′ (27). Expression analyses of the transgenes were performed by reverse transcription-PCR (RT-PCR) analysis using the above-described primers and primers specific for the β-actin endogenous mouse gene (28) and Western blotting using Sha31 (29), Pc248, or MH44 antibodies (30), as previously described (26). To comparatively assess PrP localization, immunohistochemical analyses were performed on brain slices of $tg1^M$ and $tg1^D$ transgenic mice and of FVB/N *Prnp* knockout and tg143 transgenic mice as controls using Sha31 antibody (29), as previously described (31).

Scrapie or BSE isolate inoculation and determination of incubation period. 127S, LA19K scrapie, and BSE prion strains were obtained through serial transmission and subsequent biological cloning by limiting dilution of field isolates to tg338 mice expressing the VRQ allele of ovine PrP (32). Analysis of the mouse susceptibility was assessed on the newly created $tg1^D$ and $tg1^M$ lines and on previously obtained tgPrP9.21 (27), tg143, and tg335 (26) transgenic lines that express similar levels of wild-type ovine PrP in the brain. Six- to 8-week-old animals were inoculated intracerebrally in the right hemisphere with 20 μl of a 10% (wt/vol) brain homogenate solution in 5% glucose. Back-passaging to reporter tg338 mice was performed when necessary using the same protocol. Mice were examined every day following injection for the appearance of neurologic dysfunctions. Animals with clear clinical signs or at the end of life were euthanatized by cervical dislocation, and the brains were collected to analyze the presence of PrP^{Sc} by immunoblotting.

Analysis of brain PrP^{res} accumulation by immunoblotting. Proteinase K-resistant PrP^{Sc} (PrP^{res}) was extracted from 20% (wt/vol) brain homogenates with a Bio-Rad TeSeE detection kit, as previously described (33). Briefly, aliquots were digested with proteinase K (200 μg/ml final concentration) for 10 min at 37°C before buffer B (Bio-Rad TeSeE) precipitation and centrifugation at 28,000 × *g* for 15 min. Pellets were resuspended in sample buffer, denatured, run on 12% Bis-Tris gels (Bio-Rad), electrotransferred onto nitrocellulose membranes, and probed with 0.1 μg/ml biotinylated anti-PrP monoclonal Sha31 antibody (29). Immunoreactivity was visualized by chemiluminescence (GE Healthcare).

Analysis of regional distribution of PrP^{res} in the brain by histoblotting. Brains were rapidly removed from euthanized mice and frozen on dry ice. Cryosections were cut at 8 to 10 μm, transferred onto Superfrost slides, and kept at −20°C until use. Histoblot analyses were performed as described previously (33), using the 12F10 anti-PrP antibody. Analysis was performed with a digital camera (Coolsnap; Photometrics) mounted on a binocular glass (SZX12; Olympus). The sections presented are representative of the analysis of three brains samples.

Vacuolation profiles. The vacuolation procedure was previously described (34). Brains were fixed in neutral-buffered 10% formalin (4% formaldehyde) before paraffin embedding. After deparaffinization, 2-μm-thick sections were stained with hematoxylin-eosin. Vacuolation profiles were established according to the standard method described by Fraser and Dickinson (35), using three brains per experiment.

Generation and 127S infection of RK13 cells expressing N-terminally mutated ovine PrP^C. A sheep *PRNP* open reading frame (ORF) encoding ovine PrP^{VRQ} was cloned into a pTRE plasmid (Clontech) (36), and mutations were performed by site-directed mutagenesis (QuikChange II mutagenesis kit; Stratagene) using the following mutagenic primers: for the residues at positions 26 and 27, 5′-GCCAGG TTTTGGGTGCTGCTTGCAGAGGCC-3′ and 5′-GGCCTCTGCAAG CAGCACCCAAAACCTGGC-3′, respectively; for the residue at position 29, 5′-CTCCGCCAGGGTGTGGGTGCTGC-3′ and 5′-GCAG CACCCACACCCTGGCGGAG-3′. The sequences of all the mutant constructs were verified by sequencing. Each plasmid was introduced by transfection into RK13 cells as described previously (36), and puromycin-resistant cell clones were selected and tested for doxycycline (Dox)-inducible expression of PrP^C. The cultures were maintained at 37°C in 5% CO_2 in Opti-MEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 U of penicillin · ml^{-1}, and 10 μg of streptomycin · ml^{-1}. Cell lines were split at a 1:4 dilution each week. To induce PrP^C expression in the clones, 1 μg of Dox · ml^{-1} was added to the culture medium. Confluent cultures grown in 12-well plates were incubated in culture medium containing 2.5% infected brain homogenates from 127S terminally sick tg338 mice. After 2 days, the medium was removed, and the cells were rinsed with phosphate-buffered saline (PBS) and split into two 25-cm^2 flasks. Each week, one flask was used for subcultivation, while the other was used to prepare a cell lysate for PrP^{res} content, as previously described (36). Biotinylation of cell surface PrP was performed as described previously (37), by using 0.5 mg/ml biotin (EZ-Link Sulfo-NHS-LC-biotin [sulfosuccinimidyl-6-biotinamido-hexanoate]; Pierce).

RESULTS

Generation and expression analysis of transgenic mice with deletions or mutations in the N-terminal polybasic region of ovine PrP. The $tg1^D$ and the $tg1^M$ inserts (Fig. 1A) were microinjected into the pronuclei of FVB/N *Prnp* knockout mouse eggs. These transgenes are based on the murine phgPrP half-genomic construct and encode ovine PrP^{VRQ} lacking aa 23 to 26 (KKRP) or mutated within the N-terminal polybasic region (KQHPH instead of KKRPK for aa 23 to 27, similarly to a previously described mutant mouse PrP [13]). Following injection of the $tg1^D$ and $tg1^M$ inserts, four and three transgenic founders were obtained out of 17 and 16 live-born pups, respectively. Of the four $tg1^D$ founders, only three transmitted their transgenes to their offspring. The three $tg1^M$ founders transmitted their transgenes to the next generation.

Expression analyses performed on 6- to 8-week-old offspring revealed that only two out of the three $tg1^D$ ($tg1^{D5}$ and $tg1^{D16}$) and two out of the three $tg1^M$ lines expressed detectable levels of PrP^C in their brains ($tg1^{M10}$ and $tg1^{M18}$). The relative PrP expression levels in the brain from each line were quantitated by immunoblotting. PrP expression levels compared to those in sheep brain were 1-fold for $tg1^{D5}$, $tg1^{D16}$, and $tg1^{M18}$ and 1.5-fold for $tg1^{M10}$ (Fig. 1B and Table 1). Immunohistochemical analysis of mouse brain sections did not reveal obvious alteration in the pericellular localization of the mutated PrPs (Fig. 1C and data not shown), as already described for similarly mutated mouse PrP proteins (10, 22).

The tissue distribution of the expression of the transgenes was assessed by RT-PCR analysis and found to be similar between these lines and to that previously observed with other phgPrP-based transgenes (26). Of note, while expression was detected in various organs, such as brain, liver, kidney, heart, testis, lung, and surrenal, it was consistently not detectable in the spleen, a characteristic of the transcriptional regulation of this half-genomic vector (data not shown).

These mice lived (up to 2 years) and reproduced normally with no apparent abnormal behavior compared to that of their wild-type FVB/N counterparts.

Limited impact of a deletion of residues 23 to 26 on prion pathogenesis. To examine the impact of deleting the N-terminal polybasic region of ovine PrP on the susceptibility to prions, $tg1^D$ lines were inoculated intracerebrally with 127S, LA19K, and BSE prions. 127S is a prototypal fast scrapie prion strain which induces disease within 60 days in ovine PrP^{VRQ} tg338 line (32). Scrapie LA19K and BSE prions propagate at a slower pace in tg338 mice, inducing disease in 130 and 135 days, respectively (32). As con-

TABLE 1 Survival time of transgenic mice expressing wild-type or deleted or mutated forms of ovine PrP after inoculation with prions

Transgenic line	PrP form	Ovine PrP expression level (fold)[a]	Survival time (days ± SEM)[b]		
			127S	LA19K	BSE
tgPrP9.21	Wild type	1	140 ± 4 (5/5)	NT	NT
tg143	Wild type	1.5	111 ± 3 (7/7)	325, 429 (2/2)	453 ± 12 (11/11)
tg335	Wild type	1.2	148 ± 3 (5/5)	415 ± 13 (6/6)	
tg1^{D16}	Δ23–26	1	137 ± 2 (10/10)	469 ± 18 (5/5)[c]	703 ± 46 (5/5)[c]
tg1^{D5}	Δ23–26	1	158 ± 3 (10/10)[c]	NT	692 ± 15 (5/5)[c]
tg1^{M10}	^{23}KQHPH	1.5	415 ± 16 (5/5)[c]	500–750 (0/7)	NT
tg1^{M18}	^{23}KQHPH	1	343–611 (0/9)[d]	461–629 (0/8)	421–700 (0/9)[e]

[a] Brain expression level is relative to that of normal sheep brain (VRQ allele).
[b] For mice with negative results, the range of survival times is given. Values in parentheses represent the number of mice with neurological signs and positive for PrP^{res} in the brain by immunoblotting/the number of inoculated mice. NT, not tested.
[c] Statistically significant differences in mean survival times compared with those for all wild-type transgenic mice inoculated with the same prion strain ($P < 0.05$, by analysis of variance).
[d] Individual cull dates: 343, 453, 478, 499, 551, 551, 568, 598, and 611 days. Thus, only one mouse was culled before 415 days, i.e., the mean incubation time of 127S in tg1^{M10} mice.
[e] Individual cull dates: 421, 482, 504, 518, 545, 630, 658, 700, and 700 days. Thus, only one mouse was culled before 450 days, i.e., the mean incubation time of BSE in tg143 mice.

trols, transgenic mouse lines expressing ovine PrP^{VRQ} (tgPrP9.21, tg143, and tg335) with expression levels in the range of tg^{D} and tg^{M} were inoculated. PrP expression levels compared to those in sheep brain were 1-fold for tgPrP9.21, 1.5-fold for tg143, and 1.2-fold for tg335 (Fig. 1B and Table 1). tgPrP9.21 was obtained using the phgPrP-based transgene injected into an FVB/N *Prnp* knockout genetic background (27). tg143 was obtained using a phgPrP-based transgene injected into C56BL/6 × CBA *Prnp* knockout mouse eggs and back-passaged for five generations in an FVB/N *Prnp* knockout genetic background (26; also data not shown). tg335 was obtained by injection of a 125-kb bacterial artificial chromosome (BAC) insert on a C56BL/6 × CBA *Prnp* knockout genetic background (26).

All inoculated tg^{D} mice developed typical prion disease following injection with the three prion strains. The clinical signs were dominated by lethargy, irreversible dorsal decubitus hindlimb paresis, and priapism. The mean survival times of the tgPrP9.21, tg335, tg1^{D5}, and tg1^{D16} mice infected with 127S prions were within the same range, around 140 to 160 days postinoculation (Table 1). tg1^{D16} mice showed a slight increase in the mean survival time compared to that for tg335 controls after inoculation with LA19K prions (~470 versus 415 days) (Table 1). tg1^{D16} and tg1^{D5} mice inoculated with BSE prions became sick in ~700 days, compared to 450 days for tg143 mice, with a 1.5-fold excess of wild-type PrP (Table 1). This survival time extension is proportional to that observed when 127S-inoculated tg1^{D16}, tg1^{D5}, and tg143 mice are compared (Table 1), suggesting that overexpression mostly contributed to the observed delay.

The strain-specific electrophoretic patterns of proteinase K-resistant PrP^{Sc} (PrP^{res}) were strictly conserved among animals with deletions of prion residues 23 to 26 and wild-type animals of the different lines at the terminal stage of disease (Fig. 2A). However, PrP^{res} accumulation levels in tg^{D} transgenic mouse brains relative to those of their wild-type counterparts were globally decreased for all the strains inoculated, as summarized in Fig. 2B. After inoculation with 127S prions, levels were similar in tg^{D16} and tg^{D15} mice and decreased by approximately 3-fold compared to the level in tgPrP9.21 mice, which succumbed at the same time period. Levels were, however, increased compared to those in tg143 mice (Fig. 2A and B), which died earlier. After inoculation with LA19K and BSE prions, levels in tg^{D} brains were decreased more than 10-fold compared to those in wild-type tg335 or tg143 mice.

Histoblot analyses allowed study of PrP^{res} distribution in the brain, a well-established method to discriminate between prion strains (38). The PrP^{res} distribution patterns in wild-type, tg^{D16}, and tg^{D5} lines inoculated with 127S prions were comparable in the four standard anteroposterior regions analyzed (Fig. 3). The protein was mostly present in the septum, in several thalamic nuclei, notably the ventral posterolateral nucleus, in the oriens layer of the hippocampus, and in the pons. The substantia nigra appeared specifically decorated in the three genetic backgrounds.

Collectively, these observations suggest that deleting residues 23 to 26 in the N-terminal region of the ovine PrP protein, although affecting the overall level of PrP^{res} accumulation at the terminal-disease stage, had a limited impact on prion propagation.

Impaired or delayed prion pathogenesis in mice with the KQHPH mutation in the region of residues 23 to 31. The KQHPH mutation in the polybasic region of residues 23 to 31 had a more dramatic impact on prion replication than the deletion of residues 23 to 26. Only tg1^{M10} mice inoculated with 127S prions developed symptoms of disease, yet the time to development of disease was significantly increased by 3-fold compared to that in tg^{D} or wild-type mice (Table 1). All tg1^{M18} mice, which express lower PrP^{C} levels (1-fold versus 1.5-fold for tg1^{M10}), inoculated with 127S prions and all tg1^{M} mice inoculated with LA19K and BSE prions remained asymptomatic until the end of life. The brains of these mice were negative for the presence of PrP^{res} (Fig. 2), further suggesting that the disease pathogenesis was severely delayed.

At the terminal stage of the disease, 127S-inoculated tg1^{M10} mice accumulated 10-fold reduced PrP^{res} levels but exhibited similar PrP^{res} glycoform patterns (Fig. 2) and PrP^{res} distribution patterns (Fig. 3) in their brains compared to those in wild-type animals. To further confirm the absence of 127S strain evolution after passage in tg1^{M10} mice, brain homogenates from 127S-inoculated sick tg1^{M10} mice were inoculated to reporter tg338 transgenic mice, and the disease phenotype was analyzed. All inoculated tg338 mice developed the disease in approximately 60 days (Table 2), as for 127S prions (39). The original biochemical (Fig. 2C) and histopathological properties of 127S, including PrP^{res} deposition (Fig. 3) and vacuolation profile (Fig. 4), were conserved (39), indicating that 127S prion strain properties were unaltered despite intermediate passage in tg1^{M10} mice.

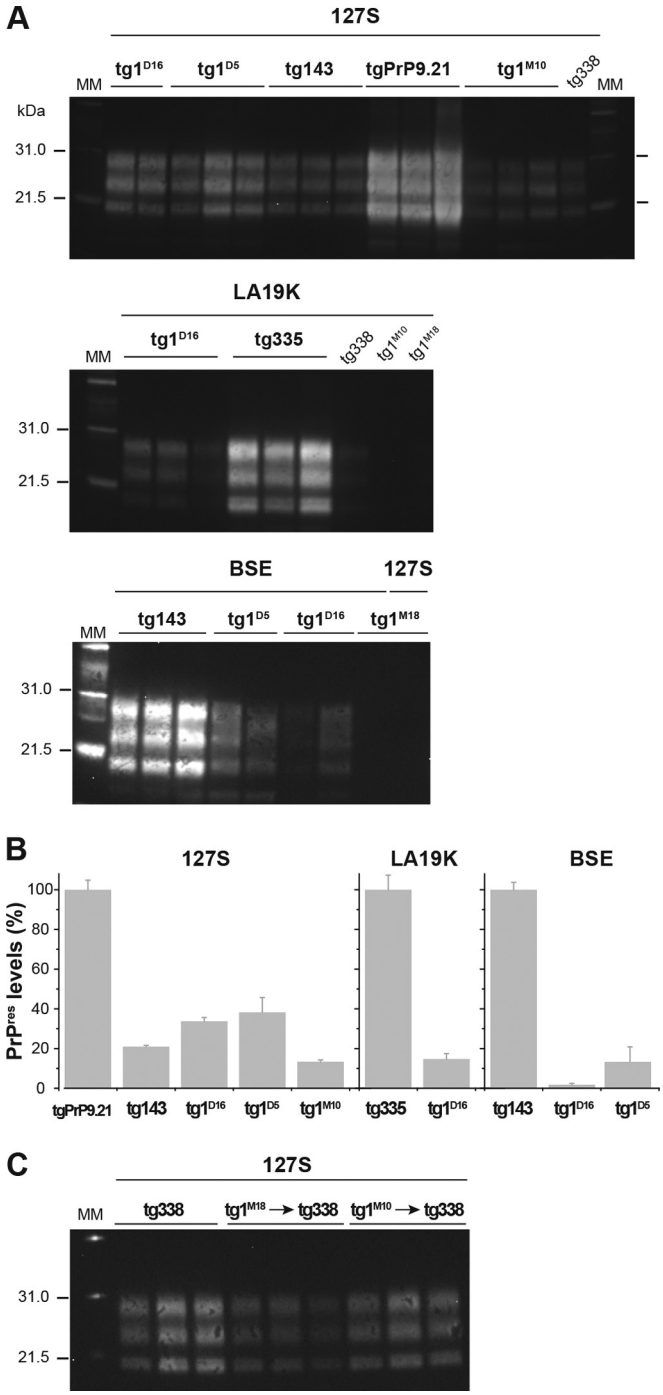

FIG 2 PrP^{res} accumulation in the brain of tg^D and tg^M lines inoculated with prions. (A) Immunoblots comparing PrP^{res} electrophoretic patterns in the brains of tg^D and tg^M mice with that of their wild-type counterparts upon inoculation with 127S, LA19K, or BSE prions. Except for $tg1^{D16}$ mice, where 6 to 7 mg was used, 2 mg of brain tissue equivalent was loaded in each lane. (B) Graph of PrP^{res} levels in the brains of tg^D and tg^M mice compared to those in their wild-type counterparts upon inoculation with 127S, LA19K, and BSE prions. (C) Immunoblot showing PrP^{res} electrophoretic pattern of 127S in tg338 mice with or without intermediate passage in $tg1^{M10}$ and $tg1^{M18}$ mice, as indicated. For the analysis, 0.5 mg of brain tissue equivalent was loaded. Lane MM, molecular mass marker.

To investigate further the deleterious effect of the KQHPH mutation on 127S propagation in $tg1^{M18}$ mice, we back-passaged a 127S-inoculated $tg1^{M18}$ brain extract to tg338 mice. Disease occurred in all tg338 mice in 95 days, indicating the presence of infectivity in asymptomatic $tg1^{M18}$ mice (Table 2). Reporting this value to a 127S infectious dose/survival time curve (32) indicates infectivity levels in the brains of tg^{M18} mice 10^5 lower than those in the brains of tg338 mice at the terminal stage of disease. Disease occurred within 60 days on the second passage to tg338 mice (Table 2). The electrophoretic pattern (Fig. 2C) and distribution patterns (Fig. 3) of PrP^{res} and vacuoles (Fig. 4) were similar in the diseased mouse brains and 127S-inoculated tg338 mice. Collectively, these data suggest that bona fide 127S prions have replicated or persisted at a low, subclinical rate in tg^{M18} mice.

Cells expressing PrP with mutation of KQHPH in the region of aa 23 to 31 are permissive to 127S prions. The N-terminal polybasic region has been involved in many functions that could be responsible for the delayed pathogenesis observed in mice; in particular, mutations (albeit with different amino acids) were associated with inefficient endocytosis (13). We thus expressed in RK13 cells (36) the same mutated ovine PrP and examined their susceptibility to 127S prions and trafficking. Among the clones analyzed, six were found to express detectable levels of mutated PrP^C (Fig. 5A). Further analysis of these independent clones revealed that the protein was properly addressed at the cell surface, as observed in the transgenic mice. Incubation of these cells in the presence of 127S prions resulted in the formation of PrP^{Sc} in an amount comparable to that observed for RK13 cells expressing the wild-type ovine PrP (Fig. 5B). These data highlighted the convertibility of the mutated PrP.

We next assessed whether internalization of the mutated protein was affected using reversible biotinylation, a technique that proved useful to detect endocytosis of cell surface PrP^C, even under conditions of aberrant PrP^C trafficking (37). No detectable difference was observed in the endocytic rates between the mutated and wild-type ovine PrPs (Fig. 5C). Similar results were obtained whether biotinylation was performed for 15 min or was copper-induced (data not shown). Collectively, these results indicate that PrP^C with the KQHPH mutation is not defective in endocytosis and is convertible into PrP^{Sc} in RK13 cells.

DISCUSSION

Several recent studies agree on the critical importance of the extreme N-terminal polybasic sequence of mouse PrP (aa 23 to 31) in modulating the toxicity of some PrP internal-deletion variants and on PrP^C-to-PrP^{Sc} conversion efficiency and the ability to sustain prion propagation in living animals. Here, we extended these observations to other species/prion strain combinations by transgenic modeling. We found that deletion of the N-proximal part of the polybasic region had limited impact on prion pathogenesis, whereas mutation of 3 aa, most likely at position 27 (see below), severely delayed the disease.

Deleting aa 23 to 26 on the ovine PrP^{VRQ} backbone had limited impact on the propagation of three distinct ovine prion strains. Only LA19K prions exhibited a slightly increased survival time compared to that of mice expressing similar levels of wild-type ovine PrP^C. This deletion reduced PrP^{res} accumulation in the brain of the diseased mice relative to that in their wild-type counterparts, as previously reported with mouse PrPΔ23–31 (10). However, overexpression of mouse PrPΔ23–31 or of longer seg-

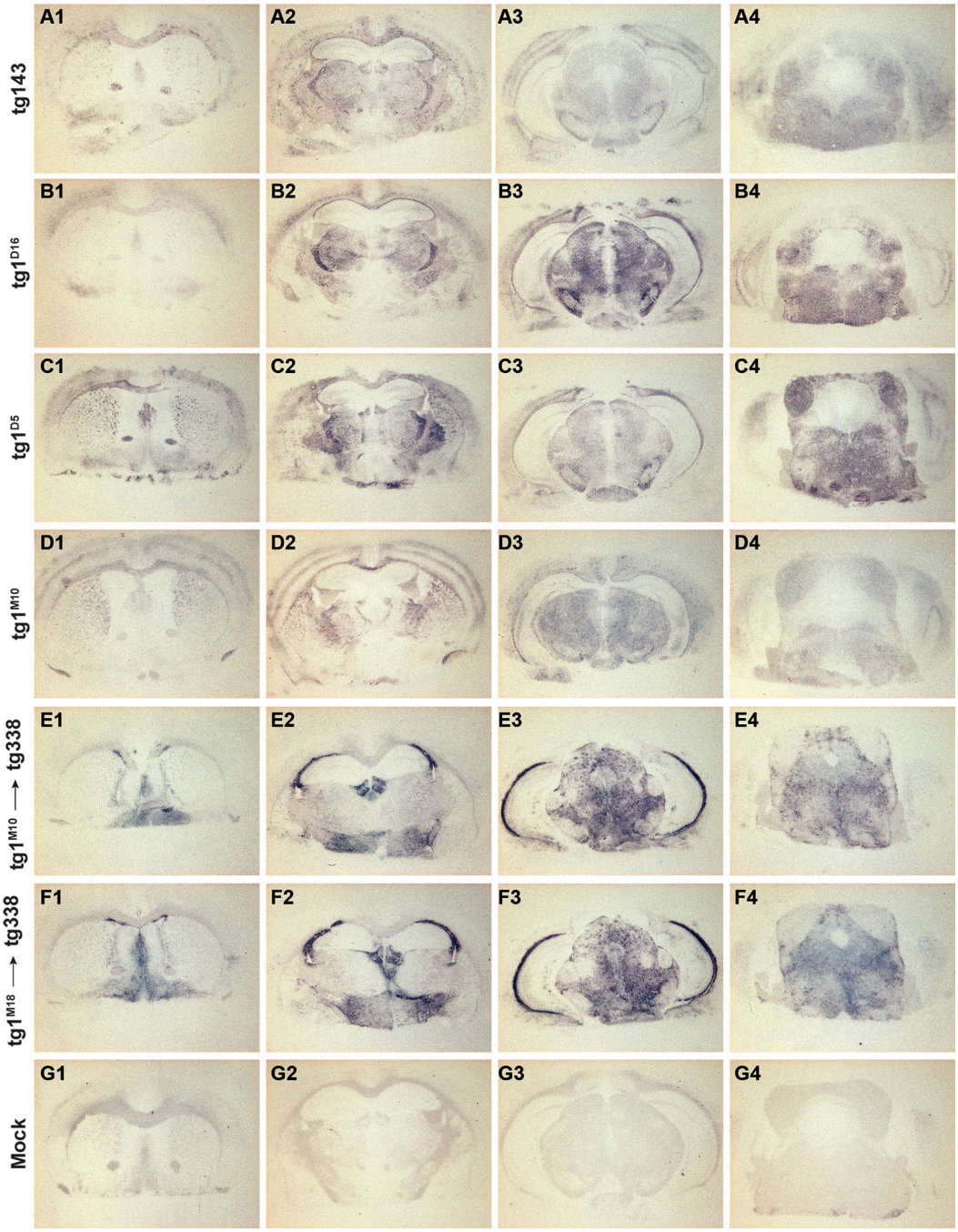

FIG 3 Regional distribution of PrP^{res} in the brains of transgenic mice expressing wild-type, mutated, or deleted forms of ovine PrP inoculated with 127S prions. Histoblot sections from 127S-infected mice of the indicated genotypes were stained for PrP^{res}. Representative staining is shown at the level of the septum, hippocampus, midbrain, and brainstem (columns 1 to 4, respectively). Images in row A are from tg143 mice, those in rows B and C are from tg1^{D16} and tg1^{D5} mice, respectively, those in row D are from tg1^{M10} mice, those in rows E and F are from tg338 mice after intermediate passage of 127S prions in tg1^{M10} and tg1^{M18} mice, respectively, and those in row G are from uninfected tg1^{M10} mice (mock).

ments extending to the C terminus (11) doubled mouse RML prion incubation time (10). Expression at physiological levels as in our mouse lines even prevented occurrence of disease. The PrP sequence at aa 23 to 31 is strictly conserved between mouse and sheep. Because our deletion is shorter, it may have allowed PrP^{Sc} to accumulate and the disease to develop, thus suggesting that the C-proximal part of the region of aa 23 to 31, aa 27 to 31, is rather key in controlling prion propagation in mice. Mutation of ovine aa 24, 25, and 27 at near physiological levels had a markedly stronger impact, delaying significantly 127S pathogenesis and preventing transmission of LA19K and BSE prions. While transmission of 127S was possible in one of the generated transgenic lines, it operated subclinically in another one expressing lower levels of PrP^{C}, as shown by retrotransmission to tg338 indicator mice. Alto-

TABLE 2 Survival time of ovine PrP transgenic mice (tg338) inoculated with 127S prions after an intermediate passage in mouse lines mutated in the N terminus of ovine PrP

Inoculum	Mean survival time ± SEM (days)[a]	
	Passage 1	Passage 2
127S → $tg1^{M10}$	60 ± 1 (5/5)	NT
127S → $tg1^{M18}$	95 ± 2 (5/5)	57 ± 1 (5/5)

[a] Values in parentheses represent the number of mice with neurological signs and positive for PrP^{res} in the brain by immunoblotting/the number of inoculated mice. NT, not tested.

gether, these observations suggest that mutation of aa 27 (from K to H) might be responsible for the resulting outcome. The dramatic impact of this single mutation resembled that observed after deletion of the whole sequence of aa 23 to 31 (10).

Expression of mutated mouse PrP at the same residues (albeit with A) in N2a cells resulted in impaired Cu^{2+}-dependent PrP endocytosis (13). Here, mutated ovine PrP appears to be correctly endocytosed in RK13 cells, thus excluding such type of impairment in our transgenic lines that would explain the disease delay. The K residue at position 27 is located between two prolines. Such motifs are known to impose, due to steric constraints, a certain degree of structure (40) to peptidic sequence. Replacing the amino acid or removing one of the proline residues may conceivably affect the whole segment of aa 23 to 31 (resulting in a loss of rigidity) and alter its interactions with other molecules or with incoming PrP^{Sc}. Because conversion of $_{23}$KKQHPH mutated ovine PrP^{C} appeared possible in RK13 cells, it is possible that mutation in mice has impaired or allowed interaction with a brain-specific cofactor. The impact of the mutation in RK13 cells may also have been minimized by PrP overexpression, which is greater than that in the tg^{M} mouse lines.

Previous studies and models suggest the involvement of a second, putatively less favorable, domain during PrP^{C}/PrP^{Sc} interactions, either the central polybasic domain located at aa 98 to 110 (8, 9, 12) or a domain within the amyloid core of the protein (9,

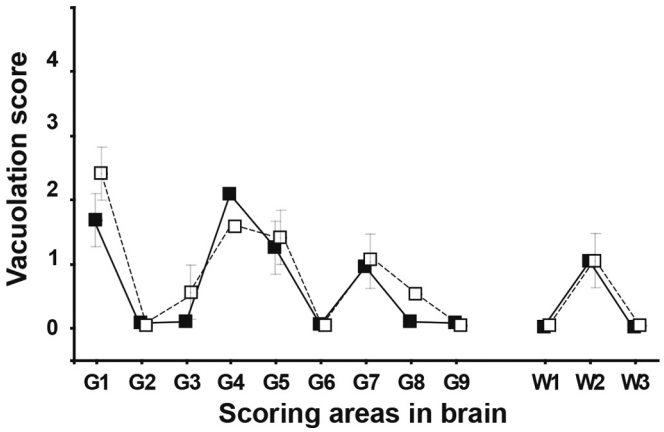

FIG 4 Distribution of vacuolation in tg338 mouse brains upon inoculation of 127S prions passaged in transgenic mice with a deletion of or mutation in ovine PrP. A lesion profile in the brain of tg338 mice infected with 127S prions passaged in $tg1^{M10}$ (dotted line) or $tg1^{M18}$ (black line) mice is shown. The intensity of vacuolation was scored as means ± standard errors of the means (error bars) in standard gray (G1 to G9) and white (W1 to W3) matter areas. These areas are as follows: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, medial thalamus; G6, hippocampus; G7, septum; G8, medial cerebral cortex at the level of the thalamus; G9, medial cerebral cortex at the level of the septum; W1, cerebellar white matter; W2, white matter of the mesencephalic tegmentum; and W3, pyramidal tract. The profiles obtained were comparable to those previously reported for back-passage of 127S prions in tg338 mice (see, e.g., references 41 and 42).

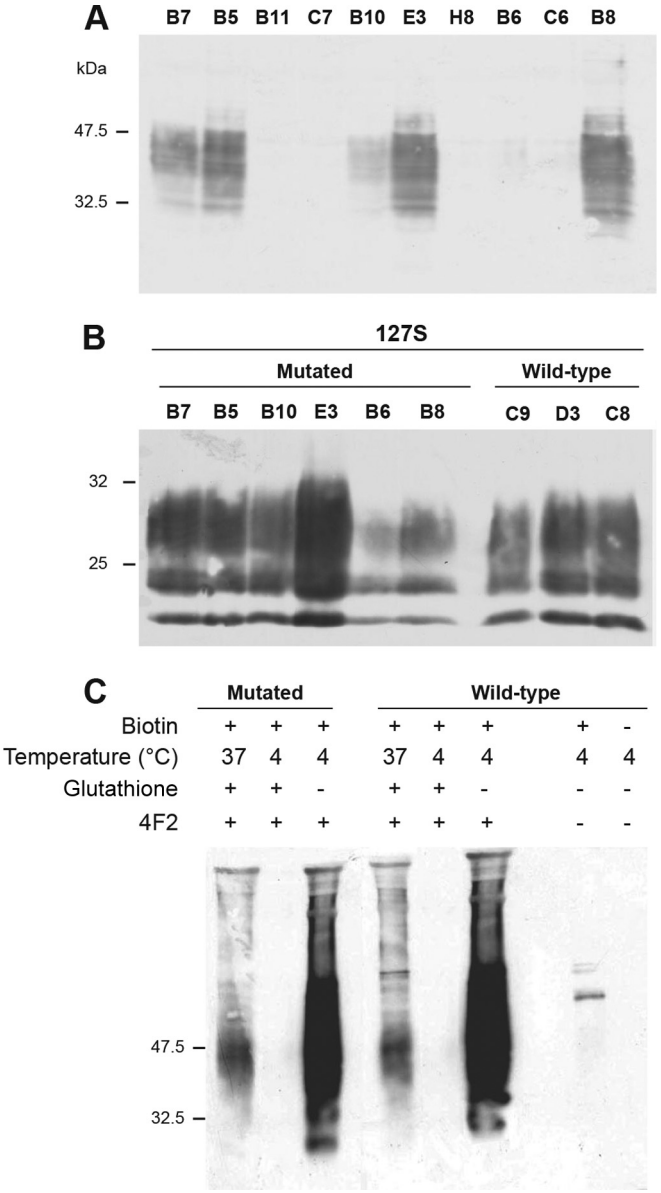

FIG 5 Endocytosis and conversion of mutated PrP in RK13 cells. (A) Immunoblot (4F2 antibody) showing detectable levels of PrP^{C} mutated within the N-terminal polybasic region in different RK13 clones (the clone designations are indicated on the top of the gel). (B) Clones expressing mutated or wild-type forms of PrP^{C} were challenged with 127S prions. Cells were analyzed for the presence of PrP^{res} after proteinase K treatment using Sha31 antibody. (The clone designations are indicated on the top of the gel). (C) Cells expressing mutated (E3 clone) or wild-type (C9 clone) forms of PrP^{C} were treated with 0.5 mg/ml of NHS-LC-biotin to label plasma membrane proteins. After incubation at 4°C or 37°C for 30 min, cells were then treated with glutathione to eliminate biotin attached to proteins still at the cell surface. PrP^{C} was specifically immunoprecipitated with the 4F2 antibody. The biotinylated fraction was revealed by immunoblotting using streptavidin peroxidase.

10). Alternatively but not exclusively to the aforementioned hypothesis suggesting that the amino acids deleted or mutated are key to the effect observed, the ovine PrP^{VRQ} allele may have a second domain with an increased affinity toward PrP^{Sc} compared to that of the mouse PrP^{a} allele (the PrP allotype present in most inbred mouse lines) that reduces the impact of the deletion/mutation. The VRQ allele is known to confer high susceptibility toward scrapie in naturally infected species (6) and in transgenic mouse models (26, 33, 39). The location of the polymorphic amino acids (aa 136, 154, and 171) makes it compatible to be within the secondary putative domain of interaction between PrP^{C} and PrP^{Sc} (9).

In conclusion, the N-terminal polybasic region may universally modulate prion pathogenesis with regard to strain and species combination, and we may narrow the most important amino acids in this region to the 27th residue onwards (KPGGW). This reinforces the view that this region might be a therapeutic target to interfere with prion propagation and, in a broader view, with other misfolded proteins (23).

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