Glycan-Controlled Epitopes of Prion Protein Include a Major Determinant of Susceptibility to Sheep Scrapie†

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Received 17 March 2004/Accepted 19 April 2004

A key feature of prion encephalopathies is the accumulation of a misfolded form of the host glycoprotein PrP. Cell-free and cell culture studies have shown that the efficiency of conversion of PrP into the disease-associated form is influenced by its amino acid sequence and also by its carbohydrate moiety. Here, we characterize four novel glycoform-dependent monoclonal antibodies raised against prion recombinant sheep PrP. We demonstrate that these antibodies discriminate the PrP monoglycosylated species, since two of them recognize molecules that have the first Asn glycosylation site occupied (mono1) while the other two recognize molecules glycosylated at the second site (mono2). Remarkably, the recognition of PrP by the anti-mono2 antibodies was strongly influenced by the amino acid present at position 171, i.e., either Gln or Arg. This polymorphism is known to be the main determinant of susceptibility and resistance to scrapie in sheep. Altogether, our findings lead us to propose that each glycan chain controls the accessibility of PrP determinants located close upstream from their attachment site. The monoglycoform-assigned and the allotype-restricted antibodies described here, the first to date, should provide further opportunities to investigate the involvement of each glycan chain in PrP conversion in relation to prion strain diversity and the basis of the resistance conferred by the Arg-171 amino acid.

Transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative diseases that include Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle (8). They are caused by infectious agents thought to be of proteinaceous nature, or prions (30). A hallmark of TSE is the accumulation of a misfolded form of the host glycoprotein PrP. PrPsc is essential for the infectivity to propagate and the neurological disorders to occur (5). The conversion of PrPc into PrPsc appears to involve a physical interaction between the two forms (6, 30). PrPsc, as yet the only identified component of prions, exhibits elevated β-sheet content and increased resistance to proteolytic digestion, leading to PrPres.

PrPc is a cell surface glycosylphosphatidylinositol-anchored glycoprotein with a still-elusive function (14). Owing to the potential implications of the sugar moiety in protein biogenesis and function, PrP glycosylation, whether in a physiological or a pathological context, is being actively investigated (35). PrP contains two consensus sites for Asn-linked glycosylation, at residue positions 180 and 196 in the mouse sequence. Both sites are located in a highly structured region of the protein, within the disulfide-bridged helix 2-loop-helix 3 domain (33, 34). Since they are variably occupied (12), mature PrP appears as three major bands on denaturing polyacrylamide gels, corresponding to molecules with zero, one, or two oligosaccharide chains (7, 28, 34). The two monoglycosylated species are usually not distinguished because their electrophoretic mobilities differ only slightly (38). Mass spectrometry analysis of the N-glycans in mouse brain PrP has revealed some site-specific processing, with N196 containing tri- and tetra-antennary glycans in higher proportions than N180 (39). Tissue-specific processing, resulting in a variation in the proportion of each glycoform and the sizes of the attached carbohydrates, has also been shown in various brain regions (2, 10, 23, 38) and non-neural tissues (24).

Major questions regarding the implications of the PrP N-glycan chains in prion propagation and strain phenotype variation remain largely unanswered. Multiple prion strains, distinguishable by the disease incubation time and neuropathology, can be propagated in the same host (4). PrPres molecules associated with such strains may differ in their molecular sizes and also in their relative amounts of glycoforms (9, 17, 26). The molecular basis of glycoform variation and whether this heterogeneity plays a role in the differential propagation of prions remain controversial issues (9, 38). There is in vivo evidence that the final glycosylation pattern of PrPres is controlled by host-, tissue-, and strain-dependent factors (38). Recent cell-free conversion data (43) have shown that the strain-specific glycoform variation cannot be fully explained by the targeting of distinct nerve cell types (2, 9, 10) or by the induction of different alterations of the cellular glycosylation process (36, 38) by the agent. Finally, whether unglycosylated PrPc molecules are associated with prion infectivity is unknown.

Susceptibility to sheep scrapie, the most widely spread TSE, is tightly controlled by polymorphism of the ovine Prnp gene. Amino acids at positions 136, 154, and 171 have been found to be major determinants of susceptibility to scrapie (11, 13). Strikingly, the presence of an arginine at position 171 has been

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† This article is dedicated to the memory of E. Treguer, who died in August 2003.
associated with complete resistance to scrapie in homozygous animals (13, 45). Increasing the frequency of the corresponding allele (named ARR) in sheep flocks through selective breeding forms the basis of scrapie eradication plans that have been launched in several European countries (1). However, it has recently been learned that the ARR-conferred resistance can be overcome, at least following intracerebral inoculation of the bovine spongiform encephalopathy agent (15), thus calling for new approaches to clarify the mechanisms underlying such resistance.

Here, we report the isolation and characterization of four monoclonal antibodies (MAbs) raised against recombinant sheep PrP which all preferentially recognize underglycosylated forms of the protein in different species. We show that they actually fall into two categories, each being specific for one of the two monoglycosylated species. These antibodies potentially delineate two distinct domains of the PrP protein, both masked by a glycan chain, with one of them encompassing a primary determinant of susceptibility to sheep scrapie.

(Par of these results was presented at the International Conference on Transmissible Spongiform Encephalopathies [Edinburgh, Scotland, September 2002].)

MATERIALS AND METHODS

Production and characterization of anti-PrP MAbs. PrP0/0 mice (5) were immunized with recombinant ovine VRO-PrP (recPrP) purified from Escherichia coli (32). One intraperitoneal injection of 25 μg of protein in complete Freund’s adjuvant was followed 4 months later by a boost of 25 μg in phosphate-buffered saline buffer and complete Freund’s adjuvant. Spleen cell fusion with Sp2-O-Ag14 myeloma and selection and growing of clones were performed as usual. Hybridoma supernatants were screened by Western blotting using both recPrP and enriched sheep brain PrPc. All of the clones studied were derived from the same animal. V5, V31, and V61 are immunoglobulin G1 (IgG1) isotypes, and V14 is an IgG2a isotype (determined with Isostat from Dynex).

Epitope scanning was performed on a series of synthetic nonapeptides derived from the ovine sequence with a shift of two residues from the N-terminal to the C-terminal end, customized by Chiron. During synthesis, each peptide was grafted through its N-terminal end on a flexible Biotin-GSGS amino acid sequence allowing fixation to streptavidin-coated 96-well plates for enzyme-linked immunosorbent assay. Binding of MAbs to the peptides was performed in the usual enzyme-linked immunosorbent assay buffer and detected by anti-mouse antibody rabbit immunoglobulins conjugated to alkaline phosphatase (Bioxy, Compiègne, France).

Other anti-PrP antibodies used in this study. The anti-octarepeat MAb P248 has been described (24). The MAb 2D6 and the polyclonal antibody MH44 were obtained by immunization of mice with a synthetic peptide (146 to 182 in the ovine sequence) (32) and of rabbits with recPrP (24).

Brain homogenates. Brains from 2-year-old, presumed healthy ewes were collected at the INRA-Jouy slaughterhouse and frozen at −80°C until they were used. The Prnp genotype at codons 136, 154, and 171 was determined by Labogena (Jouy, France) as described previously (11), using DNA prepared from Prnp used. The availability of transgenic mice expressing mouse PrP muc was VRQ-PrP) to brain PrPc from four mammalian species. Unlike most anti-PrP MAbs, the V5 and V61 MAbs specifically detected bands migrating at the level of the monoglycosylated forms of PrP. Strikingly, V5 failed to recognize PrP from species other than sheep. MAbs V14 and V31 also displayed preferential reactivity toward both monoglycosylated species but still detected biglycosylated species, except for human PrP. Since these two MAbs reproducibly produced identical patterns in various assays, only the data obtained with one of them (V14) will be presented. PNGase-treated glycosylated sheep PrPc was shown to migrate as a single species, with the same electrophoretic mobility as unglycosylated PrPc, whatever antibody was used (Fig. 1B).

RESULTS

New MAbs detect brain PrPc in a glycosylation-dependent manner. Four antibodies that recognized PrP, depending on its glycosylation state, were identified by Western blot analysis of hybridoma clones raised against ovine PrP. Figure 1A shows the reactivities of these MAbs named V-Mabs, as the immunogen was VRQ-PrP) to brain PrPc from four mammalian species. Unlike most anti-PrP MAbs, the V5 and V61 MAbs produced identical patterns in various assays, only the data obtained with one of them (V14) will be presented. PNGase-treated glycosylated sheep PrPc was shown to migrate as a single species, with the same electrophoretic mobility as unglycosylated PrPc, whatever antibody was used (Fig. 1B).

V61, V5, and V14, V31 MAbs discriminate monoglycosylated PrP species. Closer inspection of the observed PrPc molecular patterns revealed that the distances between the mono- and unglycosylated species differed among these antibodies. To test whether this could be due to preferential recognition of one of the two monoglycosylated forms of PrP, i.e., with either the N-terminal site (site 1; N184 in the sheep sequence) or the C-terminal site (site 2; N200) occupied, we took advantage of the availability of transgenic mice expressing mouse PrP mutated at each site on a PrP0/0 background (E. Neuendorf, A. Weber, A. Saalmüller, H. Schatzl, and M. H. Groschup, unpublished data). Figure 2 shows a Western blot analysis performed on brain material derived from such mice. As visualized with a control MAb (Pc248) (Fig. 2A), the monoglycosylated species, called monol and monol2, produced in mouse T198A and T182N, respectively, had distinct mobilities, with a faster-migrating band associated with monol. Thus, clear evidence was obtained for a dual reactivity pattern among the four MAbs (Fig. 2B and C and data not shown): the V5 (Fig. 3) and V61 MAbs reacted with monol2 but failed to detect the monol species; in contrast, the V14 and V31 MAbs
reacted with mono1 and failed to detect the mono2 species. The latter finding was unexpected, since as noted above and as shown here for mouse PrP (Fig. 2C, left), these antibodies exhibit some reactivity for PrP species glycosylated on both sites (see Discussion).

We next sought to determine to what extent the glycoform specificities of these MAbs were dependent upon the conformational state of PrP. First, soluble PrPc was immunoprecipitated from sheep brain extract (171-Q/R genotype) using the relevant antibodies attached to protein G-Sepharose beads and then subjected to Western blot analysis using a polyclonal anti-PrP antibody (24). As shown in Fig. 3A, MAb V14 captured the smaller monoglycosylated species (mono1), while MAbs V5 and V61 specifically captured the mono2 species and not the biglycosylated species, consistent with our Western blotting data. Second, we compared the reactivities of MAbs V61 and V14 to PK-treated PrP prepared from the brain of a scrapie-diseased transgenic mouse expressing the VRQ allele of sheep PrP (42). Both antibodies were found to maintain their specificities for either the mono1 or mono2 species in Western blotting (Fig. 3B). Intriguingly, the reactivity of V14 for the biglycosylated species decreased when assayed against PrPres. Altogether, these data led us to conclude that the MAbs under study preferentially recognized either the mono1 or the mono2 species of PrP. In addition, they established that in the mouse, but also in other species, including sheep, the glycan chain attached to the PrPc amino-proximal site is smaller than the amino-distal glycan chain.

FIG. 1. Anti-PrP MAbs recognizing glycosylation-dependent epitopes. (A) V5, V61, V14, and V31 antibodies were tested by Western blotting on brain extracts (25 μg of protein per lane) from four species (Sh, sheep heterozygous Q/R at position 171; Hu, human; Ha, hamster; Mo, mouse [PrP0/0; PrP nullizygous mouse]). The anti-PrP octarepeat MAb Pc248 was used as a reference antibody. The positions of the bigglycosylated (B), monoglycosylated (M), and nonglycosylated (N) forms of full-length PrPc are indicated. V5 and V61 detected only mono- and nonglycosylated species. Note the specificity of V5 for sheep PrP. The molecular masses of the protein standards (43 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, soybean trypsin inhibitor) used throughout the study are indicated. (B) Immunoblots of sheep PrPc (171-Q/R genotype) untreated (−) or treated (+) with PNGase F and revealed with the indicated MAbs.

FIG. 2. MAbs V61 and V14 each recognize a different monoglycoform of PrPc. Brain homogenates (25 μg of protein) from transgenic mice expressing mutated PrP lacking a glycan chain at either site 1 (mutant T182N) or site 2 (mutant T198A) were analyzed by Western blotting using the indicated V-MAbs and Pc248 anti-octarepeat MAb (two animals for each transgenic line). Brain homogenates from mouse (tg20 line) (left) and sheep (171-Q/Q) (right) were included for comparison (2.5 and 25 μg of protein, respectively). Note the different mobilities of the mono1 (site 2 mutated) and mono2 (site 1 mutated) glycoforms. In both mouse and sheep species, V61 antibody detects the mono2 but not the mono1 species, while V14 detects the mono1 and to a lesser extent bigglycosylated species of wild-type PrP. Nonreduced samples were electrophoresed using the Invitrogen 12% acrylamide MES-NuPAGE system.
MAbs V5 and V61 discriminate PrPc according to substitution at position 171. One possible explanation for the observed discrepant behavior of the V5 and V61 antibodies toward heterologous PrPs (Fig. 1) was that each recognized a distinct sheep PrP allotype. To assess this point, 16 sheep brains were randomly taken and tested blindly with respect to their Prnp genotypes. As illustrated in Fig. 4, both antibodies yielded positive signals with the brain homogenates derived from animals heterozygous at position 171 (Q/R; 10 sheep). In contrast, the brains from homozygous animals revealed a distinct symmetrical behavior of the two MAbs: PrP from the two R/R individuals (sheep 5 and 6) was recognized by V5 only, whereas PrP from the four Q/Q individuals (only two are shown, sheep 3 and 4) was recognized by V61 only, and this was irrespective of the amino acid present at position 136 (i.e., A or V). All of the brains showed comparable PrP levels when tested with V14 (not shown) or a control MAb (Pc248) (Fig. 4). This result strongly indicated that V5 and V61 discriminate sheep brain PrPc according to the amino acid present at position 171, thus explaining the lack of cross-species reactivity of V5, since R171 is unique to sheep so far. Similar results were obtained when PrP prepared from buffy coat blood cells was assayed (not shown), which might be of interest for fast genotyping.

To confirm this finding, we examined the reactivity of this MAb pair toward PrP produced in cells genetically engineered for various sheep alleles (Fig. 5). When VRQ- or ARR-PrP protein expressed in the mammalian Rov cell system (37) was used, MAbs V5 and V61 were found to maintain their allotype specificities, together with their specificities for the mono- and unglycosylated forms. The use of PrP from E. coli cells expressing four different allotypes further confirmed the specificities of V61 and V5 for 171Q and 171R, respectively (Fig. 5A). We next determined the relative reactivity of each MAb for VRQ- and ARR-PrP using a range of purified recPrP. From data such as those shown in Fig. 5B, a ratio of differential reactivity of ~50-fold, similar for each MAb, was estimated by comparing the amount of recPrP resulting in the lowest signal for each allotype. Thus, neither V61 nor V5 had absolute allotype specificity. This explains why the V5 antibody-producing hybridoma screened positive against VRQ-PrP antigen in a Western blot assay where 200 to 300 ng of recPrP was loaded per well (i.e., ~100 times the amount of PrP loaded when, for instance, brain homogenate was analyzed).

FIG. 4. MAbs V5 and V61 recognize ovine brain PrPc in an allele-dependent manner. Western blot analysis of brain material from sheep of the indicated Prnp genotypes is shown. Samples (25 μg of brain protein/lane) were allowed to migrate on 12% acrylamide gels in triplicate and then processed in parallel using MAb V5 or V61 or MAb Pc248 as a control. V5 gives a strong signal with homozygous 171-R/R individuals (sheep 5 and 6) and no signal on Q/Q individuals (sheep 3 and 4). V61 yields a reverse picture with the same animals. The positions of the biglycosylated (B), monoglycosylated (M), and nonglycosylated (N) forms of full-length PrPc are indicated.

FIG. 3. Monoglycoform-specific recognition of native PrPc and of PrPres by V-MAbs. (A) PrPc was immunoprecipitated from sheep brain extract (171-Q/R genotype) using the indicated V-MAbs and Pc248 as a reference antibody. The immunoprecipitated PrP material was revealed by Western blotting using MH44 polyclonal antibody. A double-band material binds nonspecifically to protein G-Sepharose beads in the absence of antibodies (lane control). V5 and V61 selectively recognize the mono1 glycoform, which has a lower mobility than the mono2 glycoform recognized by V14. The positions of the biglycosylated (B), monoglycosylated (M), and nonglycosylated (N) forms of full-length PrPc are indicated. (B) PrPres-enriched material (2.5-mg protein/lane) were allowed to migrate on 12% acrylamide gels in the absence of antibodies (lane control). V5 and V61 selectively recognize the mono1 glycoform, which has a lower mobility than the mono2 glycoform recognized by V14. The positions of the biglycosylated (B), monoglycosylated (M), and nonglycosylated (N) forms of full-length PrPc are indicated. (B) PrPres-enriched material (2.5-mg protein/lane) were allowed to migrate on 12% acrylamide gels in the absence of antibodies (lane control). V5 and V61 selectively recognize the mono1 glycoform, which has a lower mobility than the mono2 glycoform recognized by V14. The positions of the biglycosylated (B), monoglycosylated (M), and nonglycosylated (N) forms of full-length PrPc are indicated.
inhibited two sheep PrP allotypes conferring fairly different susceptibilities to scrapie.

Although antibodies with preferential recognition of specific mouse PrP glycoforms have been reported, their specificities for either mono1 or mono2 glycoforms have either not been recognized (41, 47) or not been formally assessed (2). Incidentally, bacterially expressed protein was used as an immunogen in these and in our studies, implying that PrP lacking the carbohydrate moiety actually increases the probability of raising antibodies against underglycosylated forms. By using brain PrPc from transgenic mice individually expressing each monoglycoform, we provided direct evidence that the MAb pair V5 and V61 recognizes a determinant(s) expressed by the mono2 species. Conversely, the MAb pair V14 and V31 recognizes a determinant(s) expressed by the mono1 species.

What information do we have about the locations of the V-MAb cognate epitopes? Peptide scanning failed to localize them on the PrP sequence, indicating that they may be conformation dependent (see Materials and Methods) (data not shown). Comparatively large and intrinsically dynamic, the glycan chains can shield two orthogonal faces of the PrP structured core almost completely, but they are unlikely to be involved in long-range interactions with the negatively charged surface of the protein (33, 35). Our findings indeed support the view that these epitopes involve amino acid residues located in close proximity to the glycan attachment site. First, the binding efficiency of the V5 and V61 antibodies to PrP molecules lacking the site 1 (N184) chain is markedly affected by the amino acid residue present at position 171, which is polymorphic in the sheep species. Indeed, V5 and V61 selectively bind PrP with arginine (R171) or glutamine (Q171), respectively. Screening of a few additional MAbs that preferentially detected mono2 PrP in our assay led to the identification of two more antibodies with allotype preferences (MAbs supplied by J. Grassi, CEA-Saclay, Grif sur Yvette, France) (data not shown). These results indicate that the presence of glycan chain 1 affects residues around position 171. However, residue 171 is unlikely to be a contact point for V5 (anti-R) antibody, since the latter was generated using 171Q molecules as the immunogen. The allotype specificities of these MAbs would thus merely reflect local conformational changes associated with the Q-to-R substitution at this position (31). Second, a striking feature of the anti-mono1 V14 and V31 antibodies is their ability to bind fully glycosylated species while they fail to detect the mono2 species. We thus postulate the existence of a repulsive interaction between the two flexible glycan chains, leading to a partial unmasking of the region located between their respective attachment sites. Hence, the epitopes recognized by the anti-mono1 antibodies are likely to involve residues belonging to the 184- to 200-amino-acid threonine-rich stretch.

Altogether, the above observations argue that the V-MAbs all target structural determinants situated on the PrP polypeptide ribbon close upstream from the attachment site of one or the other glycan chain, which influences the immunoreactivity of these determinants through steric hindrance or local conformational change (Fig. 6). Importantly, we found that the V-MAb epitopes are conserved on nondenatured PrPc, as well as on the pathological PrPres form. From a general viewpoint, we suggest that the screening of MAbs specific to monoglycosylated isoforms could facilitate the identification of antibodies discriminating between PrP allotypes (e.g., a and b in mouse, which involves the T189V substitution) or between wild-type and mutated PrP associated with familial TSE in humans (e.g., D178N).

A number of studies indicate that the conversion process of PrPc into PrPres in infected neuroblastoma cells is influenced by its carbohydrate moiety. The inhibition of glycosylation promotes the formation of PK-resistant PrP (40, 46). Unglycosylated PrP with mutations allowing trafficking to the cell surface is converted with a higher efficiency than wild-type PrP upon exposure to multiple prion strains (19). Cell-free conversion
studies also support the notion that unglycosylated PrP molecules may be a preferred substrate for PrPsc (3, 18). It is noteworthy that unglycosylated ARR ovine PrP is converted to some extent, unlike its glycosylated counterpart (3). N-terminal truncation of PrP affects the conversion of glycosylated molecules, but not that of unglycosylated molecules (21), and unglycosylated PrPc improves cross-species conversion (29). However, the reasons why underglycosylated PrP would be more prone to conversion remain obscure and may imply lower stability, a favored interaction with either PrPsc or cellular factors, and a different subcellular distribution of these PrPc glycoforms (22, 35, 40, 43, 48).

The issue of whether the monoglycoforms are equal substrates for conversion to PrPsc has never been addressed. Our glycoforms are equal substrates for conversion to PrPsc has not been addressed. Our glycoforms are equal substrates for conversion to PrPsc may be a preferred substrate for PrPsc (3, 18). It is noteworthy that unglycosylated ARR ovine PrP is converted to some extent, unlike its glycosylated counterpart (3). N-terminal truncation of PrP affects the conversion of glycosylated molecules, but not that of unglycosylated molecules (21), and unglycosylated PrPc improves cross-species conversion (29). However, the reasons why underglycosylated PrP would be more prone to conversion remain obscure and may imply lower stability, a favored interaction with either PrPsc or cellular factors, and a different subcellular distribution of these PrPc glycoforms (22, 35, 40, 43, 48). The issue of whether the monoglycoforms are equal substrates for conversion to PrPsc has never been addressed. Our finding that the first glycan chain controls access to residue 171 is of potential relevance in this regard, given that this amino acid is a prime determinant of the conversion efficiency of PrP and susceptibility to scrapie (13, 27, 45) and is also part of an epitope proposed to interact with a cell factor that is putatively rate limiting for conversion (16).

The antibodies described here provide an opportunity to learn about differential conversion efficiencies of the monoglycoforms in scrapie-infected tissues or cultures. Discrete PrPres glycopatterns are associated with prion strains having distinct biological phenotypes (20, 38), and such patterns are presently used as markers to document TSE strain variation in naturally affected species (25, 25a, 44). Therefore, looking for any strain-dependent variation in the PrP monoglycoform ratio might be a way to improve our present knowledge of prion strain diversity.

In conclusion, we introduced a set of anti-PrP antibodies whose specificities are of potential interest to further investigate the role of glycan chains in PrP conversion and prion strain variation. One of these antibodies that discriminates ovine PrP-ARR from other allotypes may also facilitate studies aimed at understanding the mechanisms underlying the resistance conferred by this allele on sheep at the cell level.

ACKNOWLEDGMENTS

We thank Didier Vilette and Vincent Beringue for stimulating discussions and Anne-Lise Haenni for carefully revising the manuscript. We acknowledge Jacques Grassi and Jean-Jacques Hauw for providing us with anti-PrP antibodies and human brain tissue, respectively.

ADDENDUM IN PROOF

The location of the V14 epitope proposed in this study is fully confirmed by the tridimensional structure of sheep PrP cocrystallized with V14 MAb, as established by Eghiaian et al. (F. Eghiaian, J. Grosclaude, S. Lesceu, P. Debey, B. Doublet, E. Treguer, H. Rezaei, and M. Knossow, Proc. Natl. Acad. Sci. USA 101:10254–10259, 2004).

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12668: 12668–12668.

13363: 13363–13363.

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22866: 22866–22866.

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ERRATUM

Glycan-Controlled Epitopes of Prion Protein Include a Major Determinant of Susceptibility to Sheep Scrapie

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