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Scrapie susceptibility-linked polymorphisms modulate the *in vitro* conversion of sheep prion protein to protease-resistant forms

(allelic variants/protein conformation/spongiform encephalopathy/proteinase K resistant)

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ABSTRACT **Prion diseases are natural transmissible neurodegenerative disorders in humans and animals. They are characterized by the accumulation of a protease-resistant scrapie-associated prion protein (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^{C}) mainly in the central nervous system. Polymorphisms in the PrP gene are linked to differences in susceptibility for prion diseases. The mechanisms underlying these effects are still unknown. Here we describe studies of the influence of sheep PrP polymorphisms on the conversion of PrP^{C} into protease-resistant forms. In a cell-free system, sheep PrP^{Sc} induced the conversion of sheep PrP^{C} into protease-resistant PrP (PrP-res) similar or identical to PrP^{Sc}. Polymorphisms present in either PrP^{C} or PrP^{Sc} had dramatic effects on the cell-free conversion efficiencies. The PrP variant associated with a high susceptibility to scrapie and short survival times of scrapie-affected sheep was efficiently converted into PrP-res. The wild-type PrP variant associated with a neutral effect on susceptibility and intermediate survival times was converted with intermediate efficiency. The PrP variant associated with scrapie resistance and long survival times was poorly converted. Thus the *in vitro* conversion characteristics of the sheep PrP variants reflect their linkage with scrapie susceptibility and survival times of scrapie-affected sheep. The modulating effect of the polymorphisms in PrP^{C} and PrP^{Sc} on the cell-free conversion characteristics suggests that, besides the species barrier, polymorphism barriers play a significant role in the transmissibility of prion diseases.**

Prion diseases such as Creutzfeldt–Jakob disease, Gerstmann–Sträussler–Scheinker syndrome (GSS), bovine spongiform encephalopathy, and scrapie manifest as infectious, sporadic, and/or inherited disorders (1). They are characterized by the accumulation of an abnormal isoform (PrP^{Sc}) of the host-encoded cellular PrP(PrP^{c}) mainly in the central nervous system of mammals. This protease-resistant PrP^{Sc} arises from protease-sensitive PrP^{C} by a posttranslational process (2, 3) involving profound conformational changes of mainly α-helical (PrP^{C}) into β-sheeted (PrP^{Sc}) structure (4, 5). The prion agent has been proposed to be composed largely, if not entirely, of these PrP^{Sc} molecules (6, 7).

Several PrP polymorphisms of humans have been associated with incidence, susceptibility, and pathology of the disease (1, 8). For sheep, eight mutually exclusive PrP polymorphisms have been described (9–15), resulting in nine different allelic variants. The allelic variants with polymorphisms at codons 112, 137, 141, 154, or 211 are rare and have not been significantly associated with any disease phenotype yet. In contrast, the PrP^{VQ} allele (polymorphic amino acids at positions 136 and 171 are indicated by superscript single-letter code) is associated with high susceptibility to scrapie and short survival times of scrapie-affected sheep (9–12, 15–18), whereas the PrP^{AR} allele is associated with resistance or incubation times that span beyond the lifetime of sheep (9–12, 16, 17). In breeds where PrP^{VQ} is rare, e.g. the Suffolk breed, the wild-type PrP^{AQ} allele is associated with susceptibility to scrapie, although with a low or incomplete penetrance (18, 19). The mechanisms by which the different PrP allelic variants contribute to differences in scrapie susceptibility and survival time are not yet understood. However, it is possible that the various PrP^{C} variants differ in their conversion kinetics into PrP^{Sc}. Such differences may be due to differences in expression levels, in cotranslational or posttranslational modifications, and/or differences in conformational structures of the various PrP variants.

Recent *in vitro* studies have demonstrated that in a cell-free system hamster PrP^{C} can be converted into protease-resistant forms that are at least similar, if not identical, to PrP^{Sc} without the synthesis of new macromolecules (20). Further biochemical studies with this cell-free system have shown that there is strain and species specificity in the PrP^{C}–PrP^{Sc} interaction that could account for the observed differences between prion strains and the barriers to interspecies transmission of prion agents, respectively (21, 22). Species specificity *in vitro* was determined by specific amino acids between positions 113 and 188 of the hamster/mouse PrP sequence (22). Species specificity between human and mouse, as determined *in vivo* using transgenic mice carrying chimeric human/mouse PrP genes, seems to be dependent on amino acid substitutions between positions 97 and 167 (23). In a reciprocal manner using murine scrapie-infected neuroblastoma cells, the conversion of mouse PrP^{C} into PrP^{Sc} could be blocked by a single hamster-specific amino acid at position 138 of the murine PrP sequence (24). *In vivo* studies with transgenic mice carrying chimeric human/mouse PrP genes with single amino acid mismatches at position 109, 129, or 200 demonstrated that single amino acid substitutions in PrP can lead to an altered susceptibility to prions (25). In addition, transmission of human Creutzfeldt–Jakob disease and fatal familial insomnia to human transgenic mice also indicated that polymorphisms in the PrP gene may lead to distinct PrP properties (26). All these findings indicate that polymorphisms in the PrP gene might lead to differences in the PrP^{C}–PrP^{Sc} interaction and/or conversion of PrP^{C} into PrP^{Sc}.

In the present study, we explore whether sheep PrP^{C} can be converted *in vitro* to protease-resistant forms using a cell-free

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Abbreviations: PrP, prion protein; PrP^{C}, cellular PrP; PrP^{Sc}, scrapie-associated PrP; ShPrP, sheep PrP; PrP-res, protease-resistant PrP; PK, proteinase K; Gdn·HCl, guanidine·hydrochloride; GSS, Gerstmann–Sträussler–Scheinker syndrome; N2a cells, neuroblastoma cells.

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system. In addition we investigated whether the various sheep PrP (ShPrP) allelic variants have different cell-free conversion characteristics and whether these characteristics reflect the observed differences in sheep scrapie susceptibility and the observed differences in survival times of scrapie-affected sheep *in vivo*.

MATERIALS AND METHODS

Cell Lines and PrP Constructs. The ShPrP allelic variants PrP^{VQ}, PrP^{AQ}, and PrP^{AR} were cloned and analyzed as described previously (10). PrP ORFs were subcloned between the β-globin intron and β-globin polyadenylylation sequences downstream the human cytomegalovirus (hCMV) promoter of expression vector pECV7, a derivative of expression vector pECV6 (27) in which the Rous sarcoma virus promoter has been substituted for the hCMV promoter. Mouse neuroblastoma cells (N2a cells; Hubrechts Laboratory, Utrecht, The Netherlands) were stably transfected with these constructs by electroporation (28), hygromycine B (500 μg/ml)-resistant single-cell clones were isolated, and high PrP-expressing clones were selected by immunoperoxidase monolayer assay using the antipeptide antibody R521–7 (peptide corresponds to amino acids 94–105 of the ShPrP sequence) (29). These cell lines were used for the isolation of the various $ShPrP^{C}$ variants.

Isolation of ^{35}S-PrP^{C}. Cells expressing the different PrP variants were radiolabeled as initially described (30) using 1 mCi of [^{35}S]methionine/[^{35}S]cysteine Tran^{35}S-label (ICN) per 70–80% confluent 25-cm^2 flask and ^{35}S-labeled proteins were methanol-precipitated from detergent cell lysates and subsequently sonicated in 0.7 ml DLPC buffer [0.05 M Tris·HCl, pH 8.2/0.15 M NaCl/2% (wt/vol) *N*-lauryl sarcosine/0.4% (wt/vol) lecithin (from soybean)] containing protease inhibitors (25 μg/ml Pefabloc SC, 0.7 μg/ml pepstatin, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM EDTA). ^{35}S-PrP^{C} was immunoprecipitated using the R521–7 antibody (1:100) and 10 μl of 50% (vol/vol) protein A-Sepharose beads per μl of antibody. Nonglycosylated ^{35}S-PrP^{C} was obtained by radiolabeling in the presence of 15 μg/ml tunicamycin D. PrP^{C} was finally eluted 20 min at room temperature from complexes of antibody and protein A beads in 0.1 M acetic acid, pH 2.8, containing protease inhibitors (G.J.R. and B.C., unpublished work). Eluates were stored on ice until further use.

Isolation of PrP^{Sc}. Proteinase K (PK)-treated PrP^{Sc} was isolated from brains of genotyped sheep (30, 31) using sarkosyl homogenization, ultracentrifugation, and PK digestion. After pelleting through a 20% sucrose cushion, the pellet was sonicated in 400 μl of 0.1% sulfobetaine (SB 3–14) in Tris-buffered saline and stored in small portions at 4°C. Quantification (silver staining and Western blotting) of the PrP^{Sc} revealed that the $PrP^{Sc(VQ/VQ)}$ and the $PrP^{Sc(AQ/AQ)}$ isolates contained about 135 μg of PrP^{Sc} per 24 g equivalent of brain. Both isolates were further diluted to a final concentration of 0.325 μg/ml and were briefly sonicated using a cuphorn sonicator before use.

Cell-Free Conversion Reaction. PrP^{Sc} isolates in siliconized tubes were partially or more completely denatured for 2.5 h at 37°C in 2.5 M or 6 M guanidine·hydrochloride (Gdn·HCl), respectively. Aliquots of denatured PrP^{Sc} (3.3 μg) and 25 kcpm of purified ^{35}S-PrP^{C} (≈5–10 ng) were mixed and further diluted to a volume of 35 μl at 1 M Gdn·HCl in conversion buffer (50 mM sodium citrate, pH 6.0/5 mM cetylpyridinium chloride/1% *N*-lauryl sarcosine/protease inhibitors). Conversion reactions were performed for 2 or 5 days at 37°C, and the reaction mixtures were subsequently diluted to 100 μl in 50 mM sodium citrate, pH 6.0, and digested with 35 μg/ml PK at 37°C for 1 h. Thereafter PK inhibitor (Pefabloc SC; Boehringer Mannheim) was added, and all proteins were precipitated with 4 vol of methanol at −20°C using 20 μg of thyroglobulin as a carrier protein. Precipitated proteins were boiled and briefly sonicated in Laemmli sample buffer with 4 M urea, and 1/10 vol was stored separately to be analyzed by Western blotting. All samples were separated by 15% SDS/PAGE, the gels were fixed and subsequently enhanced using Amplify (Amersham), ^{35}S-labeled proteins were visualized on x-ray film, and integrated intensities of bands were measured using the Intelligent Quantifier (Bio-Image, Ann Arbor, MI). Comparable results were obtained between different sets of conversions, and only data of representative experiments are shown.

Western Blotting. Western blotting was performed by standard methods on nitrocellulose membranes, and protease-resistant PrP was visualized using (1:1,000) R521–7 antibody and (1:1,000) alkaline phosphatase-goat anti-rabbit IgG (Zymed).

RESULTS

Expression of $ShPrP^{C}$ in Cell Lines. Plasmid constructs encoding the wild-type $ShPrP^{AQ}$, the $ShPrP^{VQ}$, and the $ShPrP^{AR}$ allelic variants were used to generate stably transfected N2a cells. Single-cell clones that showed intensive and equal staining with the R521–7 antibody in an immunoperoxidase monolayer assay were selected for further use. These PrP^{C}-expressing clones contained about 4–6 random integrated copies of the expression vector (data not shown), and almost equal amounts of the various radiolabeled PrP^{C} variants could be isolated. The various PrP^{C} variants were labeled with [^{35}S]methionine/[^{35}S]cysteine in the presence or absence of tunicamycin D and analyzed by radioimmunoprecipitation, using the R521–7 antipeptide antibody. In the absence of tunicamycin, the PrP proteins were glycosylated and showed predominant bands with molecular masses of 38–39 kDa and 32–33 kDa in contrast to the 26–27-kDa unglycosylated form of PrP as shown in the tunicamycin D-treated sample (compare Fig. 1, lanes 1 and 2). PrP^{Sc} and PrP^{C} isolated from sheep brain normally have molecular masses of 35 kDa, 31 kDa, and 27 kDa (Fig. 1, lane 4). Because the molecular mass of the unglycosylated PrP produced by the N2a cell line is similar to the molecular mass of the unglycosylated PrP from sheep brain (compare Fig. 1, lanes 1 and 4), we concluded that the N2a cell lines produced overglycosylated or less sialylated PrP^{C}. The R521 antibody used to isolate the PrP^{C} variants is specific for sheep PrP and did not precipitate the endogenous mouse PrP (Fig. 1, lane 3). This eliminated the possibility of interference by mouse PrP^{C} in the sheep conversion reactions. The three different variants of PrP^{C}: PrP^{CAQ}, PrP^{CVQ}, and PrP^{CAR} ex-

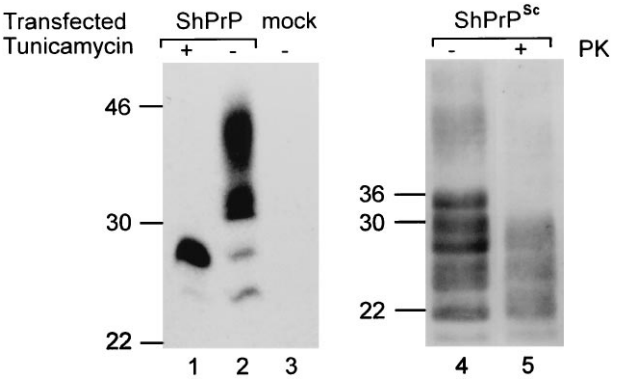

FIG. 1. Migration of PrP^{C} and PrP^{Sc}. Radioimmunoprecipitation of PrP^{C} from stably transfected N2a cells with pECV7 (mock, lane 3) or with pECV7-PrP (lanes 1 and 2). Proteins were radiolabeled in the presence (lane 1) or absence (lanes 2 and 3) of tunicamycin D. A Western blot of sheep brain PrP^{Sc} before and after PK treatment is shown in lanes 4 and 5. Lane 4 shows the migration of PrP^{Sc+C} (27–35 kDa) including the naturally N-terminally truncated forms of PrP^{Sc} (21–29 kDa). Molecular mass markers (kDa) are indicated at the left.

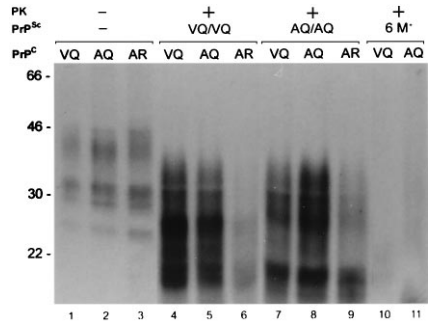

FIG. 2. Cell-free conversion of ^{35}S-PrP^{C} into PK-resistant forms. PrP^{Sc} from sheep with different PrP genotypes were pretreated in 2.5 M Gdn·HCl, except for reactions in lanes 10 and 11 in which PrP^{Sc} was denatured in 6 M Gdn·HCl. After incubation for 5 days at 37°C (1 M Gdn·HCl), the samples were PK-digested and analyzed by 15% SDS/PAGE. Molecular mass markers (kDa) are indicated at the left.

pressed in N2a cells did not show notable differences in posttranslational modifications, and all three PrP^{C} variants could be reduced to a single band of 27 kDa by inhibiting glycosylation with tunicamycin D (compare Fig. 2, lanes 1–3 with Fig. 3*a*, lanes 1–3).

Conversion of Sheep PrP^{C} to Protease-Resistant Forms. To define the most optimal partial renaturation conditions of the ShPrP^{Sc} for successful conversions, we first pretreated ShPrP^{Sc} under various Gdn·HCl conditions. By measuring PK-resistant PrP^{Sc} on Western blots we found >95% denaturation and >95% renaturation of at least the R521 epitope (amino acid residues 94–105) if denatured in 2.5 M Gdn·HCl for 2–24 hr at 37°C and subsequently renatured for 5 days in 0.75–1.0 M Gdn·HCl at 37°C.

To explore whether ShPrP^{C} could be converted to protease-resistant forms in a cell-free system, as shown for hamster and mouse PrP^{C} by Kocisko *et al.* (20, 22), ^{35}S-ShPrP^{CVQ} was incubated at 37°C for 5 days (1 M Gdn·HCl) with partially denatured (>2.5 h in 2.5 M Gdn·HCl at 37°C) ShPrP$^{Sc(VQ/VQ)}$ and ^{35}S-ShPrP^{CAQ} was incubated under the same conditions with partially denatured ShPrP$^{Sc(AQ/AQ)}$. After PK digestion, PK-resistant ^{35}S-ShPrP bands were detectable in both conversion reactions (Fig. 2, lanes 4 and 8). ShPrP^{Sc} more completely denatured with 6 M Gdn·HCl induced very little conversion to PK-resistant forms in similar reactions (Fig. 2, lanes 10 and 11).

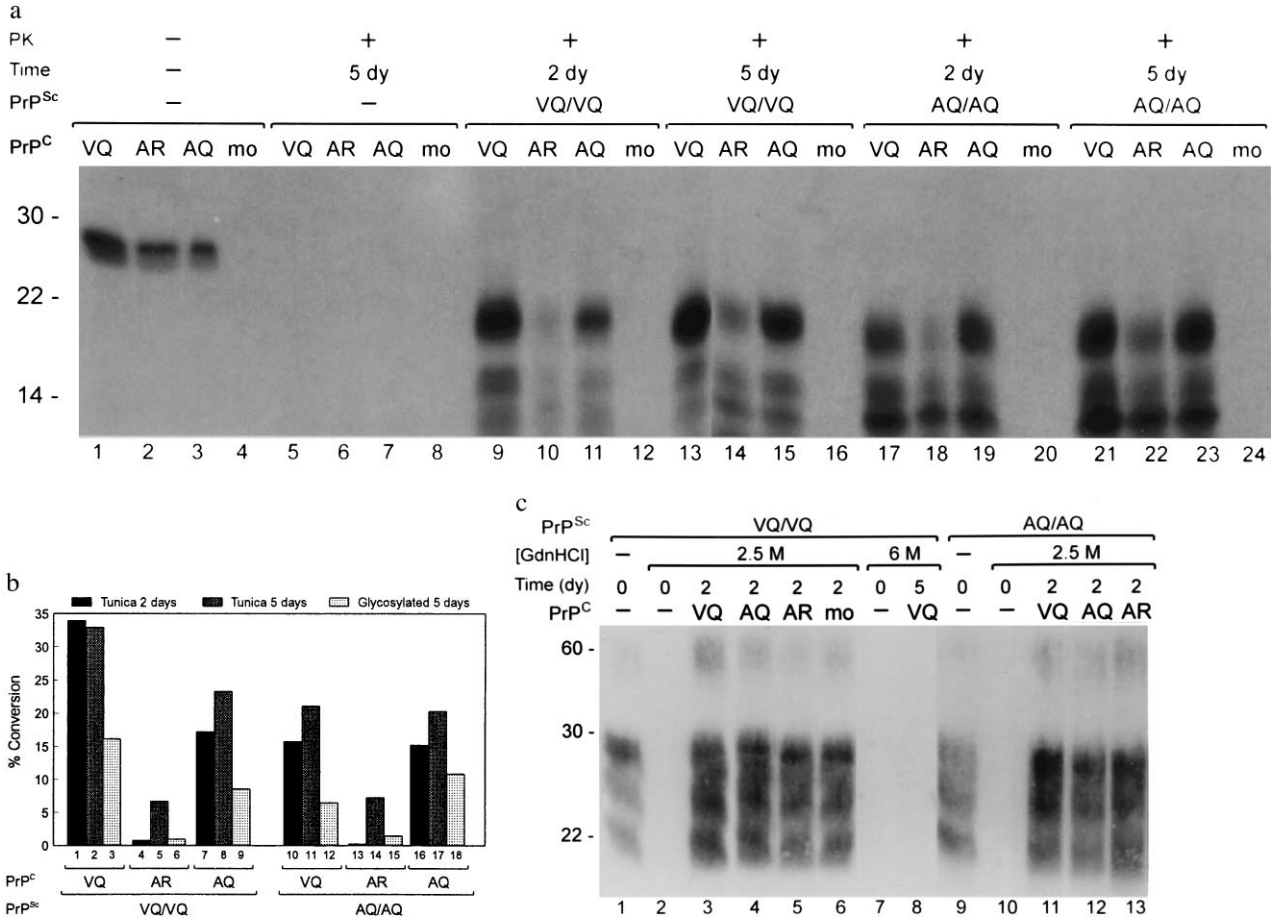

FIG. 3 (*a*) Cell-free conversion of nonglycosylated ^{35}S-PrP^{C} into protease-resistant forms. PrP^{Sc} from sheep with different PrP genotypes were pretreated in 2.5 M Gdn·HCl. The conversions were started by adding the different allelic forms of ^{35}S-PrP^{C} or adding controls (mo) and dilution to 1 M Gdn·HCl. Reactions were incubated for 2 or 5 days at 37°C and subsequently PK-digested (except lanes 1–4). Lanes 1–4 (starting material) contain approximately 10% of the input material for conversions shown in lanes 5–24. Samples were analyzed by 15% SDS/PAGE. Molecular mass markers (kDa) are indicated at the left. (*b*) Percent conversion of the different allelic forms of PrP^{C} into PrP-res using different ShPrP^{Sc} isolates. Conversion percentages from *a* were determined by dividing the integrated intensities of bands at 21 kDa (lanes 5–24) by the integrated intensities of bands at 27 kDa (lanes 1–4) and multiplication by 10 (starting material = 10%). To give an indication of conversion efficiencies in conversions with glycosylated PrP^{C}, the bands between 27–39 kDa (Fig. 2, lanes 1–3) and between 21–33 kDa (Fig. 2, lanes 10–15) were also quantified. The different types of conversions using either nonglycosylated PrP^{C} or glycosylated PrP^{C}, as well as the length of the incubation time in days (dy) and the allelic forms of PrP^{Sc} and PrP^{C}, are indicated. (*c*) Immunoblot analysis using the R521–7 antibody after PK digestion of 1/10 vol of the conversion reactions shown in *a*. Indicated are the different allelic forms of PrP^{Sc} and PrP^{C}, the renaturation time, and the concentration (M) of Gdn·HCl used to pretreat the PrP^{Sc}. Untreated PrP^{Sc} (starting material) is shown in lanes 1 and 9. Molecular mass markers (kDa) are indicated at the left.

Although the gels revealed a smear rather than discrete bands, predominant ^{35}S-labeled PK-resistant bands with molecular masses of 32–33 kDa, 26–27 kDa, and 20–21 kDa were detectable, indicating a downward shift in molecular mass by PK digestion of about 6 kDa as expected for bonafide PrP-res products (compare Fig. 1, lanes 4 and 5).

Polymorphisms Modulate the Cell-Free Conversion of PrP^C to Protease-Resistant Forms. Although the amounts of conversion products are not easy to quantify from a smear of ^{35}S-labeled PK-resistant PrP, it is obvious from Fig. 2 that if using different allelic forms of PrP^C (PrP^{CVQ}, PrP^{CAQ}, or PrP^{CAR}) or different PrP^{Sc} isolates (PrP$^{Sc(VQ/VQ)}$ or PrP$^{Sc(AQ/AQ)}$), different amounts of PrP-res are formed. For example in the ShPrP$^{Sc(VQ/VQ)}$-induced reactions the PrP^{CVQ} to PrP-res conversion was the most efficient one, the PrP^{CAQ} to PrP-res conversion was intermediate, and the PrP^{CAR} to PrP-res conversion was poor (Fig. 2, lanes 4–6). In the PrP$^{Sc(AQ/AQ)}$-induced reactions, the three PrP^C allelic variants also converted with different efficiencies into PrP-res (Fig. 2, lanes 7–9).

To be able to quantify the conversion products more accurately and to address if N-linked glycosylation plays a role in determining the differences in conversion efficiency between the three PrP^C variants, we repeated the above experiments with unglycosylated PrP^C variants that were radiolabeled in the presence of tunicamycin D to obtain more discrete and quantifiable PrP bands (Fig. 3*a*, lanes 1–3). From the hamster cell-free conversions it was already known that the unglycosylated form of hamster PrP^C converted more efficiently into protease resistant forms than the glycosylated form of hamster PrP^C (20, 22). Mock transfected cell lines did not show discrete labeled material indicating the absence of endogenous mouse PrP^C in the preparations (Fig. 3*a*, lane 4). The radiolabeled PrP^C products did not convert into protease-resistant products when incubated for 5 days under conversion conditions without PrP^{Sc} (Fig. 3*a*, lanes 5–8). However, incubation of nonglycosylated PrP^{CVQ}, PrP^{CAQ}, or PrP^{CAR} under conversion conditions for 2 or 5 days with either PrP$^{Sc(VQ/VQ)}$ or PrP$^{SC(AQ/AQ)}$ resulted in discrete and readily quantifiable protease-resistant bands of predominantly 20–21 kDa (Fig. 3*a*, lanes 9–11, 13–15, 17–19, and 21–23). As expected, the material from the mock transfected cells did not produce such PK-resistant bands (Fig. 3*a*, lanes 12, 16, 20, and 24). The downward shift in molecular mass by PK digestion was about 6 kDa, which is equal to the downward shift found for the converted glycosylated PrP^C variants (Fig. 2) and PrP^{Sc} isolated from sheep brain (Fig. 1, lanes 4 and 5). The bar diagram (Fig. 3*b*) shows the percentages of PrP^C that are converted into PrP-res by quantification of the 20–21-kDa PK-resistant conversion products and comparison with the 27-kDa input PrP^C. For quantification of the conversions with glycosylated PrP^C the region between 21 and 33 kDa of the conversion products (Fig. 2) was used (only to give a relative indication of these conversion efficiencies). PrP^{VQ}, which is associated with high susceptibility to scrapie and short survival times in scrapie-affected sheep, is overall the allelic form of PrP^C that is most efficiently converted to PrP-res (Fig. 3*b*, bars 1, 2, 10, and 11). The homologous conversions with this allelic form (Fig. 3*b*, bars 1 and 2) seem more efficient than the heterologous conversions (Fig. 3*b*, bars 10 and 11). PrP^{AQ}, which is associated with intermediate susceptibility to scrapie (with an incomplete penetrance) and with intermediate survival times in scrapie-affected sheep, is less efficiently converted to PrP-res (Fig. 3*b*, bars 7, 8, 16, and 17). PrP^{AR}, which is associated with resistance to scrapie and with incubation times that span beyond the lifetime of sheep, is poorly converted to PrP-res (Fig. 3*b*, bars 4, 5, 13, and 14).

The conversion data obtained with the different allelic forms of PrP^{Sc} revealed that not only the polymorphisms in PrP^C determine the conversion efficiencies. Differences in conversion efficiencies were also obtained using PrP^{Sc} isolates from sheep with different PrP genotypes (Fig. 3*b*, bars 1–9 and 10–18). The PrP$^{Sc(VQ/VQ)}$ induced conversion with homologous PrP^{CVQ} was the most efficient reaction in which about 35% of the initial PrP^C was converted into 20–21 kDa PrP-res (Fig. 3*b*, bars 1 and 2). The PrP$^{Sc(VQ/VQ)}$-induced conversion with heterologous PrP^{CAQ} resulted in an intermediate (17–24%) conversion into PrP-res (Fig. 3*b*, bars 7 and 8). In contrast, the PrP$^{Sc(AQ/AQ)}$-induced conversions with either heterologous PrP^{CVQ} or homologous PrP^{CAQ} resulted in almost equal intermediate conversion efficiencies (Fig. 3*b*, bars 10, 11, 16, and 17). PrP^{CAR} was poorly converted into PrP-res by both PrP^{Sc} isolates (Fig. 3*b*, bars 4, 5, 11, and 14).

The intriguing efficiency differences between the conversions of nonglycosylated PrP^{CVQ}, PrP^{CAQ}, and PrP^{CAR} were consistent with the relative efficiency differences observed with (the inaccurate quantifiable) glycosylated forms of PrP^C (compare Fig. 3*b*, bars 2, 5, 8, 11, 14, and 17 with bars 3, 6, 9, 12, 15, and 18). Therefore glycosylation of PrP^C seems to be of minor or no importance in determining the differences in conversion efficiencies between the various PrP^C variants.

To conclude that the differences in amounts of conversion products are only the result of the polymorphisms present in PrP, it is important to have as close to identical concentrations of PrP^{Sc} and PrP^C in each reaction. All conversions with the same PrP^C variant have identical PrP^C concentrations on the basis of protein content, because we aliquoted equal volumes from one batch into the different conversion reactions. All conversions with different PrP^C variants have equal amounts of PrP^C on the basis of radiolabel and equal immunoperoxidase monolayer staining of the PrP^C-expressing cells. The content of PrP^{Sc} in each conversion reaction and the rate of unfolding/refolding was compared by Western blotting 1/10 vol of each conversion reaction (Fig. 3*c*). This blot shows that the PrP^{Sc} isolates contained about the same quantity of protease-resistant PrP (Fig. 3*c*, lanes 1 and 9). At least the R521 epitope of PrP^{Sc} became PK-sensitive after pretreatment in 2.5 M Gdn·HCl (Fig. 3*c*, lanes 2 and 10) and recovered PK resistance after 2 days of renaturation (Fig. 3*c*, lanes 3–6 and 11–13). Renaturation in the presence of different allelic forms of PrP^C did not detectably inhibit the refolding of PrP-res (Fig. 3*c*, lanes 3–5 and 11–13). The denaturation of PrP^{Sc} with 6 M Gdn·HCl was not reversible (Fig. 3*c*, lane 7 and 8). We concluded that, because the amounts of PrP^{Sc} and PrP^C of the different allelic forms were similar in each conversion, differences in quantity of conversion products were probably solely an effect of the primary ShPrP amino acid sequence.

DISCUSSION

In this paper we report, for the first time to our knowledge, the cell-free conversion of sheep PrP^C into protease-resistant forms similar or identical to ShPrP^{Sc}. In addition we report that polymorphisms that are associated with differences in scrapie susceptibility and differences in survival times of scrapie affected sheep also account for comparable differences in cell-free conversion efficiencies. This suggests that the PrP conversion kinetics are directly related to scrapie susceptibility and the length of survival times of sheep affected by natural scrapie. Because there is a good correlation between *in vitro* cell-free conversion data and *in vivo* scrapie susceptibility data thus far (9–12, 16, 17), this assay may be useful for determining the relative susceptibility of individual allelic forms of PrP to different prion sources and/or the relative transmissibility of these prion sources.

The efficiency of the cell-free conversion reaction was strongly dependent on both the type of PrP^C variant and on the source of PrP^{Sc} used to induce the conversion. The PrP^{CVQ} variant, which is associated with high susceptibility and short survival times of scrapie-affected sheep, was very efficiently converted into protease-resistant forms. The wild-type PrP^{CAQ} variant, which is associated with a neutral effect on suscepti-

bility and intermediate survival times, was converted into protease-resistant forms with intermediate efficiency. The PrP^{CAR} variant, which is associated with resistance and long survival times, was poorly converted into protease-resistant forms. Although in some breeds, i.e. Suffolk and Romanov, PrP^{AQ} is associated with an incomplete penetrance to scrapie susceptibility, probably due to the low incidence of PrP^{VQ} (16, 19, 32), PrP^{VQ} carriers of these breeds still have the shortest scrapie survival time (16, 32). Another point of interest is the finding that PrP^{CAR} can be converted, although with a very low efficiency, into protease-resistant forms suggesting the possibility of scrapie agent replication in PrP^{AR}-carrying sheep as has been described by Ikeda *et al.* (32).

Not only the primary PrP^{C} sequence was found to determine the conversion characteristics but also the primary amino acid sequence of PrP^{Sc}. $PrP^{C(VQ/VQ)}$ converted PrP^{CVQ}, PrP^{CAQ}, and PrP^{CAR} with decreasing efficiencies. In contrast, $PrP^{Sc(AQ/AQ)}$ converted PrP^{CVQ} almost as efficiently as the PrP^{CAQ} variant. The PrP^{CAR} variant was poorly converted by both PrP^{Sc} isolates. This suggests that scrapie susceptibility is not only determined by the PrP genotype of the acceptor animal but also by the PrP genotype of the animal that produced the infectious PrP^{Sc}. This is consistent with the finding that the SSBP/1 scrapie isolate obtained from PrP^{VQ} NPU-Cheviot sheep is best transmitted to PrP^{VQ} sheep (12, 17). It is also consistent with the striking behavior of the CH1641 scrapie isolate, which was primarily isolated from a positive line (mainly PrP^{VQ}-carrying) NPU-Cheviot sheep, when passaged in positive-line or negative-line (non-PrP^{VQ}) Cheviot sheep. The first (primary) intracerebral passage of this positive-line material to positive-line Cheviot sheep resulted in short incubation times. Passage of the primary CH1641 isolate into negative-line Cheviot sheep resulted in longer incubation times (33) probably due to polymorphism barriers. If the negative-line passaged isolates were subsequently passaged in negative-line Cheviot sheep the incubation times in this line of sheep decreased (17, 33). A subsequent passage from these negative-line to positive-line Cheviot sheep increased the incubation times dramatically (17, 33) again probably due to the polymorphism barrier.

Modification of scrapie isolate properties were also found in mice scrapie transmission experiments in which the properties of PrP^{Sc} could be modified by passage of scrapie isolates through mice with different PrP^{C} amino acid sequences (34). Further support is derived by the transmission of human Creutzfeldt–Jakob disease or GSS to mice expressing chimeric mouse/human PrP transgenes carrying specific mutations. Mice carrying the Glu-to-Lys mutation at position 200 (E200K) were resistant to human prions from a patient with GSS carrying a Pro-to-Leu mutation at position 102 (P102L) but were susceptible to prions from familial Creutzfeldt–Jakob disease patients harboring the E200K mutation. However, mice carrying the mouse/human transgene with the P102L mutation were susceptible to GSS prions (24).

Interestingly, a homogenate of bovine spongiform encephalopathy, of which the primary amino acid sequence (at the polymorphic amino acid positions of sheep PrP) is best comparable with the sheep PrP^{AQ} genotype, gives the shortest incubation times in PrP^{AQ} sheep if inoculated by the intracerebral route. If inoculated via the longer oral route however, PrP^{VQ} sheep have the shortest incubation time (17). Probably inoculation via the oral route, compared with inoculation by the intracerebral route, extends the incubation time long enough to overcome the polymorphism barrier and subsequently allows the agent to spread more quickly using PrP^{CVQ} instead of PrP^{CAQ}.

Preliminary data from cell-free conversion experiments with the three PrP^{C} variants using PrP^{Sc} isolated from a $PrP^{VQ/AQ}$ sheep suggest that this PrP^{Sc} isolate mainly consists of PrP^{CVQ} because this $PrP^{Sc(VQ/AQ)}$ isolate converted PrP^{CVQ} at least three times as efficiently as PrP^{CAQ} into protease-resistant forms (Fig. 4). This again is consistent with the finding that PrP^{CVQ} is more readily converted into PrP-res than PrP^{CAQ}. Thus in sheep containing the mutant PrP^{VQ} allele, it is likely that the PrP^{CVQ} variant will be the preferred converted variant, similar to what has been found for the mutant human PrP allele in GSS (35). Consequently, after infection of flocks of sheep having the PrP^{VQ} allele, the agent pool would be predicted to become enriched for PrP^{VQ}.

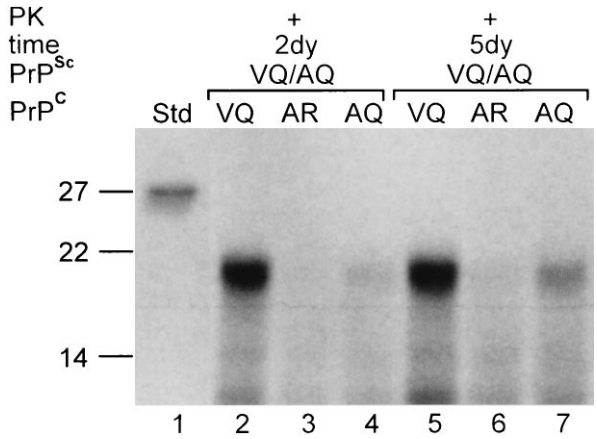

FIG. 4. Cell-free conversion of nonglycosylated ^{35}S-PrP^{C} into protease-resistant forms. PrP^{Sc} from a heterozygous sheep was pretreated in 2.5 M Gdn·HCl. Reactions were incubated for 2 or 5 days at 37°C (1 M Gdn·HCl) and subsequently PK-digested (except lane 1). Lane 1 contains approximately 2–5% of the input material (Std) for conversions shown in lanes 2–7. Samples were analyzed by 15% SDS/PAGE. Molecular mass markers (kDa) are indicated at the left.

This study shows that the cell-free system is an excellent system to measure the relative transmissibility of a prion source to animals or humans with known PrP genotypes. Although the mechanism by which PrP^{C} is converted into PrP^{Sc} and the mechanism by which polymorphisms in PrP modulate the conversion efficiency is not yet clear, studies with the cell-free conversion reaction (36) and small synthetic PrP peptides (37) are consistent with a nucleated polymerization mechanism (38, 39). The conversion of PrP^{C} to PrP^{Sc} involves a transition from a state that is predominantly α-helical to one that is largely β-sheet (4, 5, 40). PrP^{C} may rapidly interchange between these two conformations in its normal monomeric state but only be stabilized and accumulated in the β-sheet conformation by binding to a preformed PrP^{Sc} polymer (37, 38, 41). Alternatively, the transition to the PrP^{Sc} conformation may only be induced (catalyzed) upon direct binding of PrP^{C} to the PrP^{Sc} polymer. PrP polymorphisms may influence the equilibrium between the α-helical and β-sheet conformations in PrP^{C} and/or the ease with which PrP^{Sc} induces PrP^{C} to switch to the β-sheet conformation. Polymorphisms that destabilize the α-helical conformation of PrP^{C} would be expected to have these effects.

In this study we have tested the cell-free conversion of three (PrP^{VQ}, PrP^{AQ}, and PrP^{AR}) of the nine PrP variants found in sheep, including the two allelic variants that are associated with the extremes in susceptibility to scrapie (highly susceptible or resistant). From the other six allelic variants: PrP^{T112AQ}, PrP^{AT137Q}, PrP^{AF141Q}, PrP^{AH154Q}, PrP^{AH}, and PrP^{AQQ211}, it is not known whether they are significantly associated with susceptibility to natural or experimental scrapie in sheep. Using the recently published high-resolution NMR structure of the mouse PrP^{C} domain containing residues 121–232 together with Novotny secondary structure predictions, it might be possible to rationalize the effects of certain of the sheep PrP polymorphisms on PrP^{C} conformation. At least two other polymorphisms in the sheep PrP gene could be associated, by these predictions, with scrapie susceptibility. The PrP^{AT137Q} variant

could be grouped with the PrP^{VQ} variant, because both give a prediction of more β-sheeted structure and a change in hydrophobicity in the loop between β-sheet-1 and α-helix-1, which may indicate helix breaking or hydrophobic core destabilizing properties as found in theoretical studies of the Ala to Val mutation at position 117 in the human PrP sequence (42). The $PrP^{AH_{154}Q}$ variant is protective against scrapie, and no scrapie-affected sheep with this genotype have been found (10, 12, 15, 32). This variant could be grouped with the PrP^{AR} variant, because both involve a charge inversion compared with the wild-type PrP^{AQ} variant. The latter two polymorphisms are located in the loops between α-helix-1 and β-sheet-2, and between β-sheet-1 and α-helix-3, respectively, and may influence the stabilization of the hydrophobic core or the dipolar character of PrP^{C}. The other four alleles did not show differences in Novotny secondary structure predictions other than the PrP^{AQ} variant and therefore probably may be grouped with this variant. Additional cell-free conversion data with all known sheep PrP^{C} variants may enable us in the near future to determine more exactly the relative scrapie susceptibility between sheep having different PrP alleles.

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