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Sheep scrapie susceptibility-linked polymorphisms do not modulate the initial binding of cellular to disease-associated prion protein prior to conversion

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Conversion of the host-encoded protease-sensitive cellular prion protein (PrP^C) into the scrapie-associated protease-resistant isoform (PrP^{Sc}) of prion protein (PrP) is the central event in transmissible spongiform encephalopathies or prion diseases. Differences in transmissibility and susceptibility are largely determined by polymorphisms in PrP, but the exact molecular mechanism behind PrP conversion and the modulation by disease-associated polymorphisms is still unclear. To assess whether the polymorphisms in either PrP^C or PrP^{Sc} modulate the initial binding of PrP^C to PrP^{Sc}, several naturally occurring allelic variants of sheep PrP^C and PrP^{Sc} that are associated with differential scrapie susceptibility and transmissibility [the phylogenetic wild-type (ARQ), the codon 136Val variant (VRQ) and the codon 171Arg variant (ARR)] were used. Under cell-free PrP conversion conditions known to reproduce the observed *in vivo* differential scrapie susceptibility, it was found that the relative amounts of PrP^C allelic variants bound by various allelic PrP^{Sc} variants are PrP-specific and have comparable binding efficiencies. Therefore, the differential rate-limiting step in conversion of sheep PrP variants is not determined by the initial PrP^C–PrP^{Sc}-binding efficiency, but seems to be an intrinsic property of PrP^C itself. Consequently, a second step after PrP^C–PrP^{Sc}-binding should determine the observed differences in PrP conversion efficiencies. Further study of this second step may provide a future tool to determine the mechanism underlying refolding of PrP^C into PrP^{Sc} and supports the use of conversion-resistant polymorphic PrP^C variants as a potential therapeutic approach to interfere with PrP conversion in transmissible spongiform encephalopathy development.

INTRODUCTION

Transmissible spongiform encephalopathy (TSE) diseases are fatal neurodegenerative disorders and include (among others) familial, sporadic and variant Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep. TSEs (or prion diseases) are characterized by the formation and accumulation of protease-resistant prion protein (PrP^{Sc}) mainly in tissues of the central nervous system. Formation of PrP^{Sc} is a post-translational process and involves refolding (conversion) of the host-encoded prion protein (PrP^C) into partially protease-resistant forms (PrP^{Sc}) (DeArmond & Prusiner, 2003).

Scrapie in small ruminants (e.g. sheep) is one of the best documented models for natural TSE transmission. Polymorphisms in PrP have been shown to be of importance in both interspecies and intraspecies transmissibilities (Bossers *et al.*, 2003). Susceptibility of sheep to scrapie seems mainly dictated by polymorphisms in the gene encoding the prion protein itself, and to date over 20 different naturally occurring polymorphisms (only one mutation per allele) of PrP have been described (Goldmann *et al.*, 1990, 1991; Belt *et al.*, 1996; Bossers *et al.*, 1996; Junghans *et al.*, 1998; Elsen *et al.*, 1999; Thorgeirsdottir *et al.*, 1999; Tranulis *et al.*, 1999; O'Rourke *et al.*, 2000). The effects of polymorphisms in ovine PrP on the relative susceptibility of sheep to scrapie have been gauged in epidemiological studies of natural scrapie outbreaks, in experimental transmissions to and from sheep, and in cell-free conversion assays (Goldmann *et al.*, 1994; Bossers *et al.*, 1996, 1997, 1999, 2000; Hunter *et al.*, 1996). Polymorphisms at sheep PrP aa 136, 154 and 171 have been shown to be most relevant in association with differential TSE susceptibility. Several studies have shown that an alanine at position 136, arginine at position 154 and glutamine at position 171 (ARQ) to be the phylogenetic wild-type (wt) PrP, with intermediate susceptibility to scrapie. The polymorphism associated with increased susceptibility to scrapie is the substitution of alanine with valine at codon 136 (VRQ; 136V) and thus far the only polymorphism shown to be associated with decreased susceptibility or even resistance to natural scrapie is the substitution of glutamine with arginine at codon 171 (ARR; 171R). Cell-free conversion of PrP^C provides an excellent

in vitro model, in which relative amounts of produced proteinase K (PK)-resistant PrP reflect important biological aspects of TSEs at the molecular level (Caughey *et al.*, 1995; Bossers *et al.*, 1997, 2000, 2003; Raymond *et al.*, 1997, 2000; Bossers, 1999). In sheep scrapie, this technique has shown that 136V and wt-PrP^C are readily converted into PK-resistant PrP by various types of PrP^{Sc} isolated from sheep having different PrP genotypes. In contrast, 171R-PrP is hardly converted into PK-resistant PrP (Bossers *et al.*, 1997, 2000, 2003; Bossers, 1999; Raymond *et al.*, 2000).

Studies on the conversion of hamster and mouse PrP^C isoforms resulted in indications that diminished acquisition of PK resistance is not due to lack of binding of PrP^C to PrP^{Sc} (Horiuchi *et al.*, 2000). However, no data on binding efficiencies of ovine PrP^C to PrP^{Sc} are available to date. Furthermore, whereas differences in susceptibility of- and transmissibility in sheep can entirely be explained at the molecular level by the effects of single polymorphisms in PrP^C or PrP^{Sc} on PrP conversion, the exact molecular mechanism determining these differences is still unknown (Bossers *et al.*, 2000; Dubois *et al.*, 2002; Tranulis, 2002; Sabuncu *et al.*, 2003).

In the present study, sheep scrapie susceptibility-linked polymorphisms were used to determine whether differential binding efficiencies of sheep PrP^C to PrP^{Sc} determine the observed differential conversion efficiencies of sheep PrP (Bossers *et al.*, 1997, 2000; Raymond *et al.*, 1997, 2000).

METHODS

PrP^C constructs and expression. The three sheep PrP^C variants used (136V, wt and 171R) were cloned, expressed and characterized as described previously (Bossers *et al.*, 1997). Briefly, PrP open reading frames (ORF) were subcloned into the eukaryotic expression vector pECV7. The vectors containing PrP ORF were stably transfected into Chinese hamster ovary (CHO) cells. High and stable expressing single-cell-clones were selected by immunoperoxidase monolayer assay and Western blotting, using rabbit anti-peptide antiserum R521-7 (van Keulen *et al.*, 1995).

Radiolabelling and purification of PrP^C. Radiolabelling and purification of the three PrP^C variants were performed as described previously (Raymond *et al.*, 1997; Bossers *et al.*, 2000). Briefly, single cell clones expressing the different PrP^C variants were starved for 30–60 min in label medium and subsequently labelled with 1 mCi (37 MBq) [^{35}S]methionine/cysteine TRAN^{35}S-label (ICN Biomedicals). Cells were lysed in lysis buffer containing Triton X-100 (0·5 %; ICN Biomedicals) in the presence of protease inhibitors (1 nM Pefabloc SC, 1 nM leupeptine, 1 nM pepstatin and 0·15 nM aproprotin). ^{35}S-labelled PrP^C was immunopurified by PrP-specific antiserum R521-7 captured by protein A–Sepharose (10 % w/v), which was eluted in 0·1 M acetic acid.

Radiolabelling and purification of classical swine fever virus (CSFV) glycoprotein E2. For labelling, an expression vector containing the gene encoding glycoprotein E2 of CSFV transfected into SK6 cells (van Gennip *et al.*, 2002) was used. Radiolabelling and purification of E2 were essentially performed as described above, albeit on a larger scale. ^{35}S-labelled E2 was immunopurified using monoclonal antibody V3 (Wensvoort, 1989) and eluted in 0·1 M acetic acid.

PrP^{Sc} purification and analysis. PrP^{Sc} was isolated from brain tissue of clinically ill scrapie sheep with either homozygous alleles for 136V-PrP or wt-PrP. PrP genotypes were determined by Sanger sequencing of the full PrP ORF as described previously (Bossers *et al.*, 1996). PrP^{Sc} was purified by ultracentrifugational pelleting from Sarkosyl-homogenated brains as described previously (Caughey *et al.*, 1995; Bossers *et al.*, 1997). The final pellets were sonicated in PBS containing 1·0 % zwitter-reagent (SB 3-14). Yields of PrP^{Sc} were quantified by SDS-PAGE (12 % NuPAGE; Invitrogen) and Western blotting using antiserum R521-7.

Conversion-binding assay. Conversion and binding efficiencies were determined by double volume cell-free conversion reactions essentially as described previously (Horiuchi *et al.*, 2000; Priola & Lawson, 2001) and adapted to ovine cell-free conversion conditions as used before (Caughey *et al.*, 1995; Bossers *et al.*, 1997, 2000). Briefly, PrP^{Sc} was partially denatured in 2·5 M guanidinium-hydrochloride (GdnHCl) for at least 2·5 h at 37 °C. Aliquots of denatured PrP^{Sc} (2–4 µg per reaction) were mixed with 10 000–20 000 c.p.m. purified ^{35}S-labelled PrP^C (~20–40 ng ^{35}S-labelled PrP^C) and further diluted to a final concentration of 1·0 M GdnHCl in conversion buffer (50 mM sodium citrate, pH 6·0, 5 mM cetylpyridinium chloride, 1 % *N*-lauroylsarcosine and protease inhibitors). Reactions were incubated for 3 days at 37 °C (or shorter for the kinetic experiments). After incubation, the reaction volume was split in two equal aliquots in separate siliconized tubes. One aliquot was used for binding analysis and centrifuged for 30 min at 17 500 ***g*** at room temperature, the supernatant was transferred to a separate siliconized tube (unbound fraction) and the pellet (bound fraction) dissolved in 1 % SDS by sonication. From the second aliquot, 1/10 volume was transferred to a separate siliconized tube (reference fraction) and the remaining 9/10 volume was treated with 35 µg PK ml^{-1} for 1 h at 37 °C. PK was inactivated by the addition of Pefabloc-SC (Roche).

All the samples were methanol-precipitated and the pellet was dried and dissolved in Laemmli SDS-PAGE sample buffer containing 5 % (v/v) 2-mercaptoethanol and 4 M urea. Samples were run on SDS-PAGE (12 % NuPAGE; Invitrogen), the dried gels were visualized by phosphorimaging and analysed using a STORM-840 imager and the ImageQuant 5.1 software (Molecular Dynamics). Binding percentages were calculated by dividing the amount of labelled PrP^C (molecular mass between 24 and 28·5 kDa) of each fraction [pellet (p) and supernatant (s)] by the total amount of labelled PrP^C (p + s). Conversion percentages were calculated by dividing the amount of labelled PrP left after PK digestion (molecular mass between 19 and 21·5 kDa) by the amount of labelled PrP^C in the reference fraction (molecular mass between 24 and 28·5 kDa).

Statistical analysis of binding efficiencies. Statistical calculations were performed using the GenStat 6.1 program. To compensate for differences in possible variance as a result of a fixed scale, variance stabilizing angular transformation of the binding percentages was utilized (section 4·1·3; McCullagh, 1983). Absolute amounts of bound ^{35}S-labelled PrP^C varied, probably as a result of the aggregated state of the PrP^{Sc} isolate used; therefore binding patterns were compared by the analysis of variance method to determine whether significant differences occurred in these binding patterns. Comparisons of binding patterns were made separately for the three PrP^C variants and for the two different PrP^{Sc} isolate groups. In order to determine significant differences in binding patterns, the least significant difference (LSD; the minimum amount needed to demonstrate a significant difference) was calculated and compared with the observed differences between the mean binding percentages for either the PrP^C variants or the PrP^{Sc} isolate groups.

RESULTS

Binding efficiencies of PrP^C to PrP^{Sc}

In total, six independent PrP^{Sc} isolates from six sheep homozygous for 136V-PrP^C and six independent PrP^{Sc} isolates from six sheep homozygous for wt-PrP^C were isolated and tested for binding affinities to three natural allelic variants of sheep PrP^C; 136V-PrP^C (VRQ), wt-PrP^C (ARQ) and 171R-PrP^C (ARR). At least two independent reaction duplicates were analysed from each PrP^{Sc} isolate. Binding efficiencies of the individual PrP^{Sc} isolates were determined using the conversion-binding assay, in which cell-free conversion conditions used were identical to previous studies, showing significant differential conversion of sheep PrP^C variants (Bossers *et al.*, 2000). Because aggregates were pelleted by spinning for 30 min at 17 500 ***g***, we needed to take into account that not all of the PrP^{Sc} is actually pelleted at this 'low' speed. However, most of the PrP^{Sc} was pelleted (~86·4 % of the total input). Therefore, it can be assumed that the amount of ^{35}S-labelled PrP^C found in the pellet fraction is representative of most if not all of the actual bound ^{35}S-labelled PrP^C. The addition of PrP^{Sc}, isolated from sheep homozygous for 136V-PrP (Fig. 1a) or isolated from sheep homozygous for wt-PrP (Fig. 1b), resulted in recovering most of the labelled PrP^C in the bound pellet (p) fraction (Fig. 1a and b, lanes 1, 3 and 5) and only a small amount of labelled PrP^C remained in the unbound supernatant (s) fraction (Fig. 1a and b, lanes 2, 4 and 6) for each PrP^C tested (Table 1; 136V-PrP^{Sc} and wt-PrP^{Sc} isolates).

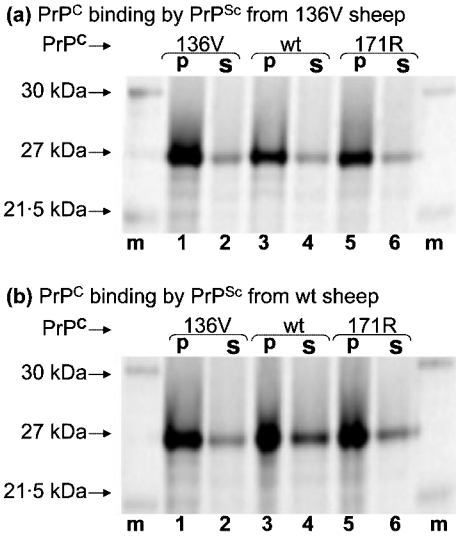

Fig. 1. Phosphorimage of SDS-PAGE showing an example of binding assay samples obtained with PrP^{Sc} isolated from a sheep homozygous for 136V-PrP allele (a) or from a sheep homozygous for wt-PrP allele (b). Lanes containing ^{14}C-marker (Amersham Biosciences) are marked 'm', sizes of the marker bands are 30 and 21·5 kDa. Non-glycosylated PrP is indicated by the 27 kDa marker. Lanes 1 and 2 represent results with 136V-PrP^C, lanes 3 and 4 represent results with wt-PrP^C and lanes 5 and 6 represent results with 171R-PrP^C of which the odd lanes contain the pelleted (bound) fraction (p) and the even lanes contain the supernatant (unbound) fraction (s).

Table 1. Mean percentages of bound ^{35}S-labelled PrP^C determined for PrP^{Sc} isolates from sheep

Isolate	Type*	*n*†	Mean binding (%) PrP^C‡			Mean binding (%) PrP^{Sc} isolate group ± SEM
			136V ± SEM	ARQ ± SEM	171R ± SEM	
553237	136V	3	83·3±11·6	91·3±2·7	89·4±2·1	
558998	136V	6	89·2±2·8	86·4±5·4	89·7±5·3	
577275	136V	2	97·0±0·5	98·4±0·5	89·2±2·5	**86·2±1·7**
601936 (343)	136V	3	81·9±8·5	83·7±4·6	91·1±1·5	
606316	136V	4	86·3±2·2	74·5±4·8	84·1±4·2	
609619	136V	5	84·0±1·1	67·5±4·4	85·7±0·4	
514076	wt (ARQ)	3	88·1±4·7	81·2±10·7	88·9±9·1	
523900	wt (ARQ)	4	80·1±5·3	85·0±6·9	70·3±9·8	
532643	wt (ARQ)	2	89·2±2·3	86·5±3·9	74·3±19·7	**87·3±1·8**
577331	wt (ARQ)	3	91·0±1·6	87·6±6·0	85·2±4·1	
603397	wt (ARQ)	3	87·9±7·2	87·4±1·4	93·5±1·0	
614116	wt (ARQ)	2	97·9±1·4	98·8±0·3	98·2±0·8	
Mean binding (%) PrP^C variant			**88·0±1·6**	**85·6±2·5**	**86·6±2·2**	

*PrP^{Sc} isolated from sheep with a homozygous allele for either 136V-PrP^C or wt-PrP^C.

†The number of repeated (independent) reactions for each PrP^{Sc} isolate.

‡Absolute amounts of bound ^{35}S-labelled PrP^C ranged from ~60 to 99 %, depending on the aggregated state of a PrP^{Sc} isolate. Therefore, analysis of variance on the observed binding patterns between PrP^C variants and PrP^{Sc} isolate groups was performed.

Comparison between repeated measurements of a PrP^{Sc} isolate or between different PrP^{Sc} isolates showed that the absolute amount of bound PrP^{C} was linked to the isolated batch of PrP^{Sc} [probably due to differences in preparation of PrP^{Sc} aliquots (sonication) or 'age' of an isolate]. Therefore, the absolute binding percentages (per measurement) of bound ^{35}S-labelled PrP^{C} were not compared, but rather the mean binding percentages of repeated measurements (Table 1).

Binding percentages were compared by variance analysis, after variance stabilizing angular transformation of the binding percentages. Firstly, binding patterns were compared between the PrP^{C} variants and no significant differences were found (Fig. 2a) since the LSD between PrP^{C} variants was calculated to be 4·5 %, which was higher than the maximum difference of 2·4 % between the mean binding percentages of 136V-PrP^{C}, wt-PrP^{C} and 171R-PrP^{C} (88·0$\pm$1·6, 85·6$\pm$2·5 and 86·6$\pm$2·2 %, respectively). Secondly, binding patterns were compared between the PrP^{Sc} groups and again no significant differences were shown (Fig. 2b) since the LSD between PrP^{Sc} variants was 5·8 %, which is again higher than the maximum difference of 1·1 % between the mean binding percentages for the wt/wt (homozygous wt-PrP) and 136V/136V (homozygous 136V-PrP) PrP^{Sc} isolate groups (86·2$\pm$1·7 and 87·3$\pm$1·8 %, respectively).

To gain insight into the dynamics of the binding reaction, binding percentages were also determined at shorter incubations (1 h, 1 day, 2 days and the standard 3 days). At each time point, binding percentages were determined of the three PrP^{C} variants to PrP^{Sc} (n=4; two wt/wt and two 136V/136V isolates). No significant differences were found between the three PrP^{C} variants and the overall mean binding percentages at the four time points (Fig. 3).

Binding specificity of PrP^{Sc} to PrP^{C}

In order to exclude potential non-specific binding or aggregation features of PrP^{C}, we performed several controls. First of all, ^{35}S-labelled PrP^{C} was incubated without addition of PrP^{Sc} to determine whether 'self aggregation' and spontaneous pelleting of PrP^{C} occurred. Exclusion of PrP^{Sc}, by replacing with demineralized water (SQ), did not result in significant amounts of ^{35}S-labelled PrP^{C} in the pellet fraction, leaving on average 93·6$\pm$1·3 % of the ^{35}S-labelled PrP^{C} variants in the supernatant fraction (Fig. 4a, lanes 1 and 2). Therefore, self-aggregation of PrP^{C} is not responsible for recovering significant amounts of labelled PrP^{C} in the pellet. Furthermore, it shows that the presence of aggregated protein (PrP^{Sc}) is a prerequisite for pelleting ^{35}S-labelled PrP^{C} under conditions maintaining cell-free conversion specificity.

To determine whether ^{35}S-labelled PrP^{C} could be non-specifically 'captured' and pelleted by any aggregated protein, PrP^{Sc} was replaced in the binding assay with keyhole limpet haemocyanin (KLH), a very large mainly aggregated

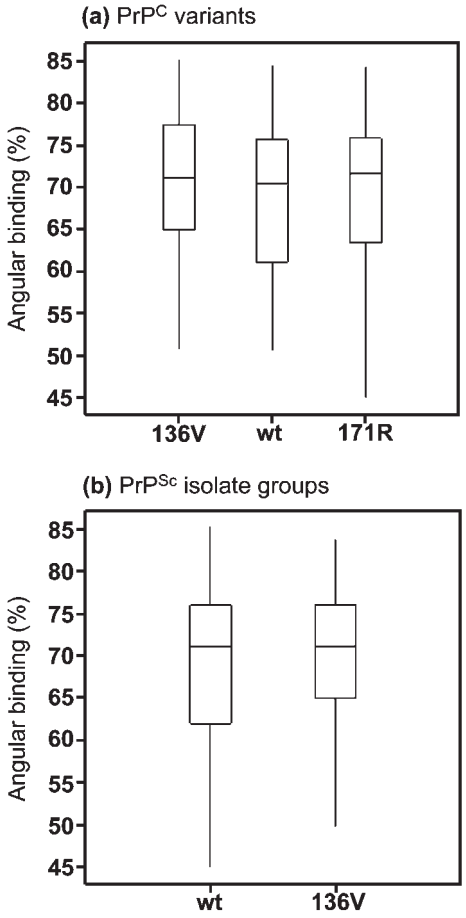

Fig. 2. Boxplots of binding patterns after angular transformation of binding percentages. The binding percentages have been plotted against the PrP^{C} variants (a) and PrP^{Sc} isolate groups (b). The boxplot shows the total spread of all determined binding percentages, with the box representing 95 % of all measurements and the line in the box representing the mean value of the measurements. Analysis of variance of the binding percentages shows that neither the PrP^{C} variant nor the PrP^{Sc} isolate group have a significant effect on the binding patterns obtained.

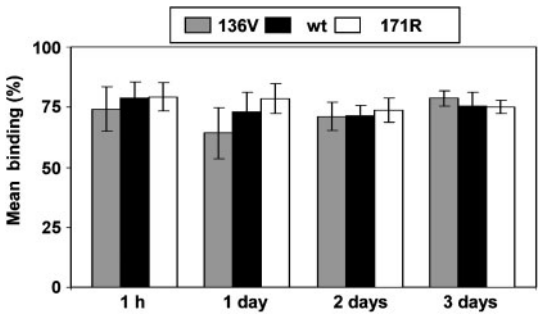

Fig. 3. Kinetics of the binding efficiencies of the three PrP^{C} variants to PrP^{Sc} homozygous 136V and homozygous wt. Mean binding percentages for each PrP^{C} variant are indicated including the error bars (SEM) for the repeated experiments after incubation for the indicated time.

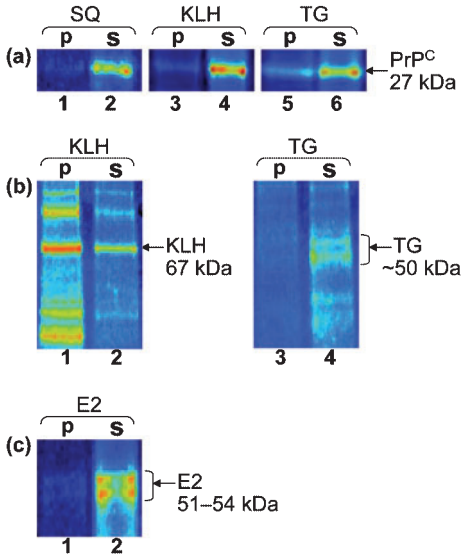

Fig. 4. Examples of control reactions for determining specificity of PrP^{C}–PrP^{Sc} interaction within the binding assay. (a) Phosphorimage of SDS-PAGE (indicated in pseudo intensity staining) analysis of samples obtained when PrP^{Sc} was replaced with either water (SQ; lanes 1 and 2), KLH (lanes 3 and 4) or TG (lanes 5 and 6). (b) Sypro Orange total protein staining of SDS-PAGE (indicated in pseudo intensity staining) containing conversion/binding samples having KLH (lanes 1 and 2) and TG (lanes 3 and 4) instead of PrP^{Sc} under the specific conversion conditions used. (c) Phosphorimage of SDS-PAGE (indicated in pseudo intensity staining) containing samples obtained when ^{35}S-labelled PrP^{C} was replaced with ^{35}S-labelled E2 protein of CSFV. All odd lanes contain the pelleted (p) (bound) fraction and all even lanes contain the supernatant (s) (unbound) fraction.

protein. A surplus of KLH was added (about 5 μg per reaction) to favour KLH aggregation. No significant amounts of ^{35}S-labelled PrP^{C} were detected in the pellet fraction. On average 94·3±2·3 % of the ^{35}S-labelled PrP^{C} remained in the unbound fraction (Fig. 4a, lanes 3 and 4), which is about the same as the amount of PrP^{C} in the pellet fraction of assays without any aggregated protein (see above). To ensure that KLH remained aggregated under the specific conversion conditions used, soluble and pellet fractions were also analysed on SDS-PAGE by total protein staining (Sypro Orange; Molecular Probes). On average 79·8±1·0 % of the added KLH was recovered in the pellet fraction (Fig. 4b, lanes 1 and 2), which is comparable to the percentage of aggregation determined for KLH (75·9±2·1 %) in storing buffer (0·1 M sodium phosphate buffer, pH 7). Since no increase in PrP^{C} pelleting was observed, and although only one other aggregated protein was tested for non-specific capture of PrP^{C}, this indicates that ^{35}S-labelled PrP^{C} is probably not a 'sticky' protein binding to any aggregate.

To determine whether ^{35}S-labelled PrP^{C} could non-specifically bind to other large soluble proteins, resulting in significant amounts of precipitation, PrP^{Sc} was replaced by thyroglobulin (TG), a large unrelated soluble protein frequently used as a carrier in protein precipitation methodologies. A surplus of TG was added (about 5 μg per reaction) but no significant amounts of ^{35}S-labelled PrP^{C} were detected in the pellet fraction; on average 90·6±2·6 % remained in the supernatant fraction (Fig. 4a, lanes 5 and 6). To ensure that TG remained largely soluble under the specific conversion conditions used, soluble and pellet fractions were also analysed on SDS-PAGE by total protein staining (Sypro Orange). On average 94·4±2·5 % of the TG remained in the supernatant (Fig. 4b, lanes 3 and 4) under the specific conversion conditions used. Even though only one large soluble protein was tested, this indicates that ^{35}S-labelled PrP^{C} is not significantly precipitated by binding to any other large soluble heterologous protein like TG.

To determine whether any labelled soluble protein would bind to the added aggregated PrP^{Sc}, ^{35}S-labelled PrP^{C} was replaced with ^{35}S-labelled E2 of CSFV, an unrelated but similarly processed protein (membrane bound partially *N*-glycosylated protein of about 51–54 kDa). No significant amounts of ^{35}S-labelled E2 protein were found in the bound fraction, while 91·9±3·5 % of the ^{35}S-labelled E2 protein remained in the unbound fraction (Fig. 4c, lanes 1 and 2). This indicates that binding of ^{35}S-labelled PrP^{C} by PrP^{Sc} is PrP-specific.

In summary, we have shown that PrP^{C} binds efficiently to PrP^{Sc} with no significant differences in binding patterns between PrP^{C} variants and PrP^{Sc} isolate groups, under conditions maintaining cell-free conversion specificity (Fig. 5). Furthermore, we have shown that PrP^{C} does not spontaneously aggregate due to the specific conversion condition used, does not stick to unrelated aggregated protein like

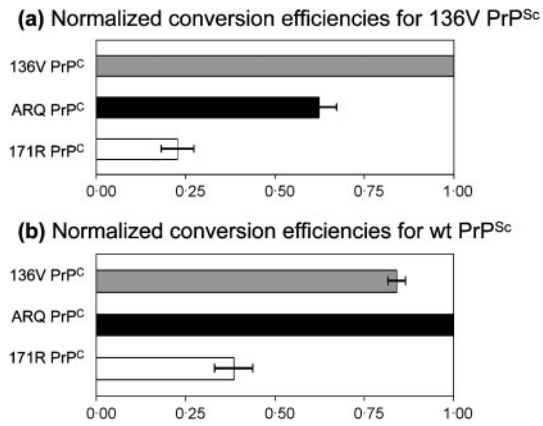

Fig. 5. Observed relative normalized conversion efficiencies of the combined conversion-binding reactions. Reactions were normalized to the most efficient homologous reactions. (a) Conversion efficiencies of the three PrP^{C} variants induced by 136V-PrP^{Sc}. (b) Reactions induced by wt-PrP^{Sc}. Mean normalized efficiencies and the corresponding error bars (SEM) are indicated.

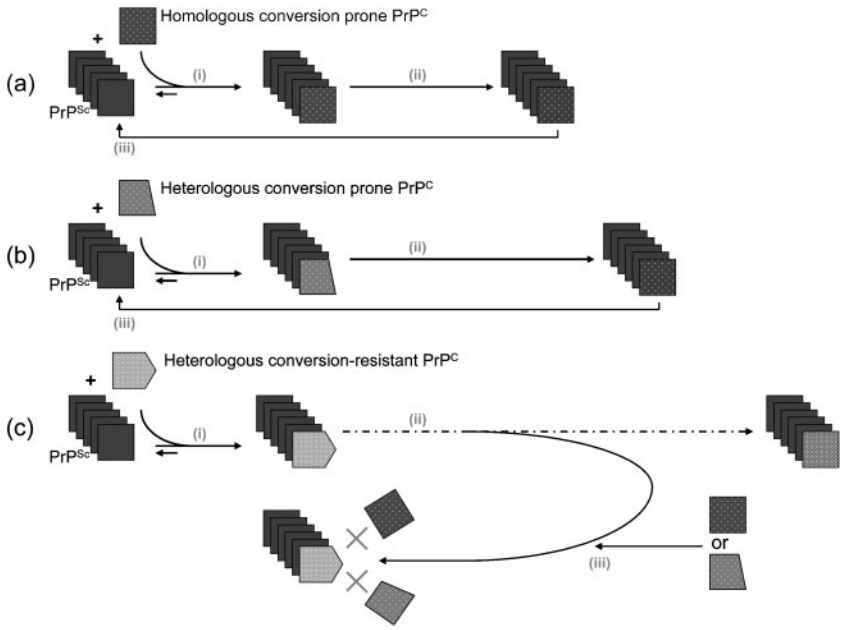

Fig. 6. Schematic representation of PrP^C conversion. (a) Conversion of homologous, conversion prone PrP^C (wt, 136V): efficient binding (i), highly efficient conversion (ii) and 're-seeding' (iii) possible. (b) Conversion of heterologous, conversion prone PrP^C (wt, 136V): efficient binding (i), efficient conversion (ii) and 're-seeding' (iii) possible. (c) Conversion of heterologous, conversion-resistant PrP^C (171R): efficient binding (i), highly inefficient or blocked conversion (ii) and 're-seeding' (iii) possibly blocked.

KLH and does not precipitate with other large soluble proteins like TG. Additionally, we have shown that PrP^{Sc} does not bind to unrelated labelled soluble protein (E2). Therefore, we can conclude that PrP^{Sc}-associated pelleting of ^{35}S-labelled PrP^C represents a PrP^C–PrP^{Sc}-specific interaction and that addition of aggregated protein (PrP^{Sc}) is a prerequisite for pelleting ^{35}S-labelled PrP^C under conditions maintaining cell-free conversion specificity. Since no differences were detected in the binding patterns of the tested PrP^C variants to the different PrP^{Sc} isolates, the rate-limiting step determining the observed differential conversion efficiencies of PrP^C variants has to be during a subsequent step in the conversion after binding of PrP^C to PrP^{Sc} (Fig. 6).

DISCUSSION

The aim of this study was to gain insight into the mechanism underlying the modulation of sheep scrapie susceptibility by polymorphisms in PrP^C or PrP^{Sc}. Bossers *et al.* (2000) showed that the *in vitro* conversion assay is a representative tool for assessing modulating effects of scrapie-associated polymorphisms. Other studies have shown that the conversion of PrP^C by PrP^{Sc} is induced by the aggregated forms of PrP^{Sc} (Caughey *et al.*, 1995, 1997). These aggregates can be pelleted by high-speed centrifugation. The amount of ^{35}S-labelled PrP^C that is bound by PrP^{Sc} and subsequently recovered from the pellet should give an indication of whether the disease-associated polymorphisms modulate binding of PrP^C by PrP^{Sc} or whether these polymorphisms have their modulating effects in a subsequent step after the initial binding during the conversion.

By applying a conversion-binding assay (Horiuchi *et al.*, 2000), binding efficiencies of sheep PrP^C variants to sheep PrP^{Sc} variants have been measured. Since no significant differences in binding efficiencies were measured between any of the variants, the initial binding efficiencies cannot account for the observed differential conversion efficiencies of sheep scrapie susceptibility-linked variants of PrP. We show for instance that 171R-PrP^C binds to PrP^{Sc} as efficiently as 136V-PrP^C or wt-PrP^C, whereas conversion efficiencies differ remarkably. Therefore, a second (or) further step in the conversion process, in which the disease-associated polymorphisms have their modulating effect, seems to be involved in the conversion of the PrP protein (Fig. 6). These findings are corroborated by a study in which interactions between heterologous forms of prion protein have been studied *in vitro* using mouse and hamster PrP isoforms (Horiuchi *et al.*, 2000), in which is shown that PrP^C of different species (hamster and mouse) bind equally efficiently to PrP^{Sc} of mouse while preserving conversion specificity, also indicating that a second step in the conversion after initial binding should determine the species specificity.

In this study, we also show that PrP^C does not precipitate spontaneously, does not stick to unrelated aggregated protein (KLH), and does not precipitate with other large soluble proteins (TG). In addition, we showed that PrP^{Sc} does not bind unrelated labelled soluble protein CSFV E2. Therefore, we conclude that PrP^{Sc}-associated pelleting of ^{35}S-labelled PrP^C represents a PrP^C–PrP^{Sc}-specific interaction and that addition of aggregated PrP^{Sc} is a prerequisite for pelleting ^{35}S-labelled PrP^C under conditions maintaining cell-free conversion specificity. These results are in conjunction with results in which no spontaneous PK-resistant PrP was formed under the cell-free conversion conditions when PrP^{Sc} was replaced by SQ water (Bossers *et al.*, 1997, 2000). The inability of PrP^{Sc} to significantly bind ^{35}S-labelled E2 additionally confirms that binding of ^{35}S-labelled PrP^C to PrP^{Sc} is PrP-specific and does not solely

depend on post-translational modifications or non-specific 'sticky' properties of PrP.

Since 171R-PrP^C seems to bind as efficiently to PrP^{Sc} as wt-PrP^C and 136V-PrP^C, the 171R-PrP^C variant may be valuable in firstly, providing clues for designing new therapeutic strategies by determination of the mechanism underlying the refolding process of PrP^C into PrP^{Sc}, for example, by using the 171R-PrP^C variant to determine sites involved in binding and/or conversion or by comparing protein properties (i.e. stability, unfolding/refolding kinetics). Secondly, since conversion-resistant 171R-PrP^C binds efficiently to PrP^{Sc} it may provide a future tool to block prion conversion through direct interference or blocking of PrP^{Sc} polymer growth as hypothesized before by Bossers *et al.* (1999). In addition, results from literature show that heterozygosity for PrP is a protective factor against TSE development as demonstrated by studies *in vitro* (Priola *et al.*, 1994; Holscher *et al.*, 1998; Horiuchi *et al.*, 2000) or *in vivo* for sheep (Goldmann *et al.*, 1994; Belt *et al.*, 1995; Clouscard *et al.*, 1995; Bossers *et al.*, 1996; Hunter *et al.*, 1996) and humans (Collinge *et al.*, 1991; Palmer *et al.*, 1991). This is in conjunction with our results showing that various differentially converting PrP^C variants bind equally efficiently to PrP^{Sc} but have different conversion efficiencies. In heterozygotes, this 'inhibition' of conversion by heterologous PrP variants might explain why heterozygotes have longer incubation times than their homozygous counterparts. This is corroborated by the fact that resistance in heterozygous sheep is not caused by preferential allelic use (Caplazi *et al.*, 2004).

The coupled *in vitro* cell-free conversion efficiencies (Fig. 5) reflect results as described before (Bossers *et al.*, 2000), where susceptibility to scrapie was linked to the modulating effects of polymorphisms on the conversion of sheep PrP. In addition to these *in vitro* cell-free conversion assays, PrP polymorphisms have been shown to tightly control sheep prion replication in cultured cells (Sabuncu *et al.*, 2003). Furthermore, it has been shown that polymorphisms in PrP determine both interspecies and intraspecies transmissibilities (Bossers, 1999; Bossers *et al.*, 2003) and/or the stability of the PrP^C molecule itself (Rezaei *et al.*, 2002).

By correlating conversion- and binding-patterns, we showed that 171R-PrP^C binds to PrP^{Sc} as efficiently as conversion prone variants like wt (ARQ) and 136V-PrP^C. Since naturally occurring polymorphisms of sheep PrP^C seem not to have a significant modulating effect on the initial binding of PrP^C to PrP^{Sc}, these could somehow modulate a subsequent step in the conversion process (Fig. 6). Both 136V and 171R are polymorphisms that affect PrP^C stability and are close to the region that supposedly is involved in refolding of PrP^C to PrP^{Sc} (Rezaei *et al.*, 2002; Eghiaian *et al.*, 2004). This region is composed of the two small β-sheets [sheep aa 129–134 (S1) and 163–167 (S2)], which are the positions from where the first α-helix [aa 146–158 (H1)] is converted into an anti-parallel organized β-sheeted structure. It could also be that the 171R polymorphism results in increased protease sensitivity of 171R-PrP^C itself due to destabilization of the PrP^C molecule (Rezaei *et al.*, 2002), thus resulting in slower amyloidogenesis because the 171R-PrP^C molecule could internalize and be degraded by the PrP-expressing cell more rapidly than the other variants before the actual polymerization can take place. In contrast, the 136V polymorphism could stabilize the PrP^C molecule, resulting in an elongation of the survival of 136V-PrP^C and thereby supporting the subsequent conversion.

Since disease-associated polymorphisms of sheep PrP do not have an effect on binding properties of PrP^C to PrP^{Sc}, dominant-negative inhibition of the 171R polymorphism on prion conversion (Bossers, 1999; Bossers *et al.*, 1999, 2000; Perrier *et al.*, 2002) is therefore not due to lack of interaction between PrP variants as suggested by Perrier *et al.* (2002), but more probably due to a more subtle mode of modulation by the PrP polymorphism on the conversion or on the interaction with chaperone proteins under natural conditions.

This study shows that the interaction between PrP^C and PrP^{Sc} in the conversion-binding assay is PrP-specific. Whether PrP^C binds to the so-called nucleation site of PrP^{Sc} only or whether it can bind to other sites of PrP^{Sc} aggregates is under investigation. The next logical step, currently under investigation, is to find out whether conversion-resistant (natural or artificial) PrP variants can effectively interfere with the process of PrP conversion and thereby therapeutically block or significantly delay TSE development.

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