Biochemical and Biophysical Research Communications **281,** 101–108 (2001)
doi:10.1006/bbrc.2001.4326, available online at http://www.idealibrary.com on IDEAL®

Molecular Specificities of Antibodies against Ovine and Murine Recombinant Prion Proteins

D. Betemps and Th. Baron
Agence Française de Sécurité Sanitaire des Aliments, Lyon, France

Received January 11, 2001

The prion proteins (PrP) from sheep and mouse were produced in large quantities of full-length protein in *Escherichia coli* after fusion with a carboxy-terminal hexahistidine sequence. Both recombinant proteins were recognized, at variable levels, in ELISA using a panel of antibodies recognizing different parts of the PrP molecules, from the octo-repeat region (79–92 human sequence), to the C terminal end of the protein. We show that these recombinant proteins enable polyclonal antisera to be produced in $PrP^{0/0}$ mice, the sheep prion protein being strongly immunogenic, using either native or guanidium hydrochloride-treated recombinant protein. Sera produced against the sheep protein also reacted in Western blot with bovine, ovine, and murine PrP res, but showed higher reactivity with sheep PrP res. Interestingly, when compared to an antiserum produced against bovine 106–121 peptidic sequence (RB1), we found strikingly different ratios of the PrP res glycoforms, in both cattle with BSE and sheep with natural scrapie, but not in scrapie infected mice. Such results further demonstrate that the assessment of PrP res glycoform ratios, using different antibodies, may depend on antibodies species-specificities. © 2001 Academic Press

Key Words: **prion; sheep; mice; recombinant protein; BSE; scrapie.**

Prion diseases, or transmissible spongiform encephalopathies, are neurodegenerative diseases that affect both humans and animals (1). All prion diseases share the same molecular pathogenic mechanism that involves conversion of the normal cellular prion protein (PrP^{c}) from the host of the disease into a form that is insoluble in non ionic detergent and partially resistant to proteases (PrP^{res}). Attempts at further biochemical studies have been hampered by difficulties in obtaining large quantities of purified protein. In recent years, the availability of recombinant full-length mouse (mPrP) (2), Syrian hamster (3), bovine (4), and human PrPs (5), or of their C-terminal domains, namely mouse (121–231) (6), human (90–231) (7), bovine (121–230) (8), and Syrian hamster PrPs (90–231) (9) has facilitated structural analysis of the prion protein. NMR studies indicated that the N terminal portion of murine PrP between residues 29 and 124 was highly flexible (10), whereas the C-terminal residues 121–231 form a globular domain with three α-helices and two short antiparallel β-sheets.

The generation of antibodies against PrP has been hindered, in the past, by immunotolerance in the immunized animals, associated with the highly conserved amino acid sequences among species. $PrP^{0/0}$ mice (11) lack these immunotolerance characteristics and develop an excellent polyclonal antibody response when immunized with PrP^{sc} (sc for scrapie) (12).

In this report we describe the reactivities of a panel of antibodies, directed against different sequences of the PrP protein, against a sheep and a murine full length recombinant protein produced in *Escherichia coli*. We also show that these recombinant proteins enable polyclonal antisera to be produced in null mice, the sheep prion protein being strongly immunogenic. These antisera showed different electrophoretic profiles, for bovine and ovine PrPres, compared to that observed with a serum directed against the 106–121 bovine peptidic sequence.

MATERIAL AND METHODS

Cloning of murine and ovine PrP genes. Recombinant sheep PrP (sh rPrP) (amino acid Lys 25-Ser 234) was expressed in *E. coli* after fusion with a carboxy-terminal hexahistidine sequence, as described previously (13).

Recombinant murine PrP (mu rPrP) was prepared in the same way using pMH79 vector (14) derived from pPROK plasmid (Clontech) (kindly provided by V. Cheynet, UMR 103 CNRS Biomérieux, Lyon).

Briefly, the murine PrP insert of 786 base pairs, containing the complete sequence encoding the 254 amino acids of the proteins, was obtained after PCR amplification of DNA from N_2A mouse neuroblastoma cells, using primers P1 and P2 derived from the 5′ and 3′ coding regions of the murine PrP gene, corresponding to Prn-p nucleotides 95–114 and nucleotides 881–860 respectively (P1, 5′-TCATCATGGCGAACCTTGGC-3′ and P2, 5′-AAGCAGGAAGGCCTCCCTCATC-3′).

Cloning procedures were performed as described previously (15) to

0006-291X/01 $35.00

ligate the PCR product into the PCR-Script SK(+) vector (Stratagene). A further amplification using primers P3 (5′-*ATC-GAT*TAAATAAGGAGGAATAACAT**ATG**AAAAAGCGGCCA-3′) and P4 (5′-*GAATTC***CTA**ATGGTGATGGTGATGGTGGCTGCTGGA-TCTTCTCC-3′), with P3 containing a *Cla*I restriction site (in italic) and an initiation codon ATG (in bold type), and P4 the hexahistidine sequence (underlined), a TAG stop codon (in bold type) and an *Eco*RI restriction site (in italic), gave a 686 pb insert. This insert subcloned between the same restriction sites, thus contained the complete sequence encoding the amino acid Lys 23 to Ser 232 from mouse PrP, without the signal peptide or the C-terminal end.

Expression and purification of poly-histidine fusion proteins. Both proteins, induced from transformed XL1-Blue MRF′ *E. coli* (Stratagene) by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 4 h at 37°C, were solubilised by adding 8 M urea in lysis buffer (NaH_2PO_4 50 mM, Tris–HCl 10 mM, NaCl 100 mM, pH 8.0) and purified by adsorption to TALON metal affinity resin (Clontech), according to the manufacturer's instructions. Elution was performed using 20 mM PIPES (Piperazine-*N*,*N*′-bis (2-ethanesulfonic acid)) (Sigma) at pH 5.0.

The fractions containing the fusion protein were pooled for further applications, and the protein concentration was determined using the Lowry method (16), with a DC Protein Assay kit (Biorad).

Proteinase K sensitivity assays. Proteinase K (0.06 μg) was added to 1 μg of His PrP (4) in 25 μl final volume of 10 mM Tris–HCl, pH 8.0, 130 mM NaCl, 1 mM $CaCl_2$. Digestion was carried out for 15 min at 37°C, then stopped by adding 100 μg/ml PMSF (phenylmethylsulfonyl fluoride) and heating in a boiling water bath for 5 min. The digestion products were examined by Western blot.

Extraction of PrPres from brain. PrP res was obtained from bovine, sheep, or mouse brains homogenized at 10% (wt/vol) in a 5% glucose solution. The bovine and ovine samples came from natural bovine spongiform encephalopathy and scrapie isolates identified in France. The murine PrPres originated from the C506M3 scrapie agent strain passaged into the C57BL/6 mice (kindly supplied by D. Dormont, CEA Fontenay-aux-Roses, France) (17). Homogenates were forced through a 0.4 mm diameter needle before being treated at 37°C for 1 h with 10 μg Proteinase K (Roche)/100 mg tissue. After the addition of N-Lauroylsarkosyl (Sigma) (final concentration 10% v/v), samples were incubated for 15 min at room temperature and then centrifuged at 465,000*g* for 2 h on a 10% sucrose cushion (Beckman TL 100 ultracentrifuge) to pellet the PrPres. These pellets were resuspended in 50 μl of denaturing buffer (SDS 4%, β-mercaptoethanol 2%, glycine 192 mM, Tris 25 mM, sucrose 5%), heated for 5 min at 100°C and recentrifuged at 12,000*g* for 15 min at 20°C. Finally, the pellets were discarded and the supernatants run on SDS–PAGE.

Western blotting. Proteins were separated in 15% SDS–PAGE, then transferred to nitrocellulose membranes (Biorad) using a 25 mM Tris, 192 mM Glycine, 10% Isopropanol transfer buffer at 400 mA and 100 V for 1 h. For immunoblotting, the membranes were blocked with 5% nonfat dried milk in PBS-Tween 20 1%, then incubated for 1 h at room temperature with anti PrP sera (1:1000), or monoclonal anti PrP antibodies (at the appropriate dilutions) in 0.05% nonfat dried milk in PBS-Tween 20 1%. After 3 washes in PBS-Tween 20 0.1%, the membranes were incubated in Peroxidase Conjugate Anti-Rabbit or Anti-Mouse IgG (1:2500) (Clinisciences) in PBS-Tween 20 0.1% for 30 min at room temperature. Blots were finally washed in PBS-Tween 20 0.1% and the bound antibodies visualized onto Biomax films (Kodak) by chemiluminescence (ECL kit, Amersham), or colored with DAB substrate (Roche). The PrPres were glycotyped by directly quantifying the signal intensities corresponding to the di-, mono-, and unglycosylated forms from the membranes scanned using the Fluor S MultiImager (BioRad). The values obtained for each band were expressed as percentages of the total signal. The means and SD for each individual PrPres sample, were calculated on the basis of five separate runs. The scores were statistically analysed using nonparametric tests. The reactivities of the sera were compared by an analysis of variance (ANOVA) at the 5% level.

Generation of antisera. Four-week-old prion protein-deficient Prn-$p^{0/0}$ female mice (kindly provided by C. Weissman, University of Zürich, Switzerland) were first immunized subcutaneously with 6.6 μg of recombinant ovine or murine PrPc in complete Freund's adjuvant, under native or denatured conditions, with PBS or guadinine hydrochloride 1 M heated for 5 min at 80°C (rediluted in PBS before inoculation), respectively. After three intraperitoneal boosters with the same antigens in incomplete Freund's adjuvant 21, 42, and 48 days after the initial inoculation, the mice were sacrificed after 51 days and the sera recovered.

ELISA. Indirect enzyme-linked immunosorbent assay (ELISA) was used to test a panel of antibodies as described elsewhere Polystyrene microdilution plates (Maxisorp. Nunc) were coated with 100 ng of purified recombinant prion protein per well, in 0.05 M carbonate buffer pH 9.6, previously denatured or not in guanidium hydrochloride (19). Briefly, samples of purified recombinant prion proteins were diluted to a final concentration of 4 M guanidium hydrochloride and heated 5 min at 80°C (denatured rPrP), before dilution in 0.05 M carbonate buffer at pH 9.6. The plates were incubated overnight at 4°C. After washing in PBS Tween 20 0.1%, followed by incubation of the plates with bovine serum albumine (BSA) 1% and gelatine 1% used as blocking reagents, 100 μl of each antibody was added per well in PBS-Tween 20 0.1%, gelatine 1% and BSA 1%, and at the same dilutions as in the Western blot studies, then incubated for 2 h at room temperature. After additional washings, anti-rabbit or murine IgG conjugated with horseradish peroxidase (1:5000) (Clinisciences) were added to each well. *O*-phenylenediamine was used for the detection and optical densities ($A_{492\ nm}$) were obtained with an automatic microtiter plate reader at 492 nm.

Antibodies. The antibodies used to characterize the recombinant proteins in the Western blot and ELISA techniques included: (i) rabbit antisera RS1, RB1 and RM1, prepared against the sheep, bovine and murine sequences respectively, corresponding to amino acids of the hypervariable region 95-110 of the human prion protein (THSQWNKPSKPKTNMK) (20). (ii) mAbs 4F2, 3B5, 8G8 and 12F10 produced against human recombinant PrP using DNA-mediated immunization techniques in $PrP^{0/0}$ mice (21), corresponding to amino acids 79–92 (3B5, 4F2), 95–110 (8G8), or 142–160 (12F10) of the human prion protein (kindly supplied by J. Grassi, SPI/CEA, Saclay, France). (iii) mAbs SAF 15, SAF 70 and SAF 84 obtained in $PrP^{0/0}$ mice immunized with SAFs (Scrapie Associated Fibrils) from infected hamster brain, and mAbs Pri 917 obtained by immunizing mice with synthetic human peptides (kindly supplied by J. Grassi, SPI/CEA, Saclay, France) (22). These recognized epitopes located in the 79–92 (SAF 15), 142–160 (SAF 70), 126–164 (SAF 84) or 214–230 (Pri 917) regions. The locations of the prion protein regions recognized by these antibodies are summarized in Fig. 1. (iv) antisera produced in prion-deficient Prn-$p^{0/0}$ mice (see § Generation of antisera) immunized with recombinant Gdn HCl treated or nontreated ovine PrPrec (for serum MSr2 and MSr1, respectively), and recombinant Gdn HCl treated or nontreated murine PrPrec (for serum MMr2 and MMr1, respectively).

RESULTS

Expression and Purification of His-PrP

The recombinant protein (rPrP) of 210 residues corresponding to the sheep Lys 24 to Ser 234 prion sequence expressed and purified from *E. coli* has been described (13). Large quantities of recombinant murine prion protein (mu rPrP) were produced in the same way in this study. Sequencing of the murine clone,

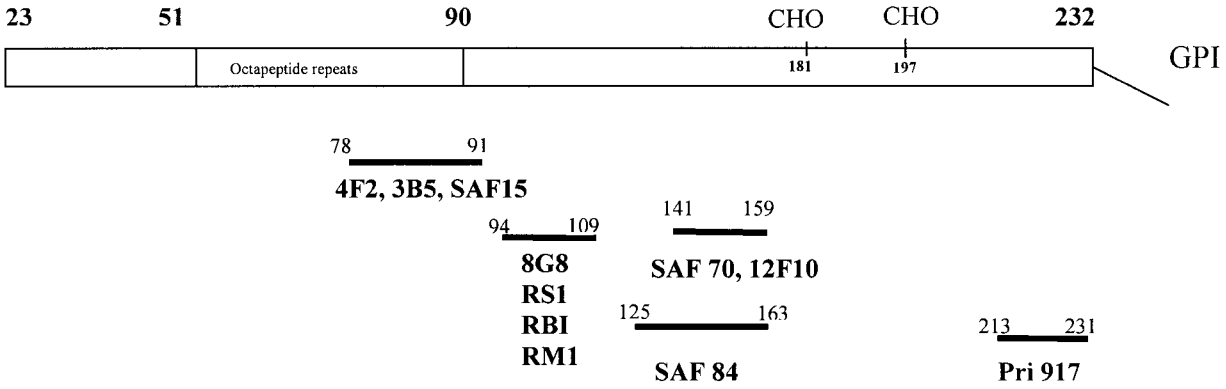

FIG. 1. Epitope mapping of anti PrP antibodies. Diagrammatic representation of epitope locations in the 23-232 PrP^{c} murine sequence.

produced from N2a neuroblastoma cells, demonstrated the sequence of the Prn-p^{a} allele of the prion gene, corresponding to a short incubation period in mice infected with most scrapie strains, with the amino acids L and T, at codon 108 and 189 respectively. A valine was noted at aa 177 instead of the aspartic acid reported in the sequence published by Wopfner (23).

The elution fractions obtained after purification of the protein induced from PrP::His-transformed bacterial clones of both ovine and murine origin contained two detectable bands with mobilities of 29 and 26 kDa, as shown by Coomassie blue staining on SDS–PAGE. (Fig. 2A). Both 29 and 26 kDa proteins were recognized, after Western blotting, by antibodies against the prion protein (Fig. 2B), and by a monoclonal antibody against the hexahistidine sequence (anti-His) (ascitic fluid 1:5000) (Clontech) (data not shown).

We thus obtained large amounts of sh rPrP (25–234) and mu rPrP (23–232) which accumulated in inclusion bodies. The protein from the inclusion bodies was solubilized in 8 M urea, and the recombinant proteins purified by single step metal affinity chromatography. In both cases, yields of about 20 mg of purified recombinant proteins were obtained per liter of bacterial culture.

Protease Sensitivity of His-PrP

No protein could be detected after proteinase K digestions by Western blotting (500 ng per lane) (data not shown) for either murine or sheep PrP (0.06 μg proteinase K/1 μg protein), thus supporting the notion that His-PrP contains the normal cellular isoform of PrP (PrP^{c}).

Recombinant Ovine and Murine Proteins Are Differently Recognized in ELISA by Polyclonal and Monoclonal Anti-PrP Antibodies

These mAbs were tested in ELISA with recombinant prion proteins diluted in ELISA dilution buffer (100 mM phosphate buffer pH 7.4 containing 150 mM NaCl, 0.1% bovine serum albumin (BSA), and 0.01% sodium azide). The observed signals were weak with both pro-

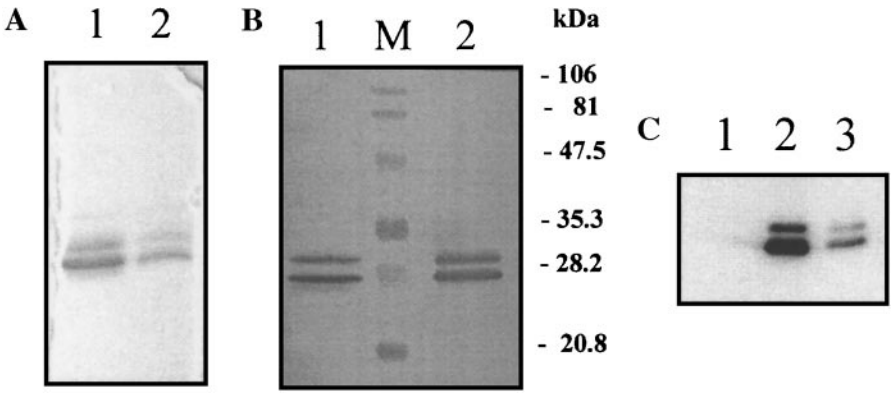

FIG. 2. Characterization of sheep (lane 1) and murine (lane 2) recombinant PrP. (A) Proteins were visualized by SDS-PAGE on a 15% polyacrylamide gel after Coomassie blue staining. (B) Western blot with RB1 antibody. Lane M, molecular weight markers. (C) Reactivity of different Histidine-Tag recombinant proteins. Purified recombinant BIV (lane 1), ovine PrP (lane 2) and murine PrP (lane 3) were analysed by Western blot with sera of $PrP^{0/0}$ mice immunized with recombinant GdnHCl treated ovine PrP (MSr2), and diluted 1:1000.

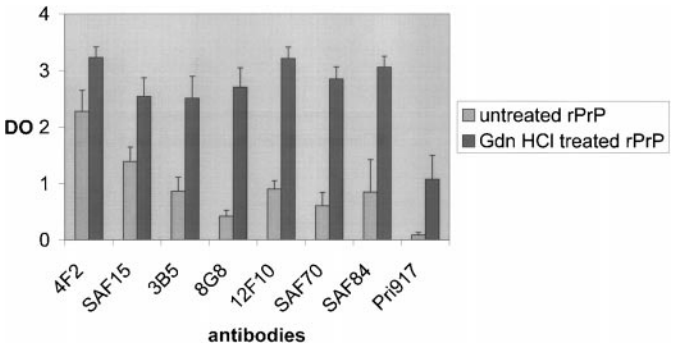

FIG. 3. Reactivities of monoclonal antibodies tested in ELISA against untreated or Gdn HCl treated ovine recombinant prion proteins.

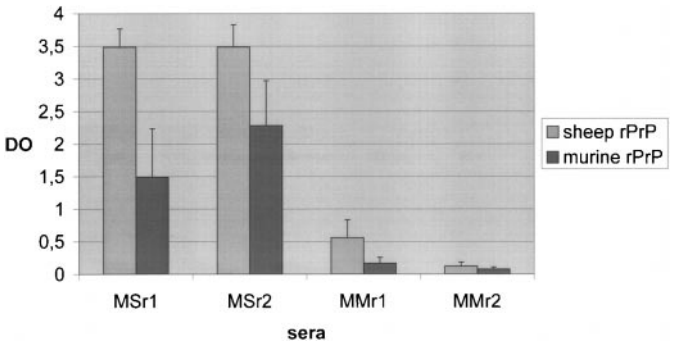

FIG. 5. Reactivities of mice $PrP^{0/0}$ polyclonal antibodies tested in ELISA with Gdn HCl treated ovine and murine recombinant prion proteins.

teins with some mAbs, as shown for the sheep rPrP (Fig. 3), so the recombinant prion proteins were denatured in guanidium hydrochloride for use in further ELISA studies. The results obtained with Gdn HCl treated ovine and murine PrP are shown in Fig. 4. Both recombinant proteins were recognized in ELISA with rabbit polyclonal antisera RS1, RB1 directed against the (98–113) sheep amino acid sequence and (106–121) bovine homologous amino acid sequence, respectively, but very faintly with rabbit polyclonal serum RM1 (94–109 murine homologous amino acid sequence). The signals were nevertheless consistently much higher with the ovine PrP rec.

Eight monoclonal antibodies recognized the sheep recombinant prion protein in all the regions tested, with a lower signal in the case of mAb Pri 917. The antibody reactivity against the murine recombinant prion protein was lower with all mAbs, but especially with mAbs 3B5, 8G8, 12F10, and Pri 917. Indeed, monoclonal antibodies 4F2 and SAF 15 (human PrP (hPrP) 79–92), SAF70 (hPrP 142–160), and SAF 84 (hPrP 126–164) gave similar high reactivities with both proteins.

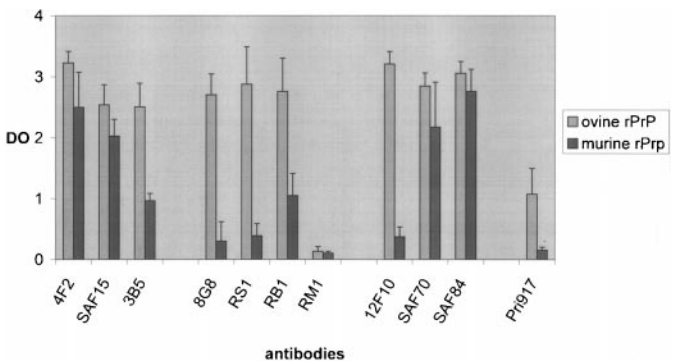

FIG. 4. Reactivities of monoclonal antibodies and rabbit antisera tested in ELISA with Gdn HCl treated ovine and murine recombinant prion proteins.

Generation of Antisera in Mice

We then attempted to produce mouse polyclonal antibodies against the two recombinant prion proteins in prion protein-deficient $Prn\text{-}p^{0/0}$ mice. These $Prn\text{-}p^{0/0}$ immunized mice developed a polyclonal antibody response against prion proteins depending on the recombinant protein, and on its chemical treatment (native or treated with guanidium hydrochloride).

The anti sheep sera, MSr1 (for **M**ouse inoculated with **S**heep untreated **r**ecombinant prion protein) and MSr2 (for **M**ouse inoculated with **S**heep Gdn HCl treated **r**ecombinant prion protein) strongly reacted in ELISA with both ovine and murine rPrP, more with the ovine PrP, whether the protein was denatured (MSr2) or not (MSr1) before immunization (Fig. 5). In Western blot (Fig. 2C), serum MSr2 showed the two bands previously described with serum RB1, when tested against ov rPrP (lane 2) and mu rPrP (lane 3). No signal was observed with mice sera obtained prior to immunization, thus confirming the specificity of the signal (data not shown).

Sera obtained after the immunization of mice with mu rPrP, MMr1 (for **M**ouse inoculated with **M**urine untreated **r**ecombinant prion protein) and MMr2 (for **M**ouse inoculated with **M** Gdn HCl treated **r**ecombinant prion protein), respectively, gave low reactivities in ELISA, especially when the mu rPrP was previously treated with guanidium hydrochloride. No signal was observed with mice sera obtained prior to inoculation (data non shown). A faint signal was observed in Western blot with serum of mouse inoculated with the recombinant murine prion protein, but only from mice immunized with non denatured PrP and only against the homologous murine PrPres (data not shown).

In addition, neither of the two anti-sheep sera showed any reactivity in ELISA (data not shown) and Western blot (Fig. 2C) with another His tagged protein of retrovirus, i.e., BIV capsid protein produced in our laboratory (24), showing that no antibodies directed against the His part of the recombinant protein were detectable in these sera.

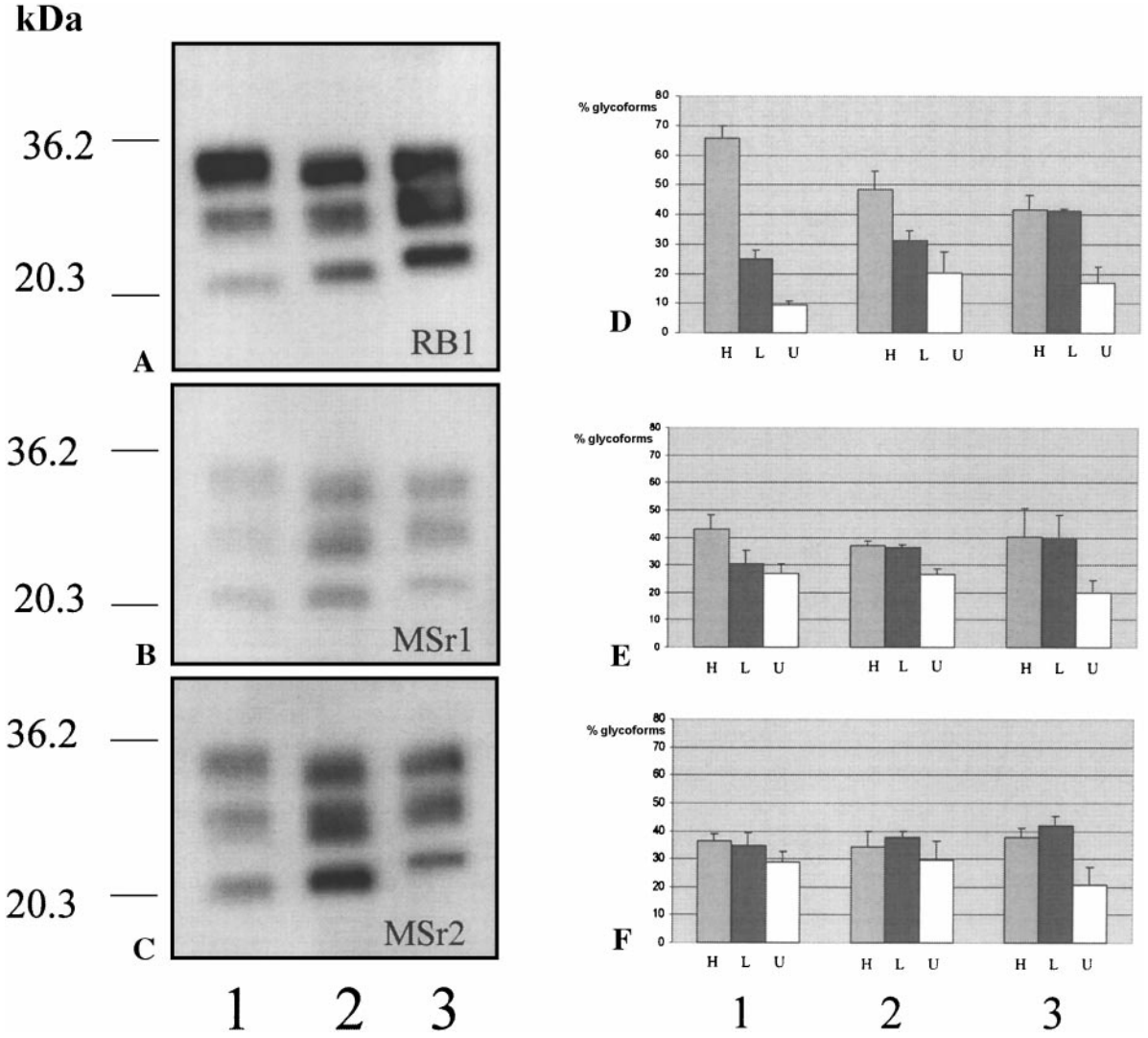

FIG. 6. Western blot studies (A, B, C) and glycosylation type (D, E, F) i.e., relative ratios of the three PrPres glycoforms of PrPres using antibodies against the prion protein RB1 (A, D), MSr1 (B, E), and MSr2 (C, F) obtained after Western blotting of bovine (1), ovine (2), and murine (3) PrPres. Results were quantified using a Fluor S MultiImager (Biorad), and expressed as mean + SD (standard deviation). H, high glycosylated; L, low glycosylated; and U, unglycosylated forms of PrPres.

Species and Molecular Specificities of Mouse Antisera

Sera of mice inoculated with native (MSr1) or denatured (MSr2) recombinant ovine prion protein clearly reacted in Western blot with PrP res of bovine, ovine and murine species, as did serum RB1 (Figs. 6A, 6B, and 6C). Comparable quantities of PrPres were loaded on each lane to compare the signals identified on the Fluor S Imager for antisera RB1, MSr1 and MSr2. A stronger reactivity was observed against ovine PrPres with sera against sheep recPrP, thus showing the higher species-specificity of the MSr1 and MSr2 sera, compared with RB1. We then analysed the glycotypes, i.e., the relative ratios of the three PrPres glycoforms obtained after Western blotting with antisera RB1, MSr1 and MSr2 against bovine, ovine and murine PrPres (Fig. 6B). Different profiles were obtained for bovine and ovine PrPres, between rabbit antisera RB1 and the murine antisera MSr1 and MSr2. These differences were statistically highly significant at the 5% level (for example, $P = 0.0001$, 0.003, and 0.0001 for the diglycosylated, monoglycosylated and unglycosylated forms of the bovine isolate respectively, and $P = 0.0008$ and 0.0013 for the diglycosylated and monoglycosylated forms of the ovine isolate, respectively). Both sera obtained with untreated or Gdn-HCl-treated sheep rec PrP recognized the unglycosylated and monoglyosylated forms better and the diglycosylated less well than did the antiserum RB1 (Figs. 6D–6F). In contrast no statistically significant differences in the glycoform ratios at the 5% level were observed for the murine PrPres. Comparative analysis of the electrophoretic profiles could not be obtained for antiserum

against murine PrP rec, on account of the weak signals noticed in Western blot.

The apparent molecular mass of the unglycosylated PrPres from the BSE isolate was slightly lower than that of the scrapie isolate, which was also lower than that observed in the murine scrapie C506M3 PrPres (Figs. 6A, 6B, and 6C), whatever the sera used.

DISCUSSION

Our aim here was to study the immunological characterization of recombinant sheep and mouse prion proteins produced in *E. coli*. Both proteins were produced as the whole sequence of mature PrPc, fused with a hexahistidine sequence at the C-terminal end of the protein, as described previously for the sheep protein (13). Whereas this latter was shown to correspond to the PrP A_{136} R_{154} Q_{171} allele (13) at the three main susceptibility codons for natural scrapie, sequencing of the murine clone showed that the Prn-p^a allele of the prion gene corresponded to a short incubation period with most scrapie strains (25), with the amino acids L and T at codons 108 and 189 respectively. It is presently unknown whether the 177 Val amino acid, rather than the 177 Asp reported in previously published sequences (23), was initially encoded in the Prn-p gene of the N2a originating cells.

These proteins were first characterized using a series of monoclonal or polyclonal antibodies against different regions of the protein spanning the PrP sequence from the octo-repeat region to its C-terminal end. The sh rPrP was strongly recognized in ELISA by all antibodies except Pri 917 Mab, which showed a lower reactivity, and RM1 that almost failed to react. Among the antibodies against the octapeptide region (4F2, SAF 15, 3B5), MAb 4F2 also gave a strong signal with both sheep and murine proteins, consistent with previously obtained results (21). In contrast, 8G8, 12F10 and Pri 917 showed little reaction with the murine protein. The mAb 8G8 antibody (directed against the human 95–110 sequence), had already been shown to detect cattle, sheep and human proteins but failed to bind any other mammalian cellular prion proteins tested by Krasemann (21). Antisera RS1 and RB1 produced against this same sequence, from sheep or cattle respectively, also showed moderate or faint reactivity with this mouse protein. Different antibody reactivities, depending on the species-specific sequence, have already been described within this hypervariable region of the protein (26) that could explain these results with RS1, RB1 and 8G8, obtained from sheep, bovine and human PrP sequences respectively, whereas the lack of reactivity of RM1 is clearly explained by the low titer of this antiserum against the murine peptidic sequence (13). A polymorphic site (amino acid 108 distinguishing mouse prn^a and prn^b alleles), is also located within this region, which differs from the methionine encoded in both human, sheep and cattle prion genes. As for the 12F10 mAb produced against human recombinant PrP that recognizes the 142–160 human sequence, Krasemann (21) observed faint signals in Western blot against different species which included ovine, bovine, murine, hamster and human cellular prion protein. However, SAF 70 mAb directed against this same region, produced using hamster SAF, reacted more strongly in ELISA with recombinant murine protein than 12 F10. We should emphasize that the hamster sequence is closer to the murine sequence (1 amino acid different) in the region 142–160, than the human sequence (3 amino acids different).

The recombinant proteins in our study were unfolded with guanidine hydrochloride (Gdn HCl) before the ELISA, as described by Safar for the detection of PrPres (19), since low reactivities with certain antibodies had been obtained in preliminary tests with a buffer containing 0.1% bovine serum albumin (BSA). These data contrast with most previous ELISA studies of recombinant proteins. Peretz (27) noted the same reactivity for native and denatured SHa recombinant prion proteins (90–231) treated with GdnHCl, except for two recombinant monoclonal antibodies (D13 and D4) reacting with the 95–104 region. Safar also observed similar reactivities of denatured and native recombinant SHa 90–231 recombinant protein with the 3F4 monoclonal antibody directed against the 95 and 98 amino-acids of the Syrian hamster protein. However, to the best of our knowledge, only Brown (25) used a full-length murine recombinant protein in ELISA, as in our study. Evidence of conformational plasticity of recombinant PrP, due to the unfolding of PrP with GdnHCl, was shown with hamster PrP (28), that could modify the accessibility of the epitopes depending on whether the protein was denatured or native. Alternatively, different aggregation states of the protein could also modify the results of ELISA studies according to the environmental conditions of the recombinant proteins.

We then used both recombinant proteins to produce mouse antibodies in PrP$^{0/0}$ mice. Whereas immunisation with sheep recombinant protein readily produced antisera that strongly reacted with both sheep and murine recombinant proteins, the mouse protein was poorly immunogenic. It had previously been shown that PrP$^{0/0}$ mice produced antibodies when immunised with purified mouse or hamster SAFs as deletion of the PrP gene abrogated the immunotolerance of mice (12). By immunizing PrP$^{0/0}$ mice with recombinant murine prion protein (PrP^c), Zanusso (29) also obtained a panel of mAbs specific for the murine 23–231 sequence; most of them recognizing epitopes located within the C-terminal region of the protein (145–220). Our data confirm that null mice can be used to produce antibodies against PrP, but the reason why murine PrP is less immunogenic is not clearly understood. However, in

line with previous studies (29), the two antisera MSr1 and MSr2 against sheep PrP also showed a marked species-specificity with homologous ovine PrPres suggesting that antibodies preferentially recognize regions of the prion protein different in sheep and mouse. Sera obtained after inoculation of $PrP^{0/0}$ mice with Gdn HCl treated recombinant prion proteins reacted more strongly with PrPres in Western blot. Since previous works showed that certain antibodies reacted poorly against the untreated proteins, it is possible that, when mice are immunized with Gdn HCl treated PrP, either a large number of epitopes are also immunogenic in mice, or some of the epitopes are more strongly immunogenic.

These two antisera against sheep rec PrP recognize the various PrP glycoforms of pK resistant PrP differently, compared to the RB1 antiserum against a bovine 106–121 PrP peptidic sequence. RB1 antiserum preferentially recognizes the highly glycosylated form of bovine or sheep PrP res, but reacts weakly with the unglycosylated isoform, as previously described for RS1 antiserum against the sheep homologous sequence (30). It has been proposed that the predominance of diglycosylated PrPres is a signature of BSE in humans and in other species (31, 32). Here, we show that antisera against sheep PrP rec reacted more faintly with the diglycosylated PrPres of ruminants, whereas similar profiles were observed with the mouse PrPres compared to those observed with RB1 antiserum. Although different reactivities of different monoclonal antibodies have already been described for the three glycoforms of the cellular form of PrP, some differences depending on the species origin of PrP^{c} have been observed (29). Some differences in the glycoform ratio, depending on the antibodies used, have also been described for the pK resistant form of PrP (33). It was proposed that such differences in PrPres from sheep with scrapie compared to bovines with BSE could lead to different abilities of different antibodies in distinguishing prion strains. In our study, we observed differences similarly affecting PrPres both from cattle with BSE and from sheep with natural scrapie, but not at all the PrPres from mice infected with a scrapie strain. These results suggest that antibodies specified-differences in the glycoform ratios of PrP res may be species-dependent rather than indicative of the involved prion strain. In contrast, both antisera showed the same differences in the apparent molecular mass of PrP res, that was shown to be indicative of differences, at least between BSE and some scrapie strains, in the site of cleavage with proteinase K and possibly associated with different strain-specified conformations of the prion protein (34).

In conclusion, potent antibodies can be raised in mice to pathological bovine, ovine and murine PrP using recombinant proteins and immunization of $PrP^{0/0}$ mice. These antibodies could serve as sensitive and specific probes for the detection and characterization of PrP in TSEs.

ACKNOWLEDGMENTS

We gratefully thank S. Philippe for statistical analysis of the data and Diana Warwick for careful reading of the manuscript.

REFERENCES

1. Prusiner, S. B. (1998) Prions. *Proc. Natl. Acad. Sci. USA* **95,** 13363–13383.
2. Hornemann, S., Korth, C., Oesch, B., Riek, R., Wider, G., Wüthrich, K., and Glockshuber, R. (1997) Recombinant full-length murine prion protein, mPrP (23-231): Purification and spectroscopic characterization. *FEBS Lett.* **413,** 277–281.
3. Donne, D. G., *et al.* (1997) Structure of the recombinant full-length hamster prion protein PrP (29–231): The N-terminus is highly flexible. *Proc. Natl. Acad. Sci. USA* **94,** 13452–13457.
4. Negro, A., De Filippis, V., Skaper, D., James, P., and Sorgato, M. C. (1997) The complete mature bovine prion protein highly expressed in Escherichia coli: Biochemical and structural studies. *FEBS Lett.* **412,** 359–364.
5. Zahn, R., *et al.* (2000) NMR solution structure of the human prion protein. *Proc. Natl. Acad. Sci. USA* **97,** 145–150.
6. Hornemann, S., and Glockshuber, R. (1996) Autonomous and reversible folding of a soluble amino-terminally truncated segment of the mouse prion protein. *J. Mol. Biol.* **261,** 614–619.
7. Swietnicki, W., Petersen, R., Gambetti, P., and Surewicz, W. K. (1997) pH-dependent stability and conformation of the recombinant human prion protein PrP(90-231) [In Process Citation]. *J. Biol. Chem.* **272,** 27517–27520.
8. Lopez Garcia, F., Zahn, R., Riek, R., and Wuthrich, K. (2000) NMR structure of the bovine prion protein. *Proc. Natl. Acad. Sci. USA* **97,** 8334–8339.
9. Mehlhorn, I., *et al.* (1996) High-level expression and characterization of a purified 142-residue polypeptide of the prion protein. *Biochemistry* **35,** 5528–5537.
10. Riek, R., Hornemann, S., Wider, G., Glockshuber, R., and Wüthrich, K. (1997) NMR characterization of the full-length recombinant murine prion protein mPrP(23-231). *FEBS Lett.* **413,** 282–288.
11. Büeler, H., *et al.* (1992) Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein [see comments]. *Nature* **356,** 577–582.
12. Prusiner, S. B., *et al.* (1993) Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. *Proc. Natl. Acad. Sci. USA* **90,** 10608–10612.
13. Baron, T., Betemps, D., Martin, H., Groschup, and Madec, J.-Y. (1999) Immunological characterization of the sheep prion protein expressed as fusion proteins in *Escherichia coli. FEMS Immunol. Med. Microbiol.* **25,** 379–384.
14. Cheynet, V., Verrier, B., and Mallet, F. (1993) Overexpression of HIV-1 proteins in *Escherichia coli* by a modified expression vector and their one-step purification. *Protein Exp. Purif.* **4,** 367–372.
15. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) (C.S.H.L.P., Ed.), USA.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.
17. Lasmézas, C. I., Deslys, J. P., Demaimay, R., Adjou, K. T., Hauw, J. J., and Dormont, D. (1996) Strain specific and common patho-

genic events in murine models of scrapie and bovine spongiform encephalopathy. *J. Gen. Virol.* **77,** 1601–1609.

18. Clavijo, A., and Thorsen, J. (1995) Bacterial expression of the caprine arthritis-encephalitis virus gag and env proteins and their use in enzyme-linked immunosorbent assay. *Am. J. Vet. Res.* **56,** 841–848.
19. Safar, J., Wille, H., Itrri, V., Groth, D., Serban, H., Torchia, M., Cohen, F. E., and Prusiner, S. B. (1998) Eight prion strains have PrPSc molecules with different conformations. *Nat. Med.* **4,** 1157–1165.
20. Madec, J.-Y., Vanier, A., Dorier, A., Bernillon, J., Belli, P., and Baron, T. (1997) Biochemical properties of protease resistant prion protein PrPsc in natural sheep scrapie. *Arch. Virol.* **142,** 1603–1612.
21. Krasemann, S., Groschup, M. H., Harmeyer, S., Hunsmann, G., and Bodemer, W. (1996) Generation of monoclonal antibodies against human prion proteins in PrP-0-0 mice. *Mol. Med. (Cambridge)* **2,** 725–734.
22. Demart, S., *et al.* (1999) New insight into abnormal prion protein using monoclonal antibodies. *Biochem. Biophys. Res. Commun.* **265,** 652–657.
23. Wopfner, F., Weidenhofer, G., Schneider, R., vonBrunn, A., Gilch, S., Schwarz, T. F., Werner, T., and Schatzl, M. (1999) Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *J. Mol. Biol.* **289,** 1163–1178.
24. Betemps, D., Mallet, F., Cheynet, V., and Baron, T. (1999) Overexpression and purification of an immunologically reactive His-BIV capsid fusion protein. *Protein Expr. Purif.* **15,** 258–264.
25. Brown, D. R., *et al.* (2000) Functional and structural differences between the prion protein from two alleles prnp(A) and prnp(B) of mouse. *Eur. J. Biochem.* **267,** 2452–2459.
26. Groschup, M. H., and Pfaff, E. (1993) Studies on a species-specific epitope in murine, ovine and bovine prion protein. *J. Gen. Virol.* **74,** 1451–1456.
27. Peretz, D., *et al.* (1997) A conformation transition at the N terminus of the prion protein features in formation of the scrapie isoform. *J. Mol. Biol.* **273,** 614–622.
28. Zhang, H., Stockel, J., Mehlhorn, I., Groth, D., Baldwin, M. A., Prusiner, S. B., James, T. L., and Cohen, F. E. (1997) Physical studies of conformational plasticity in a recombinant prion protein. *Biochemistry* **36.**
29. Zanusso, G., *et al.* (1998) Prion protein expression in different species: Analysis with a panel of new mAbs. *Proc. Natl. Acad. Sci. USA* **95,** 8812–8816.
30. Baron, T. G. M., Madec, J.-Y., and Calavas, D. (1999) Similar signature of the prion protein in natural sheep scrapie and bovine spongiform encephalopathy-linked diseases. *J. Clin. Microbiol.* **37,** 3701–3704.
31. Collinge, J., Sidle, K. C. L., Meads, J., Ironside, J., and Hill, A. F. (1996) Molecular analysis of prion strain variation and the aetiology of "new variant" CJD. *Nature* **383,** 685–690.
32. Hill, A. F., Sidle, K. C. L., Joiner, S., Keyes, P., Martin, T. C., Dawson, M., and Collinge, J. (1998) Molecular screening of sheep for bovine spongiform encephalopathy. *Neurosci. Lett.* **255,** 159–162.
33. Sweeney, T., Kuczius, T., McElroy, M., Parada, M. G., and Groschup, M. H. (2000) Molecular analysis of irish sheep scrapie cases. *J. Gen. Virol.* **81,** 1621–1627.
34. Baron, T., and Biacabe, A. G. (2001) Molecular analysis of the abnormal prion protein during coinfection of mice by bovine spongiform encephalopathy agent and scrapie strain. *J. Virol.*, in press.