PrP glycoforms are associated in a strain-specific ratio in native PrP\textsuperscript{Sc}

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Prion diseases involve conversion of host-encoded cellular prion protein (PrP\textsuperscript{C}) to a disease-related isoform (PrP\textsuperscript{Sc}). Using recombinant human \(\beta\)-PrP, a panel of monoclonal antibodies was produced that efficiently immunoprecipitated native PrP\textsuperscript{Sc} and recognized epitopes between residues 93–105, indicating for the first time that this region is exposed in both human vCJD and mouse RML prions. In contrast, monoclonal antibodies raised to human \(\alpha\)-PrP were more efficient in immunoprecipitating PrP\textsuperscript{C} than PrP\textsuperscript{Sc}, and some of them could also distinguish between different PrP glycoforms. Using these monoclonal antibodies, the physical association of PrP glycoforms was studied in normal brain and in the brains of humans and mice with prion disease. It was shown that while PrP\textsuperscript{C} glycoforms can be selectively immunoprecipitated, the differentially glycosylated molecules of native PrP\textsuperscript{Sc} are closely associated and always immunoprecipitate together. Furthermore, the ratio of glycoforms comprising immunoprecipitated native PrP\textsuperscript{Sc} from diverse prion strains was similar to those observed on denaturing Western blots. These studies are consistent with the view that the proportion of each glycoform incorporated into PrP\textsuperscript{Sc} is probably controlled in a strain-specific manner and that each PrP\textsuperscript{Sc} particle contains a mixture of glycoforms.

INTRODUCTION

The prion diseases, which include Creutzfeldt–Jakob disease (CJD) in humans, and scrapie and bovine spongiform encephalopathy (BSE) in animals, are a group of fatal, transmissible, neurodegenerative conditions. The human prion diseases are unique in that they may have a sporadic, inherited or transmitted aetiology (Collinge, 2001), and there is evidence that BSE-like prions have infected humans (Collinge et al., 1996; Bruce et al., 1997; Hill et al., 1997). A common feature of all prion diseases is the post-translational conversion of the normal cellular prion protein (PrP\textsuperscript{C}), a host-encoded, glycosylphosphatidylinositol (GPI)-anchored sialoglycoprotein (Stahl et al., 1987), into an abnormal isoform termed PrP\textsuperscript{Sc} (Prusiner, 1991). There are no known covalent post-translational modifications between PrP\textsuperscript{C} and PrP\textsuperscript{Sc} (Stahl et al., 1993), the difference between the two being conformational. While PrP\textsuperscript{C} can be completely digested by the serine protease, proteinase K (PK), only 90–100 aa are removed from the N terminus of PrP\textsuperscript{Sc} by PK, depending upon prion strain-type, leaving a large protease resistant C-terminal fragment. Both circular dichroism and Fourier transform infrared spectroscopic methods show PrP\textsuperscript{C} to be rich in \(\alpha\)-helix, whereas PrP\textsuperscript{Sc} is predominantly \(\beta\)-sheet (Pan et al., 1993). It has been postulated that PrP\textsuperscript{Sc} may act as a template promoting the conversion of PrP\textsuperscript{C} into PrP\textsuperscript{Sc}. Using PrP-specific monoclonal antibodies (mAbs), it has been reported that residues 90–120, accessible in PrP\textsuperscript{C}, are largely cryptic in the protease resistant core of PrP\textsuperscript{Sc} (denoted PrP\textsuperscript{127–130}) and only become exposed after denaturation (Peretz et al., 1997). Using the same mAbs and recombinant Syrian hamster (SHA) PrP\textsuperscript{23–231}, it was suggested that residues 90–115 are involved in the conformational rearrangement of PrP\textsuperscript{Sc} formation (Leduc et al., 2001).

Similar NMR structures of PrP\textsuperscript{C} have been reported for mouse (Riek et al., 1996), hamster (James et al., 1997) and human (Hosszu et al., 1999a; Zahn et al., 2000) recombinant protein residues \(\sim 120–231\). In \textit{vitro} studies have shown that PrP can adopt different conformations depending upon solvent pH and redox potential (Swietnicki et al., 1997; Hornemann & Glockshuber, 1998). At low pH following reduction of the disulphide bond, the folded C-terminal domain of the human prion protein can exist as a soluble monomeric \(\beta\)-sheet structure (Jackson et al., 1999a). It is possible that the conversion of the \(\alpha\)-helical form, \(\alpha\)-PrP, to the \(\beta\)-sheet form, \(\beta\)-PrP, caused by reduction and mild acidification is relevant to the conditions that PrP\textsuperscript{C} might encounter within the cell (Shyng et al., 1993).
Distinct prion strains can be serially propagated, producing characteristic clinical features and neuropathology in defined hosts. Although the molecular basis of strains is unknown, PrPSc can be distinguished by differing physicochemical properties of the protein (Bessen & Marsh, 1992; Collinge et al., 1996). After PK digestion of brain homogenates from different CJD clinical phenotypes, variation in PrPSc fragment length has been observed (Collinge et al., 1996; Parchi et al., 1996) and glycosylation can also be used to further distinguish strains (Collinge et al., 1996). PrP is post-translationally modified by the addition of two N-linked glycosylation sites at positions 181 and 197 in the human sequence, either or both of which can be occupied to give monoglycosylated or diglycosylated isoforms, or remain unglycosylated. The type and combination of sugar molecules can produce a high degree of heterogeneity (Endo et al., 1989), potentially affecting the conformation and intermolecular interactions. Crucially, both PrPSc fragment size (Collinge et al., 1996; Telling et al., 1996) and glycoform ratios (Collinge et al., 1996), following PK cleavage, can be maintained on serial passage in experimental animals, arguing that these intrinsic properties of PrPSc may encode prion strain diversity. Several human PrPSc types have been identified that are associated with different phenotypes of CJD (Parchi et al., 1996). Based both on PrPSc fragment sizes and the ratios of the three PrP glycoforms, four different PrPSc types are readily distinguishable (Collinge et al., 1996, 1997; Wadsworth et al., 1999; Hill & Collinge, 2002).

Although all the PrP glycoforms are present in PrPSc, it is not known if they can be physically or chemically separated, except when denatured, since existing mAbs do not readily immunoprecipitate PrPSc, i.e. we cannot yet determine whether a single PrPSc particle contains mixed glycoforms or only a single species. Prnp0/0 mice have been used by many groups to raise anti-PrP mAbs (Krasemann et al., 1996; Korth et al., 1997; Zanusso et al., 1998; Williamson et al., 1998; Nakamura et al., 2003), but mAbs that can readily immunoprecipitate PrPSc or recognize different glycoforms of PrP are rare. We have also used Prnp0/0 mice to raise anti-PrP mAbs, using a soluble a-helical monomer of the human PrP sequence 91–231 (a-PrP91–231) as antigen. We also used the novel immunogenic recombinant human b-PrP91–231, identical in amino acid sequence but folded into a soluble monomeric conformation and partially resistant to PK (Jackson et al., 1999b), as antigen and produced mAbs that could efficiently immunoprecipitate PrPSc. Here, we describe these mAbs and the findings on the physical association of PrP glycoforms in normal and prion-diseased brains. We also show that mAbs raised to b-PrP91–231 and reactive to residues 93–105, can immunoprecipitate native PrPSc.

**METHODS**

**mAb production.** mAbs were produced in Prnp0/0 mice (Bueler et al., 1993) back-crossed onto an FVB/N background. Briefly, the mice were immunized subcutaneously with 100 µg human recombinant a- or b-PrP91–231 (Jackson et al., 1999b) in complete Freund’s adjuvant and in incomplete Freund’s adjuvant on days 21 and 42, then boosted intraperitoneally on day 50 with 50 µg antigen in PBS. Three days later, single-cell suspensions of spleenocytes were prepared and fused with the NS0 myeloma cell line, using conventional methodology. Hybridomas were selected after screening culture supernatants by ELISA against a- and b-PrP91–231, by dot blot against human and mouse brain homogenates and by flow cytometry for recognition of human and mouse PrPSc (data not shown). We studied mAbs ICSM-4, ICSM-10 and ICSM-18 that were produced from immunization with a-PrP91–231, and ICSM-35 and ICSM-37 produced from immunization with b-PrP91–231. After affinity purification using protein-A or G-Sepharose column chromatography, the antibodies were isotypically adapted with a polyclonal of anti-mouse Ig subclass-specific mAbs (Pierce). The mAb epitopes were mapped by ELISA, using IgG at 1–2 µg ml–1 and human 20 aa synthetic peptides, spanning the PrP sequence 91–231. Animal care conformed to national and institutional guidelines.

**Preparation of brain homogenates.** Brain homogenates (10% w/v) from un inoculated Prnp0/0, wild-type and RML-prion terminally scrapie-ill Prnp0/0/FVB/N mice were prepared, either in cold lysis buffer (PBS, 0.5% sodium deoxycholate, 0.5% NP40, pH 7.4) or in Dulbecco’s Mg2+/Ca2+-free PBS (Gibco-BRL). Normal human and CJD brains were homogenized to 10% (w/v) in Mg2+/Ca2+-free PBS. The brain homogenates prepared in PBS were diluted 1:2 with 4% N-laurylsarcosine, and after 10 min at 37 °C, DNase (Benzonase; 50 U ml–1) (Roche) and 1 mM MgCl2 were added and further incubated for 30 min at 37 °C, then diluted in PBS (PBS, 0.1% (w/v) Tween 20), and used as antigen. To denature PrPSc, the brain homogenate, 1% (w/v) in 4% (w/v) SDS, was incubated at 100 °C for 14 min then diluted in PBST.

**Western blotting.** The brain homogenates were electrophoresed through 16% gels using conventional methods. Following transfer onto Immobilon-P membranes (Millipore), non-specific binding was blocked with 5% (w/v) milk proteins in PBST, and membranes were diluted 1:2 with 4% SDS, were placed in cold lysis buffer (PBS, 0.5% sodium deoxycholate, 0.5% NP40, pH 7.4) or in Dulbecco’s Mg2+/Ca2+-free PBS (Gibco-BRL). Normal human and CJD brains were homogenized to 10% (w/v) in Mg2+/Ca2+-free PBS. The brain homogenates prepared in PBS were diluted 1:2 with 4% N-laurylsarcosine, and after 10 min at 37 °C, DNase (Benzonase; 50 U ml–1) (Roche) and 1 mM MgCl2 were added and further incubated for 30 min at 37 °C, then diluted in PBS (PBS, 0.1% (w/v) Tween 20), and used as antigen. To denature PrPSc, the brain homogenate, 1% (w/v) in 4% (w/v) SDS, was incubated at 100 °C for 14 min then diluted in PBST.

**Immunoprecipitation.** Immunoprecipitation was carried out either directly, using antibody covalently cross-linked to beads to capture PrP, or indirectly, using beads to capture antibody–antigen immune complexes. Diseased and normal brain homogenates were used, matched for their PrP signal.

For the direct method, protein-A Dynabeads or M-280 streptavidin beads (Dynal Biotech) were used. Protein-A beads have a maximum binding capacity of mouse IgG 100 µg beads ml–1 (~2.7 x 108 beads ml–1). For maximum binding, we incubated 100 µg IgG per 100 µl beads, and the immobilized immunoglobulin was cross-linked to the beads using 20 mM dimethyl pimelamide (Sigma-Aldrich). M-280 streptavidin beads (binding capacity of 50–100 µg biotinylated-IgG ml–1) were used with biotinylated mAbs. mAbs IgG were biotinylated by incubating 500 µl IgG (1 mg ml–1) with Biotin-LC-NHS ester (2 mg ml–1) (IGEN International), at a 1:20 molar ratio, and quenching the reaction with 20 µl 2 M glycine. Free biotin was removed using a PD-10 column (Amersham Bioscience). To allow
binding of both high and low affinity mAbs, IgG-adsorbed streptavidin or cross-linked protein-A beads (20–25 μl) were incubated with 100–200 μl of 0.5–0.1% (w/v) brain homogenates for 8–12 h at 4°C, then washed briefly, 6–8 times in PBST (1% Tween 20). To determine the PK-resistance of the bead-adsorbed PrP, these beads were treated with PK (20 μg ml⁻¹ for mouse and 50 μg ml⁻¹ for human PrPC), for 1 h at 37°C, and the reaction was stopped with 20 mM PMSF, or AEBSF [4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochlo- ride] (Roche). For a direct capture of PrPC²⁻³⁵, the brain homogenates were treated with PK before immunoprecipitation.

The indirect immunoprecipitation method was carried out by incubating anti-PrP mAbs IgG at a concentration of 2–3 μg ml⁻¹ with brain homogenate [200 μl 0.5–0.1% (w/v)] containing a complete protease inhibitor cocktail (Roche), for 12–14 h at 4°C to allow even the weak antibodies to bind. Protein-G Dynabeads (20–25 μl) (Dynal Biotech), with a binding capacity of IgG ~ 200 μg ml⁻¹, were then added for 2 h at room temperature, to immunoprecipitate the immune complexes. The beads were then washed four times with PBST (2% Tween 20), four times with PBS containing 2% Tween 20 and 2% NP40, and once with PBS to remove the detergents. The captured PrP was visualized by Western blot.

Adsorption of PrP to beads was optimized by varying the incubation time, temperature, adsorption buffer, salt concentration, pH and detergent. Both isotype control negative mAb- and PBS-adsorbed beads were negative when using normal brain, but a small amount of non-specific binding occurred with scrapie-affected brain homogenate. To reduce this non-specific binding, beads were negative when using normal brain, but a small amount of non-specific binding occurred with scrapie-affected brain homogenate. The other form, denoted PrPC, had been refolded in the reduced state, i.e. with the disulphide bridge intact, and adopts the predominantly α-helical PrPC conformation. The PrPC form of the protein is in the normal cellular conformation and is characterized by being highly sensitive to digestion by PK. The other form, denoted β-PrP, is refolded in the reduced state, i.e. with the disulphide bridge broken. The physical properties of β-PrP share some similarity with ex-vivo PrPSc. The PrPSc form of the protein accumulates in prion-infected brain tissue, and is characterized by its resistance to proteolytic degradation by PK.

Three of the five antibodies (ICSM-4, ICSM-10 and ICSM-18) were raised to α-PrP and two (ICSM-35 and ICSM-37) to β-PrP (Table 1). As previously described (Khalili-Shirazi et al., 2005) using a peptide ELISA, ICSM-18 recognized PrP residues 142–153, ICSM-35 residues 93–105 and ICSM-37 residues 97–105. ICSM-4 and ICSM-10 could not be epitope-mapped by peptide ELISA. The isotype of ICSM-4, ICSM-10 and ICSM-18 was IgG1, ICSM-35 was IgG2b and ICSM-37 was IgG2a (data not shown).

**Anti-PrP mAbs raised to α-PrP differentiate denatured PrP glycoforms in normal and diseased states**

When either PrPC or PrPSc were subjected to Western blot analysis, i.e. the proteins are in a denatured state after SDS-PAGE.
treatment, ICSM-18, ICSM-35 and ICSM-37 recognize three PrP bands corresponding to the unglycosylated, monoglycosylated and diglycosylated forms (Fig. 1a). Immunization with α-PrP produced ICSM-4 and ICSM-10 that could differentiate between the three PrP glycoforms, whereas none of the mAbs produced to β-PrP could discriminate between them. ICSM-4 can only recognize the unglycosylated form of PrP and detects just a single, principal band on Western blots of both normal and infected brain homogenates of PrP<sup>Sc</sup> and PrP<sup>Sc</sup>, either before or after digestion with PK (Table 1 and Fig. 1a). ICSM-10 was characterized as binding to both the unglycosylated and monoglycosylated bands, but not the diglycosylated species (Table 1 and Fig. 1a). Although ICSM-4 and ICSM-10 were negative in the peptide ELISA, their epitopes are likely to span areas around residues 181 and 197, the known sites of N-linked glycosylation of PrP.

The ratio of PrP glycoforms is a consistent feature of different prion strains, which can be differentiated by Western blotting with mAb 3F4 (Wadsworth et al., 2003). ICSM-35 (epitope 93–105) (Khalili-Shirazi et al., 2005), like 3F4 (epitope 104–113) (Kanyo et al., 1999), also recognized all three glycoforms of PrP in the correct proportions in Western blots of human CJD brains, containing PrP<sup>Sc</sup> types 1–4 (Fig. 1b). All of the negative control mAbs, as well as the controls in which primary antibody is omitted, were negative (data not shown). Anti-PrP mAbs were also negative when tested against Prnp<sup>0/0</sup> brain homogenates (data not shown).

In wild-type mouse brain homogenate, a truncated PrP band ~18 kDa was also recognized by ICSM-4, ICSM-10 and weakly by ICSM-18 and ICSM-37, and a truncated band at 20–22 kDa was recognized in scrapie-infected mouse brain homogenate by all the mAbs (Fig. 1a). Both the 18 kDa and 20–22 kDa truncated bands were PK-sensitive and, since they were both recognized by ICSM-4, they are probably unglycosylated. A PK-sensitive 18 kDa truncated band (C1) and a PK-resistant 20–22 kDa band (C2) in CJD brain have been reported previously (Chen et al., 1995), as have minority C-terminal smaller fragments that would not be visualized by the majority of our antibodies with more N-terminal epitopes.

**PrP glycoforms are associated in native PrP<sup>Sc</sup> but not in PrP<sup>C</sup>**

To investigate whether an individual PrP<sup>Sc</sup> particle in infected brain consists of a single glycoform or a mixture, we used our glycoform-specific mAbs, ICSM-4 and ICSM-10, to capture PK-resistant PrP from infected brain homogenates. The unique properties of ICSM-4 and ICSM-10 allowed the individual PrP glycoforms, which comprise native PrP<sup>C</sup>, to be selectively captured from homogenates of normal murine brain. ICSM-4 immunoprecipitated a single band corresponding to the unglycosylated form of PrP, whereas ICSM-10 captured two principal bands corresponding to the unglycosylated and monoglycosylated isoforms (Fig. 2a). In contrast, immunoprecipitation from scrapie-infected homogenates by ICSM-4 and ICSM-10 was either negative or the three PK-resistant PrP glycoforms were only weakly captured (Fig. 2a). PrP<sup>Sc</sup> glycoforms could not be separated, regardless of the glycoform specificity of the mAb used. Thus, ICSM-4 and ICSM-10 could selectively immunoprecipitate PrP<sup>C</sup> glycoforms from normal brain homogenates, whereas PrP<sup>Sc</sup> glycoforms always co-immunoprecipitated as a complex from prion-infected brain homogenates.
The inability of ICSM-4 to differentially immunoprecipitate PrPSc glycoforms from infected brains was conditional upon the addition of SDS and then subjected to immunoprecipitation, ICSM-4 captured only the unglycosylated PrP band, although ICSM-18 continued to capture all three PrP glycoforms (Fig. 2b). Confirmation that PrPSc was successfully denatured was provided by treating with PK, before and after SDS denaturation: thus PrPSc was PK-resistant prior to denaturation, whereas once denatured, PrPSc became sensitive to PK digestion (Fig. 2b). Despite their ability of recognizing only a subset of the glycoforms, using the direct immunoprecipitation method, ICSM-4 and to a lesser extent ICSM-10 weakly immunoprecipitated PrPSc as a complex of all three glycoforms from a vCJD-affected brain homogenate (Fig. 3a). These immunoprecipitations were very weak, suggesting a very low affinity for mAb–PrPSc interaction, perhaps involving only a small subset of PrPSc molecules.

Using the indirect method, ICSM-4 (3 μg ml⁻¹) only immunoprecipitated a major single band representing unglycosylated PrPc from normal human brain homogenate, whereas in contrast, it weakly immunoprecipitated all three glycoforms comprising PrP²⁷⁻³⁰ from PK-treated vCJD-affected brain homogenate (Fig. 3b). The capture of the three native PrP²⁷⁻³⁰ glycoforms was improved when a higher concentration of ICSM-4 IgG (20 μg ml⁻¹) was used (Fig. 3c). The presence of the three intact native PrP²⁷⁻³⁰ glycoforms in the sample was confirmed by their immunoprecipitation by ICSM-35 (Fig. 3b and c). In addition, when an aliquot of the same brain homogenate PrPSc was denatured, ICSM-4 exclusively recognized unglycosylated PrP, while all three PrP glycoforms were present and recognized by ICSM-18 (Fig. 3d). This confirms that it is association of the three glycoforms of PrP in the quaternary structure of PrPSc that allows immunoprecipitation of the complex via recognition of only a single component. ICSM-4 has a high affinity for unglycosylated PrP, and it could deplete a 0.1% (w/v) normal mouse brain homogenate of its unglycosylated PrPc after immunoprecipitation (Fig. 4a). However, it did not have the same effect in depleting PrPSc as immunoprecipitation by ICSM-4 was so weak that PrPSc signal from the 0.25% (w/v) vCJD brain homogenate remained almost unchanged (Fig. 4b). The weak binding of ICSM-4 to native PrPSc indicates that its epitope, on the unglycosylated PrP incorporated into the native prion polymer, is partially hidden and revealed as the unglycosylated protein dissociates upon denaturation. It is also possible that ICSM-4 only immunoprecipitates a subset of the PrPSc polymers, as sequential immunoprecipitation from the same brain homogenate by ICSM-4 led to subsequently weaker or negative capture of PrP (data not shown). Whatever the explanation, the ICSM-4 to PrPSc interaction is of low affinity and alternative methods of coupling ICSM-4 to different magnetic beads did not improve its ability to immunoprecipitate PrPSc (data not shown).

**ICSM-18 has a higher affinity for native PrPc than PrPSc**

ICSM-18 was raised against the α-conformer of recombinant human PrP¹⁹¹⁻²³¹, which may explain its higher affinity.
for native PrP<sup>C</sup> than PrP<sup>Sc</sup>. We have shown that ICSM-18 strongly binds denatured-PrP derived from both PrP<sup>C</sup> and PrP<sup>Sc</sup> (Fig. 1), and we have also shown that it efficiently immunoprecipitates native PrP<sup>C</sup> from normal murine brain homogenates, whereas immunoprecipitation of native PrP<sup>Sc</sup> was weak (Fig. 2). The immunoprecipitation of native PrP<sup>Sc</sup> from a vCJD-affected brain homogenate was more successful if PK-treatment was performed after PrP capture (Fig. 3a) rather than before (Fig. 3b), although ICSM-18 strongly recognizes human denatured PrP<sup>27–30</sup> on a Western blot (Fig. 3d). It is possible that ICSM-18 may only bind a subset of native PrP<sup>Sc</sup>

The high affinity of ICSM-18 for PrP<sup>C</sup> was demonstrated not only by its strong recognition of native (Fig. 3b) and denatured PrP<sup>C</sup> (Fig. 4c), but also by its depletion of native PrP<sup>C</sup> from a normal human brain homogenate after immunoprecipitation (Fig. 4c).

**Residues 93–105 in PrP<sup>Sc</sup> are resistant to proteolysis with PK, but are exposed to antibody binding**

mAbs raised to β-PrP, such as ICSM-35 and ICSM-37, could immunoprecipitate both native PrP<sup>C</sup> and PrP<sup>Sc</sup>. Indeed, ICSM-35 had a higher affinity for native PrP<sup>Sc</sup> than PrP<sup>C</sup>, while ICSM-37 had a high affinity for both native PrP<sup>C</sup> and PrP<sup>Sc</sup> (Fig. 2a). ICSM-35 and ICSM-37 immunoprecipitate all three PK-sensitive PrP<sup>C</sup> and three PK-resistant PrP<sup>Sc</sup> bands from normal and prion-infected mouse (Fig. 2a) and human brain homogenates (Fig. 3a). As previously described (Khalili-Shirazi et al., 2005), we showed by ELISA that these β-PrP-derived mAbs are directed to epitopes comprising residues 93–105 and 97–105 of PrP<sup>C</sup>, which are unstructured in PrP<sup>C</sup>. The structure of PrP<sup>Sc</sup> is unknown, but this N-terminal region of PrP<sup>Sc</sup> is believed to be important in conferring some strain-specific properties of prions, and is thus likely to have at least a degree of conformation that may vary between strains. Structural heterogeneity within the N terminus may explain the different affinities observed for ICSM-35 and ICSM-37, for native PrP<sup>C</sup> and PrP<sup>Sc</sup>.

ICSM-35 robustly immunoprecipitated native human PrP<sup>Sc</sup> regardless of the method used, whether the vCJD brain homogenate was treated with PK after immunocapture (Fig. 3a) or before (Fig. 3b and c). Although both ICSM-35 and 3F4 immunoprecipitated native PrP<sup>C</sup> from normal and vCJD human brain homogenates (Fig. 3b), ICSM-35 and 3F4 singularly failed to immunoprecipitate PrP<sup>Sc</sup> at IgG concentrations of 3 μg ml<sup>-1</sup> (Fig. 3b) or 20 μg ml<sup>-1</sup> (Fig. 3c).
The glycoform profile of PrPSc immunoprecipitated by ICSM-35 exactly matched the known glycoform profile of the infected brain. Diglycosylated dominant PrPSc bands from vCJD-affected brains and monoglycosylated dominant PrPSc from sporadic CJD-affected brain homogenates were immunoprecipitated by ICSM-35 (Fig. 5). This suggests that PrPSc aggregates are highly ordered, and the glycoform ratios specific for each strain are reflected in the prion polymer itself.

**Anti-PrP mAbs recognize PrPSc plaques in the brain**

Serial sections of brain from mice infected with mouse passaged-BSE stained with ICSM-4, ICSM-10, ICSM-18 and ICSM-35 (Fig. 6) showed areas of PrPSc reactive plaques or deposits, particularly in the corpus callosal area of mice inoculated with mouse-passaged BSE (Fig. 6). These sections demonstrated classical histological features of spongiform change, consistent with typical prion disease. Age-matched, saline-inoculated controls had normal histology and were negative when tested with these mAbs (data not shown).

The PrP deposits in the brains of mice with terminal prion disease as shown by a WB of the homogenate (10 μl of 0.1% (w/v)), showing all the PrPC glycoforms (pre-ICSM-4), becomes depleted of unglycosylated PrPC after immunoprecipitation by ICSM-4 (post-ICSM-4). The direct immunoprecipitation method was used (see Methods) with biotinylated ICSM-4 adsorbed streptavidin beads (20 μl) and N mouse brain homogenate [100 μl of 0.1% (w/v)]. (b) WB of a vCJD brain homogenate [20 μl of 0.25% (w/v)] shows all the PrP glycoforms before (−) and after (+) PK digestion (pre-PrP capture). ICSM-35 covalently coupled protein-A beads (25 μl) strongly immunoprecipitated (IP) PrP27–30 from PK treated (+) vCJD brain homogenate [150 μl of 0.25% (w/v)], using the direct immunoprecipitation method. There was no non-specific binding by negative control (−) mAb and a very weak binding by ICSM-4 beads. WB of the vCJD brain homogenate (20 μl) after ICSM-4 capture of PrP27–30 (post-ICSM-4) showed a strong PrP27–30 signal remaining in the brain homogenate, while PrP27–30 was almost depleted after immunoprecipitation by ICSM-35 (post-ICSM-35). Captured PrP was eluted from the beads and visualized by Western blotting using ICSM-18 as primary antibody. (c) WB of human N brain homogenate [10 μl of 0.5% (w/v)] shows the presence of all the PrP glycoforms (pre-ICSM-18). ICSM-18 (20 μg of IgG) strongly immunoprecipitated PrPC from an aliquot of this brain homogenate [200 μl of 0.5% (w/v)], almost depleting it of PrPC as shown by a WB of the homogenate (10 μl) after immunoprecipitation by ICSM-18 (post-ICSM-18). The indirect immunoprecipitation method was used (see Methods). A cartoon of diglycosylated (filled box), monoglycosylated (grey box) and unglycosylated (open box) PrP bands for brain homogenates ‘−PK’ and ‘+PK’ is outlined.

**Fig. 4.** Depletion of PrPC by ICSM-4 and ICSM-18, and of PrPSc by ICSM-35 from human and mouse brain tissues. (a) Western blot (WB) of mouse normal (N) brain homogenate [20 μl of 0.1% (w/v)], showing all the PrPC glycoforms (pre-ICSM-4), becomes depleted of unglycosylated PrPC after immunoprecipitation by ICSM-4 (post-ICSM-4). The direct immunoprecipitation method was used (see Methods) with biotinylated ICSM-4 adsorbed streptavidin beads (20 μl) and N mouse brain homogenate [100 μl of 0.1% (w/v)]. (b) WB of a vCJD brain homogenate [20 μl of 0.25% (w/v)] shows all the PrP glycoforms before (−) and after (+) PK digestion (pre-PrP capture). ICSM-35 covalently coupled protein-A beads (25 μl) strongly immunoprecipitated (IP) PrP27–30 from PK treated (+) vCJD brain homogenate [150 μl of 0.25% (w/v)], using the direct immunoprecipitation method. There was no non-specific binding by negative control (−) mAb and a very weak binding by ICSM-4 beads. WB of the vCJD brain homogenate (20 μl) after ICSM-4 capture of PrP27–30 (post-ICSM-4) showed a strong PrP27–30 signal remaining in the brain homogenate, while PrP27–30 was almost depleted after immunoprecipitation by ICSM-35 (post-ICSM-35). Captured PrP was eluted from the beads and visualized by Western blotting using ICSM-18 as primary antibody. (c) WB of human N brain homogenate [10 μl of 0.5% (w/v)] shows the presence of all the PrP glycoforms (pre-ICSM-18). ICSM-18 (20 μg of IgG) strongly immunoprecipitated PrPC from an aliquot of this brain homogenate [200 μl of 0.5% (w/v)], almost depleting it of PrPC as shown by a WB of the homogenate (10 μl) after immunoprecipitation by ICSM-18 (post-ICSM-18). The indirect immunoprecipitation method was used (see Methods). A cartoon of diglycosylated (filled box), monoglycosylated (grey box) and unglycosylated (open box) PrP bands for brain homogenates ‘−PK’ and ‘+PK’ is outlined.

We conclude that residues 93–105 of PrP, comprising the epitope for ICSM-35, are exposed in human and mouse PrPSc. The efficiency of ICSM-35 binding to native PrPSc was evidenced by its ability to almost deplete a vCJD brain homogenate (0.25% w/v) of its PrPSc signal (Fig. 4b). The high affinity of ICSM-35 for native PrPSc was also confirmed, when no measurable dissociation of mouse-scrapie brain-derived PrPSc was observed from the PrPSc-bound covalently coupled ICSM-35-protein-A beads, after washing the beads in excess PBST for 12 h at 4°C (data not shown). A titration of scrapie-affected mouse brain homogenate also showed ICSM-35 capturing PrPSc from as little as 0.01% (w/v) brain homogenate (data not shown).

**Fig. 5.** Immunoprecipitation of different glycoform strain-specific PrPSc from vCJD and sporadic CJD brains by ICSM-35. ICSM-35 and negative control (−) mAb covalently coupled protein-A beads (30 μl) were used to immunoprecipitate PrPSc from 300 μl of 0.2% (w/v) of a human vCJD or a sporadic CJD brain homogenate, using the direct immunoprecipitation method (see Methods). PrP-captured beads were treated with PK (+), PrP27–30 was eluted from the beads and visualized by Western blotting, using 3F4 mAb as primary antibody. A cartoon of diglycosylated (filled box), monoglycosylated (grey box) and unglycosylated (open box) PrP bands for brain homogenates ‘+PK’ is outlined.
Fig. 6. Immunocytochemistry of PrP-reactive plaques in scrapie-affected mouse brain. PrPSc was visualized by immunostaining serial paraffin sections of BSE-infected SJL mouse brain with ICSM-18 (10 μg IgG ml⁻¹), ICSM-35 (0.2 μg IgG ml⁻¹), ICSM-4 (100 μg IgG ml⁻¹) and ICSM-10 (neat culture supernatant at 75–100 μg IgG ml⁻¹), using DAB as chromogen. Coomassie blue was used to counterstain. The insert in ICSM-35 slide represents ×400 magnification of the section. No staining was observed with the negative control (−) anti-D mAb (10 μg IgG ml⁻¹). Bar, 10 μm.

DISCUSSION

Glycoform-specific mAbs, such as ICSM-4 and ICSM-10, were produced from immunization with α-PrP, a conformation of the prion protein that is PK-sensitive and, thus more easily processed for presentation to B cells than the partially PK-resistant β-PrP (Jackson et al., 1999b). The mAb ICSM-4, which recognizes unglycosylated PrP, is a valuable tool for further structural studies. Through the use of ICSM-4, we have demonstrated that while there is no detectable intermolecular association between the major PrP glycoforms in native PrPSc, the glycoforms of PrPSc are associated either strongly together, or associated with an unidentified protein that prevents their differential immunoprecipitation in the native state. Indeed, regardless of glycoform specificity of the mAbs used, such as ICSM-4 or ICSM-35, native PrPSc could only be immunoprecipitated as a complex of glycoforms, with proportions of diglycosylated, monoglycosylated and unglycosylated PrP, similar to that visualized for denatured PrP in Western blots and detected by ICSM-18. Since differential immunoprecipitation of denatured, but not native, PrPSc was possible by ICSM-4, this suggests that the intimate association of individual PrP glycoforms, comprising PrPSc aggregates, occurs only in the native state. Also, as glycoform-specific mAbs, ICSM-4 and ICSM-10 were unable to preferentially immunoprecipitate subspecies of PK-resistant PrPSc enriched in unglycosylated and/or monoglycosylated PrPSc from human vCJD or scrapie-affected mouse brain homogenates, we concluded that the composition of immunoprecipitated native PrPSc, regardless of the mAb used, is an assembly of glycoforms and is homogeneous.

Having shown that PrPSc particles in a given prion strain are composed of a characteristic spectrum of glycoforms, it is interesting to consider mechanisms that can account for this observation. With respect to the formation of the PrPSc particles, there are two mechanisms that can lead to a defined characteristic ratio of components which constitutes a prion strain. In a stoichiometric assembly process, the formation of prion particles occurs by the exact packing of the three glycoforms. For instance, a given PrP conformation and packing arrangement, which constitutes a strain, might only allow direct 2:4:2 stoichiometry of the three glycoforms, i.e. there is a discreet, whole-number molecular stoichiometry. By contrast, in a statistical or probabilistic process, a strain will be characterized by having different on and off rates for addition of each glycoform to the growing prion particle. For instance, assuming a negligible rate of dissociation, the ratio of the on-rates for each glycoform to the growing prion particle. However, unlike the case of the stoichiometric model, there would not be an exact packing arrangement that only allows precise stoichiometries; rather there would be a given probability of finding a particular glycoform at a particular location in the structure of the fibril.

Studying the appearance of PrPSc deposits in the brains of mice with terminal scrapie using our panel of mAbs showed that, regardless of the mAb’s glycoform specificity, large PrP-reactive plaques were successfully recognized in the corpus callosum of diseased brain. These brain sections were treated such that native PrPSc was destroyed and PrPSc was denatured; hence, the mAbs should recognize their specific PrPSc glycoforms if their epitope is available. A higher concentration of ICSM-4 than ICSM-18 or ICSM-35 was required to detect the plaques, possibly reflecting lower unglycosylated PrP expression in the brain or that the disease were mainly extracellular and when associated with cells they were adjacent to, or within neurons and, to a lesser extent, astrocytes. Although both ICSM-18 and ICSM-35 recognize all three PrPSc glycoforms by Western blot, ICSM-35 was more sensitive than ICSM-18 in detecting PrPSc plaques and deposits in the diseased brain. The ability of the mAbs to detect PrPSc in brain sections was different: ICSM-35 at IgG 0

\[ \text{g IgG ml}^{-1} \]

was required to detect the plaques, possibly reflecting lower unglycosylated PrP expression in the brain or that the
epitope of ICSM-4 is partially occluded or destroyed by tissue processing, but remains an integral component of disease related PrP. ICSM-35 was more sensitive in detecting PrP\textsuperscript{Sc} in the diseased brain than ICSM-18, although they both recognized all three PrP\textsuperscript{Sc} glycoforms. It is possible that the 93–105 epitope of ICSM-35 is more frequently exposed in the plaques and PrP\textsuperscript{Sc} deposits in the brain than the 142–153 epitope of ICSM-18.

Finally, we found that native PrP\textsuperscript{Sc} could strongly be immunoprecipitated from human and mouse prion-infected brain homogenates by ICSM-35 and ICSM-37, reactive to PrP residues of 93–105 and 97–105. Our findings with ICSM-35 and ICSM-37 are in contrast to the previous report (Peretz et al., 1997), suggesting that residues 90–120 are accessible in PrP\textsuperscript{Sc}, but cryptic in PrP\textsuperscript{Sc}. In that study, mAbs 3F4 (epitope aa 104–113) and D13 (epitope 96–104) immunoprecipitated native Syrian hamster (SHa) PrP\textsuperscript{C} and denatured SHa PrP\textsuperscript{27–30}, but were incapable of immunoprecipitating native SHa PrP\textsuperscript{27–30}, and it was deduced that the 90–120 region is exposed in PrP\textsuperscript{C} but is largely cryptic in PrP\textsuperscript{27–30} (Peretz et al., 1997). In agreement with previous findings, we found that 3F4 could successfully immunoprecipitate native human PrP\textsuperscript{C}, but failed to capture native PrP\textsuperscript{27–30} from vCJD brain. ICSM-35, raised to human PrP\textsuperscript{27–30} (types 1–4) recognition as 3F4 in Western blots of native PrP\textsuperscript{Sc} in this region. In contrast, recombinant human PrP\textsuperscript{27–30} (Peretz et al., 1997) was found to be a highly antigenic epitope for mAbs raised to \(\beta\)-PrP\textsuperscript{91–231}.

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