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Conformation-Dependent High-Affinity Monoclonal Antibodies to Prion Proteins

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Prion diseases are fatal, neurodegenerative illnesses caused by the accumulation of PrPSc, an aberrantly folded isoform of the normal, cellular prion protein. Detection of PrPSc commonly relies on immunochemical methods, a strategy hampered by the lack of Abs specific for this disease-causing isoform. In this article, we report the generation of eight mAbs against prion protein (PrP) following immunization of Prnp-null mice with rPrP. The eight mAbs exhibited distinct differential binding to cellular prion protein and PrPSc from different species as well as PrP-derived synthetic peptides. Five of the eight mAbs exhibited binding to discontinuous PrP epitopes, all of which were disrupted by the addition of 2-ME or DTT, which reduced the single disulfide bond found in PrP. One mAb F20-29 reacted only with human PrP, whereas the F4-31 mAb bound bovine PrP; the K₀ values for mAbs F4-31 and F20-29 were ∼500 pM. Binding of all five conformation-dependent mAbs to PrP was inhibited by 2-ME in ELISA, Western blots, and histoblots. One conformation-dependent mAb F4-31 increased the sensitivity of an ELISA-based test by nearly 500-fold when it was used as the capture Ab. These new conformation-dependent mAbs were found to be particularly useful in histoblotting studies, in which the low backgrounds after treatment with 2-ME created unusually high signal-to-noise ratios. The Journal of Immunology, 2010, 185: 000–000.

A ccumulation of the alternatively folded isoform (PrPSc) of the normal, cellular prion protein (PrPc) causes fatal neurodegeneration in humans and animals (1–3). The prion diseases include Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, chronic wasting disease (CWD) in elk and deer, bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy, and feline spongiform encephalopathy. Conversion of PrPc into PrPSc results in a profound conformational change, including a reduction in the α-helical content and an increase in the β-sheet content of the molecule (4–7). Initially, PrPSc was distinguished from PrPc by its resistance to limited proteolysis. Discovery of PrPSc and the subsequent production of Abs to the protein made possible the definitive identification of BSE in cattle and variant Creutzfeldt-Jakob disease (vCJD) in humans (8, 9). More importantly, immunoassays of brainstems from cattle destined for the human food supply have helped to reduce exposure of people to bovine prions (10). Compelling evidence has accumulated that vCJD results from the consumption of prion-infected bovine tissue (11–13); fortunately, cases of vCJD are dwindling. Notably, four cases of vCJD have been traced to transfused blood from donors who later developed vCJD (14, 15).

Despite numerous attempts to produce PrPSc-specific Abs that can be used in rapid immunoassays, none have been identified. Such PrPSc-specific Abs would react with native PrPc and not with PrPSc; several Abs that immunoprecipitate native PrPSc have been reported (16, 17). In addition, Abs engineered with peptide grafts corresponding to prion protein (PrP)89–112 or PrP36–158 were reported to selectively bind native PrPSc in prion-infected mouse, human, and hamster tissues (18, 19). The need for PrPSc-specific Abs has increased with the discovery that not all forms of PrPSc are resistant to limited proteolysis. Prion-sensitive PrPSc has been identified in humans, domestic animals, and laboratory rodents.

Herein, we describe studies designed to generate conformation-dependent Abs. PrP-ublated (Prnp0/0) FVB mice were immunized with recombinant bovine or human PrP. After multiple immunizations, spleens were harvested from the Prnp0/0/FVB mice with detectable serum anti-PrP Abs. Fusions of splenocytes with mouse myeloma cells were performed to produce hybridomas, from which eight clones producing IgG mAbs were isolated. Five of the eight mAbs exhibited binding to discontinuous PrP epitopes, all of which were disrupted upon reduction of the single disulfide bond with 2-ME or DTT. The F20-29 mAb exhibited high-avidity binding to human PrP with a K₀ of ∼500 pM; the F4-31 mAb bound bovine PrP with similar avidity. The binding of all five conformation-dependent mAbs to PrP was inhibited by 2-ME or...
DTT in ELISA, Western blots, and histoblots. F4-31 was found to increase the sensitivity of an ELISA-based test by nearly 500-fold when it was used as the capture Ab. In histoblotting studies, these new conformation-dependent mAbs were particularly useful when the low backgrounds after treatment with 2-ME created unusually high signal-to-noise ratios.

Materials and Methods

Recombinant protein

Recombinant bovine PrP [rBoPrP(102–241)] and recombinant human PrP [rHuPrP(90–231)] were purified as previously reported, with slight modifications (19, 20). We used pBS1 secretion vector using the alkaline phosphatase (AP) promoter and the periplasmic bacterial signal peptide from Shigella toxin II. The transfected bacteria were grown in 2- or 10-l fermenters and harvested 24 h after inoculating the fermentation media. The recombinant protein was released by disruption of bacteria in 8 M GdnHCl/100 mM DTT (pH 8). Protein was purified by size-exclusion chromatography on a HiLoad 26/60-cm Superdex 200 column (GE Healthcare, Piscataway, NJ), followed by reverse phase-HPLC, using a Jupiter C-4 preparative column (25/250 mm; Phenomenex, Torrance, CA) and linear acetonitrile gradients.

Brain homogenates

Brain homogenates (10% w/v) were prepared from bovine (Bo), human (Hu), sheep (Ov), mouse (Mo), Syrian hamster (SHa), and mule deer (MD) in calcium- and magnesium-free PBS. Brain tissue was homogenized with a PowerGen homogenizer (Fisher Scientific, Pittsburg, PA) on ice by three 15-s strokes. The homogenate was cleared by a short centrifugation at 500 × g for 5 min, and the supernatant was used for the preparation of the samples.

Ab production

Six- to eight-week-old Prnp±/± female FVB mice were immunized with rBoPrP(102–241) or rHuPrP(90–231) prepared in RIBI adjuvant (Corixa, Hamilton, MT) at 1 mg/ml. The amino acid numbering used in this article is based on the alignment described by Prusiner (22). Animals were maintained in DMEM with high glucose and L-glutamine (without sodium pyruvate) (Life Technologies; #11965-092), supplemented with 10% FCS and penicillin/streptomycin. Myeloma cells (viability ≥99%) were gently disrupted, and 1 ml a 50% polyethylene glycol solution per 108 cells was added while stirring. Then, the cell mixture was centrifuged (500 × g, 5 min). The cell pellet was slowly suspended in serum-free growth medium and incubated for 20 min at 37˚C. The supernatant was aspirated, and the plate was washed and blocked with 5% NFDM in blocking buffer. This second blocking step was critical to reduce background fluorescence, presumably resulting from binding of subsequent reagents to any unoccupied combining sites of the goat anti-mouse IgC Ab. The plates were decanted, rHuPrP(90–231) or rBoPrP(102–241) at 2 μg/ml in blocking buffer was added, and the plates were sealed and incubated at 37˚C for 2 h. The plates were washed three times, incorporating a 1-min soak per cycle, and patted dry. Next, 200 μl/well of Eu-conjugated HuM-P, at 0.25 μg/ml in DELFIA assay buffer (#4002-0010; Perking-Elmer, Waltham, MA), was added, and the plates were incubated for 2 h at 37˚C. Finally, the plates were washed seven times with a 1-min soak per cycle, and 200 μl/well DELFIA-enhancement solution (#1244-105; PerkingElmer) was added and incubated for 10 min at room temperature (RT). The time-resolved, fluorescence intensity was measured using a Packard Discovery fluorometer. In some cases, a dsELISA was used when HRP-conjugated HuM-P was substituted for Eu-conjugated HuM-P, and the assay was developed with a colorometric substrate. Ab isotype analysis was performed using isotype-specific, AP-conjugated anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) in an ELISA format and using a one-step, lateral-flow-mouse isotype assay (Roche). The CDI was as described (24).

Western blots

Aggs for Western blots were 10% (w/v) brain homogenates prepared from prion-diseased hamsters, mice, humans, cattle, sheep, and deer. Weanling SHa and CD1 mice were inoculated with 50 μl scrape isolate Sc237 and 30 μl mouse-passaged Rocky Mountain Laboratory (RML) prions, respectively. Hamsters and mice were monitored for signs of neurologic dysfunction, and were euthanized when disease was imminent (25, 26). Brain homogenates (10% (w/v) brain tissues in calcium- and magnesium-free PBS. Brain tissue was homogenized with a PowerGen homogenizer on ice by three 15-s strokes of a PowerGen homogenizer (Fisher Scientific). The homogenate was cleared by a short centrifugation at 500 × g for 5 min, and the supernatant was used for the preparation of the samples.

Screening

A multistep screening strategy was used: first, a direct-binding ELISA was performed, followed by a double-sandwich (ds) dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) and, finally, Western immunoblotting using normal brain homogenates from various species. Hybridoma cells from wells containing medium that tested positive in all three screening steps were expanded and cloned (at least three times), as described (23). Ascites tumors were generated by i.p. injection of 0.5–2 ml hybridoma supernatant into 0.1 ml peritoneal cavity. Hybridoma supernatant was used for the preparation of the samples.

ELISA methods

The direct-binding ELISA used rHuPrP(90–231) or rBoPrP(102–241) peptide antisera and 96-well microtiter plates (#3455; ThermoScientific, Waltham, MA). Supernatants or other test solutions were added and incubated for 2 h at 37˚C. The plates were washed, and AP-conjugated anti-mouse Fab(2)/2 (#31324; ThermoScientific) IgG was added. After a final set of washes, substrate p-nitrophenyl phosphate disodium salt (pNPP) (#-9389; Sigma-Aldrich, St Louis, MO) was added, and the plate was read in a 96-well spectrophotometer at 405 nm.

The dsDELFIA was used at an early stage in the screening process (analysis of media from the original 96-well fusion cultures) to identify Abs that might function as capture reagents in the conformation-dependent immunoassay (CDI). Eu-labeled, chimeric human-mouse (HuM) rFab P (24) was used as the reporting Ab. The primary Ab was monoclonal goat anti-mouse IgG (Fc) (Pierce), diluted 1:5000 in TBST. ECL detection reagent (GE Healthcare) was used as substrate. The blots were exposed to Amersham Hyper-Film ECL (GE Healthcare).
Immunoprecipitation

Protein A or protein G-Sepharose (100 μl; #10-1041 and #10-1242; Invitro- gen, Carlsbad, CA) was mixed with 2 μg Ab in a final volume of 0.5 ml in TBS. The pH values for protein A and protein G were 9 and 7.5, respectively. The binding of the Ab to Sepharose was done on a rugged rotator for 1 h at 4˚C. After washes with TBS (pH 7.5), the mixture was blocked with 10% BSA in TBS (pH 7.5). The Abs were 10% human brain homogenates prepared with or without PK from uninfected mice, RML-infected mice, uninfected humans, or CJD cases, as previously described. We added 5 μl of the Ag to the Sepharose/Ab mixture, in 1 ml TBS (pH 7.5), and 0.3% Sarkosyl and then incubated the mixture on a rugged rotator for 1 h at 4˚C. After three consecutive washes with TBS (pH 7.5) and 0.25% Sarkosyl, the samples were boiled in sample buffer without reducing agent and analyzed by Western blot. The mouse samples were detected with rFab HuM-D18-HP-AB (27); the human samples were detected with HuM-P-HP (24).

Epitope determination

Two series of peptides were synthesized: one series spanned BoPrP(95–241), 12 aa in length with an eight-residue overlap, and one series spanned HuPrP(88–226), 10 aa in length with a seven-residue overlap (Sigma-Genosys, Woodlands, TX) (Supplemental Tables I, II). Three lysine residues were added to the N-termini to improve solubility; a single biotin molecule was also added to the N-terminal lysine residue. The C-termini were amidated. Peptides were dissolved in 10% acetonitrile and diluted to 1 mM in 10% ethanol.

On the day of the experiment, peptides were diluted 200-fold in TBST (pH 7.4) to give a final peptide concentration of 5 μM. Streptavidin-coated microtiter wells (Pierce) were blocked for 60 min at RT by incubation with 300 μl of 5% blocking buffer (NDFD or BSA with TBST). The blocking solution was decanted, 50 μl of the 5 μM peptide solution was added, and the plates were incubated for 1 h at RT. Anti-PrP Abs were added, and the plate was incubated for 1 h at 37˚C. Following three washes with 200 μl well/TBST, 100 μl well/HRP-conjugated, goat anti-mouse IgG+ A+M (#65-6420, Invitrogen), diluted 1:2000 in 5% NFDM-TBST buffer, was added, and the plate was incubated for 60 min at RT. Finally, the plates were washed three times with TBST, 100 μl well/ K-blue substrate (Neogen, Lansing, MI) was added, and the reaction was stopped with 0.1 N HCl. Color development was recorded at 650 nm in a 96-well plate reader. Alternately, we used an AP-labeled secondary Ab and the substrate was pNPP.

Evaluation of rFab HuM-P Ab binding to peptides necessitated the substitution of 100 μl of a 1:5000 dilution of HRP-conjugated anti-human IgG (Fab) (#31414; Thermofisher) for the HRP-conjugated anti-mouse IgG.

ELISA of reduced PrP

PrP, at 5 μg/ml in coating buffer (0.1 M NaHCO₃, [pH 8.6]), was mixed with the reducing agent, DTT, at final concentration of 10 mM. The mixture was plated in a 96-well microtiter plate and incubated overnight at 4˚C. The plate was washed; nonreactive sites on the plastic were blocked with 0.25% BSA in TBST buffer, and the appropriate Ab was titrated on the coated wells. The assay was developed with an AP-conjugated goat anti-mouse IgG-Fab’/2 (Thermofisher). The substrate used was pNPP.

Sandwich CDI for human and bovine PrP Sc

The CDI data described in this article were generated with rFab HuM-P (24) labeled with Eu-chelate of N-(p-isothiocyanatobenzyl)-diethylenetriamine-N₁,₉,N₂,N₃,N₄-tetraacetic acid (DTTA) at pH 8.5 for 16 h at RT, as described (28). Additional data were generated with Eu-labeled mAb 3F4 (29, 30).

The principle, development, calibration, and calculation of PrP Sc concentrations were described (24, 28, 30, 31). Briefly, each concentration from CDI data were described (24, 28, 30, 31). Briefly, each sample was divided into two aliquots: untreated (designated native [N]) or treated (designated D). The principle, development, calibration, and calculation of PrP Sc concentrations were described (24, 28, 30, 31). Briefly, each concentration from CDI data were described (24, 28, 30, 31). Briefly, each sample was divided into two aliquots: untreated (designated native [N]) or treated (designated D). The concentration of PrP Sc was directly proportional to (O–D) N value and was calculated from a previously published equation (24, 28, 31).

Affinity measurements

Ab-affinity measurements for F4-31 and F20-29 were obtained using two independent methods, by ELISA and by surface plasmon resonance (SPR). ELISA. The K_D values in solution were measured by ELISA using the method described by Friguet et al. (32). Data analysis and calculation of K_D used the following relationships as modified by Bobrovnik (33):

\[
(Ao - A)/A = k_o Li
\]

\[
k_o = 1/k_a.
\]

Ao equals the absorbency measured when no Ag was present (100% Ab binding to the plate), A equals the absorbency measured when different concentrations of rPrP (Li) were present, k_o equals the on rate, and k_a equals the off rate. A minimum of five rPrP concentrations, ranging between 0.1 to 50 (×10⁻⁷ M), was used in each analysis. A constant concentration of Ab (0.2 μg/ml) was used in these experiments. The Ab was mixed with the Ag in solution and incubated for up to 18 h at RT. Incubation time in the ELISA was optimized (15 min) so as not to disturb the Ab/Ag complex at equilibrium. The K_D value was determined graphically as the slope of the linear relationship described in Equation 1.

SPR measurements. SPR measurements were obtained with a Biacore 2000 (GE Healthcare) instrument. Determination of the affinity constants of IgG in SPR is best accomplished by coupling the Ab to the chip. Because of the physicochemical properties of PrP, we were unable to regenerate the chip surface between sequential injections of Ag solution. Therefore, the single-cycle kinetic method (34) was used, and data analyzed by the Biacore control software method, using the BiaEvaluation software, fit the algorithm kindly provided by Kevin Lindquist (GE Healthcare). Briefly, Ab (10 μg/ml) was coupled to a CM5 chip (Biacore) using 10 μM acetate buffer at optimum pH (pH 4, 4.5, 5, and 5.5 from Biacore BR 100349, 100350, 100351, and 100352, respectively), resulting in ~1000 relative units being immobilized on the CM5 chip using amine-coupling chemistry, as described by the manufacturer.

Three sample cycles were performed for each determination. The first two sample cycles consisted of injections of running buffer (HEPES-buffered saline with 3 mM EDTA and 0.005% (v/v) surfactant P20 [GE Healthcare], 0.05% Tween 20, 0.1 mg/ml BSA [pH 7.4]), followed by injection of the Ag solutions. Five Ag solutions in running buffer, ranging between 1 and 150 nM, were injected sequentially from lowest to highest concentration, with a 1-min stabilization period between each injection. After the last injection, the Ag was allowed to dissociate for 30 min. Background (blank sample cycle data trace) was subtracted, and data were analyzed with the BiaEvaluation software to generate a calculated on-rate (k_o) and off-rate (k_a). K_D values were calculated by dividing k_o/k_a.

HistobLOTS

Frozen coronal sections (10 μm) from Tg(BoPrP+/+)/Prnp0/0/FVB4092 and Tg(HuPrP/M129)Prnp0/0/FVB440 mice, as well as from normal human and bovine cerebrum, were transferred into noncellulose membranes for overnight incubation with 10% 2-ME (Sigma-Aldrich). After reduction, immunohistochemistry was performed by incubating membranes for 2 h with F20-29 and F4-31 (1:1000) Abs, followed by a 1-h incubation with anti-mouse, AP-conjugated secondary Ab (Promega, Madison, WI). The signal was developed using NBT/5-bromo-4-chloro-3-indolyl phosphate (Roche) substrate.

Results

Ab production

High serum titers were observed following immunization of Prnp0/0/FVB mice (35) with huHuPrP(90–231) or roBoPrP(102–241). Western blot analyses using normal brain homogenates from mice, SHAs, humans, cattle, sheep, and deer suggested that the Prnp0/0/FVB mice were producing Abs reactive with PrP Sc (data not shown). Following multiple cell-fusion experiments, hundreds of putative hybridomas were obtained in the direct-binding ELISA (a response of three to five times above background was considered positive). However, only a few were positive in the direct-binding ELISA and the dsDELFLIA (Table I).
Eight mAbs (F4-31, F10-26, F20-29, F20-89a, F20-108a, and F20-130a) were selected for further study. Western blots using brain homogenates from normal control animals demonstrated a diverse set of binding patterns (Table I). Two mAbs (F4-31 and F10-26) were isolated from Prnp\(^{0/0}\)/FVB mice immunized with rBoPrP(102–241), whereas the remaining six mAbs were isolated from Prnp\(^{0/0}\)/FVB mice immunized with rHuPrP(90–231). Isotype analysis indicated that these eight Abs are of subclass IgG1 with \(k\) L chains. Three mAbs (F20-108a, F20-130a, and F20-80) showed binding to PrP\(C\) across all species tested (cattle, human, sheep, mouse, hamster, and mule deer). F4-31 bound to all but human PrP\(C\), whereas F20-29 bound only to human PrP\(C\).

Ab specificity

To ascertain the patterns of immunoreactivity of the eight mAbs with PrP\(^{Sc}\), brain homogenates containing BSE, sporadic Creutzfeldt-Jakob disease (sCJD), sheep scrapie, mouse scrapie, or Syrian hamster scrapie prions were digested with PK for 1 h at 37˚C. The undigested and digested samples were subjected to Western blotting using the eight mAbs (Fig. 1). Our data showed binding behavior similar to that observed with uninfected brain homogenates from MD with CWD was not tested. Each value represents the average of three independent measurements in triplicate. The dissociation constants for F4-31 mAb binding to rBoPrP(102–241) and F20-29 mAb binding to rHuPrP(90–231), the \(K_D\) value for F4-31 mAb binding to rMoPrP(89–231) obtained by ELISA was nearly 40-fold higher than that found by SPR (Table II).

Differential Ab binding

Immunoprecipitation experiments, as well as immunostaining of frozen brain sections, called histoblots, showed that the F4-31 and F20-29 mAbs bound to bovine brain homogenate digested with PK, whereas the F20-80 mAb bound to human PrP\(C\). The F20-80 mAb did not bind to PrP\(C\) in uninfected mouse or hamster brain, suggesting that the affinity for these PrP Ags may be low (Fig. 1).

To quantify the avidity of F4-31 for PrP from various mammals, Ab binding to PrP was analyzed using dsDELFIA (Fig. 2). The most avid binding was seen with the F4-31 Ab with recombinant polypeptides: rBoPrP(102–241), rMoPrP(89–231), and rSHaPrP(90–231). Approximately 10-fold weaker binding was observed to rOvPrP (23–231) polypeptide, and the lowest activity was observed using rHuPrP(90–231). Measurements of the \(K_D\) values for F4-31 mAb binding to rBoPrP(102–241) in solution, by ELISA, and for immobilized Ag, by SPR, gave values of 630 pM and 255 pM, respectively (Fig. 3, Table II). Each value represents the average of three independent measurements in triplicate. The dissociation constants for F20-29 mAb binding to rHuPrP(90–231) by ELISA and SPR were 276 pM and 463 pM, respectively. Although the dissociation constants determined by ELISA and SPR were in good agreement for F4-31 mAb binding to rBoPrP(102–241) and F20-29 mAb binding to rHuPrP(90–231), the \(K_D\) value for F4-31 mAb binding to rMoPrP(89–231) obtained by ELISA was nearly 40-fold higher than that found by SPR (Table II).

![FIGURE 1](http://www.jimmunol.org/)

Western blots show the binding of each of the eight mAbs to PrP in infected brain homogenates from different species. Samples were prepared from the obex of a cow with BSE (paired lanes 1), human with sCJD (paired lanes 2), sheep with scrapie (paired lanes 3), mouse infected with RML prions (paired lanes 4), and SHa infected with Sc237 prions (paired lanes 5); samples were undigested (–) or digested with 20 \(\mu\)g/ml PK (+) for 1 h at 37˚C. Brain homogenates from PrP-knockout mice are shown as controls, lane C. Western blots were developed with 1 \(\mu\)g/ml of the mAb indicated and peroxidase-labeled anti-mouse secondary Ab, followed by the addition of a chemiluminescent substrate. The Mr of the protein fragments are shown in kDa.
F20-29 mAbs bound to native PrP\(^\text{C}\) but not to native PrP\(^\text{Sc}\) (Fig. 4A, 4B, Supplemental Fig. 1). The F4-31 and F20-29 mAbs (Fig. 4A, 4B, lanes 1) immunoprecipitated native PrP\(^\text{C}\) in uninfected brain homogenates prepared from an FVB mouse and a normal human control, respectively. The F4-31 and F20-29 mAbs (Fig. 4A, 4B, lanes 2) immunoprecipitated native PrP in prion-infected brain homogenates prepared from an RML prion-infected mouse and an sCJD case, respectively. Neither F4-31 nor F20-29 (Fig. 4A, 4B, lanes 2) immunoprecipitated native PrP\(^\text{Sc}\) in prion-infected brain homogenates prepared from an RML prion-infected mouse and an sCJD case, respectively. Prior to immunoprecipitation, these samples were digested with 20 \(\mu\)g/ml PK for 1 h at 37°C to remove PrP\(^\text{C}\) and the N terminus of PrP\(^\text{Sc}\) to form PrP 27-30. Staining of PrP 27-30 was confirmed by immunohistochemistry (data not shown).

In ELISA studies, F4-31 bound PrP in undigested, nondenatured brain homogenates of RML-infected mice (Fig. 4C), but the signal disappeared when brain samples were digested with PK. When the RML-infected brain homogenates were denatured with 4 M GdnHCl after limited digestion with PK (Fig. 4C) or without digestion, the F4-31 mAb gave similar ELISA signals. Similar to the immunoprecipitation and histoblot findings, the ELISA results showed that the F4-31 mAb binds to native PrP\(^\text{C}\), denatured PrP\(^\text{C}\), and denatured PrP\(^\text{Sc}\), but not to native PrP\(^\text{Sc}\), in RML-infected mouse brain homogenates. Comparable findings were obtained using the anti–HuPrP-specific F20-29 mAb with sCJD brain homogenates (Fig. 4D). When the sCJD brain homogenates were denatured with 4 M GdnHCl after limited digestion with PK (Fig. 4D) or without digestion, the F20-29 mAb gave similar ELISA signals. Moreover, F20-29 bound human PrP\(^\text{C}\) in undigested, nondenatured sCJD brain homogenate (Fig. 4D), but the signal disappeared when brain samples were digested with PK.

Disruption of the disulfide bond interferes with Ab binding

PrP contains a single intramolecular disulfide bond (36). In rBoPrP (102–241), this disulfide bond is located between Cys\(^{190}\) and Cys\(^{225}\), linking \(\alpha\) helices B and C. We investigated the effect of reducing the disulfide in rBoPrP (102–241) on Ab binding. The PrP Ag was absorbed onto microtiter plates in the absence or presence of 10 mM DTT. Then the plates were blocked and immediately used to determine Ab binding by ELISA (Fig. 5). Although the binding of F20-89a and F20-49 to oxidized and reduced rBoPrP (102–241) was similar, the F20-29 and F4-31 mAbs were unable to bind the reduced rHuPrP (90–231) and rBoPrP (102–241), respectively (Fig. 5A, 5B). The ratios of Ab binding to oxidized and reduced PrP (O/R) were determined for the eight mAbs (Table I).

These observations were confirmed by electrophoretic analysis. Silver staining revealed that reduced rBoPrP (102–241) migrated more slowly on SDS-PAGE than the nonreduced polypeptide (Fig. 5E, lanes 1, 2). Abs F4-31, F10-26, and rFab HuM-P were used in Western blots. The HuM-P Fab recognized reduced and nonreduced rBoPrP (Fig. 5E, lanes 3, 4), which was expected because its linear epitope is remote from the disulfide bond; therefore, it should not be influenced by its reduction. The F4-31 mAb bound nonreduced rBoPrP (102–241) but not reduced BoPrP (Fig. 5E, lanes 5, 6). In contrast, the F10-26 mAb detected oxidized and reduced BoPrP (Fig. 5E, lanes 7, 8), reflecting its O/R binding ratio of 1.2 (Table I). The inability of the F20-29 and F4-31 mAbs to bind reduced HuPrP and BoPrP, respectively, argues that these two Abs bind to discontinuous epitopes that are conformation dependent.

Further evidence that the F20-29 and F4-31 mAbs bind to conformation-dependent epitopes comes from histoblot analyses (37). Coronal sections were taken from the brains of uninfected cattle, humans, and FVB, Tg(BoPrP\(^{+/-}\))4092, and Tg(HuPrP)440 mice. These frozen brain sections were developed with F4-31 or F20-29 mAbs. The F4-31 mAb bound to sections from cattle, FVB mice, and Tg(BoPrP\(^{+/-}\))4092, and Tg(HuPrP)440 mice. Exposure of the brain sections to 10% 2-ME to reduce the PrP disulfide abolished the Ab binding (Figs. 6, 7, Supplemental Fig. 1).
**F4-31 as a capture Ab in ELISA**

To test the ability of mAb F4-31 to function as a capture Ab, we compared its fluorescence signals to those of the capture Ab HuM-D18 rFab using brain homogenates from BSE-infected cattle, scrapie-infected sheep, CWD-infected deer, Sc237-infected SHa, RML-infected mouse, and sCJD-infected human brain (Fig. 8A).

Using the CDI format (28), we measured the differential Ab binding to native PrP versus chemically denatured PrP. The CDI uses Abs that recognize an epitope available on PrP\(^{Sc}\) but buried in native PrP\(^{Sc}\); chemical denaturation of PrP\(^{Sc}\) reveals the epitope, thereby allowing detection of PrP\(^{Sc}\), even in the presence of PrP\(^{C}\). Formatted as a sandwich immunoassay, we asked whether the CDI might be improved by substituting the F4-31 mAb for HuM-D18 rFab in the detection of BSE and CWD prions (24).

In the CDI format, mAb F4-31 showed higher fluorescence signals for all species tested, except for human PrP, to which it does not bind. With Sc237-infected hamster brain, we observed a 4-fold increase in signal from F4-31 compared with D18. Additionally, F4-31 demonstrated an ~3-fold increase in signal with infected cattle and sheep brain homogenates and a 2-fold increase in signal with infected deer and mouse samples (Fig. 8A).

Next, we compared the sensitivity of the CDI for detection of BoPrP\(^{Sc}\) in BSE-infected bovine brain using D18 or F4-31 as capture Ab (Fig. 8B). BoPrP\(^{Sc}\) in BSE-infected bovine brain was serially diluted into uninfected brain homogenate, and HuM-D18 rFab or F4-31 mAb was used as the capture Ab. Eu-conjugated HuM-P rFab was used as the detection Ab. The (D–N) difference was directly proportional to the concentration of PrP\(^{Sc}\) in the sample. Compared with HuM-D18 rFab, the F4-31 mAb increased the sensitivity ~500-fold for the detection of BSE prions.

### Table II. \(K_D\) values (pM) for mAbs F4-31 and F20-29

| mAb  | ELISA  |  |  |  |  |
|------|--------|  |  |  |  |
|      | rHuPrP | rMoPrP | rBoPrP | rHuPrP | rMoPrP | rBoPrP |
| F4-31 | 1700 ± 200 | 630 ± 4.0 |  | 463.3 ± 52.6 | 5.5 | 255.3 ± 47.4 |
| F20-29 | 276 ± 5.0 |  |  |  |  |  |

**FIGURE 4.** F4-31 and F20-29 mAbs recognize native PrP\(^{C}\) and denatured PrP\(^{Sc}\) but not native PrP\(^{Sc}\). Immunoprecipitation shows F4-31 binding to MoPrP (A) and F20-29 binding to HuPrP (B) in uninfected, control brain homogenates (lanes 1) and undigested, infected brain homogenates (lanes 2). F4-31 and F20-29 mAbs did not bind to native rPrP\(^{Sc}\) in PK-digested, infected brain homogenates (lanes 3). Apparent molecular masses based on the migration of protein standards are shown in kDa.

**FIGURE 5.** Binding of mAb F20-29 to reduced (●) and nonreduced (○) rPrP(90–231) (A) and binding of mAbs F4-31 (B), F20-89a (C), and F20-49 (D) to reduced (●) and nonreduced (○) rBoPrP(102–241), as measured by ELISA. Reduced samples were incubated overnight with 10 mM DTT at 4°C. E, Western blot of reduced (odd-numbered lanes) and nonreduced (even-numbered lanes) rBoPrP(102–241), probed with rFab HuM-P (lanes 3, 4), mAb F4-31 (lanes 5, 6), and F10-26 (lanes 7, 8). PrP was silver stained as a control (lanes 1, 2). Apparent molecular masses based on the migration of protein standards are shown in kDa.
Ab epitope mapping

To identify the epitopes of the F4-31 mAb, we evaluated Ab binding to a series of peptides with overlapping residues by ELISA. For BoPrP, we synthesized 12-residue peptides with a 7-aa overlap, spanning BoPrP(95–241) (Supplemental Table I). Binding of rFab HuM-P to these peptides served as a positive control. Binding of F4-31 was not observed to any of these peptides, whereas strong binding was observed to the intact rBoPrP(102–241) polypeptide (Supplemental Table I). However, binding of F4-31 to longer bovine peptides (BoPrP sequences 102–190, 186–212, 110–242, 205–237, 222–241, 186–212, 206–237, and 222–241) was negative (data not shown). In contrast, mAb F10-26, which failed as a capture Ab in the dsDELFIA and CDI (Table I), demonstrated strong binding by ELISA to the bovine peptides (Supplemental Table I). Together, these data suggest binding at a linear epitope within BoPrP(107–116), corresponding to the epitope motif HSQWNKPSKP, similar to the epitope for HuM-P (24). This finding explains the failure of mAb F10-26 to capture BoPrP in the dsDELFIA and CDI when the Hu-M-P rFab was used as the detection (sandwich) Ab. In these assays, the detection and capture Abs must bind to different epitopes on PrP to give positive responses. In competitive-binding experiments (data not shown), preincubation of rBoPrP(102–241) with mAb F10-26 inhibited subsequent binding of rFab HuM-P, again suggesting that these Abs share an identical or overlapping epitope.

We performed similar experiments to determine the epitopes of F20-89a on HuPrP. We synthesized 10-residue peptides, overlapping by 7 aa, spanning HuPrP(88–226) (Supplemental Table II). Binding of Ab 3F4 was used as a positive control. Of the mAbs generated using the rHuPrP immunogen, only F20-49 (data not shown) and F20-89a (Supplemental Table II) bound specific peptides. These data suggest an epitope motif of HuPrP(108–115) (MKHMAAGA) for F20-49 and HuPrP(97–100) (SQWN) for F20-89a.

When mAbs F20-29, F20-108a, F20-130a, and F20-80 were tested on the individual peptides, no binding was observed, which suggests a nonlinear epitope for these mAbs (Table I).

Discussion

Once PrP 27-30 was discovered (38, 39), a substantial effort led to the production of polyclonal Abs (40, 41) and, later, mAbs (42). Western blotting and immunohistochemistry opened new avenues of research, including the demonstration that amyloid plaques in prion disease contain PrP (40), in accord with earlier studies showing PrP 27-30 polymerized into rod-shaped structures that were indistinguishable ultrastructurally and tinctorially from amyloid (43). Localization of PrPSc in brain sections was made possible by development of the histoblotting technique (37) that was used in this study to show that reduction of the disulfide bond of PrPSc in situ abolished immunostaining with the conformation-dependent F20-29 and F4-31 mAbs (Figs. 6, 7, Supplemental Fig. 1).

Although anti-PrP Abs have been crucial in advancing prion research, bioassays in rodents and cultured cells continue to be essential (25, 44, 45). Using transgenic mice expressing luciferase under control of the glial fibrillary acidic protein promoter, it has been possible to correlate the increase in bioluminescence with the production of PrPSc and prion infectivity long before clinical signs of neurologic dysfunction appear (46). A nonimmunoassay-based procedure has been developed using the amyloid-seeding properties of PrPSc and is capable of detecting protease-resistant and -sensitive forms of PrPSc (47).

mAbs were generated by immunizing mice with the recombinant polypeptides rHuPrP(90–231) and rBoPrP(102–241), sequences...
that correspond to the PK-resistant cores of human and bovine prions, respectively. We used a tiered screening strategy that identified, early in the screening process, which mAbs are highly active as capture reagents. Initial fusion experiments resulted in several hundred positive signals. However, only six candidates were identified that consistently gave high signals when used as a capture reagent. These Abs were subsequently cloned, resulting in mAbs F4-31, F20-29, F20-49, F20-89a, F20-108a, and F20-130a. Two additional mAbs were selected (F10-26 and F20-80), which gave strong signals in the ELISA but failed as capture Abs in the CDI. Results are presented as the (D–N) difference in time-resolved fluorescence signals, measured in cpm. B, Measurement of BoPrPSc in BSE-infected bovine brain samples diluted into normal bovine brain homogenate, using HuM-D18 rFab (C) or F4-31 mAb (C) as the capture Ab in the CDI. In both experiments, Eu-conjugated HuM-P rFab was used as the detection Ab. The (D–N) difference is directly proportional to the concentration of PrPSc in the sample. Data points and bars represent the averages and standard deviations, respectively, from three independent measurements.

FIGURE 8. Improved detection of BSE prions. A, Detection of PrPSc in infected brain homogenates of different species, as indicated, using F4-31 mAb (shaded bars) or HuM-D18 rFab (open bars) as the capture Ab in the CDI. Results are presented as the (D–N) difference in time-resolved fluorescence signals, measured in cpm. B, Measurement of BoPrPSc in BSE-infected bovine brain samples diluted into normal bovine brain homogenate, using HuM-D18 rFab (C) or F4-31 mAb (C) as the capture Ab in the CDI. In both experiments, Eu-conjugated HuM-P rFab was used as the detection Ab. The (D–N) difference is directly proportional to the concentration of PrPSc in the sample. Data points and bars represent the averages and standard deviations, respectively, from three independent measurements.

References

Disclosures
The authors have no financial conflicts of interest.

HIGH-AFFINITY mAbs TO PrP

virtually eliminated (Figs. 5–7, Supplemental Fig. 1, Table I). In contrast, binding of the other three Abs was not reduced by removal of the disulfide bond. Differences in the O/R ratios argue linear or discontinuous epitopes for the Abs, the latter of which are conformation dependent (Table I, Supplemental Tables I, II). F10-26, F20-49, and F20-89a had O/R ratios near 1.0 and bound to short, overlapping peptides, which indicate linear epitopes (Table I). The other five Abs had O/R ratios ≥ 3.0 and did not bind any overlapping peptides, arguing for nonlinear, conformation-dependent epitopes. Reduction of the disulfide bridge disrupted the conformational or discontinuous epitopes, but it did not affect the linear epitopes, for binding. The atomic structures of the epitopes for F4-31, F20-29, F20-108a, F20-130a, and F20-80 mAbs remain to be determined.

F4-31 was selected for further study because of its strong binding to BoPrP, its broad cross-species reactivity, and its ability to function as a capture Ab. F4-31 binds to native PrPSc and Gdn-denatured PrPSc but not to native PrPSc (Fig. 4A, 4C). F4-31 gave higher signals in the CDI compared with HuM-D18 rFab for all PrP sequences tested, except for HuPrP (Fig. 8A), demonstrating a substantial improvement for prion detection. Experiments in which BSE-infected brain homogenates were diluted into normal bovine brain homogenate demonstrated a >500-fold increase in sensitivity when F4-31 was used as the capture reagent in the CDI (Fig. 8B). It seems likely that the improved performance that we observed in the CDI using F4-31 results from its high affinity to BoPrP and its conformational epitope, the atomic structure of which remains to be elucidated. Nevertheless, our data indicate that the F4-31 mAb can be used as an excellent capture mAb for the highly sensitive detection of PrP.

This article reported a novel set of conformation-dependent mAbs, the activities of which can be abolished by reducing the single disulfide bond in PrP. The extremely low backgrounds after reduction of PrP in various testing formats suggest that these conformation-dependent mAbs are likely to find application in a wide variety of immunoassays.