Novel Epitopes Identified by Anti-PrP Monoclonal Antibodies Produced Following Immunization of *Prnp*^{0/0} Balb/cJ Mice with Purified Scrapie Prions

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Prions, or infectious proteins, cause a class of uniformly fatal neurodegenerative diseases. Prions are composed solely of an aberrantly folded isoform (PrP^{Sc}) of a normal cellular protein (PrP^C). Shared sequence identity of PrP^{Sc} with PrP^C has limited the detection sensitivity of immunochemical assays, as antibodies specific for the disease-causing PrP^{Sc} isoform have not been developed. Here we report the generation of three new monoclonal antibodies (MAbs) to PrP, which were isolated following immunization of *Prnp*^{0/0} Balb/cJ mice with highly purified PrP^{Sc} isolated from brain lipid rafts. Epitope mapping using synthetic PrP peptides revealed that the three MAbs bind different epitopes of PrP. The DRM1-31 MAb has a conformational epitope at the proposed binding site for the putative prion conversion co-factor "protein X." The DRM1-60 MAb binds a single linear epitope localized to the β 2- α 2 loop region of PrP, whereas DRM2-118 binds an epitope that includes sequences within the octarepeat region and near the site of N-terminal truncation of PrP^{Sc} by proteinase K. Our novel anti-PrP MAbs with defined PrP epitopes may be useful in deciphering the conformational conversion of PrP^C into PrP^{Sc}.

Introduction

PRION DISEASES ARE A FAMILY of fatal neurodegenerative disorders caused by the progressive accumulation of the alternatively folded prion protein (PrP^{Sc}) in the brain.^(1,2) Normal, endogenous PrP^C shares the same amino acid sequence as PrP^{Sc} but differs in its conformation. Different conformers of PrP^{Sc} impart distinct biochemical properties that create specific neuropathologic changes.⁽³⁻⁷⁾ Infectious prions are propagated by conversion of α -helical PrP^C into β -sheet-rich PrP^{Sc} by a template-mediated process that is not well understood.^(8,9)

Unlike PrP^C, much of the PrP^{Sc} molecule is resistant to limited proteolysis with proteinase K (PK). Thus, in the presence of PK, the protease-sensitive PrP^C is hydrolyzed whereas resistant PrP^{Sc} is N-terminally truncated and a readily detectable C-terminal fragment (PrP 27-30) remains.⁽¹⁰⁾ Current immunoassays for PrP^{Sc} exploit this difference, and detection of PrP 27-30 is used to corroborate disease diagnosis. Antibodies used for these tests bind common epitopes present in both PrP^C and PrP^{Sc} as no conformation-specific antibody has been generated that can directly distinguish PrP^{Sc} from PrP^C.⁽¹¹⁾ Although antibodies that immunoprecipitate PrP^{Sc} have been reported,^(12,13) none appear to possess the selectivity necessary for development into a reliable immunoassay. Recent studies have described in-

fectious PrP that is not PK resistant, further demonstrating the need for a PrP^{Sc}-specific antibody.⁽¹⁴⁾

Efforts to generate PrP^{Sc}-selective antibodies have been disappointing; this lack of success is likely a result of the physiochemical properties shared between PrP^C and PrP^{Sc} that render the two isoforms indistinguishable immunologically. As expected, wild-type (wt) animals are tolerant to the normal protein PrP^C.^(15,16) This tolerance probably accounts for the lack of an immune response to PrP^{Sc} during prion infection. The generation of the *Prnp*^{0/0} mice overcame the barrier associated with self-recognition of PrP^{Sc} as these mice elicit a robust immunoreponse when challenged with either PrP^C or PrP^{Sc}.^(17,18) However, the immune system may produce antibodies that recognize epitopes shared by both PrP^C and PrP^{Sc}. Likewise, immunization with synthetic PrP peptides or recombinant PrP^C produced antibodies that bind both PrP^C and denatured PrP^{Sc}.^(17,19,20) Therefore, antibodies that can distinguish PrP^{Sc} may recognize rare epitopes that are only occasionally presented to the immune system. In such a case, exploitation of hybridoma technology to dissect the immunological response will be critical for isolating rare monoclonal antibodies (MAbs) with conformational epitopes.

In this report, we describe a series of new anti-PrP MAbs isolated from *Prnp*^{0/0} Balb/cJ mice⁽¹⁸⁾ immunized with a

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highly purified PrP^{Sc} immunogen derived from prion-infected hamster brain. Furthermore, we describe a direct-binding ELISA that uses prion-enriched detergent-resistant membranes (DRMs) from normal and Sc237-infected hamster brain as part of an overall hybridoma screening strategy to favor identification of anti-PrP MAbs. We report here the characterization of these new anti-PrP MAbs and identify novel binding epitopes, which have been implicated in the conversion of PrP^C into PrP^{Sc}.

Materials and Methods

Reagents

All reagents were of the highest grades commercially available. Antibodies were diluted in 10 mM Tris-buffered saline with 1% Tween-20 (TBST) containing 1% IgG-free BSA (Jackson Immunochemicals, West Grove, PA) or TBST containing 3–10% nonfat dry milk (NFD) powder. Secondary antibodies include: goat-anti-mouse-HRP and goat-anti-rabbit-HRP (diluted 1:10,000; Pierce, Rockford, IL) or goat-anti-mouse-HRP (diluted 1:5000; Sigma, St. Louis, MO). Recombinant Syrian hamster (recSHa) PrP(90–231), recombinant mouse (recMo) PrP(89–230), recombinant ovine (recOv) PrP(23–231), and recombinant human (recHu) PrP(90–231) were generated at UCSF as previously described.^(20–22) A series of peptides was synthesized, each of 12 amino acids in length with a 7-residue overlap spanning bovine PrP(25–241) (Table 1). Three lysine residues were added to the N-terminus of each peptide to improve solubility; a single biotin molecule was also added to the N-terminal lysine residue. The C-termini were amidated (Sigma Genysis, Woodlands, TX). Peptides were dissolved in 10% acetonitrile and diluted to 1 mM in 10% ethyl alcohol.

Animals

Animals were housed as previously described⁽¹⁸⁾ in compliance with the USDA Western Regional Research Center's Institutional Animal Care and Use Committee approved protocols. Hamster-passaged Sc237 scrapie prions (PrP^{Sc}) were propagated in female Syrian golden hamsters,^(18,23) and experimental procedures using infectious prions were conducted in BSL2 laboratories. Generation and immunization of *Prnp*^{0/0} Balb/cJ mice were previously described.⁽¹⁸⁾

Immunogen

PrP^{Sc} was first isolated in DRM fractions from Sc237-infected Syrian hamster brain homogenate and processed to obtain a purified PrP 27–30 immunogen as previously described.⁽¹⁸⁾ Briefly, hamster brains were homogenized (10% w/v) on ice in 25 mM MES (pH 6.5) with 150 mM NaCl, 1% Triton X-100, 60 mM n-octyl-glucoside, 10 mM PMSF, and protease inhibitors (Complete mini; Roche, Nutley, NJ). Brain homogenates were fractionated by ultra-centrifugation using a discontinuous sucrose gradient and the DRM fraction digested with 150 µg/mL of PK for 1 h at 60°C. Protease digestion was then inhibited by 10 mM PMSF. PrP^{Sc} was concentrated by phosphotungstic acid (PTA) precipitation, and the PK-resistant PrP^{Sc} further separated from the PK and proteolytic products by size exclusion chromatography on a Sephadex G100 column. PrP^{Sc} was recovered in the void

TABLE 1. PEPTIDE SEQUENCES AND NUMBERING ACCORDING TO BoPrP (NCBI ACCESSION # CAA39368)

Peptide no.	Amino acid nos.	Amino acid sequence
1	25–36	KKRPKPGGGWNT
2	30–41	PGGGWNTGGSRY
3	35–46	NTGGSRYPGQGS
4	40–51	RYPGQGSPPGGR
5	45–56	GSPGGNRYPPQG
6	50–61	NRYPQGGGGWG
7	55–66	QGGGGWGQPHGG
8	60–71	WGQPHGGGGWGP
9	65–76	GGGWGQPHGGGW
10	70–81	QPHGGGGWGQPHG
11	75–86	GWGQPHGGGGWQ
12	80–91	HGGGGWGQPHGGG
13	85–96	GQPHGGGGWGQPH
14	90–101	GGGWGQPHGGGGW
15	95–106	PHGGGGWGQGGT
16	100–111	GWGQGGTHGQWN
17	105–116	GTHGQWNKPSKP
18	110–121	WNKPSKPKTNMK
19	115–126	KPKTNMKHVAGA
20	120–131	MKHVAGAAAAGA
21	125–136	GAAGAGAVVGGGL
22	130–141	GAVVGGGLGGYML
23	135–146	GLGGYMLGSAMS
24	140–151	MLGSAMSRPLIH
25	150–161	IHFSGDYEDRY
26	155–166	DYEDRYRENMH
27	160–171	YYRENMHRYPNQ
28	165–176	MHRYPNQVYRYP
29	170–181	NQVYRYPVDQYS
30	175–186	RPVDQYSNQNNF
31	180–191	YSNQNNFVHDCV
32	185–196	NFVHDCVNITVK
33	190–201	CVNITVKEHTVT
34	195–206	VKEHTVTTTTKG
35	205–216	KGENFTETDIKM
36	210–221	TETDIKMMERVV
37	215–226	KMMERVVEQMCI
38	220–231	VVEQMCIQYQR
39	225–236	CITQYQRESQAY
40	230–241	QRESQAYYQARGA

Three lysine residues were added to the N-terminus of each peptide, with the N-terminal lysine labeled with biotin, and the C-terminus was amidated.

volume, concentrated by a second PTA precipitation, and resuspended in buffer. This material (referred to as the immunogen) was previously shown to be infectious and to migrate as a single Coomassie staining protein band with a molecular mass consistent with the PK-resistant PrP^{Sc} core (PrP 27–30).

Immunization

Prnp^{0/0} Balb/cJ mice (25 day old) were immunized with two bilateral intraperitoneal (i.p.) inoculations of 100 µL of purified hamster PrP^{Sc} immunogen (11.6 µg, measured by BCA, Pierce) in adjuvant (Sigma Adjuvant System; Sigma) separated by 10 days.⁽¹⁸⁾ A third immunization boost was performed 10 days after the second injection without adjuvant and 3 days prior to cell fusion.

Antibody generation

Cell fusions were performed as previously described.⁽²⁴⁾ Ten to 14 days following cell fusion, media from culture wells containing hybridoma cells were screened using a direct-binding ELISA for antibody binding to prion-enriched DRM preparations obtained from diseased hamster brain homogenates (PrP^{Sc}-DRM).⁽¹⁸⁾ Since the majority of hybridoma cells were not expected to produce antibodies, the averaged ELISA activity on a plate was taken to represent background. Cells from positive wells, those with a response at least five times the background, were transferred to 24-well culture plates, allowed to grow for at least 24 h, and the media analyzed by ELISA for antibody binding to rec-SHaPrP(90–231) and to DRM fractions obtained from normal (PrP^C-DRM) and diseased (PrP^{Sc}-DRM) brain treated with and without PK. Finally, hybridoma cells from ELISA-positive wells were cloned at least twice by limiting dilution and media evaluated by Western blot analysis. Cloned hybridoma cells were used to generate ascites in Balb/cJ mice (Covance Immunology Services, Denver, PA) and MAbs purified by affinity chromatography on protein-G Sepharose (Pierce).

ELISA

Antigens were diluted in 0.1 M sodium bicarbonate buffer (pH 9.4) and 100 μ L absorbed to 96-well Maxisorb plates (Nunc, Rochester, NY) overnight at 4°C. Plates were washed in TBST, blocked in 10% NFDm for 1 h, incubated for 1 h with primary antibody, washed, incubated for 1 h with HRP-conjugated secondary antibody, washed, resolved by chemiluminescence (Supersignal; Pierce) detection using a Victor² plate reader (PerkinElmer, Waltham, MA), and results expressed as relative light units (RLU). Isotype analysis was determined by ELISA using isotype-specific antibodies (Southern Biotechnology, Binghamton, AL).

Antibody sequencing

The mRNA coding for the PrP MAbs were extracted and purified from hybridoma cells, converted to cDNA, and the heavy (Hc) and light (Lc) chains of each antibody amplified using polymerase chain reaction (PCR).⁽²⁵⁾ Circularized vector pCR2.1 (Invitrogen, Carlsbad, CA) was digested with *EcoRV* and treated with calf intestinal phosphatase (New England BioLabs, Bethesda, MD). PCR products were gel-purified and treated with polynucleotide kinase (New England BioLabs) ligated into vector pCR2.1 and transformed into TOP10 cells (Invitrogen) according to manufacturer's instruction. Colonies were picked and grown aerobically in 5 mL of Luria-Bertani (LB) medium at 37°C supplemented with 100 μ g/mL ampicillin.⁽²⁶⁾ Plasmids were purified using the QuickClean 5M range of kits (GenScript Corp, Piscataway, NJ). Automated DNA sequencing was performed using the Big Dye Terminator Version 3.1 and XTerminator reagents, and a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Primers M13F (GTAAAACGACGGCCAG) and M13R (CAGGAAACAGCTATGAC) were used for sequencing the DNA insert in plasmid pCR2.1.⁽²⁷⁾

All Lc clones were amplified with the same primers, 3' Kc and 5' Mk, and the Hc primers are as follows: DRM1-15, 5' MH2 and 3' IgG2b; DRM1-26, 5' MH2 and 3' IgG2b; DRM1-31, 5' MH2 and 3' IgG2b; DRM1-60, 5' MH2 and 3' IgG2b; and DRM2-118, 5' MH2 and 3' IgG1 as described.⁽²⁵⁾

Western blot analysis

Western immunoblotting was performed as described.⁽²³⁾ Briefly, samples (brain homogenates and DRM preparations) were separated by electrophoresis through 4–12% Bis-Tris gels with MES running buffer (Novex, Invitrogen). Proteins were transferred to nitrocellulose (Bio-Rad, Hercules, CA), washed in TBST, blocked with 10% NFDm, probed with antibodies, protein bands resolved by ECL (Supersignal, Pierce),

FIG. 1. Direct-binding ELISA results from the initial screening of a cell-fusion experiment using splenocytes of PrP^{Sc}-immunized mice. A microtiter plate was coated with purified PrP^{Sc}-DRM following PK digestion. Since the majority of wells of a screening plate are not expected to contain hybridomas secreting specific antibody, the plate background was calculated as the average (dashed line) relative luminescence units (RLU), 0.15×10^6 RLU for the plate shown.

and imaged on a Fluorochem HD documentation system (Alpha Innotech, San Leandro, CA).

Epitope determination

A series of 12-residue peptides spanning bovine PrP(25–241) (NCBI accession # CAA39368), which corresponds to hamster sequences 23–231 (NCBI accession # P04273) (see Table 1), was synthesized and used in ELISA, as previously described,⁽²⁰⁾ to localize antibody binding to specific epitopes. Each peptide contained 12 amino acids of the PrP sequence with a 7-residue overlap. Three lysine residues were added to the N-terminus of each peptide to improve solubility, the N-terminal lysine residue was biotinylated, and the C-termini were amidated. Two peptides in this series corresponding to ^{Bo145}MSRPLIHFGSDY¹⁵⁶ and ^{Bo200}VTTT²⁰⁰TKGENFTE²¹¹ failed in the synthesis and were not available.

Peptides were dissolved in 10% acetonitrile and diluted to 1 mM in 10% ethyl alcohol. On the day of the experiment, streptavidin-coated microtiter wells (Pierce) were blocked for

60 min at room temperature (RT) by adding 300 μ L/well of 5% NFDMS-TBST. Peptides were diluted in TBST (pH 7.4) to a final concentration of 5 μ M, then added (50 μ L) to the plates and incubated for 1 h at RT. Anti-PrP antibodies were then added (50 μ L) and the plate incubated for 1 h at 37°C. Following three washes with 200 μ L/well of TBST, 100 μ L/well of HRP-conjugated, goat anti-mouse IgG (Pierce), diluted 1:10,000 in 1% BSA-TBST, were added and the plates incubated for 1 h at RT. Finally, the plates were washed three times with TBST; then 100 μ L/well of substrate (Supersignal, Pierce) was added and luminescence measured with a Victor³ plate reader (PerkinElmer). Peptide sequences were aligned for maximum sequence conservation.

Results

Antibody production

Recent studies demonstrated production of high-titer anti-PrP sera following immunization with highly purified PK-

FIG. 2. ELISA binding of five MAbs—(A) DRM1-15, (B) DRM1-26, (C) DRM1-31, (D) DRM1-60, and (E) DRM2-118—to titrated recombinant PrP harboring different species sequences: recSHaPrP(90–231), open red circle; recMoPrP(89–230), open blue diamond; recOvPrP(23–231), open pink triangle; and recHuPrP(90–231), open green square. Data points and error bars represent mean and standard error of the mean (SEM) from three independent experiments. Concentration of MAb at 1 μ g/mL.

resistant hamster PrP^{Sc} in recently bred *Prnp*^{0/0} on a Balb/c background but not wt mice.⁽¹⁸⁾ We used splenocytes from *Prnp*^{0/0} Balb/c mice immunized with a purified PrP^{Sc} immunogen derived from brain DRMs to generate hybridomas. The hybridomas were subsequently screened for production of anti-PrP antibodies using a direct-binding ELISA on microtiter plates coated with PrP^{Sc}-DRM preparations purified from brain homogenates of prion-infected Syrian hamsters. Hybridoma cells from wells with chemiluminescence signals greater than five times the plate background (Fig. 1; dotted line) were selected, and antibody binding further characterized using a panel of antigens consisting of recSHaPrP(90–231), PrP^C-DRMs, and PrP^{Sc}-DRMs (before and after PK treatment). Those cells producing antibodies suggestive of binding PrP^{Sc} (i.e., minimal binding on recSHaPrP(90–231) and PrP^C-DRMs, but greater than five times binding on PrP^{Sc}-DRMs) were cloned by limiting dilution. Subsequent ELISA tests suggested that these MABs bound both PrP^C and PrP^{Sc}. Monoclonal antibodies were then produced as ascites and purified by protein-G affinity chromatography. Five MABs were identified and designated DRM1-15, DRM1-26, DRM1-31, DRM1-60, and DRM2-118. Isotype analyses revealed that DRM1-15, 26, 31, and 60 have IgG2b heavy chains while DRM2-118 is an IgG1 antibody, all with kappa light chains.

Antibody specificity

The binding specificity of the five MABs to recombinant PrP was evaluated in ELISA titration experiments; we used recPrP harboring the sequences from SHa (90–231), mouse (Mo; 89–230), sheep (Ov; 23–231), and human (Hu; 90–231) (Fig. 2). These titration studies revealed binding by all of the MABs to recSHaPrP(90–231), demonstrating that they lack the PrP^{Sc} specificity suggested in the initial screening (Fig. 2). Antibodies DRM1-15 and DRM1-26 showed nearly identical binding patterns with the strongest binding to recSHaPrP(90–231), approximately 10-fold lower binding to recMoPrP(89–231), at least 200-fold lower binding to recOvPrP(23–230), and virtually no binding to recHuPrP(90–231) (Fig. 2A, B). The

binding profile of DRM1-31 was similar to those of DRM1-15 and DRM1-26, but slightly better binding to recOvPrP(23–231) was observed (Fig. 2C). In contrast, MAB DRM1-60 had a distinctly different binding profile: strongest binding to recSHaPrP(90–231), slightly less binding to mouse and ovine PrP, and weaker but detectable binding to recHuPrP(90–231) (Fig. 2D). Antibody DRM2-118 also demonstrated strongest binding to recSHaPrP(90–231) and only slightly weaker binding to recMoPrP(89–230), but moderate binding to recHuPrP(90–231) and weakest binding to recOvPrP(23–230) (Fig. 2E). Western blot analyses of recPrP with the five MABs showed binding patterns similar to those observed with ELISA (data not shown). These results suggest that the five MABs represent at least three distinct epitope groups, and that neither the GPI anchor nor the carbohydrate groups present on native PrP (the antigen used for screening) are required for binding.

Antibody binding to full-length PrP^C from brain homogenates derived from eight different species was compared by Western blot analysis (Fig. 3). As expected, all five MABs recognized denatured, full-length, glycosylated SHaPrP^C containing the GPI anchor (Fig. 3). MAB DRM2-118 recognized PrP^C from all the species tested except for cervid (Fig. 3; top panel). Antibody DRM1-60 detected PrP^C from hamster, mouse, sheep, cow, pig, and white-tailed deer, but not horse or human PrP^C (Fig. 3; second panel). DRM1-31, DRM1-26, and DRM1-15 showed strong binding to SHaPrP^C and weak but detectable binding to MoPrP^C and CerPrP^C (Fig. 3, bottom three panels).

Antibody variable region sequences

The binding patterns observed with MABs DRM1-15, DRM1-26, and DRM1-31, to both recombinant PrP and native PrP^C derived from normal brain homogenates of different species, suggest that they bind the same or a closely related epitope. To clarify their relationship to each other, the nucleic acid sequence and derived amino acid sequences of the variable region for both the Lc and Hc of each antibody were determined (sequences available from GenBank: accession #

FIG. 3. Western blot detection of PrP^C in 40 μg of normal brain homogenates with the five MABs. Homogenates were prepared from uninfected whole Syrian hamster brain (SHa); whole mouse brain (Mo); sheep cortex (Ov); horse cortex (Eq); cow cortex (Bo); pig cortex (Po); whole human brain (Hu); and white-tailed deer obex (Ce). For each MAB, 2 μg/mL was used. Molecular masses of protein standards are shown in kilodaltons (kDa).

DRM1-15_Lc HQ218926; DRM1-26_Lc HQ218927; DRM1-31_Lc HQ218928; DRM1-60_Lc HQ218929; DRM2-118_Lc HQ218930; DRM1-15_Hc HQ218931; DRM1-26_Hc HQ218932; DRM1-31_Hc HQ218933; DRM1-60_Hc HQ218934; DRM2-118_Hc HQ218935). Alignment of the amino acid sequences is shown for the entire cloned region from the beginning of framework region one (FRW-1) to the end of the fourth framework region (FRW-4). The J-region is shown in bold, the amino acids corresponding to the PCR primers are underlined, and the complementarity determining regions (CDRs) are shaded (Fig. 4). The leader sequences, framework regions, CDRs, and J-regions were assigned by comparison of the Hc and Lc of these five MAbs to other antibody sequences.⁽²⁸⁻³²⁾ Antibodies DRM1-15, DRM1-26, and DRM1-31 have identical variable Hc and Lc amino acid sequences (Fig. 4),

and most likely represent independent cell fusions of a clonally expanded lymphoid population. Therefore, for these three MAbs, only DRM1-31 was further characterized. Both MAbs DRM1-60 and DRM2-118 have variable region amino acid sequences distinct from each other and from the DRM1-15, -26, and -31 group (Fig. 4).

Peptide mapping

In an effort to further define the epitopes, antibody binding to a library of synthetic peptides spanning the entire PrP sequence was evaluated. The library, used in a previous study,⁽¹⁹⁾ consisted of 12-residue peptides spanning the bovine PrP sequence (NCBI accession # CAA39368), each overlapping by seven amino acids (Table 1). Because 11 of the 16

Light Chain

	Primer	FWR-1	CDR1	
DRM1-15	<u>ELDIVITQSPSS</u> LAVSAGEKVTMSCR	SSQSLNSRTRKNYLAWYQQKP		
DRM1-26	 <u>L</u>			
DRM1-31	 <u>M</u>			
DRM1-60	 <u>L</u>	AI•S•P•R•SF•A•IG---S-IH•RT		
DRM2-118	 <u>T</u> •A•.....	L•QRATI•KA•VDYD--GDS•MN•		

	FWR-2	CDR2	FWR-3	
DRM1-15	GQSPKLLIYWASTRESGVPDRFTGSGSGTDFLT	ISSVQAEDLAVYYC		
DRM1-26				
DRM1-31				
DRM1-60	NG•R•KYG•ESI•I•S•S•N•NN•ES•I•D•			
DRM2-118	•P•A•NLE•I•A•S•N•HP•EE•A•T•			

	CDR3	FWR-4	
DRM1-15	<u>KQSYN-LYTFGGG</u> TKLEIKRADAAPTVSAC		
DRM1-26			
DRM1-31			
DRM1-60	QQTNSWPW•R•		
DRM2-118	Q•NEDPL•		

Heavy Chain

	Primer	FWR-1	CDR1	FWR-2
DRM1-15	<u>EFQVKLEQSGPEL</u> KKPGETVKISCKASSYTFT-VYSIHWVKQTPGKGLK			
DRM1-26	•E•E•			
DRM1-31	•E•			
DRM1-60	•E•	G•VI•SQSLSLT•TV•G•SI•SD•AWN•IR•F•NK•E		
DRM2-118	•E•E•	-----G•N•GMN•A•		

	CDR3	FWR-3	
DRM1-15	WMGWINTETGEPTYADDFKGRFAFSLETSASTAYLQINNLDKEDTATY		
DRM1-26			
DRM1-31			
DRM1-60	Y•Y•S•YS•STS•NPSLKS•ISITRD•KNQFF•L•SVTT•		
DRM2-118	•Y•G•F•R•N•M•		

	CDR3	FWR-4	
DRM1-15	<u>FCTRG-GTGDYWGQ</u> TTLTVSSAKTTPPSVYPLAPRS		
DRM1-26			
DRM1-31			
DRM1-60	Y•A•SRYYF•		
DRM2-118	•V•SR•S•V•	-----	

FIG. 4. Amino acid sequences for the variable regions of DRM1-15, DRM1-26, DRM1-31, DRM1-60, and DRM2-118 kappa light and heavy chains. The deduced amino acids, from the first (FWR-1) to the end of the fourth framework region (FWR-4) are shown. The three complementarity determining regions (CDRs) are shaded, J-region is shown in bold, amino acids coded for by the primers are underlined, and framework regions (FWR-1, FWR-2, FWR-3, and FWR-4) are indicated.

differences between SHa and Bo PrP sequences are strongly conserved amino acids (two represent deletions), the Bo peptide library was used as a tool to evaluate antibody binding. To improve peptide solubility, three lysine residues were added to the N-terminus of each peptide and the terminal lysine was biotinylated. The peptides were oriented and immobilized using streptavidin-coated, 96-well microtiter plates. Results of DRM antibody binding to this library are summarized in Figure 5. The simplest binding pattern was observed with MAb DRM1-60; only peptide #29 was recognized (Fig. 5B), suggesting a linear binding epitope within the sequence $^{170}\text{NQVYYRPVDQYS}^{181}$ corresponding to SHaPrP(159–170) (NCBI accession # P04273). The carboxy-terminal Ser (S) at bovine position 181 is not critical for

FIG. 5. Epitope mapping of MAbs DRM1-31 (A), DRM1-60 (B), and DRM2-118 (C) using overlapping BoPrP peptides in direct-binding ELISAs. Peptides spanned 12 amino acids, with 7-residue overlaps, corresponding to BoPrP(25–241). Dashed line represents $2\times$ background activity. Bar represents mean $\pm$ SEM from two independent experiments.

binding since the hamster sequence has an Asn (N) in this position. Antibody DRM1-31 displayed a more complex peptide-binding pattern (Fig. 5A), binding to spatially separated peptide #29, and peptides #37–39. These data suggest a discontinuous or conformational epitope, incorporating the three segments: $^{170}\text{NQVYYRP}^{176}$ (the β -2 sheet region); the amino end of the second α -helical region, $^{185}\text{NFVHDCV}^{191}$; and a segment near the carboxyl end of the third α -helical region, $^{222}\text{EQMCITQY}^{229}$. Antibody DRM2-118 bound a number of peptides located in the N-terminal non-structured region of PrP (Fig. 5C). Specifically, strong binding was observed to peptides #9, #14–#17, as well as to peptide #44, which is a modification of peptide #16, in which T106 and G108 were both substituted with Ser (Table 2). Peptide alignment that results in the largest invariant sequences suggests an epitope motif containing the sequence GWGQ (Table 2). This sequence contains elements of the octarepeat region. The motif is broadly represented in PrP sequences of different species as part of the octarepeat, which is consistent with the broad species reactivity observed (Fig. 3) and suggests the possibility of multiple binding sites for DRM2-118.

Detection of PrP^{Sc}

To determine whether the three MAbs recognize PrP^{Sc}, we evaluated their binding to purified PrP^{Sc}-DRM by Western immunoblotting (Fig. 6). As a control, binding to PrP^C-DRM was also tested. All three MAbs DRM1-31, DRM1-60, and DRM2-118 ($1\text{ }\mu\text{g/mL}$) were able to detect PrP^{Sc} by Western blot ($8\text{ }\mu\text{g/lane}$; Fig. 6). Following PK treatment, the antibodies effectively detected PK-resistant PrP 27–30, as defined by the molecular weight shift on Western blots [compare undigested samples (–) to digested (+) samples]. In contrast, PK digestion effectively abolished detection in the DRM-PrP^C samples by Western blot. In these experiments, detection of PrP 27–30 and total PrP (undigested PrP^C + PrP^{Sc}) in PrP^{Sc}-DRM samples was greater compared to PrP^C in DRMs, which likely reflects the greater amount of accumulated PrP^{Sc} in DRM preparations compared to PrP^C in DRM samples, as observed previously.⁽¹⁸⁾

Differential antibody binding

Next, we determined whether removal of the disulfide bond in PrP affected binding of the three MAbs. Brain homogenates

TABLE 2. ALIGNMENT OF PEPTIDES BINDING MAb DRM2-118

Peptide no.	PrP region	Sequence	ELISA response
9	Bo 65–76	GGGWGQPHGGGW	+++
14	Bo 90–101	GGWQPHGGGGW	+++
15	Bo 95–106	PHGGGGWGQGGT	+++
16	Bo 100–111	GWGQGGTHGQWN	+++
17	Bo 105–116	GTHGQWNKPSKP	++
44	Ov 92–103	GWGQGGSHSQWN	+++

Peptide #44 corresponds to OvPrP(92–103) with two differences from peptide #16 indicated in bold. ELISA binding from Figure 5C is summarized here for convenience. Potential epitope motif is GWGQ.

Bo, bovine (NCBI accession # CAA39368); Ov, ovine (NCBI accession # CAA04236); ++, moderate binding; +++, strong binding.

FIG. 6. Detection of PrP^C and PrP^{Sc} in DRMs by MAB DRM1-31 (A), DRM1-60 (B), and DRM2-118 (C) by Western blots. DRM samples were left undigested (-) or digested with PK (+). For each lane, 1 μ g/mL of each MAB was used to detect 8 μ g of brain DRM protein. Molecular masses of protein standards are shown in kilodaltons (kDa).

from prion-infected Syrian hamsters were prepared and boiled for 1 min in electrophoresis sample buffer containing 10% β -mercaptoethanol, then 10 μ g was loaded per lane, subjected to SDS-PAGE and Western immunoblotting (Fig. 7). Removal of the disulfide bond in PrP reduced the binding of DRM1-31 and DRM1-60, but had little effect on the binding of DRM2-118.

Discussion

We have previously demonstrated a novel method to purify and concentrate PrP^{Sc} from lipid rafts (DRM), and that immunization of a newly derived *Prnp*^{0/0} Balb/cJ mouse line with PrP^{Sc}-DRM generated antisera reactive on Western blots to PrP, but not to other proteins present in brain homogenate.⁽¹⁸⁾ The objective of this study was to use hybridoma technology to dissect the immune response in order to isolate novel antibodies that might be employed in novel diagnostics.

FIG. 7. Effect of disulfide bond disruption in PrP on MAB binding. Scrapie-infected hamster brain homogenates were treated with (+) and without PK (-) in the presence (+) or absence (-) of 10% 2-mercaptoethanol (2-ME) to reduce the single disulfide bond present in PrP. Brain homogenates were loaded at 10 μ g/lane and DRM MAB used at 1 μ g/mL. Molecular masses of protein standards are shown in kilodaltons (kDa).

The most useful antibodies for diagnostics would be those capable of directly distinguishing PrP^{Sc} from PrP^C. Thus, we employed a differential screening strategy early in the selection to identify antibodies that showed strong binding by ELISA to PK-digested PrP^{Sc}-DRM preparations derived from scrapie-infected hamster brain, and little or no binding to PrP^C-DRM preparations derived from non-diseased hamster brain homogenate. Of the nearly 100 positive wells identified in our initial ELISA screen (Fig. 1), only five were selected for cloning and further characterization following the results obtained from a secondary screening for activity toward PrP^C, both undigested and PK-digested PrP^{Sc} as well as recombinant PrP(90-231) (Fig. 2).

While the secondary screening suggested that MAbs DRM1-15, DRM1-26, DRM1-31, DRM1-60, and DRM2-118 preferentially bound PrP^{Sc}, subsequent studies demonstrated that all five MAbs also bound to PrP^C (Fig. 3). Western blots of brain homogenates generated from healthy hamster, mouse, sheep, horse, cow, pig, human, and white-tailed deer demonstrated that all five MAbs bound hamster PrP^C, but differed in their binding to PrP^C of other species. Furthermore, the binding patterns observed by our biochemical analysis indicated that the five antibodies represent three epitope groups. Sequencing of the variable regions of the Hc and Lc supported

FIG. 8. Location of DRM MAb binding mapped onto space-filled models of PrP^C. The amino acids that contribute to the epitope of DRM1-31 from each of the three peptides clusters (^{Bo170}NQVYYRPVDQYS, red; ^{Bo180}NFVHDC, green; and ^{Bo225}EQMCITQY, blue) are shown in the left panel. Amino acids ^{Bo170}NQVYYRPVDQYS (red) that contribute to DRM1-60 MAb binding are depicted in the center panel. Amino acids GWGQ (purple) that contribute to DRM2-118 binding are shown in the right panel. Space-filled models used for DRM1-31 and DRM1-60 from Protein Database (PDB) file 1DX0 and for DRM2-118 from PDB file 2KUN.

assignment into three epitope groups and further suggested that the antibodies DRM1-15, DRM1-26, and DRM1-31 most likely represent hybridomas derived from independent cell fusions of a clonally expanded lymphocyte population, whereas DRM1-60 and DRM2-118 represent distinct hybridomas (Fig. 4).

Results from epitope mapping (Fig. 5) and disulfide bond reduction (Fig. 7) experiments indicate that MAb DRM1-31 has a conformational epitope, incorporating amino acids from three separated regions: SHaPrP(159–170) (NQVYYRPVDQYS); SHaPrP(174–179) (NFVHDC); and SHaPrP(225–236) (EQMCITQY). These regions correspond to the β 2 loop, the N-terminal residues of α -helix 2, and the C-terminal region of α -helix 3, respectively.^(7,33,34) Interestingly, the β 2– α 2 loop and the C-terminal end of the α 3 helix form a continuous surface postulated to be the binding site for the hypothetical “protein

X” thought to be important in the conformational conversion of PrP^C to PrP^{Sc}.⁽³⁵⁾ The epitope motifs from each of these regions were mapped onto the 3-dimensional NMR structure of BoPrP and HuPrP (Fig. 8), demonstrating a juxtaposition of these regions.

Characterization of MAb DRM1-60 suggest a linear epitope (Figs. 5 and 7; Table 2). DRM1-60 showed strong binding to only one peptide (#29: NQVYYRPVDQYS) suggesting a linear epitope corresponding to SHaPrP(159–170), located in β 2– α 2 loop. This epitope assignment is consistent with the binding of DRM1-60 to PrP^C to all species tested, except human and horse, which both harbor substitutions in this region. V166 and Q168 in SHaPrP^C are Met and Gha in HuPrP^C, respectively. For the horse sequence, Ser is expressed at codon 170 instead of Asp (Table 3). Disruption of the disulfide bond by 2-mercaptoethanol reduced DRM 1–60 did not affect

TABLE 3. ALIGNMENT OF RESIDUES 159–170 AND 214–225 OF SHaPrP, MoPrP, BoPrP, HuPrP, EQPrP, CePrP, AND OvPrP

		160				165				170				215				220				225				
SHa	159	N	Q	V	Y	Y	R	P	V	D	Q	Y	N	214	C	T	T	Q	Y	Q	K	E	S	Q	A	Y
Mo	158	•	•	•	•	•	•	•	•	•	•	•	S	213	•	V	•	•	•	•	•	•	•	•	•	•
Bo	170	•	•	•	•	•	•	•	•	•	•	•	S	225	•	I	•	•	•	•	R	•	•	•	•	•
Hu	159	•	•	•	•	•	•	•	M	•	E	•	S	214	•	I	•	•	•	E	R	•	•	•	•	•
Eq	161	•	•	•	•	•	•	•	•	S	E	•	S	216	•	I	•	•	•	•	•	•	Y	E	•	F
Ce	162	•	•	•	•	•	•	•	•	•	•	•	•	217	•	I	•	•	•	•	R	•	•	•	•	•
Ov	162	•	•	•	•	•	•	•	•	•	•	•	S	217	•	I	•	•	•	•	R	•	•	•	•	•

SHaPrP, *Mesocricetus auratus*, NCBI accession #P04273; MoPrP, *Mus musculus*, AAA39996; BoPrP, *Bos taurus*, CAA39368; HuPrP, *Homo sapiens*, AAH22532; EqPrP, *Equine caballus*, ACG59276; CePrP, *Odocoileus virginianus*, AAQ23191; and OvPrP, *Ovis aries*, CAA04236. Only the amino acids that differ from SHaPrP are indicated; dots denote identical amino acids.

binding to PrP, an observation consistent with a linear epitope (Fig. 7).

By using a highly purified preparation of PrP^{Sc} obtained from lipid rafts and novel *Prnp*^{0/0} Balb/cJ mice, we identified three new anti-PrP monoclonal antibodies, DRM1-31, DRM1-60, and DRM2-118. While antibodies with epitopes similar to DRM2-118 have been described,⁽³⁶⁾ DRM1-60 and DRM1-31 have unique epitopes not previously reported. DRM1-60 appears to bind a linear epitope localized to the β 2- α 2 loop, while DRM1-31 has a conformational epitope located on the exposed surface formed by the close proximity of the β 2- α 2-helix loop, the N-terminus of the α 2 helix, and the C-terminus of the α 3 helix.

The antibodies described here, while clearly binding recombinant PrP in ELISA and PrP^C by Western blot, appear to have a preference for PrP^{Sc} that is increased 3-fold following limited PK digestion (PrP 27–30). PK digestion of samples may remove unstructured portions of prion aggregates, exposing more antibody-binding epitopes that results in an increase in total signal detected by immunoassay.

Conclusion

We report here the generation and characterization of three new anti-PrP MAbs obtained following immunization of *Prnp*^{0/0} Balb/cJ mice using purified PrP^{Sc}-DRM. Characterization of these antibodies revealed binding to PrP with distinct species specificity, a strong affinity for PrP^{Sc}, and binding to novel epitopes in the PrP structure not previously reported. Some of these antibodies may prove useful in identifying putative co-factors or binding partners involved in PrP^C conversion to PrP^{Sc}.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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