PrP Conformational Transitions Alter Species Preference of a PrP-specific Antibody*

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The epitope of the 3F4 antibody most commonly used in human prion disease diagnosis is believed to consist of residues Met-Lys-His-Met (MKHM) corresponding to human PrP-(109–112). This assumption is based mainly on the observation that 3F4 reacts with human and hamster PrP but not with PrP from mouse, sheep, and cervids, in which Met at residue 112 is replaced by Val. Here we report that, by brain histoblotting, 3F4 did not react with PrP of uninfected transgenic mice expressing elk PrP; however, it did show distinct immunoreactivity in transgenic mice infected with chronic wasting disease. Compared with human PrP, the 3F4 reactivity with the recombinant elk PrP was 2 orders of magnitude weaker, as indicated by both Western blotting and surface plasmon resonance. To investigate the molecular basis of these species- and conformer-dependent preferences of 3F4, the epitope was probed by peptide membrane array and antigen competition experiments. Remarkably, the 3F4 antibody did not react with MKHM but reacted strongly with KTNMK (corresponding to human PrP-(106–110)), a sequence that is also present in cervids, sheep, and cattle. 3F4 also reacted with elk PrP peptides containing KTNMKHV. We concluded that the minimal sequence for the 3F4 epitope consists of residues KTNMK, and the species- and conformer-dependent preferences of 3F4 arise largely from the interactions between Met-Val (human PrP) or Val115 (cervid PrP) and adjacent residues.

The conformational transition of the cellular prion protein (PrPc)2 into its pathologic conformer (PrPSc) is the key molecular event in the pathogenesis of prion diseases, a group of largely transmissible neurodegenerative disorders affecting both animals and humans, including bovine spongiform encephalopathy in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk, and Creutzfeldt-Jakob disease (CJD) in humans (1, 2). Currently an established diagnosis of prion diseases depends on detection of the protease K (PK)-resistant PrPSc in the brain by antibody-based immunoblotting, immunohistochemistry, or histoblotting. The 3F4 antibody is a powerful immunoreagent commonly used in the diagnosis of human prion diseases, and it has been demonstrated to be species-specific (3, 4), binding strongly to PrP from hamsters and humans but not from mice, rats, rabbits, cattle, sheep, deer, or elk. The 3F4 epitope is generally believed to comprise four amino acids: Met-Lys-His-Met (MKHM) (corresponding to residues 109–112 in human PrP). The species specificity of 3F4 was originally attributed to the substitution of Val in other animal species for Met112 that is in the PrP molecule of humans and hamsters (Table 1). By means of an antigen-competition ELISA, and characterization of the chemical nature of the epitope, the 3F4 epitope was later found to be located within 10 residues containing Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala corresponding to human PrP-(106–115) (4). Recently, through a study of the binding of 3F4 to synthetic peptides and analysis of mutated ovine PrP::GFP constructs expressed in cell culture, the epitope was found to consist of a heptapeptide, Lys-Thr-Asn-Met-Lys-His-Met, corresponding to human PrP-(106–112) (5). Although these studies differ in their specification of the amino acids that comprise the 3F4

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‡ The abbreviations used are: PrPc, cellular prion protein; PrPSc, pathological, scrapie isoform of prion protein; CJD, Creutzfeldt-Jakob disease; CWD, chronic wasting disease; PK, protease K; g5p, Fd gene 5 protein; PBS, phosphate-buffered saline; sCJD, sporadic CJD; SPR, surface plasmon resonance; Fmoc, N-(9-fluorenyl)methoxycarbonyl; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; Tg, transgenic.
epitope in a few residues, they nevertheless agree that Met$^{112}$ is one of the critical residues for its high affinity binding, which is consistent with initial reports on the species specificity of the 3F4 antibody. However, the substitution of Met$^{112}$ with other amino acids, including Val, affects the binding of 3F4 to its epitope and how the species specificity of 3F4 is determined by Met$^{112}$ are two questions that remain unresolved.

Earlier studies demonstrated that 3F4 did not distinguish between PrP$^{C}$ and PrP$^{Sc}$ (3) and the 3F4 epitope was believed to be linear (4), which might be consistent with the fact that the two molecules share the same primary structure. However, it has been well documented that several un-structured areas or $\alpha$-helical domains of the PrP$^{C}$ molecule acquire or convert to $\beta$-sheet structures during the conversion of PrP$^{C}$ into PrP$^{Sc}$ (6). Moreover, the 3F4 epitope is one of the critical areas involved in such structural transition and has been suggested to comprise part of the “nucleation domain” from which conformational change can disseminate to other parts of the protein (1, 6–8).

Antibodies against PrP, including 3F4, have been used to investigate the antigenicity of both PrP$^{C}$ and PrP$^{Sc}$ and to evaluate the structural differences between the two conformers (7, 9–11). Indeed, these studies have revealed some differences in 3F4 immunoreactivity between PrP$^{C}$ and PrP$^{Sc}$, although the molecular basis of this divergent reactivity remains poorly understood (7–11). The observation that the 3F4 epitope is exposed in the hamster PrP$^{C}$ but becomes cryptic upon the conversion of PrP$^{C}$ to PrP$^{Sc}$ implies that the 3F4 antibody is not only species-specific but also conformer-specific. However, whether the conformational change associated with the conversion of PrP$^{C}$ to PrP$^{Sc}$ affects the species specificity of the 3F4 antibody, which is predominantly linked to the single residue Met$^{112}$, is largely unknown.

The 3F4 epitope is located in a critical area of the protein, which is involved in at least six highly important molecular events known so far. First, the molecular region of human PrP-(90–120) undergoes structural transition during the conversion from PrP$^{C}$ to PrP$^{Sc}$, the key molecular event in the pathogenesis of prion diseases (1). Second, human PrP-(106–126) (called toxic prion peptide), containing the 3F4 epitope, is involved in neuronal toxicity (12). Third, the 3F4 epitope is also hidden in a so called transmembrane domain of human PrP-(104–132) (13). Fourth, human PrP-(95–110) is the area to which amyloid-$\beta$ oligomers bind, through which PrP$^{C}$ mediates impairment of synaptic plasticity by amyloid-$\beta$ oligomers in Alzheimer disease (14). Fifth, the 3F4 epitope is the endoproteolytic cleavage site for generation of the normal metabolic product of human PrP called C1 (15). Finally, it has been recently demonstrated that PrP$^{C}$-associated signal transduction is modulated by the primary sequence around the C1 cleavage site (16, 17). Therefore, the 3F4 epitope appears to be the central area within which these physiological and pathophysiological molecular events occur. Studies on the structure and binding behaviors of the 3F4 epitope would be significant in enhancing our understanding of these events.

Using histoblotting, we revealed that the immunoreactivity of 3F4 with PK-treated elk PrP$^{Sc}$ in the brain of CWD-infected cervidized transgenic mice (Tg) expressing elk PrP is virtually equal to that of two other antibodies without species specificity: 6H4 and 8H4. However, in contrast to 6H4 and 8H4, 3F4 does not react with elk PrP from uninfected cervidized Tg mice. Moreover, on the Western blots, 3F4 also reacts with PrP$^{Sc}$ enriched from elk brain infected with CWD by the g5p capture. It also reacts with recombinant elk PrP, but the affinity is as much as ~98% lower compared with that in recombinant human PrP, as detected by Western blotting and surface plasmon resonance. Using peptide arrays on the cellulose paper and competition experiments with Western blotting or ELISA, we demonstrated that the epitope residues minimally required for 3F4 binding are PrP-(106–110) (KTNNMK), not PrP-(109–112) (MKHM), and that the effect of Met$^{112}$ in human PrP or Val$^{115}$ in PrP on the species preference of the 3F4 antibody is largely modulated by the adjacent amino acids including Ala$^{115}$.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Phenylmethylsulfonyl fluoride, PK, $N,N'$-diisopropylcarbodiimide, 1-hydroxybenzotriazole, trifluoroacetic acid, piperidine, triisobutylsilane, and dichloromethane were purchased from Sigma. Amino-PEG cellulose membranes and Fmoc-amino acids were obtained from Intavis (San Marcos, CA). $N^\alpha$-Fmoc-O-benzyl-$\gamma$-phosphoserine and $N^\alpha$-Fmoc-O-benzyl-$\gamma$-phosphothreonine were purchased from AnaSpec (San Jose, CA). Reagents for enhanced chemiluminescence (ECL Plus) were obtained from Amersham Biosciences, Inc. Magnetic beads (Dynabeads M-280, tosyl activated) were from Dynal Co. (Oslo, Norway). PrP-specific murine monoclonal antibodies used were: monoclonal antibody 3F4 (3) from Signet Laboratories, Inc. (Dedham, MA), 8H4 epitope mapped to human PrP-(177–180) (18), 6H4 from Prionics Co. (Zürich, Switzerland) epitope mapped to human PrP-(144–152) (19), and 1E4 from Cell Sciences, Inc. (Canton, MA) epitope mapped to human PrP-(97–102) (8). Recombinant full-length mouse PrP was purified from Prionics Co. Synthetic peptides based on the cervid or human PrP amino sequence were synthesized following established Fmoc procedures as described (20). The amino termini were amidated, and the carboxyl termini were acetylated. Peptides were purified up to 95% purity by high performance liquid chromatography using 215 nm spectrophotometric estimation and checked for their identity using mass spectrometric analysis.

**Generation and Prion Inoculation of Transgenic Mice** (Tg(ElPrP-132M)Prnp0/0) Expressing Elk PrP—The transgenic mice were generated in a previous study as described (21). Briefly, the elk PrP-132M transgene construct based on the murine half-genomic PrP vector (22) was microinjected into fertilized FVB/NJ eggs. Founders were screened by tail DNA PCR, bred with FVB/Prnp0/0 mice (22) to obtain Tg mice in PrP-null background. The inoculation of Tg mice was performed as described previously (21). In brief, after anesthetization with isoflurane, 30 $\mu$L of 1% brain homogenate (in PBS) from elk infected with CWD was injected into each mouse brain with a 26-gauge needle inserted to a depth of ~2 mm at the left parietal region of the cranium. All animal experiments in this study were approved by the Institutional Animal Use and Care Committee and the Institutional Biosafety Committee.

**Preparation of the Recombinant Human and Elk PrP**—Recombinant human PrP-(23–231) were prepared as previously...
described (23). In brief, cDNA coding for human PrP-(23–231) was amplified from a plasmid pVZ21 by polymerase chain reaction. The final constructs coded for appropriate PrP fragments were fused to the N-terminal linker containing the His6 tail and a enterokinase cleavage site. A Gly-Ser-Asp-Pro extension at the N terminus remained after cleavage of the linker. DNA sequences of all constructs were verified by automated DNA sequencing. The purity of the final products was greater than 98% as judged by SDS-PAGE. The identity of each protein was further confirmed by mass spectrometry. For preparation of recombinant elk PrP-(25–233), the coding sequence of elk PrP between codons 25 and 233 was amplified by PCR with the forward primer including a 4-base pair sequence (CACC) on the 5’ end and the reverse primer to clone into a pET100/D-TOP® (Invitrogen). The construct was sequenced using the CEQ 8000 Genetic analysis system (Beckman Coulter, Fullerton, CA). Transformed BL21 Star™(DE3) expressed elk PrP-(25–233) fragments fused to the N-terminal His6 tag and a enterokinase cleavage site with a Asp-His-Pro-Phe-Thr extension at the N terminus remained after cleavage of the His tag. Purification was performed using nickel-nitrirotiazide acid column (Qia-gen, Valencia, CA) according to the manufacturer’s instructions. Purified protein was refolded by dialysis in 20 mM of sodium acetate buffer (pH 4.5). Protein concentrations were determined spectrophotometrically by measuring absorbance at 280 nm and using the molar extinction coefficient ε280 of 56,795 M⁻¹ cm⁻¹ for human PrP (23) and 59,485 M⁻¹ cm⁻¹ for elk PrP (ExPASy CA). Tools > Primary structure analysis > ProtParam).

Preparation of Gene S Protein (g5p)—The recombinant g5p was isolated from Escherichia coli, transformed with an Fd gene 5-containing plasmid and purified using DNA cellulose affinity plus Sephadex G-75 sizing columns as described (24). The purity was >99% as determined by quantitation of Coomassie Blue-stained bands on SDS-PAGE.

Preparation of Brain Homogenates—The 10% (w/v) brain homogenates were prepared in 9 volumes of lysis buffer (10 mM Tris, 100 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 10 mM EDTA, pH 7.4). For PK digestion, samples were incubated with the designated amounts of PK at 37 °C for 1 h and the reaction terminated through the addition of phenylmethylsulfonyl fluoride at a final concentration of 3 mM and boiling in SDS sample buffer (3% SDS, 2 mM EDTA, 4% β-mercaptoethanol, 10% glycerol, 50 mM Tris, pH 6.8) for 10 min.

Histoblotting—Histoblot analysis was performed generally as described previously (25, 26), with the following modifications: the cryosections were 12 μm thick, and the sections were treated with 100 μg/ml of proteinase K for 4 h at 37 °C, incubated with monoclonal antibodies 3F4 (1:10,000 dilution), 8H4 (1:3,000 dilution), or 6H4 (1:1000 dilution) overnight at 4 °C, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1:500; DAKO), and developed with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium solutions (Sigma).

Epitope Mapping by Peptide Membrane Arrays—The general methods for preparing multiple overlapping peptides bound to cellulose membranes have been described in detail (27). After being blocked with 5% skim milk in TBST (150 mM NaCl, 0.05% Tween 20, 10 mM Tris-HCl, pH 7.6) at 37 °C for 2 h, the prion peptide membrane was probed with 1E4 at 1:500 in 1% skim milk for 2 h at 37 °C. The membrane was washed with TBST, and then incubated at 37 °C with 1:4000 horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG for 1 h. After a final wash and developing with ECL Western blotting detection reagent (Amersham Biosciences), the membrane was visualized by using Bio-Rad Fluorescent Imager. The control membrane was probed only with HRP-conjugated sheep anti-mouse IgG without 3F4 antibody.

Competitive ELISA—An ELISA was used where 10-mer peptides were tested for their capacity to block binding of antibody 3F4 to coated human PrP (a generous gift from T. Sklaviadis, Aristotle University, Thessaloniki, Greece). In brief, polystyrene plates (NUNC, Maxisorp) were coated with the recombinant human PrP at 0.2 μg/ml in 6 μm guanidine in phosphate-buffered saline (PBS) (pH 7.2) overnight at 4 °C. The coated plates were washed subsequently with 0.05% TWEEN in water. A range of 11 synthetic 10-mer elk PrP peptides with overlapping sequences amply covering the epitope region of 3F4 and a 19-mer human PrP peptide (PrP-(101–119)) were combined in 10-fold dilution series with constant amounts of 0.5 μg/ml of 3F4 (all in PBS-T). These peptide/antibody mixtures were applied to the coated plates and incubated at room temperature for 60 min. After being washed, the plates were further incubated at room temperature with rabbit antibodies to mouse Ig conjugated with HRP (DAKO), 1:1000, diluted in PBS-T. We detected peroxidase activity by adding tetramethyl benzidine as substrate and terminated after 20 min with 0.75 M H₂SO₄. The absorbance values of the samples were determined at 450 nm in an ELISA plate reader.

Competitive Western Blotting—Brain homogenate (10%) from sCJD case with PrPSc type 2 was subjected to SDS-PAGE and the protein was then transferred onto the polyvinylidene fluoride membrane after PK treatment and denaturing as described below. The 3F4 antibody was preincubated with various elk prion peptides in 5% milk in TBST blocking buffer for 1 h at room temperature prior to incubation with the Immobilon-P membrane (polyvinylidene fluoride, Millipore) containing the protein. The other procedures of Western blotting are described below.

Specific Capture of Abnormal PrP by g5p—The g5p molecule (100 μg) was conjugated to 7 × 10⁸ tosyl-activated magnetic beads in 1 ml of PBS at 37 °C for 20 h (8, 28). The g5p-conjugated beads were incubated with 0.1% bovine serum albumin in PBS to block nonspecific binding. The prepared g5p beads were stable for at least 3 months at 4 °C. The specific capture of PrPSc by g5p was performed as described (8, 28) by incubating S1 fractions and g5p-conjugated beads (10 μg g5p/6 × 10⁷ beads) in 1 ml of binding buffer (3% Tween 20, 3% Nonidet P-40 in PBS, pH 7.5). After incubation with constant rotation overnight at room temperature, the PrP-containing g5p beads were collected with an external magnetic force and all unbound molecules in the solution were removed. Following three rinses in the wash buffer (2% Tween 20 and 2% Nonidet P-40 in PBS, pH 7.5), the g5p beads were resuspended in SDS sample buffer (3% SDS, 2 mM EDTA, 10% glycerol, 50 mM Tris, pH 6.8) and heated at 95 °C for 5 min to release bound proteins.
The membranes were incubated for 2 h at room temperature with 3F4 (1:10,000), 6H4 (1:3,000), 8H4 (1:6,000), or 1E4 (1:500) as primary antibodies for probing the PrP molecule. Following incubation with HRP-conjugated sheep anti-mouse IgG at 1:3,000, the PrP bands were visualized on Kodak film by the ECL Plus in accordance with the manufacturer’s protocol.

RESULTS

Detection of PrP in Cervidized Tg Mice by Histoblotting—Antibodies such as 6H4 and 8H4 with epitopes localized in human PrP-(145–152) and PrP-(177–180), respectively, have been used to detect PrP from cervidized Tg mice using Western blotting and immunohistochemistry (21). By histoblotting, 8H4 and 6H4 detected PrP in both uninfected and CWD-infected brains from Tg mice (Fig. 1, C–F), although the intensity of the PrP signal was higher in the CWD-infected Tg mouse brain samples (Fig. 1, D and F). At variance with 8H4 and 6H4, 3F4 did not detect any signal in the histoblots from the brain of uninfected Tg mice (Fig. 1, A and G), but surprisingly, it exhibited intense reactivity in those from Tg mice infected with CWD (Fig. 1B). Moreover, the signal after PK treatment was as much intense as signals revealed by 8H4 and 6H4 (Fig. 1, H, J, and L).

Detection of PrP in Both Uninfected and CWD-infected Elk and Cervidized Tg Mice by Western Blotting and Dot Blotting—PrP from uninfected and CWD-infected elk brain was probed by Western blot analysis with both 3F4 and 6H4. Although no PrP bands were detected with 3F4 in samples from normal or CWD-affected elk (CWD1 and CWD2) (Fig. 2A, upper panel), the same samples were readily detectable using 6H4 (Fig. 2A, lower panel). Similarly, 3F4 showed no detectable immunoreactivity with PrP from uninfected and CWD-infected cervidized Tg mice (data not shown). Moreover, by dot blotting, undenatured PrP in uninfected and CWD-infected Tg mouse brain homogenates was undetectable with 3F4 but detectable with 6H4 (data not shown). However, when PrP preparations enriched with g5p were used, 3F4 detected PrP in brain homogenates of the two CWD-infected elk cases either with or without PK digestion (Fig. 2B) as well as very faint PrP bands in homogenates from uninfected elk in the absence of PK digestion (data not shown).

Detection of the Recombinant Elk and Human PrP by Western Blotting—The above observations raised intriguing questions about whether Met to Val substitution at residue 112 only partly eliminates the 3F4 immunoreactivity of PrP and whether the conformational change observed in the pathologic PrP conformer can alter species specificity of 3F4. To address these issues, the first necessary step would be to determine

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Surface Plasmon Resonance (SPR)—SPR experiments were performed using Biacore 3000 instrument (GE Healthcare). The surfaces of flow channels on a CM5 chip were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide. Antibody 3F4 was diluted to 20 μg/ml by 10 mM sodium acetate (pH 5.5) and immobilized on one channel of a CM5 sensor chip. All surfaces were then blocked with 1 M ethanolamine hydrochloride (pH 8.5). Another channel of the chip was processed through an identical coupling procedure without addition of ligand 3F4 and was used as reference 1. This reference channel generates a sensorgram simultaneously with the ligand channel, which is subtracted from ligand sensorgram to account for nonspecific interactions between the analyte and the CM5 chip. The running buffer contained 150 mM NaCl, 0.005% surfactant P20, 10 mM HEPES, 3 mM EDTA (pH 7.4). Regeneration of the surfaces was achieved by injection of 50 mM HCl and a solution of 0.1% Triton X-100. The proteins on the gels were transferred to polyvinylidene fluoride for 2 h at 70 V.

Dot blot Analysis—One microliter of brain homogenate (10% w/v) each from uninfected and infected cervidized Tg mice or humans was spotted onto the 0.2-μm nitrocellulose membranes. After being blocked with 5% nonfat dry milk in TBST buffer for 1 h at room temperature, the blots were incubated with 3F4 (1:10,000) or 6H4 (1:3,000) and then with sheep anti-mouse IgG conjugated with HRP after wash. The PrP spots were visualized on Kodak film by the ECL Plus from GE Healthcare in accordance with the manufacturer’s protocol.

Western Blot Analysis—Samples were resolved on 15% Tris-HCl criterion pre-cast gels (Bio-Rad) for SDS-polyacrylamide gel electrophoresis at 150 V for ~80 min. The proteins on the gels were transferred to polyvinylidene fluoride for 2 h at 70 V.

FIGURE 1. Histoblotting of PrP in brain tissue from normal and CWD-infected cervidized Tg mice treated without (A–F) or with PK (G–L), A, B, G, and H, probed with 3F4, C, D, I, and J, probed with 8H4, E, F, K, and L, probed with 6H4. Unlike 8H4 and 6H4 antibodies that detected both PrPSc and PrPSc, 3F4 detected PrP in CWD-infected brain samples (B and H) but not PrP in non-infected normal controls (A and G). The reactivity of 3F4 with untreated or PK-treated PrPSc in the brain histoblots was highly reproducible.

Detection of PrP in Both Uninfected and CWD-infected Elk and Cervidized Tg Mice by Western Blotting and Dot Blotting—

Blotting

Dot-blot Analysis—One microliter of brain homogenate (10% w/v) each from uninfected and infected cervidized Tg mice or humans was dotted onto the 0.2-μm nitrocellulose membranes. After being blocked with 5% nonfat dry milk in TBST buffer for 1 h at room temperature, the blots were incubated with 3F4 (1:10,000) or 6H4 (1:3,000) and then with sheep anti-mouse IgG conjugated with HRP after wash. The PrP spots were visualized on Kodak film by the ECL Plus from GE Healthcare in accordance with the manufacturer’s protocol.
whether 3F4 could react with the PrP species in which Met\textsuperscript{112} is replaced with Val. Like bovine and ovine PrP, elk PrP has Val at the codon corresponding to human Met\textsuperscript{112} (Table 1). Accordingly, we studied the immunoreactivity of recombinant full-length elk PrP with 3F4. Recombinant human PrP was used as a control. Different amounts of recombinant human and elk PrP ranging from 1 to 64 ng were subjected to SDS-PAGE and immunoblotting with 3F4. On the blot exposed for 2 min, 3F4 immunoreactivity with human PrP was readily detected even at as low as 1 ng, whereas elk PrP was detectable only when the protein concentration was 16 ng or higher (Fig. 3A). To exclude the possibility that the minimal binding motif and affinity might be secondary to the avidity of 3F4 with the larger peptide sequence and hence that immunoreactivity might be translated to other species, we further examined the immunoreactivity of 3F4 with the recombinant mouse PrP. Compared with human PrP-(98–120) that corresponds to mouse PrP-(97–119), there is an additional residue difference between the two species. Met at residue 109 of human PrP was replaced with Leu at residue 108 of mouse PrP (Table 1). No 3F4 immunoreactivity with mouse PrP was detectable even at a protein concentration of 64 ng (Fig. 3A), which suggests that Met\textsuperscript{109} in the human sequence corresponding to residue 112 in the elk sequence is part of the 3F4 epitope and that weak immunoreactivity of 3F4 with elk PrP is not secondary to the avidity of the antibody with the larger peptide sequence. Therefore, the immunoreactivity of 3F4 with the recombinant elk PrP was significantly lower than with recombinant human PrP (98% less, indicated by densitometric analysis), whereas mouse PrP revealed no detectable 3F4 immunoreactivity (Fig. 3B). However, when the same samples were probed with the 1E4 antibody, the intensity of elk PrP was virtually similar to that of human PrP (data not shown).

**TABLE 1**

Comparison of PrP sequence containing the 3F4 epitope between human and other species

The letters in red font represent variable amino acids among different species.

| Species   | Sequence     | Residue location |
|-----------|--------------|-----------------|
| Human     | QNNKPSKPKNMKHAGAAAAGA* | 96–120          |
| G. hamster| QNNKPSKPKNMKHAGAAAAGA  | 96–120          |
| Elk       | QNNKPSKPKNMKHAGAAAAGA  | 101–123         |
| Deer      | QNNKPSKPKNMKHAGAAAAGA  | 101–123         |
| Bovine    | QNNKPSKPKNMKHAGAAAAGA  | 101–123         |
| Sheep     | QNNKPSKPKNMKHAGAAAAGA  | 101–123         |
| Goat      | QNNKPSKPKNMKHAGAAAAGA  | 101–123         |
| Mouse     | QNNKPSKPKNMKHAGAAAAGA  | 97–119          |

**Comparison of Binding Affinity of 3F4 to Recombinant Human, Elk, and Mouse PrP by Surface Plasmon Resonance**——The binding between 3F4 antibody and human, elk, and mouse PrP was studied more quantitatively using SPR. In these experiments, the association and dissociation curves were obtained at PrP concentrations of 0.5, 1, 2, 5, 10, 25, and 50 nM. As shown in Fig. 4A, human PrP readily bound to immobilized antibody 3F4 in a dose-dependent manner. Analysis of the binding data yielded an apparent dissociation constant, $K_d$ of 1.3 nM. Compared with human PrP, the binding of elk PrP to 3F4 antibody was much weaker, with an apparent $K_d$ of ~130 nM by steady-state evaluation (Fig. 4B). SPR experiments were also performed with mouse PrP. However, in the latter case no binding to immobilized 3F4 antibody could be detected by this method (Fig. 4C). Altogether, SPR data are consistent with those obtained using Western blot analysis (Fig. 3).

**Competition Experiments Using Synthetic Elk PrP-derived Peptides**—Given that we had demonstrated by Western blotting and SPR that 3F4 indeed reacts with the recombinant full-length elk PrP, we then attempted to detect the 3F4 epitope in...
The human PrP peptide that the minimal amino acid requirement for the 3F4 epitope lighted in mainly occurred in the peptides containing KTNMK (high-resistant PrPSc). In contrast, the peptide containing Val115 (corresponding to human Met112) blocks ~80% of 3F4 binding.

To further ascertaine whether elk peptides containing MKHV can block the binding of 3F4 to human PrP, we employed a competition ELISA technique in which the 3F4 antibody was preincubated with a range of concentrations of competing peptide human PrP-(101–119), we did not detect PrP (Fig. 5A). A human PrP-(101–119) peptide (in Western blot analysis and ELISA) and a random 19-mer peptide (in Western blot analysis) were used as positive and negative controls, respectively. After preincubating 3F4 with the positive control peptide human PrP-(101–119), we did not detect PrP (Fig. 5B, lane 1). Thus, the positive control peptide had completely blocked the binding of 3F4 to the PK-resistant PrPSc transferred onto the blot. No significant differences in PrP intensity were observed with elk PrP-(101–110), PrP-(102–111), PrP-(103–112), and PrP-(110–119) (Fig. 5B), which suggests that no competition occurred with these peptides. However, PrP intensity had clearly decreased when the 3F4 antibody was preincubated with elk PrP-(104–113) and elk PrP-(109–118), as evidenced by densitometric analysis (Fig. 5B, lanes 6 and 11). The inhibition of 3F4 immunoreactivity reached levels between ~60 and ~80% with elk peptides PrP-(104–118) that contain the pentapeptide KTNMK (Fig. 5B, lanes 6–11). Thus, inhibition mainly occurred in the peptides containing KTNMK (highlighted in green in Fig. 5A). Cumulatively, these data suggest that the minimal amino acid requirement for the 3F4 epitope includes PrP-(106–110) (KTNMK). The human PrP peptide containing Met112 completely blocks the binding of 3F4 to PK-resistant PrPSc. In contrast, the peptide containing Val115 (corresponding to human Met112) blocks ~80% of 3F4 binding.

To further ascertain whether elk peptides containing MKHV can block the binding of 3F4 to human PrP, we employed a competition ELISA technique in which the 3F4 antibody was preincubated with a range of concentrations of competing peptides between 10 ng to 30 μg, before being applied to the PrP antigen. From the curves obtained for each peptide, we then determined the concentration of each peptide that would be required to inhibit 50% (IC50) of the 3F4 antibody binding to the recombinant human PrP applied to the plate (Table 2). Compared with the human peptide that blocked the binding most efficiently (IC50 of 0.004 μM), the 10-mer cervid PrP peptides between residues 106 and 118 showed blocking with IC50 values up to 0.6 μM. Thus, we confirmed that by competitive Western blot as well as by ELISA, elk PrP peptides from PrP-(104–113) up to PrP-(109–118) could partially block the binding of 3F4 to the human PrP.

Epitope Mapping by Peptide Membrane Arrays—The above finding that 3F4 can react with elk brain Prion Protein Antibody 3F4 and Its Epitope

FIGURE 3. Detection of the recombinant full-length human, elk, and mouse PrP by Western blotting. A, Western blotting of a series of concentrations of recombinant human, elk, and mouse PrP (rePrP) from 1 to 64 ng probed with 3F4. Compared with human PrP, elk PrP revealed much lower 3F4 immunoreactivity. There was no detectable 3F4 immunoreactivity with mouse rePrP. The concentration of protein was determined with a spectrophotometer (Ultrospec 3000, Amersham Biosciences). B, intensity of rePrP as a function of concentration of the protein examined by using densitometric analysis of rePrP on Western blots done by three independent experiments. The affinity of 3F4 for the elk PrP was much lower than that for human PrP (~98% less).
The reactivity of the elk peptides was compared to human peptides (Fig. 6B, upper row, human sequences 4 and 5; lower row, elk sequences 4 and 5). However, the immunoreactivity of the peptide with 3F4 almost completely disappeared when Ala^{115} was added to the peptide containing MKHV (Fig. 6C, lower row, elk sequences 6 and 7). In contrast, weak 3F4 immunoreactivity persisted with the peptides containing MKHM after the addition of Ala^{115} (Fig. 6B, upper lower, human sequences 6 and 7). Although the intensity of the peptide immunoreactivity with 3F4 decreased by ~30% compared with peptides without Ala^{115} (Fig. 6C, upper row, human sequences 6), 3F4 immunoreactivity was partially recovered when Ala^{116} was added to the peptide (decreased by only ~10% compared with immunoreactivity with peptides 3–5) (Fig. 6C, upper row, human sequence 7).

The above study indicates that the 3F4 epitope consists of KTNMK, not MKHM, and that 3F4 does react with PrP containing MKHV albeit with lower affinity. Therefore these results suggest that 3F4 should be regarded as species preferential instead of species specific. Although the peptides containing KTNMK without Met^{112} show immunoreactivity with 3F4, Met^{112} does play a role in species preference. After establishing these results, we next investigated how replacement of Met^{112} with other amino acids affects 3F4 immunoreactivity with the epitope. To ascertain the effect of this substitution, 20 16-mer peptides containing the same residues, PSKPKTNNMKHAMAGA (except that Met^{112} was replaced with 19 other individual amino acids), were probed with 3F4 (Fig. 6D). Of the 20 peptides, the one containing Met^{112} exhibited the highest PrP intensity with 3F4. But other peptides that contain different amino acids (including Val except Cys at codon 112) also revealed varied 3F4 immunoreactivity. According to the spot intensity measured by quantitative densitometric analysis, the following is the order of 3F4 immunoreactivity with peptides containing different amino acids at human PrP codon 112: Met > Phe > Ser > Trp > Lys > Arg > Gln > His > Ala > Val > Ile > Asn > Thr > Leu > Tyr > Gly > Asp > Pro > Glu > Cys (Fig. 6D).

**DISCUSSION**

Our current study presents five new findings on the commonly used antibody 3F4 and its epitope localized in a critical conversion region in the PrP molecule. First, histoblotting revealed 3F4 immunoreactivity with PrPSc from cervidized mouse brain infected with CWD that is similar to reactivity of the 6H4 and 8H4 antibodies; however, unlike 6H4 and 8H4, 3F4 does not react with PrPSc from uninfected cervidized mice. Second, by Western blotting, 3F4 can detect elk PrPSc enriched by g5p although its reactivity is much lower than that of antibodies 6H4 and 8H4. Third, 3F4 does react with recombinant elk PrP, but its immunoreactivity is about 98% less than with recombinant human PrP. Fourth, the 3F4 antibody can react with elk peptides containing the sequence KTNMKHV and its immunoreactivity with some of elk PrP peptides is only slightly lower (approximately ~7%) than with human PrP peptide containing KTNMKHV, especially Ala^{115}. Finally, human PrP-(106–110), Met^{112} was added to the human peptide (Fig. 6C, upper row, human peptide 3). The immunoreactivity of the peptide continued to increase with addition of Ala^{113} and Gly^{114} (Fig. 6C, upper row, human sequences 4 and 5; lower row, elk sequences 4 and 5). However, the immunoreactivity of the peptide with 3F4 almost completely disappeared when Ala^{115} was added to the peptide containing MKHV (Fig. 6C, lower row, elk peptides 6 and 7).
not PrP-(109–112), constitutes the minimal amino acid requirement for the 3F4 epitope. These unambiguous findings not only reveal 3F4 as a new application for detecting elk PrPSc by histoblotting, but they also enhance our understanding of the 3F4 epitope residing in the region that is crucial to the physiology and pathophysiology of PrP.

Based on previous studies (3–5), the 3F4 epitope might be expected to be a linear determinant. However, although PrPSc and PrPC share the same primary structure, it has been observed that the accessibility of the 3F4 epitope is different in native PrPSc and PrPC molecules, which suggests that changes in protein conformation during the conversion from PrPC to PrPSc alter accessibility of the 3F4 epitope. For example, epitopes (including the 3F4 epitope) within the N-terminal region of hamster PrP (residues 90–120), which are masked in PrPSc, are exposed in PrPC (9). In light of this finding, the 3F4 antibody was used to distinguish PrPSc from PrPC in an ELISA-based conformation-dependent hamster PrPSc immunoassay (10). The same might be true for human PrP as well. Indeed, compared with PrPC, much less PrPSc was precipitated by 3F4 from the human brain homogenates (28). Moreover, compared with a PrPSc-specific antibody, the recovery rate of human PrPSc by 3F4-based immunoprecipitation was much lower (29).

Cumulatively, these observations suggest that indeed the 3F4 epitope is located in an area whose accessibility is greatly affected by the conformational changes that occur during the PrPC → PrPSc conversion. Therefore, 3F4 appears to have a lower preference for the native hamster or human PrPSc conformer compared with PrPC. Moreover, the cryptic 3F4 epitope could also be present in denatured PrPSc or PrPSc-like forms on the Western blots. For example, in our recent studies Western blotting has identified a group of PK-resistant human PrP fragments exhibiting much lower reactivity with 3F4 than with 1E4, an antibody with an epitope N-terminal adjacent to the 3F4 epitope (human PrP-(97–102)) (8, 30–32). These PK-resistant PrP fragments are identified in human brains either in normal subjects, or in patients with protease-sensitive prionopathy (a newly discovered prion disease), sporadic fatal insomnia, or a subset of sporadic CJD, type 2 (8, 30–32). Although these PK-resistant PrP fragments would be expected to contain the 3F4 epitope based on their molecular size and the adjacency of the epitope to the 1E4 epitope (8), surprisingly, they are not readily detectable with 3F4 even by Western blotting under denaturing conditions. It is worth noting that human PrP treated with reducing and alkylating reagents prior to Western blotting were undetectable with the 6H4 antibody against human PrP-(144–152) but remained detectable with other antibodies against either the N or C terminus of the 6H4 epitope (33). Because no chemical modifications were detected by mass spectrometry, it has been proposed that the treated PrP might acquire some structures during transfer of the protein onto polyvinylidene fluoride membranes, although PrP was denatured prior to SDS-PAGE and Western blotting (33). The molecular area containing the 3F4 epitope in native human PrPSc or in native hamster PrPSc is cryptic, probably because of the presence of some structures and is thus undetectable with 3F4. But the structure becomes unfolded and is detectable after denaturing...
Prion Protein Antibody 3F4 and Its Epitope

prior to Western blotting with 3F4. For those PrPSc treated with PK and detergents but still undetectable with 3F4 after denaturation, the structure in the 3F4 epitope might be reformed or be resistant to denaturation.

By Western blot analysis, antibody 3F4 can react with recombinant elk PrP, synthetic elk PrP peptides, and g5p-enriched elk PrPSc; however, the affinity of 3F4 for elk PrP decreased significantly compared with the affinity of 6H4 or 1E4. In contrast, by histoblot analysis, the elk PrPSc molecule revealed an intensity of 3F4 immunoreactivity that was similar to that with 6H4. However, in contrast to the 6H4 antibody, 3F4 did not react with elk PrPC on the histoblots. Thus in the histoblots, 3F4 appeared to exhibit preference for the elk PrPSc conformer, which is opposite to the hamster or human PrPSc conformer mentioned above. This effect might also be reflected in the immunohistochemistry of some forms of human PrPSc. For example, PrPSc is readily detectable with immunohistochemistry in the tonsils of variant CJD, in the spleen, skeletal muscle, and olfactory epithelium of sporadic CJD (34, 35), and in the brains of protease-sensi-
tive priapopathy identified recently by our group (31). In contrast, to detect the PK-resistant PrPSc by Western blotting with 3F4 in these tissues, an extra step is necessary, specifically, pre-enrichment of PrPSc by phosphotungstate or g5p, PrPSc-specific binding reagents (31, 34–36). In general, in the detection of human PrPSc the results of Western blotting and those of immunohistochemistry agree. Where results differ, it is difficult to claim that one method is inherently more sensitive than the other. This is because the inconsistent results may be caused by non-uniform distribution of PrPSc. If PrPSc is restricted to only a small population of cells in an area, it may be detectable by immunohistochemistry or histoblotting but not by Western blotting. However, there is a greater likelihood that the epitopes of the antibodies used are differently exposed under the two conditions, because 3F4 immunoreactivity with PrPSc in the brain histoblots of CWD-infected cervidized Tg mice is highly intense and extensive. If this is the case, it would be the first indication that the 3F4 conformer preference could be species dependent. In other words, 3F4 binds to human and hamster PrPC more predictably than to their PrPSc conformers; at the same time, it binds to elk PrPSc but not elk PrPC. Remarkably, our data may provide the first evidence that abnormal protein folding eliminates the species preference of the 3F4 antibody. Although the molecular basis underlying the difference in the accessibility of the 3F4 epitope between PrPSc and PrPSc of elk on the histoblots is uncertain at present, our study suggests that 3F4 can be used for the diagnosis of CWD by histoblotting. Whether it holds true for other animal conditions like scrapie and bovine spongiform encephalopathy remains to be investigated.

The minimal affinity of 3F4 to elk PrP may raise a possibility that perhaps the amino acid sequence motif defined for human PrP interacting with 3F4 is based on monovalent binding (affinity), whereas the motifs for PrP molecules from the other species are based on multivalent binding (avidity). If so, the precise
The nature of the immunoassay might influence whether 3F4 detectably binds a given PrP molecule, thereby increasing the possibility of inconsistent results, erroneous diagnostic outcomes, and confusion. Thus, the differentiation between affinity and avidity is crucial. Antibody affinity, sometimes referred to as “intrinsic affinity,” formally refers to the equilibrium association constant that characterizes the interaction between a single antibody paratope (i.e. combining site) and a single epitope (i.e. antigenic determinant). Thus, affinity constitutes a quantitative measure of the strength of a monovalent interaction. On the other hand, avidity, sometimes called “functional affinity,” formally refers to the equilibrium association constant that characterizes the interaction between an antibody (always bivalent or multivalent in physiological contexts) and an antigen that is multivalent with respect to that antibody (37). This quantitative measure of antibody binding strength takes into account simultaneous interactions between two or more (e.g. with IgA and IgM antibodies) paratopes and two or more epitopes on the same antigen molecule or molecular complex. Our quantitative analysis of immunoreactivity and equilibrium association and dissociation constants of human and elk PrP with 3F4 by Western blot analysis and SPR demonstrated that 3F4 immunoreactivity is dramatically affected by the two residues: Met at residues 109 and 112 in human PrP corresponding to residues 113 and 116 in elk PrP. Particularly, because the substitution of Leu for Met at residue 109 in human PrP corresponding to residue 108 in mouse PrP completely eliminates 3F4 immunoreactivity, the Met residue is obviously part of the 3F4 epitope. Thus, it is most likely that the immunoreactivity of 3F4 with both human and elk PrP is associated with only a single epitope localized around residue 109.

The combination of peptide membrane array and peptide competition experiments with Western blotting or ELISA in the present study further pinpoints the minimal amino acid requirement for the 3F4 epitope. Based on our new data, this minimum is composed of the pentapeptide KTNMK (human PrP-(106–110)), not MKHM (human PrP-(109–112)). Our study confirms the earlier observation by other groups that Lys is the amino acid that is most critical for the epitope to react with 3F4 (4, 5). Furthermore, this newly identified requirement agrees with our finding that 3F4 can react with elk PrP containing KTNMKHV. The reactivity of antibody 3F4 with recombinant elk PrP containing KTNMKHV, albeit lower than its reactivity with recombinant human PrP, further establishes that the minimal amino acid requirement of the 3F4 epitope consists of the pentapeptide KTNMK. Our study also indicates that although Met112 is not a constituent of the minimal epitope, its presence, nevertheless, considerably increases the reactivity of 3F4; it therefore constitutes part of the complete 3F4 epitope. Besides Met112, Ala115 is another adjacent amino acid that also dramatically affects 3F4 reactivity. However, unlike Met112, which enhances 3F4 reactivity, the addition of Ala115 almost completely eliminates the reactivity of 3F4 with the elk peptide, KTNMKHAVAGA, and significantly decreases the reactivity of 3F4 with the human peptide, KTNMKHMAGA. The presence of Met at residue 112 in human PrP and hamster PrP reveals the highest 3F4 reactivity. However, substitution of Met112 with other amino acids including Val does not completely eliminate the reactivity of 3F4; lower 3F4 reactivity is still detectable. Given that Ala115 participates in formation of the unique palindromic domain PrP-(113–120) that is required for the formation of the PrPSc-PrPSc complex (38), the significant effect of Ala115 on immunoreactivity of the 3F4 epitope suggests that the interaction between PrPc and PrPSc in the prion disease might modulate the accessibility of this epitope. Moreover, this study demonstrates that 3F4 should not be considered species specific but rather species preferential. Although Met112 and Ala115 do not participate in the formation of the minimal 3F4 epitope, the species preference of 3F4 is probably attributable to the two amino acids. The effect of Met112 and Ala115 on the affinity of 3F4 might represent a common phenomenon in which one and more amino acids outside an epitope are able to interfere with antibody binding, presumably because of a closely neighboring amino acid that might affect the affinity of the epitope for its antibody. This may also explain our observation that 3F4 immunoreactivity is extremely lower (~98%) with the recombinant full-length elk PrP than with human PrP, whereas it reveals less difference (~7%) between the short synthetic elk and human peptides. Moreover, on histoblots, 3F4...
shows the full immunoreactivity with elk PrP\textsuperscript{Sc} that shares an identical sequence with elk PrP\textsuperscript{C}. It would be intriguing to ascertain whether abnormal PrP folding during PrP\textsuperscript{Sc} formation may suppress the effect of Ala\textsuperscript{115} on the binding of 3F4 to elk PrP. X-ray crystallization revealed that Met\textsuperscript{109} and Met\textsuperscript{112} in an unmodified peptide corresponding to human PrP-(104–113) (KP KartNMKHA) did bind to a pocket formed either by the light or heavy chains of the 3F4 antibody (11). It would be interesting to use this method to further determine the binding of this peptide with substitution of Met with Val at residue 112. Because we have now demonstrated that 3F4 can bind to recombinant elk PrP and to synthetic prion peptides, it is possible to establish the binding differences of 3F4 between human PrP and elk PrP species with x-ray crystallography of the recombinant full-length PrP or synthetic prion peptides. Such a study will not only enhance our understanding of the species barrier, but also provide the most direct route to capturing and characterizing PrP\textsuperscript{C}, PrP\textsuperscript{Sc}, and their converting intermediates (11). Using SPR, we confirmed that the binding affinity of 3F4 for the recombinant elk PrP is much weaker than for the recombinant human PrP. Because the immunoreactivity of 3F4 with elk brain-derived PrP\textsuperscript{Sc} was significantly stronger when compared with the normal (a-helical) conformer, it would be interesting to determine whether the change in the species preference of 3F4 induced by protein conformational transitions could be replicated using different conformers of recombinant PrP that can be generated in vitro (39).

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PrP Conformational Transitions Alter Species Preference of a PrP-specific Antibody

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