In Vivo Conversion of Cellular Prion Protein to Pathogenic Isoforms, as Monitored by Conformation-specific Antibodies*

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The central event in prion disease is thought to be conformational conversion of the cellular isoform of prion protein (PrPC) to the insoluble isoform PrPSc. We generated polyclonal and monoclonal antibodies by immunizing PrPC-null mice with native PrPC. All seven monoclonal antibodies (mAbs) immunoprecipitated PrPC, but they immunoprecipitated PrPSc weakly or not at all, thereby indicating preferential reactivities to PrPSc in solution. Immunoprecipitation using these mAbs revealed a marked loss of PrPC in brains at the terminal stage of illness. Histoblot analyses using these polyclonal antibodies in combination of pretreatment of blots dissociated PrPC and PrPSc in situ and consistently demonstrated the decrease of PrPC at regions where PrPSc accumulated. Interestingly, some mAbs showed immunohistochemical reactivities to abnormal isoforms. One group of mAbs showed reactivity to materials that accumulated in astrocytes, while the other group did so to amorphous plaques in neuropil. Epitope mapping indicated that single mAbs have reactivities to multiple epitopes, thus implying dual specificities. This suggests the importance of octarepeats as a part of PrPSc-specific conformation. Our observations support the notion that loss of function of PrPC may partly underlie the pathogenesis of prion diseases. The conversion of PrPSc to PrPC may involve multiple steps at different sites.

Prion diseases, such as scrapie in sheep and goats and Creutzfeldt-Jakob disease in humans, are transmissible neurodegenerative disorders (spongiform encephalopathy). A major component of the infectious agent responsible for these diseases is thought to be a post-translationally modified form of prion protein, termed PrPSc. Conformational differences are observed between PrPC and PrPSc. We immunized PrPC-null mice with native PrPC. All seven mAbs so far obtained recognize both PrPSc and PrPC. However, one of the mAbs (clone 15B3) was reported to be specific for PrPSc. To gain insight into conversion processes, it is important to characterize epitopes reflecting conformational differences between PrPSc and PrPC.

PrPC, expressed on the cell surface with a C-terminal glycosylphosphatidylinositol anchor, is expressed in most tissues of uninfected animals. The function of PrPC has remained obscure. However, it has been suggested that PrPC has a role for normal synaptic function. Furthermore, ablation of the prion protein gene (Prnp) caused cell death in some circumstances in vivo and in vitro. Although recent studies suggested that the ectopic expression of the Prnd gene, encoding a homolog of PrPC, was involved in cell death, these results raised the possibility that functional loss of PrPC might partly underlie the pathogenesis of prion diseases. However, the expression level of the mRNA of the Prnp gene is unaltered in cases of scrapie infection. Thus, the equilibrium of PrPC and PrPSc in the pathogenesis remained to be examined.

We immunized Prnp−/− mice with native PrPC from wild type mice (Prnp+/+) and established mAbs to PrP. These mAbs specifically recognized PrPSc in solution with a marked decrease of PrPC at the terminal stage of illness. Epitope mapping of these mAbs and polyclonal antibodies from mice immunized with native PrPC suggested the importance of octarepeats as part of PrPSc specific conformation.

EXPERIMENTAL PROCEDURES

Generation of Prnp−/− Mice—Prnp−/− mice were generated by homologous recombination, as described (20). The entire Prnp gene open reading frame as well as 3′-end of the intron 2 was replaced with a pgk-neo gene cassette. The resulting allele is almost identical to that generated by another group (16). The homologous recombinant ES clones were injected into blastocysts of C57BL/6 mice to give rise to chimeras. Mice heterozygous for the mutation (Prnp−/+) were obtained by crossing the chimeras with C57BL/6J mice. The heterozygotes (Prnp−/+ ) were further intercrossed to obtain mutation homozygotes (Prnp−/− ). The mouse line has been maintained by backcrossing to C57BL/6. Genotypes of the mice were determined by Southern blot analysis or by polymerase chain reaction analysis of DNAs prepared.
from tails of the mice. The primers used were 5′-GTACAGTAGAC-CAGTTGCTG-3′ and 5′-CAGAGTGCTCCTCTTAG-3′ for the wild-type allele and 5′-CTCGTGATTCAGTATGCG-3′ and 5′-CAGAGTGCAAGTCTCTAGT-3′ for the mutated allele.

**Antibodies.—** Fluorescein isothiocyanate-conjugated anti-mouse IgG and IgM, horseradish peroxidase (HRP)-conjugated anti-mouse IgM, fluorescein isothiocyanate-conjugated anti-rabbit IgG, and HRP-conjugated anti-rabbit IgG antibodies were purchased from Jackson Immunoresearch Laboratories. Biotinylated anti-mouse IgM antibody was purchased from Vector Laboratories. Anti-PrP peptide (position of the peptide on mouse PrP was amino acid residues 213-226) rabbit serum (Ab,v.M)-VI(21) was used as a reference antibody.

**Western Blot Analysis (WB)—** WB was carried out, as described (21). In brief, mouse brain extracts were fractionated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membrane (Millipore Corp.). After blocking the membrane with Block Ace (Dainippon Seiyaku), the blots were immersed in primary antibodies at 37 °C for 1 h. Then after washing, the blots were incubated with either HRP-conjugated anti-mouse IgG (1:5,000), anti-mouse IgM (1:5,000), or anti-rabbit IgG (1:5,000) and developed in an ECL Western blotting detection reagent (Amersham Pharmacia Biotech).

**Immunization and Fusion Protocols—** For immunization, 10% brain homogenates or 2 × 10⁷ thymocytes of Prnp+/− mice four times at 2–3-week intervals. The brains were homogenized with RPMI medium and then centrifuged at 3,000 rpm for 30 min. The supernatant fraction was emulsified with Freund’s complete and incomplete adjuvants for first and subsequent immunizations, respectively. Thymocytes were given intraperitoneally without adjuvants. The spleen cells of the immunized mice were fused to P3U1 mouse myeloma cells (53 × 63Ag8.1) and cultured, as described (22). Subclasses of the mAbs were determined using a mouse mAb isolotyping kit (Amersham Pharmacia Biotech).

**PrP recombinant Baculovirus—** Autographa californica nuclear polyhedrosis virus (AcNPV) and recombinant virus stocks were grown and assayed in monolayers of Spodoptera frugiperda ovary cells IPLB-SF-21AE (SF21AE) (23) in TC100 medium containing 10% fetal bovine serum. The Prnp open reading frame fragment was amplified by polymerase chain reaction and subcloned into the BamHI site of the transfer vector pAcYM1S. Primers used were 5′-GGATCCAGTCATCATGGCGAACCT-3′ and 5′-GGATCCACGGAATGCGAAGGAA-3′. SF21AE cells were cotransfected with AcNPV DNA and transfer vector DNA by Lipofectin (Life Technologies, Inc.), and then the recombinant baculovirus PrP-AcNPV was selected, as described (24). The expression of recombinant PrP was confirmed by indirect fluorescent assay (IFA) and WB by using Abs Mo-VI (21). PrP-AcNPV-infected cells were washed with phosphate-buffered saline and then fixed with acetone for 5 min at room temperature. These fixed cells were used for screening of hybridomas, using an IFA test (see details below). The antigenicity of the recombinant PrP was evaluated by immunizing 7–8-week-old Prnpp+/− and Prnp+/+ mice with PrP-AcNPV-infected cells.

**Scrapie Prions and Animals—** The Ohbishi strain of scrapie prion (PrPsc), which had been passaged in ICR/Slc mice more than 10 times, was prepared from infected brains homogenized in phosphate-buffered saline and intracerebrally inoculated into 3-week-old C57BL/6 (Prnp+/−) and Prnp+/− mice, as described (21). At appropriate time points, the brains were collected and used for PrPsc-extraction and immunoprecipitation. Noninfected brains of Prnp−/− and Prnp+/− mice served as controls. For histoblot analysis, we used the Sc237 strain of scrapie prion, which had been passaged through Syrian golden hamsters more than 10 times. Three-week-old hamsters were intracerebrally inoculated with Sc237 prion, as described (21). PrPsc extraction from infected animal brains and an enzyme-linked immunosorbent assay (ELISA) were done, as described (21).

**Immunohistochemical Analysis—** Mouse brains were frozen in liquid N₂ and then cut into 5-μm-thick cryosections. The sections were fixed with acetone for 10 min and then reacted with primary antibodies for 30 min. After washing three times in phosphate-buffered saline, the sections were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG or IgM. Recombinant baculovirus-infected cells were also examined using the same procedure.

**Immunoprecipitation (IP)—** The brains were homogenized (10%, w/v) in 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, 10 mM EDTA, in 10 mM Tris-HCl (pH 7.5) and then preclared by centrifugation at 11,000 × g for 30 min at 4 °C. The samples were incubated with antibodies at 4 °C for 1 h. The antigen-antibody complexes were collected on beads. Immuno Assist MG-PP (Kanto Chemical) and protein G beads (Amersham Pharmacia Biotech) were used for mAbs and Ab,Tg, respectively. After washing three times, the beads were mixed with SDS-sample buffer and boiled for 5 min. The precipitated PrP was detected by WB, using biotinylated Ab,Tg and streptavidin-HRP (Life Technologies, Inc.).

**Histoblot Analysis—** Histoblot analysis was done, as described (25). Briefly, mouse and hamster brains were frozen in liquid N₂, and 8-μm-thick cryosections were prepared and placed on glass slides. The glass slides carrying the sections were immediately pressed onto Immobilon-P membranes for 1 min. The membranes were used with one of the following pretreatments: 100 μg/ml proteinase K for 1 h at 37 °C or hydrated autoclaving pretreatment for 10 min at 115 °C. To detect PrPsc, hydrated autoclaving pretreatment was done following proteinase digestion. The pretreated membranes were incubated with Ab,Tg. Bound antibodies were detected using HRP-conjugated anti-mouse IgG and chemiluminescence, as described for WB.

**Epitope Analysis—** To determine the epitopes, antibodies were incubated with a gridded array of peptides comprising 122 polypeptides of 13 amino acids, shifted by 2 amino acids and covering the entire mouse sequence. The peptides were covalently attached at COOH termini to a cellulose support, as individual spots (Jerini Biotools, Berlin). Bound antibodies were detected using HRP-conjugated anti-mouse IgG or IgM and chemiluminescence, as described for WB.

**RESULTS**

**Generation of Mice Devoid of PrPC—** We generated mice devoid of PrPC by gene targeting. As shown in Fig. 1, the entire open reading frame and 3′-end of the second intron were replaced with the pgk-neo gene cassette. The genotype of mice was determined by either Southern blotting or polymerase chain reaction analysis of DNA prepared from tails of the mice. An example of Southern blot analysis of tail DNAs from crosses between heterozygotes is shown in Fig. 1B. Immunoblot analysis of lysates from cerebral showed the absence of PrPsc in Prnp+/− mice and a reduced level in Prnp−/− mice compared with findings in Prnp+/− mice (Fig. 1C). Homozygous mutant mice showed no obvious behavioral changes and at a young age appeared to have normal motor activity. However, the aged mice showed tremor and ataxia, as reported (16).

**Generation of Polyclonal and Monoclonal Antibodies to PrP—** Prnp+/− mice were immunized with brain homogenates and thymocytes of Prnp−/− mice. The sera from immunized
Seven clones of hybridomas were established. Noninfected SF21 cells were served as controls. Hybridomas were screened by IFA against PrP-AcNPV-infected SF-21 cells could serve as an antigen for screening mAbs. Splenocytes from mice immunized with brain homogenates were fused with P3U1 mouse myeloma cells, and the resulting hybrids were screened by IFA against PrP, in various forms (Table I). Thus, PrP-AcNPV-infected SF21 cells were killed at 0, 9, 12, 15, and 20 weeks postinfection. Brain homogenates were examined with (+) or without (−) protease K (PK) treatment. PK(−) samples represent the total amounts of PrP C and PrPSc, while PK(+) samples represent the amounts of PrPSc. Note the increase in protease K-resistant PrP at 20 weeks postinfection. PK(−) samples represent the total amounts of PrP C and PrPSc, while PK(+) samples represent the amounts of PrPSc. Proteinase K-resistant PrP was markedly decreased at 20 weeks postinfection, while Ab.Tg detected a larger amount of PrP (PrPSc) in the same sample.

Characterization of mAbs—All of these mAbs reacted to neither PrPSc nor PrP C in WB and showed only a weak reaction to PrPSc fractions, using ELISA. These mAbs were IgMs. Despite evidence that the mAbs did not react with PrP in WB, the immunoreactivity of mAbs was confirmed by immunoprecipitation. Immunoprecipitates from brain lysates with mAbs were size-fractionated, blotted onto a membrane, and probed with Ab.Tg. All mAbs efficiently immunoprecipitated PrP from brain samples of wild-type healthy mice. Two mAbs, 4A3 and 11H1, representing two distinct groups that showed different properties in IFA, as described later, were extensively characterized. Representative data from mAbs 4A3 and 11H1 are shown in Fig. 3A (lane 2). These mAbs reacted weakly to brain homogenates from scrapie-affected mice (Fig. 3A, lanes 3). No signals were detected with PrP C, brain homogenates (Fig. 3A, lanes 1), thus indicating the PrP specificity of the immunoprecipitates from samples same as in B. D, brain homogenates used for B and C were immunoprecipitated with 11H1 mAb. In C, same homogenates were immunoprecipitated with anti-pan-PrP (Ab.Tg) antibody. 11H1-reactive PrP was markedly decreased at 20 weeks postinfection, while Ab.Tg detected a larger amount of PrP (PrPSc) in the same sample.

4A3 and 11H1 suggested that the fraction precipitated by these mAbs is a subfraction of PrPSc immunoprecipitated with Ab.Tg (Fig. 4). On the other hand, sequential immunoprecipitation of Purified PrPSc was used as an antigen for ELISA. Immunoreactivities to thymocytes of Prnp+/− and Prnp−/− mice immunized with lysates of Prnp−/− mouse brains with two mAbs and Ab.Tg. Brain homogenates of Prnp−/− mice (lane 1), mock-infected Prnp−/− mice (lane 2), and scrapie-infected Prnp−/− mice (lane 3) at 20 weeks postinfection were immunoprecipitated and analyzed by WB, using biotinylated Ab.Tg. B, WB with Ab.Tg of PrP from the scrapie-infected mouse brain. Scrapie-infected mice were killed at 0, 9, 12, 15, and 20 weeks postinfection. Brain homogenates were examined with (+) or without (−) protease K (PK) treatment. PK(−) samples represent the total amounts of PrPSc and PrP C, while PK(+) samples represent the amounts of PrPSc. Note the increase in protease K-resistant PrP at 20 weeks postinoculation. C, immunoprecipitates from samples same as in B. D, brain homogenates used for B and C were immunoprecipitated with 11H1 mAb. In C, same homogenates were immunoprecipitated with anti-pan-PrP (Ab.Tg) antibody. 11H1-reactive PrP was markedly decreased at 20 weeks postinoculation, while Ab.Tg detected a larger amount of PrP (PrPSc) even in the same sample.
are specific to a subfraction of PrP\textsuperscript{C}.

**Dynamics of PrP\textsuperscript{C} to PrP\textsuperscript{Sc} Conversion in the Brain**—The findings that the mAbs immunoprecipitated small amounts of PrP from infected brains (Fig. 3A) suggested the exhaustion of PrP\textsuperscript{C} caused by the exponential conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}. Thus, we measured the equilibrium of PrP\textsuperscript{C} and PrP\textsuperscript{Sc} during the pathogenesis. Homogenates from mouse brains at 0, 9, 12, 15, and 20 weeks postinfection, immunoprecipitated with mAbs and Ab.Tg, were probed using the biotinylated Ab.Tg (Fig. 3B). The mAbs precipitated a large amount of PrP\textsuperscript{C} from the samples that had been immunoprecipitated with mAbs. On the other hand, mAbs precipitated a limited amount of PrP\textsuperscript{C} from those samples.

**Spatial Distribution of PrP\textsuperscript{C} and PrP\textsuperscript{Sc}**—To gain insight into the regional distribution of PrP\textsuperscript{C} and PrP\textsuperscript{Sc}, we attempted to define conditions to discriminate PrP\textsuperscript{C} and PrP\textsuperscript{Sc} using histoblot analysis. The pan-PrP-specific Ab.Tg was used as a probe. Without any pretreatment of the membrane, diffuse signals were observed in both scrapie-aFFECTed and mock-aFFECTed wild-type mice (Fig. 5A, *first column*). These signals disappeared by proteinase K treatment (Fig. 5A, *second column*). With autoclave pretreatment, the signal from scrapie-aFFECTed mice was enhanced (Fig. 5A, *third column*), while the signal from mock-aFFECTed mice was slightly reduced. When the autoclave pretreatment was followed by proteinase K digestion (Fig. 5A, *fourth column*), the signal from scrapie-aFFECTed mice was further enhanced. The same treatment completely eliminated the signal from the mock aFFECTed mice. No signals were detected in case of Prnp\textsuperscript{-/-} mice, regardless of pretreatment conditions. These results indicate that signals on the membrane (without pretreatment) preferentially represent PrP\textsuperscript{C}, while PrP\textsuperscript{Sc} was specifically detected on the membrane exposed to a combination of autoclaving and proteinase K digestion (Fig. 5A, *fourth column*). It should be noted that the PrP\textsuperscript{C} signal was reduced in the scrapie-aFFECTed sample, particularly in the cerebral cortex (Fig. 5A, *first column*). To better understand temporal and spatial relationships between amounts of PrP\textsuperscript{C} and PrP\textsuperscript{Sc}, we inoculated scrapie prion Sc237 into the brains of hamsters, which were killed at 38 and 80 days after inoculation, respectively. Mock-inFected hamsters were also examined, as controls. As shown in Fig. 5B, PrP\textsuperscript{C} but not PrP\textsuperscript{Sc} was present in normal hamster brains. PrP\textsuperscript{Sc} deposition was first detected in the thalamus at 38 days postinoculation, and the PrP\textsuperscript{C} signal decreased specifically in that region. At the terminal stage of illness, PrP\textsuperscript{Sc} had spread to all brain regions, and the PrP\textsuperscript{C} signal was decreased in those areas (Fig. 5B). The distribution of PrP\textsuperscript{Sc} revealed by the histoblot was consistent with the distribution of PrP\textsuperscript{Sc} plaque deposits detected by immunohistochemistry, using the reference antibody (Ab.Mo-VI) (data not shown). The results from histoblot and IP analyses consistently indicated the loss of PrP\textsuperscript{C} at the terminal stage of illness.

**Immunohistochemical Dissociation of Multiple Isoforms**—Ab.Tg recognized PrP\textsuperscript{C} in Prnp\textsuperscript{-/-} mice (Fig. 6, *panel 2*). Homogeneous signals representing PrP\textsuperscript{C} were distributed throughout the entire brain areas except for nuclei. In the scrapie-aFFECTed mouse brain, granulous and/or amorphous signals appeared (Fig. 6, *panels 6, 7, and 8*). Seven mAbs were classified into two groups in terms of reactivities to distinct materials in IFA. All mAbs showed homogeneous and weak signals in the normal unaffected mouse brain (Fig. 6, *panels 5 and 8*, and Table II) in agreement with their reactivities to PrP\textsuperscript{C} by IP. However, for the scrapie-aFFECTed samples, four (clones 1H8, 4A3, 6B5, and 9A8) showed stellar structures (Fig. 6, *panels 4 and 10*), while the others (7H8, 8G6, and 11H1) showed plaque structures in neuropil (Fig. 6, *panels 7 and 11*). Double staining of the former group together with the anti-GAP antibody indicated that stellar materials were PrPs that had accumulated in astrocytes (data not shown). There were no signals in Prnp\textsuperscript{-/-} brain samples (Fig. 6, *panels 7 and 9*). These results suggested that the mAbs recognized not only PrP\textsuperscript{C} but also abnormal isoforms in the acetone-fixed sections.

**Epitope Mapping**—A single mAb recognizes PrP\textsuperscript{C} under a certain condition (in solution) but recognizes abnormal isoforms under another condition (acetone-fixed tissue section). To
In Vivo Conversion of PrP<sub>C</sub> to PrP<sub>Sc</sub>

![Diagram](http://www.jbc.org)

**Fig. 6.** Indirect fluorescent assay with Ab.Tg and mAbs 4A3 and 11H1 to scrapie-affected Prnp<sup>+/+</sup> mice. Scrapie-affected and mock-affected Prnp<sup>+/+</sup> mouse brains were rapidly frozen and then cryosectioned. Prnp<sup>−/−</sup> mice served as controls. Brain samples were examined using Ab.Tg (panels 1–3), mAb 4A3 (panels 4–6), and 11H1 (panels 7–9) as primary antibodies. Ab.Tg detected the PrP<sup>C</sup> throughout the entire brain areas as indicated by smear signals (panel 2). In addition to similar signals, granular and amorphous signals representing PrP<sup>Sc</sup> were observed in the scrapie-affected brain (panel 1). mAbs 4A3 and 11H1 also weakly revealed PrP<sup>C</sup> in the mock-affected brain (panels 7–9). These mAbs detected stellar structures and plaques, respectively (panels 4 and 7). No signal was observed in Prnp<sup>−/−</sup> mice, using these antibodies (panels 3, 6, and 9).

**Table II**

| Antibody | IP<sup>a</sup> | WB<sup>b</sup> | ELISA<sup>c</sup> | IFA<sup>d</sup> | PrP-AcNPV |
|----------|---------------|---------------|-----------------|---------------|------------|
|          | C             | Sc            | C               | Sc            |            |
| Monoclonals |               |               |                 |               |            |
| 1H8 IgM   | +             | −             | −               | −             | +/−        |
| 4A3 IgM   | +             | −             | −               | −             | +/−        |
| 6B5 IgM   | +             | −             | −               | −             | +/−        |
| 9A8 IgM   | +             | −             | −               | −             | +/−        |
| 7H8 IgM   | +             | −             | −               | −             | +/−        |
| 8G6 IgM   | +             | −             | −               | −             | +/−        |
| 11H1 IgM  | +             | −             | −               | −             | +/−        |
| Polyclonal|               |               |                 |               |            |
| Ab.Tg IgG | +             | +             | +               | +             | +          |

<sup>a</sup> IP analysis with PrP<sup>C</sup> and PrP<sup>Sc</sup> prepared from normal (C) and scrapie-affected (Sc) mouse brains, respectively.

<sup>b</sup> WB analysis with PrP<sup>C</sup> and PrP<sup>Sc</sup> prepared from normal (C) and scrapie-affected (Sc) mouse brains, respectively.

<sup>c</sup> For ELISA we used partially purified PrP<sup>Sc</sup>.

<sup>d</sup> IFA with acetone-fixed cryosection from normal (C) and scrapie-affected (Sc) brains, and PrP-AcNPV-infected SF21AE cells. +, A, stellar signals in astrocytes; +N, amorphous signals in neurophils; +, positive; +/−, weakly positive; −, negative.

elucidate the molecular basis for these observations, we determined the epitopes recognized by mAbs and Ab.Tg, using a gridded array of synthetic peptides consisting of 122 13-residue peptides, sequentially shifted in steps of 2 amino acids and covering the whole mouse PrP sequence (Fig. 7A). Ab.Tg recognized distinct clusters of polypeptides, probably reflecting the conformation of native PrP<sup>C</sup> used as an immunogen. Four discontinuous regions appeared to constitute dominant epitopes. Interestingly, all mAbs examined recognized multiple discontinuous peptides, as summarized in Fig. 7B. Two mAbs (4A3 and 11H1) as well as Ab.Tg recognized sequences around octarepeat sequences. In the 4A3-1 segment, a pair of tripptides (T/G/S/WG; positions 55–57, 63–65, 71–73, 79–81, and 87–89) seemed to constitute an epitope. Two perfect repeats (GQPGGGSWG; positions 57–65 and 81–89), but not repeats containing a substituted residue (GQPGHGSWG; positions 65–73 and 73–81), were preferential epitopes recognized by Ab.Tg. mAb 4A3 also recognized a different region (4A3–2, GNDWEDR; positions 141–147), the sequence of which completely overlapped with the epitope of mAb 15B3-1, which is specific for PrP<sup>Sc</sup> (11). mAbs 11H1 and 8G6 also recognized segments (11H1-2: MIHFGND (positions 137–143); 8G6-1: MRSPMIHF (positions 133–145)) that partially overlapped with the 15B3-1. Furthermore, mAb 11H1 recognized the third segment (11H1-3: YRRVDAQYS (positions 161–169)), which is a complete overlap with the second epitope of mAb 15B3 (15B3-2). mAb 8G6 recognized two other segments (8G6–2: QVYYRPVDQ (positions 159–167); 8G6–3: SNQNN-FVHDCV (positions 169–179)). In addition to the octarepeat region, Ab.Tg also recognized Ab.Tg-2 (QWNKP; positions 97–101) and Ab.Tg-3 (DWEDRYRE; 143–151). The position of Ab.Tg-3 was close to that of 15B3-1. To examine the reproducibility of the experimental system, we analyzed sera from four independent immune mice. Ab.Tg-1 and -2 were detected with all sera, while Ab.Tg-3 was detected with three of four immune sera samples. These highly reproducible results indicate the reliability of our experimental system.

**DISCUSSION**

To gain insight into epitopes representing PrP<sup>C</sup> conformation and in vivo conversion processes of PrP<sup>C</sup> to PrP<sup>Sc</sup>, we immunized Prnp<sup>−/−</sup> mice with native PrP<sup>C</sup>, prepared as brain
and 9A8) recognized a stellar structure (Fig. 6, panel 7). In mock samples, a weak and diffuse signal was evident. Stellar and plaque structures were never observed (Fig. 6, panels 5 and 6). Thus, these stellar and plaque structures might represent disease-specific isoforms of PrP. mAb 4A3 recognized an isoform that accumulates in astrocytes (Fig. 6, panel 4). The PrP may be an intermediate form in converting to the fully pathogenic PrPSc. It has been reported that PrPSc first accumulates in astrocytes prior to development of neuropathological changes (26) and that the astrocyte-specific induction of PrP in Prnp−/− mice restores susceptibility to scrapie (27). These data suggest that astrocytes play an important role in scrapie pathogenesis. The mAb 4A3 may be useful to study the roles of astrocytes in scrapie disease. The mAb 11H1 may recognize the isoform closely related to the fully pathogenic PrPSc. The immunohistochemical staining pattern of the mAb 11H1 resembled results obtained with rabbit polyclonal antibodies to synthetic peptide or scrapie-associated fibrils in formalin-fixed samples treated with either hydrated autoclaving (21) or guanidine (28). Either guanidine or hydrated autoclaving pretreatment has been found essential for immunofluorescent detection of PrPSc plaques. The mAbs and Ab.Tg facilitated detecting abnormal isoforms of PrP, with solely acetone fixation. The distinct immunohistochemical staining patterns of two groups of mAbs suggests that conversion from PrPSc to PrPSc involves multiple steps at different sites in vivo.

The mAbs were preferentially reactive to PrPSc in IP but were immunohistochemically reactive to abnormal isoforms of PrP. One of the intriguing observations is that all mAbs recognized multiple discontinuous linear peptides that show no apparent similarities (Fig. 7). A similar unusual nature was noted for a PrPSc-specific clone 15B3 (11). Clone 15B3 recognized three distinct linear polypeptides. At least one of each of the epitopes recognized by three mAbs examined in this study overlapped with one of the 15B3 epitopes. Especially, the segments of 4A3-2 and 11H1-3 completely overlapped with epitope 15B3-2. The segments of 8G6-2 is closely related to the 15B3-2 epitope. We suggest that these epitopes largely contributed to reactivities of these mAbs to abnormal isoforms in acetone-fixed tissue sections. Epitopes 15B3-1/4A3-2 and 15B3-2/11H1-3/8G6-2 may represent isoforms accumulating in astrocytes and in the neuropil, respectively.

Regarding epitopes specific to PrPSc, the octarepeat region is notable. Two of three mAbs, 4A3 and 11H1, reacted to the region of octarepeats. Furthermore, results from polyclonal

**Fig. 7. Epitopes associated with PrPSc.** A, the mAbs and Ab.Tg were reacted with a gridded array of peptides comprising 122 polypeptides of 13 amino acids, shifted by 2 amino acids and covering the entire mouse sequence. Epitope numbers for each antibody are indicated by lines. Polypeptide numbers are indicated on the left. B, epitope positions are indicated with boxes under the sequences of mature murine PrPSc (positions 23–231) (31). Sequence numbers are indicated on the right. End sequence numbers are also indicated. Octarepeats (positions 51–90) are lined above the sequences.
antibody Ab.Tg suggested that this region is likely to be one of the major epitopes on PrPC. Because native PrPC was used as an immunogen, the epitopes recognized by Ab.Tg may reflect conformation of PrPC. A pair of tripeptides (T/G/S)WG; positions 55–57, 63–65, 71–73, 79–81, and 87–89) seemed to constitute an epitope of mAb 4A3. Ab.Tg recognized two perfect repeats (GQPHGGGWG; positions 57–65 and 81–89) but not other repeats that carried a substitution (GQPHGGSWG; positions 65–73 and 73–81). The substitutions at 71 and 79 residues (Gly to Ser; underlined) affect the immunoreactivity of Ab.Tg. A model suggested that the octarepeat region is constrained by four Cys(I)-coordinating histidines into a compact structure (29). The tripeptides involved in 4A3 reactivity are located at centers of loops generated by copper binding (29).

The epitopes for some mAbs generated by DNA-mediated immunization of Prnp−/− mice were also detected in a mouse colonized by PrP. We examined sera from four immune mice and observed reproducible results immunologically intolerant of PrP. We examined sera from four independent immune mice and observed reproducible results among the sera against the PrP immunogen. Epitopes 1 and 2 in Ab.Tg-1 and Ab.Tg-2 were always detected, and the third one (Ab.Tg-3) was detected in three of four immune sera, indicating their dominance as epitopes. The NH2-terminal sequences between positions 23 and 40 seldom functioned as an epitope, and therefore were unstable. These results suggested that the amino-terminal half of PrPC has a characteristic conformation, in a good agreement with findings with the mAbs we characterized. We also observed distinct epitope patterns on PrPSc fractions. This experimental strategy may aid in understanding species barrier mechanisms and differences in infectious prion strains replicated in a given animal species.

Data from experiments using these mAbs and polyclonal antibodies we developed revealed a marked decrease of PrPC in brains of animals at the terminal stage of illness, thereby supporting an immunohistochemical study suggesting that conversion of PrPC to PrPSc may involve multiple steps at different sites.

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2 T. Yokoyama and S. Itohara, unpublished observation.
In Vivo Conversion of Cellular Prion Protein to Pathogenic Isoforms, as Monitored by Conformation-specific Antibodies

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