Quantitative Analysis of Prion Protein by Immunoblotting

Kaori Takekida,[a] Yutaka Kikuchi,[b] Takeshi Yamazaki,[*,c] Motohiro Horiuchi,[d,e] Tomoshi Kakeya,[b] Morikazu Shinagawa,[e] Kosuke Takatori,[b] Akio Tanimura,[a] Ken-ichi Tanamoto,[b] and Jun-ichi Sawada[f]

[a]Showa Woman's University, 1–7 Taishido, Setagaya, Tokyo 154–8533, Japan, [b]Division of Microbiology, [c]Division of Food Additives, and [f]Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya, Tokyo 158–8501, Japan, [d]Research Center for Protozoan, and [e]Laboratory of Veterinary Public Health, Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary medicine, Inada-cho, Obihiro, Hokkaido 080–8555, Japan

(Received December 25, 2001; Accepted January 11, 2002)

Transmissible spongiform encephalopathy (TSE) is a neurodegenerative disease characterized by spongiform degeneration and accumulation of an infectious isoform (PrP^{Sc}) of the prion protein in the central nervous system. PrP^{Sc} originates from a ubiquitous cellular prion protein (PrP^{C}). We attempted to develop an easy method of quantitative analysis of PrP by immunoblotting based on densitometry data for PrP bands in immunoblots. Both PrP^{C} and PrP^{Sc} yield three bands in immunoblots, and they correspond to PrP molecules carrying two, one, and no Asn-linked sugar chains. We used bovine PrP^{C} as a model protein in the immunoblotting study. We removed the Asn-linked sugar chains from the PrP molecules with *N*-glycanase to convert all three glycoforms of PrP into a single band of the deglycosylated form and determined the PrP by densitometry calibrated with recombinant bovine PrP.

Key words —— prion protein, immunoblotting, quantification, bovine, PrP

INTRODUCTION

Transmissible spongiform encephalopathy (TSE) is a neurodegenerative disease characterized by spongiform degeneration and accumulation of an abnormal prion protein (PrP^{Sc}) in the central nervous system.[1] PrP^{Sc} is an infectious isoform of the prion protein (PrP) that originates from a normal isoform, ubiquitous cellular prion protein (PrP^{C}), and promotes conversion of PrP^{C} into PrP^{Sc}. Although the two isoforms have the same amino acid sequence, PrP^{Sc} differs significantly from PrP^{C} in the following respects. It has β-sheet-rich secondary structures, is resistant to proteinase K digestion, and forms aggregates that are not solubilized with nonionic detergents.

Detection of PrP^{Sc} by immunodetection with anti-PrP antibodies by ELISA, Western blotting, and immunohistochemistry has been used to diagnose TSE,[2] but since no antibodies discriminate between abnormal and normal isoforms of PrP, with the minor exception of those[3] that react with PrP^{C} and not with PrP^{Sc}, detection of PrP^{Sc} requires removal of PrP^{C} by proteinase K digestion. Moreover, aggregates of PrP^{Sc} require denaturation and depolymerization of PrP^{Sc} to expose antigenic epitopes to antibodies on the outside. ELISA and immunoblotting following proteinase K digestion and denaturation steps have been applied to the diagnosis of bovine spongiform encephalopathy (BSE) in the EU[4] and Japan. Immunoblotting has the advantage of a lower rate of false-positive results over ELISA, because the remaining PrP^{C}, which interferes with the detection of protease-resistant PrP^{Sc} by ELISA, can be distinguished by its size from PrP^{Sc} when immunoblotted. However, it has the disadvantage of quantitative determination by immunoblotting being more labor-intensive than ELISA. Quantification of immunoblots usually employs endpoint dilutions where the target protein band disappears to quantitate the target protein in a solution.

In this study, we attempted to develop an easy method of quantitative analysis of PrP by immunoblotting based on densitometry data for PrP bands on chemiluminescence films. Immunoblotting of both PrP^{C} and PrP^{Sc} yield three bands, which correspond to PrP molecules carrying two, one, and no Asn-linked sugar chains. We used bovine PrP^{C} as a model protein in the immunoblotting study. *N*-glycanase was used to remove the Asn-linked sugar chains from PrP molecules and convert the three glycoforms of PrP into a single band of the deglycosylated form, and PrP was then determined by densitometry calibrated with recombinant bovine PrP (rBoPrP). This method succeeded in quantitating PrP^{C} in bovine brain homogenates.

*To whom correspondence should be addressed: Division of Food Additives, National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya, Tokyo 158–8501, Japan. Tel.: +81-3-3700-1141; Fax: +81-3-3707-6950; E-mail: yamazaki@nihs.go.jp

MATERIALS AND METHODS

Anti-PrP Antibodies —— Two different anti-PrP monoclonal antibodies were used to detect PrP^C by immunoblotting: BSPX-54[5] and 6H4. BSPX-54 was prepared from the supernatant of a hybridoma culture by ammonium sulfate precipitation and Protein-A column chromatography. 6H4 was purchased from Prionics AG (Zürich, Switzerland).

Preparation of Bovine Brain Homogenates —— Brains from healthy Holstein cattle were used. Fragments of cerebral cortex were homogenized in a 6- to 9-fold volume of homogenizing buffer (0.32 M sucrose, 50 mM Tris–HCl (pH7.2), 1 mM EDTA, 1 mM PMSF), and after cleaning the homogenate by centrifugation at $1000 \times g$ for 10 min, the supernatant was used as the homogenate. The pellets were resuspended in suspension buffer (50 mM Tris–HCl (pH7.2), 1 mM EDTA, 1 mM PMSF) and centrifuged at $10000 \times g$ for 20 min, and after suspending the pellets in the suspension buffer, the suspension was used as the P2 fraction.

Preparation of Recombinant Bovine Prion Protein —— cDNA coding bovine PrP^C ($BoPrP^C$) was prepared from the cerebral cortex of Holstein cattle by the RT-PCR method as described by Yoshimoto *et al.*[6] The sense primer was: 5′-ACGGAATTCCATATGTCATCATGGTGAAAAGCCACATAGG-3′, and the antisense primer was: 5′-TTATCTCGAGGCGGCCGCAGGAAGGTTGCCCCTATCCTACTAT-3′. The cDNA was cloned in pBlueScript II SK (+) vector (Stratagene, CA, U.S.A.). The sequence was identical to that of pPCJY1 (Accession # D10613) reported by Yoshimoto *et al.*[6] The insert DNA for expression plasmids in *Escherichia coli* was prepared by PCR with the cloned cDNA as a template. The sense primer was: 5′-CTTGGACCATATGTCCAAGAAGCGTCCGAAACCTGGAGGA-3′, and the antisense primer was: 5′-TCCCAAGCTTCTCATGCCCCTCGTTGGTAATAAGCCTGG-3′. The insert DNA, which codes MetSer-BoPrP (25-241), molecular weight 23.7 kDa, was cloned between the *Nde*I and *Hin*dIII sites in pET23b vector (Novagen, WI, U.S.A.). The resulting plasmid was transfected into *E. coli* BL21(DE3)pLysS (Novagen), and rBoPrP was produced in *E. coli* in the presence of 1 mM IPTG. rBoPrP was solubilized from inclusion bodies with 8 M urea in 50 mM Tris–HCl (pH8.0) containing 1 mM PMSF. After dilution of the urea concentration to 6 M and addition of 0.05% sodium azide, the solution was allowed to stand in a refrigerator for 1 month to permit disulfide bonds to form in PrP molecules by slow air oxidation of cysteine residues. The oxidized protein was purified by Cu^{2+}-chelating column chromatography (HiTrap Chelating column, 5 ml, Amersham Pharmacia-Biotech, Buckinghamshire, UK). After charging the column with copper ion (Cu^{2+}), it was equilibrated with the elution buffer (50 mM Tris–HCl (pH8.0), 6 M urea, 0.02% sodium azide). The sample was then loaded onto the column and eluted with 0, 25, 50, 100 and 300 mM imidazole and 50 mM EDTA in the elution buffer. rBoPrP was collected mainly in the 100 mM imidazole fraction. The fraction was dialyzed in 3 M, 1 M, and 0 M urea in 50 mM Tris–HCl (pH8.0). After removing the insoluble proteins, the dialysate was concentrated to about one tenth to one twentieth. The protein concentration was determined with a BCA reagent kit (Pierce, IL, U.S.A.). The purity of the refolded soluble rBoPrP was confirmed by SDS-PAGE/silver staining, and the rBoPrP was used as the standard protein.

Immunoblot Analysis of PrP^C in Bovine Brain —— The brain homogenate and P2 fraction were treated with *N*-glycanase (PNGase F, peptide: *N*-glycosidase F; New England Biolabs, MA, U.S.A.) according to the manufactures instructions. The samples were subjected to SDS-PAGE and transferred to a nitrocellulose or PVDF membrane. PrP was detected with anti-PrP monoclonal antibody as the first antibody, HRP-labeled anti-mouse IgG antibody as the second antibody and ECL Plus (Amersham Pharmacia Biotech) as the substrate for chemiluminescence detection. The immunoreactive bands were visualized on film. The intensity of the bands was quantified by computer-assisted densitometry of film, and calibrated with rBoPrP for each membrane.

RESULTS AND DISCUSSION

Immunoblot Analysis of PrP^C in Bovine Brain with Anti-PrP Antibodies

PrP^C in the bovine brain P2 fraction was analyzed by immunoblotting with the two anti-PrP monoclonal antibodies, BSPX54 and 6H4. PrP^C yielded three bands, which corresponded to PrP molecules carrying two, one, and no Asn-linked sugar chains. The relative intensity of the three bands on the immunoblots varied with the antibody (Fig. 1), suggesting that the glycoforms of PrP^C affect the antibody's recognition to the epitopes on PrP

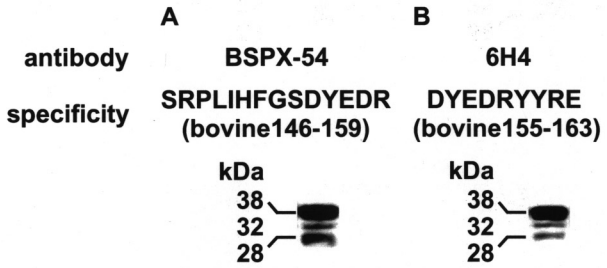

Fig. 1. Reactivity of Anti-PrP Monoclonal Antibodies with the Bovine Brain P2 Fraction.

The P2 fraction was subjected to SDS-PAGE and transferred to a PVDF membrane. PrP was detected with anti-PrP monoclonal antibody BSPX-54 (A) and 6H4 (B). Specificity for BSPX-54 means the antigen peptide sequence used to immunize mice. Specificity for 6H4 means the sequence recognized by the antibody.

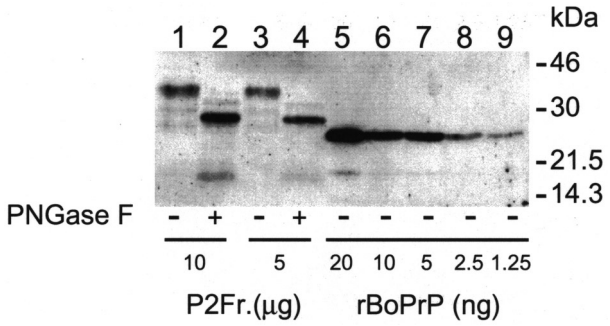

Fig. 2. Determination of PrP^C in the Bovine P2 Fraction by Immunoblotting.

The P2 fraction was treated with *N*-glycanase (PNGase F +), subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. The samples were analyzed by immunoblotting with anti-PrP monoclonal antibody BSPX-54. The intensity of the bands was quantified by calibration with rBoPrP. The values under the lanes are the amounts of rBoPrP and the amounts of total protein in the P2 fraction (P2Fr.) before *N*-glycanase treatment.

molecules. The glycoform patterns also vary with the infectious PrP strain[7] and the brain region,[8] making it necessary to use PrP carrying Asn-linked sugar chains as a calibration standard protein for quantitative determination of PrP by immunoassay. However, it is difficult to prepare large amounts of PrP with sugar chains from brains.

N-Glycanase converted the three bands of PrP^C into a single band of the deglycosylated form in the immunoblots (Fig. 2). PrP^C in brain has two or fewer *N*-glycans and one glycophosphatidylinositol (GPI) anchor, whereas rBoPrP has nor *N*-glycans or GPI anchor. Consequently, the molecular weight of the rBoPrP (apparent molecular weight 25 kDa, Fig. 2 lanes 5–9) was lower than that of the unglycosylated PrP^C (apparently 27 kDa, the third band from the top in lane 1). The deglycosylated PrP (lane 2) has a slightly higher molecular weight (apparently 29 kDa) than the unglycosylated PrP^C. The reason for this is unclear.

We compared the intensity of the PrP^C bands in the bovine brain P2 fraction before and after deglycosylation with *N*-glycanase. With the intensity of the band corresponding to deglycosylated PrP^C set equal to 100%, the intensity of the bands of the untreated diglycosylated, monoglycosylated, and unglycosylated PrP^C was 40.0 ± 12.5%, 10.7 ± 6.6% and 17.1 ± 15.2% (mean ± S.E. derived from three independent experiments), respectively. The total intensity of the three bands was 67.9 ± 13.7%. The fact that the sum of the intensities is less than 100% suggests inaccurate quantification of PrP without deglycosylation.

N-terminally truncated 18 kDa PrP^C is found in normal human and mouse brain after treatment with *N*-glycanase,[9,10] and the 18 kDa band was found in the *N*-glycanase treated P2 fraction from bovine brain. Since the ratio of the intensity of the 18 kDa band to that of the deglycosylated PrP^C band (29 kDa) was only 3–7%, not measuring the 18 kDa band has little influence on the accuracy of quantitative determination of PrP^C.

To avoid the problems described above, we quantified deglycosylated PrP^C with *N*-glycanase by using recombinant PrP derived from *E. coli* as a calibration standard protein.

Quantitative Analysis of PrP^C in Bovine Brain

The amounts of PrP^C in healthy bovine brain (cerebral cortex) homogenate and the P2 fraction were determined by immunoblotting with anti-PrP antibody BSPX-54 using the densitometry data for the bands. Deglycosylated PrP^C was quantified with *N*-glycanase. The immunoblot pattern of the P2 fraction is shown in Fig. 2. The band intensity of rBoPrP on the immunoblots depended on the amount of rBoPrP in the range between 1 to 20 ng/lane on the linear dose-response curve (data not shown). The amount of PrP^C in the P2 fraction was calculated according to the calibration curve of rBoPrP. The amount of PrP^C in the brain homogenate was also determined by similar procedures. The results are shown in Table 1. The P2 fraction, which is rich in synaptosomes, contained a higher amount of PrP^C than the homogenate, consistent with PrP^C being concentrated primarily in the synaptic fields of neurons.[11] The amount of PrP^C in bovine brain homogenate measured by immunoblotting (0.4 μg/mg of total protein) was similar to the amount measured

Table 1. PrP^C in Bovine Brain Homogenate and the P2 Fraction

Sample	PrP^C (μg/mg of total protein)[a]	
	Immunoblotting[b]	Competitive ELISA[c]
Homogenate	0.4 ± 0.1	0.3 ± 0.1
P2 fraction	0.9 ± 0.1	1.0 ± 0.1

a) The amount of PrP^C is expressed as rBoPrP equivalents. The values are mean $\pm$ S.E. ($n = 3$). *b*) PrP^C was determined by immunoblotting with anti-PrP monoclonal antibody BSPX54 as the first antibody. *c*) Unpublished data. PrP^C was determined by ELISA with the anti-PrP polyclonal antibody as the first antibody and rBo-PrP as competitor.

by competitive ELISA (0.3 μg/mg of total protein) (unpublished data). Our results are consistent with a report[12] that the amount of PrP^C detected in healthy bovine brain (thalamus) homogenate by dot blotting was about 0.2 μg/mg of the total protein in the homogenate, although the amount of PrP^C varies with the anatomic site in the brain.

In this study, we attempted to develop an easy quantitative method for analysis of PrP by immunoblotting. Some ELISA and immunoblotting systems are used to detect PrP^{Sc} in bovine brain and medulla oblongata to prevent material from cattle with BSE from entering the market. No diagnostic antibodies distinguish PrP^{Sc} from PrP^C. Some antibodies recognize denatured PrP. In other words, they do not discriminate between PrP^C and PrP^{Sc} after denaturation. Other antibodies recognize similar 3D structures in epitopes on both PrP^C and PrP^{Sc}. We therefore think that the *N*-glycanase treatment of PrP^C used in this study can be applied to the detection of PrP^{Sc}.

Acknowledgements We wish to thank Dr. Yasumasa Kido (Research Institute for Animal Science in Biochemistry & Toxicology) for providing the bovine brains. This work was supported by grants for Research on Pharmaceutical and Medical Safety and for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1) Prusiner, S. B. (1998) Prions. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 13363–13383.
2) MacGregor, I. (2001) Prion protein and development in its detection. *Transfus. Med.*, **11**, 3–14.
3) Yokoyama, T., Kimura, K. M., Ushiki, Y., Yamada, S., Morooka, A., Nakashiba, T., Sassa, T. and Itohira, S. (2001) In vivo conversion of cellular prion protein to pathogenic isoform, as monitored by conformation-specific antibodies. *J. Biol. Chem.*, **276**, 11265–11271.
4) European Commission, Unit B3 Management of Scientific Committees II (1999) *The evaluation of tests for the diagnosis of transmissible spongiform encephalopathy in bovines* (8 July 1999). (http://europa.eu.int/comm/food/fs/bse/bse12_en.pdf).
5) Horiuchi, M., Yamazaki, N., Ikeda, T., Ishiguro, N. and Shinagawa, M. (1995) A cellular form of prion protein (PrP^C) exists in many non-neuronal tissues of sheep. *J. Gen. Virol.*, **76**, 2583–2587.
6) Yoshimoto, J., Iinuma, T., Ishiguro, N., Horiuchi, M., Imamura, M. and Shinagawa, M. (1992) Comparative sequence analysis and expression of bovine PrP gene in mouse L-929 cells. *Virus Genes*, **6**, 343–356.
7) Collinge, J., Sidle, K. C. L., Meads, J., Ironside, J. and Hill, A. W. (1996) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* (London), **383**, 685–690.
8) Dearmond, S. J., Qiu, Y., Sànchez, H., Spilman, P. R., Ninchak-Casey, A., Alonso, D. and Daggett, V. (1999) PrP^C glycoform heterogeneity as a function of brain region: implications for selective targeting of neurons by prion strains. *J. Neuropathol. Exp. Neurol.*, **58**, 1000–1009.
9) Zanusso, G., Liu, D., Ferrari, S., Hegyi, I., Yin, X., Aduzzi, A., Hornemann, S., Liemann, S., Glockshuber, R., Manson, J. C., Brown, P., Petersen, R. B., Gambetti, P. and Sy, M. S. (1998) Prion protein expression in different species: analysis with a panel of new mAbs. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 8812–8816.
10) Liu, T., Zwingman, T., Li, R., Pan, T., Wong, B. S., Petersen, R. B., Gambetti, P., Herrup, K. and Sy, M. S. (2001) Differential expression of cellular prion protein in mouse brain as detected with multiple anti-PrP monoclonal antibodies. *Brain Res.*, **896**, 118–129.
11) Harris, D. A. (1999) Cellular biology of prion diseases. *Clin. Microbiol. Rev.*, **12**, 429–444.
12) Meyer, R. K., Lustig, A., Oesch, B., Fatzer, R., Zurbriggen, A. and Vandevelde, M. (2000) A monomer-dimer equilibrium of a cellular prion protein (PrP^C) not observed with recombinant PrP. *J. Biol. Chem.*, **275**, 38081–38087.