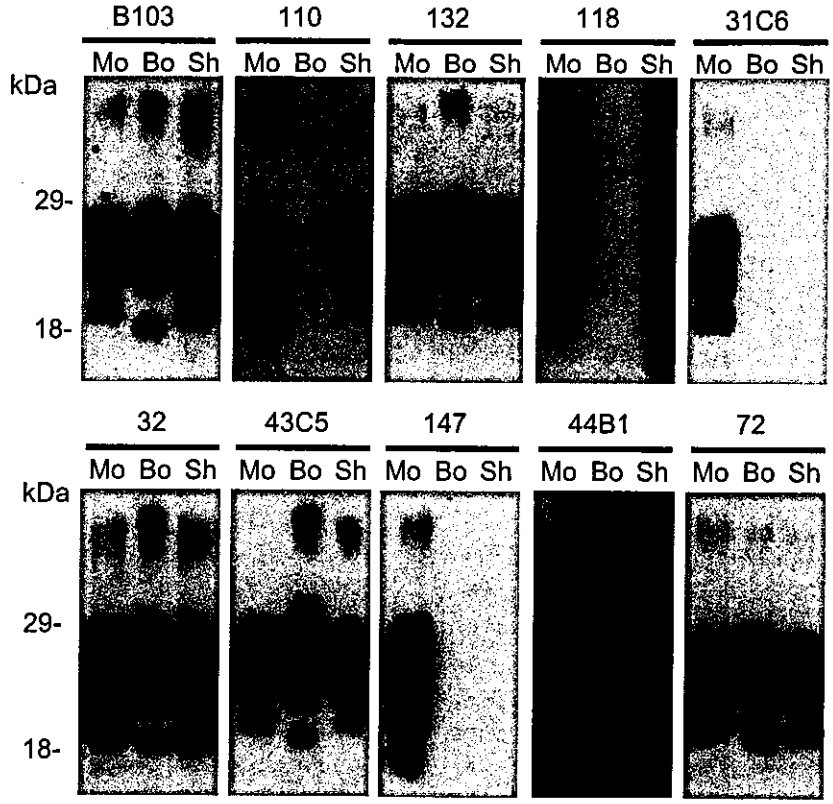

Fig. 3. Species specificity in Western blot analysis. Crude preparations of mouse (Mo), bovine (Bo), and sheep (Sh) PrP^{Sc} were prepared as described elsewhere (Grathwohl et al., 1997) and were separated by SDS-PAGE and transferred onto PVDF membranes. Brain tissue equivalents of 25 mg, 500, and 125 μg were loaded for Mo, Bo, and Sh PrP^{Sc}, respectively. The blots were stained with B-103 rabbit polyclonal antibodies (Horiuchi et al., 1995) or mAbs as indicated.

treated PrP^{Sc} is not due digestion of the PrP^{Sc}. Rather, it appears that the epitopes on PK-treated PrP^{Sc} are cryptic. A considerable amount of PK-sensitive PrP, including PrP^{C}, is co-purified during PrP^{Sc} purification in the absence of PK treatment (Caughey et al., 1995). Therefore, it is likely that reaction of the mAbs with PK-untreated non-denatured PrP^{Sc} accounts for the reaction to PK-sensitive PrP that exposes these epitopes on its accessible surface. Furthermore, the epitopes recognized by our panel of mAbs do not appear to be exposed on the surface of the PK-resistant core

Table 2
Characterization of mAbs against PrP

Group	mAb	Epitope[a]		Reactivity to PrP^{Sc} in ELISA[b]		
		position (amino acid)	L/DC	PK(−) GdnHCl(−)	PK(+) GdnHCl(−)	PK(+) GdnHCl(+)
I	8, 37, 40, 106, 110, 162	56–90	L	+	–	+
IIa	132	119–127	L	+	–	+
IIb	13, 118	137–143	L	+	–	+
IIc	31C6	143–149	L	+	–	+
IId	32, 149	147–151	L	+	–	+
III	43C5	163–169	L	+	–	+
IV	39, 147	219–229	L	+	–	+
V	66[c], 31B1, 31B5, 42B4, 42D2, 42D6, 44A2, 44A5, 44B1, 44B5[c]	155–231	DC	+(−)[c]	–	+(−)[c]
VI	23D9, 42D3, 44B2	89–231	DC	+	–	+
VII	72	89–231 (143–153)	DC	+	–	+

[a] L, linear epitope; DC, discontinuous epitope.
[b] Treatments of PrP^{Sc} are as described in Fig. 4.
[c] MAbs 66 and 44B5 reacted with rPrP but did not react with the three PrP^{Sc} preparations.

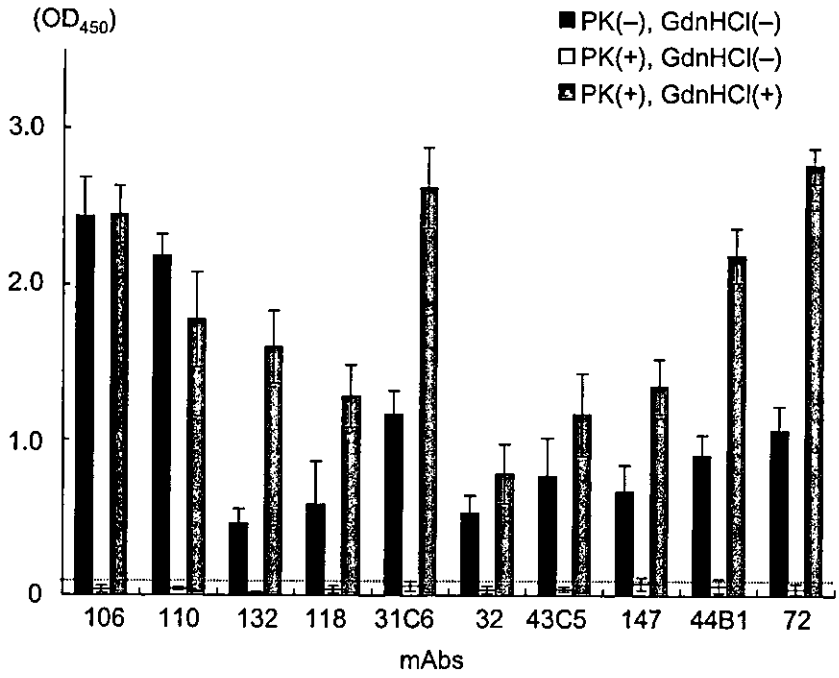

Fig. 4. Reactivity of mAbs to PrP^{Sc} fraction in ELISA. A set of three wells were used for each mAb: after the adsorption of purified PrP^{Sc} fraction, first well was neither treated with PK nor with GdnHCl [PK(–), GdnHCl(–)], second well was treated with 40 μg/ml PK for 60 min at 37 °C [PK(+), GdnHCl(–)], and third well was treated with PK and then further treated with 6M GdnHCl for 60 min to denature PrP^{Sc} [PK(+), GdnHCl(+)]. mAbs indicated in the figure were used as a representative for each group in Table 2. Dotted line indicates the cut-off value [An average OD_{450} value of negative control monoclonal antibody plus 5 times standard deviation ($n > 4$)].

of PrP^{Sc}. Instead, they become accessible to the mAbs after denaturation of PK-resistant core of PrP^{Sc}.

Reduction of PrP^{Sc} aggregate size does not expose cryptic epitopes

Purified PrP^{Sc} forms relatively large aggregates that can be precipitated by centrifugation at 10000 × *g*. We were concerned that the large aggregates themselves affect the antibody accessibility to PrP^{Sc}. To address this concern, we attempted detergent-lipid-protein complex (DLPC) treatment, which can reduce aggregate size without loss of infectivity (Gabizon et al., 1987). As shown in Fig. 5, nearly all of the purified PrP^{Sc} was present in the pellet after centrifugation at 10000 × *g* for 10 min, although more than the half of DLPC-treated PrP^{Sc} remained supernatant. Further centrifugation at 100000 × *g* of the soluble DLPC-treated PrP^{Sc} resulted in its precipitation. These results show that the DLPC treatment reduces the size of PrP^{Sc} aggregates without significant a loss of PK resistance. As described above (Fig. 4), mAbs did not react with PK-treated, DLPC-untreated PrP^{Sc} unless it was denatured (at 0 M GdnHCl in Fig. 6). Although all the mAbs except for mAbs 110, 132, and 32 faintly reacted with DLPC-treated PrP^{Sc} without denaturation (OD_{450} < 0.17 at 0 M GdnHCl), none of the mAbs showed a significant increase in reactivity to DLPC-treated PrP^{Sc} compared with DLPC-untreated PrP^{Sc}. These results suggest that the reduction of aggregate size by DLPC is not sufficient to expose cryptic epitopes on PrP^{Sc}.

Exposure of cryptic epitopes by denaturation

The reduction of PrP^{Sc} aggregate size by DLPC did not result in the efficient exposure of the hidden epitopes. In

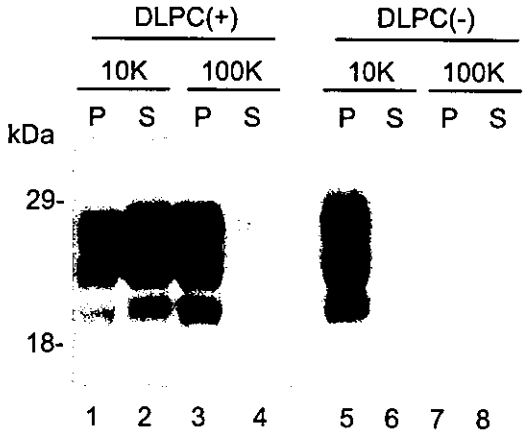

Fig. 5. Sedimentation analysis of DLPC-treated PrP^{Sc}. DLPC-treated (lanes 1–4) and untreated PrP^{Sc} (lanes 5–8) were digested by PK and then subjected to sedimentation analysis. Centrifugation at 10000 × *g* yielded pellet (lanes 1 and 5) and supernatant (lanes 2 and 6) fractions. The supernatants were further subjected to ultracentrifugation at 100000 × *g* to generate pellet (lanes 3 and 7) and supernatant (lanes 4 and 8). The PrP^{Sc} in each fraction was detected by immunoblot analysis.

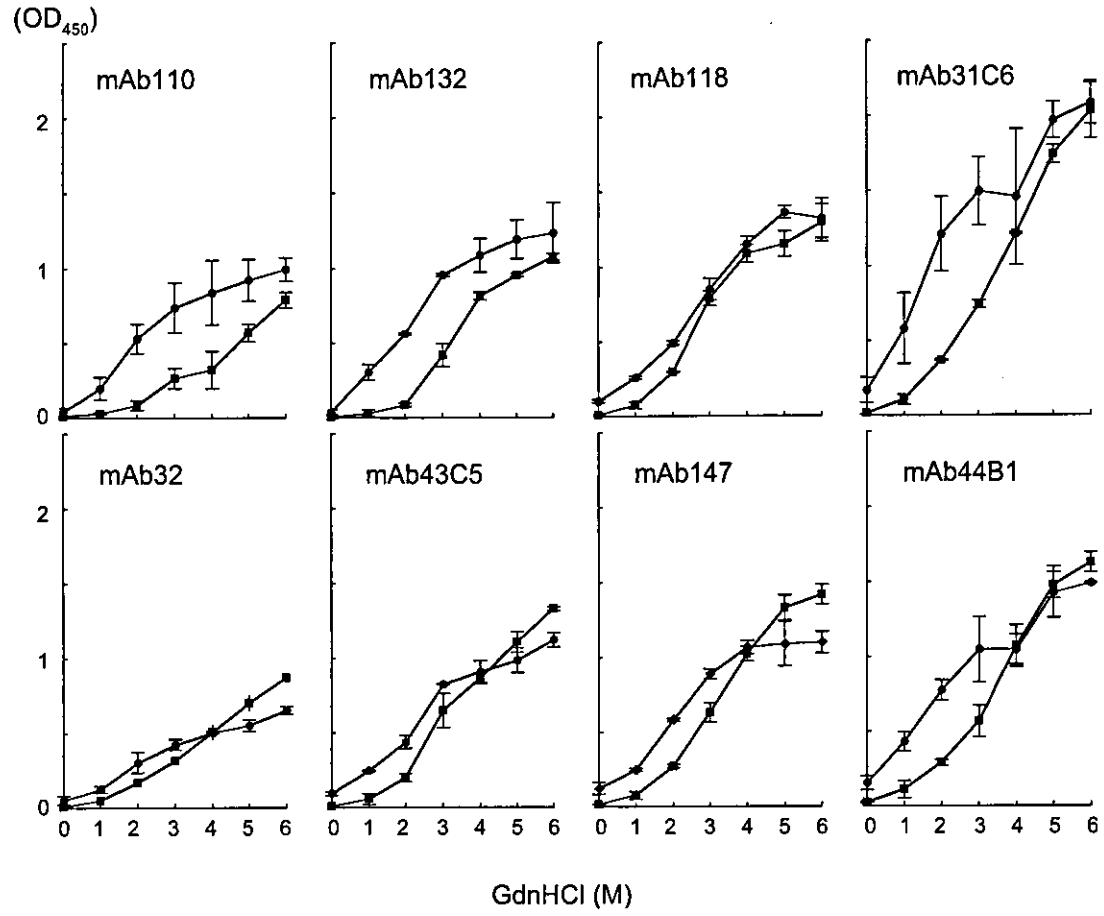

Fig. 6. Exposure of epitopes by GdnHCl treatment. DLPC-treated or untreated PrP^{Sc} was adsorbed to an ELISA plate, digested with PK, and then treated with the various concentrations of GdnHCl indicated at the bottom of the figure. Average and SD of three independent experiments were plotted. Circles indicate the DLPC-treated PrP^{Sc}, whereas squares indicate DLPC-untreated PrP^{Sc}.

contrast, Fig. 6 shows that treatment with of both PrP^{Sc} preparations to GdnHCl dramatically increased the reactivities to all mAbs. This indicates that the dissociation and denaturation of PrP^{Sc} aggregates resulted in pronounced exposure of epitopes. However, one striking difference in the reactivity of mAbs was observed especially at lower GdnHCl concentration: most of the mAbs displayed a higher reactivity to DLPC-treated PrP^{Sc} than to DLPC-untreated PrP^{Sc} at the lower GdnHCl concentrations. This suggests that DLPC-treated PrP^{Sc} may be more sensitive to denaturant than DLPC-untreated PrP^{Sc}.

PrP^{Sc} aggregates are thought to undergo partial denaturation in 2 M GdnHCl, and are believed to be almost completely denatured in 6 M GdnHCl. In parallel with denaturation of PrP^{Sc}, 2 M GdnHCl treatment does not completely abolish prion infectivity, although >4 M GdnHCl treatment drastically reduces the infectivity (Caughey et al., 1997; Prusiner et al., 1993). Table 3 shows the ratio of OD_{450} (in Fig. 6) at 2 and 6 M GdnHCl for both PrP^{Sc} preparations. In DLPC-untreated PrP^{Sc}, the ratios varied from 0.08 to 0.19, suggesting that less than 20% of the epitope for each mAb was exposed following 2 M GdnHCl treatment. In contrast, the ratios increased, varying from 0.37 to 0.58, following DLPC treatment, indicating that the reduction of PrP^{Sc} size influences the sensitivity to denaturation. The variation of the ratios may reflect the difference in the denaturation process for the specific epitopes. For example, epitopes for mAbs 118 and 43C5 appeared to be more resistant to denaturation by GdnHCl than other epitopes.

Table 3
Reactivity of mAbs to partially denatured PrP^{Sc}

mAb	DLPC-untreated		DLPC-treated	
	OD_{450} at 2M/6M GdnHCl	Ratio	OD_{450} at 2M/6M GdnHCl	Ratio
110	0.084/0.789	0.10	0.529/0.994	0.53
132	0.084/1.074	0.08	0.560/1.235	0.45
118	0.295/1.292	0.19	0.488/1.320	0.37
31C6	0.367/2.031	0.18	1.211/2.085	0.58
32	0.167/0.872	0.19	0.305/0.653	0.47
43C5	0.199/1.332	0.15	0.438/1.123	0.39
147	0.271/1.419	0.19	0.581/1.099	0.53
44B1	0.290/1.628	0.18	0.775/1.492	0.52

Discussion

To generate a diverse panel of mAbs to PrP molecules, we established a variety of hybridomas by using rMoPrP and $MoPrP^{Sc}$ purified from scrapie-affected mice brain as immunogens. According to the extensive epitope analyses using rPrP and pepspot membrane, our mAb panel contained mAbs recognizing at least seven different linear epitopes and three discontinuous epitopes. Five of seven linear epitopes were located within the N-terminal half of the PK-resistant core of PrP^{Sc} (aa 119–127, 137–143, 143–149, 147–151, and 163–169). This region is thought to undergo a major conformational change from random coil or α-helix- to β-sheet-rich structure during the conversion of PrP^{C} to PrP^{Sc}. Among the antibodies, mAb 132, recognizing the epitope aa 119–127 (AVVGGLGGY), is of particular interest. This region is adjacent to the highly amyloidogenic sequence AVAAAAVA (aa 112–119) (Gasset et al., 1992) and the first short β-strand (aa 128–131). Studies have shown that this region plays an important role in the conversion of PrP^{C} to PrP^{Sc} (Holscher et al., 1998; Muramoto et al., 1996). In addition, this region is highly conserved between mammals and birds, suggesting the importance of this region in PrP^{C} biology (Wopfner et al., 1999). Thus, mAb 132 will facilitate studies of how this region is involved in the conversion process as well as how PrP^{C} functions. The epitope for mAb 43C5 (aa 163–169) on PrP^{C} is of also interest because this region, in conjunction with its C-terminal portion, is thought to be a binding domain for an unidentified factor tentatively named protein X, which is expected to act as a molecular chaperon during the conversion process (Kaneko et al., 1997). mAb 43C5 will also be a good tool for studying how this region is involved in the intermolecular interaction. In addition, the first α-helix on PrP^{C} may undergo α to β conformational change during the conversion process, although this has not been fully clarified (Zhang et al., 1995). The mAbs in groups IIb, IIc, and IId recognizing the first α-helix and its immediate N-terminal portion will therefore contribute to understanding of structural differences in this region.

Elucidation of the PrP^{Sc} structure is an important problem to understand the identity of prion. Although the model structure of PrP^{Sc} and its aggregates were recently proposed from electron crystallography (Wille et al., 2002), their atomic structures remain to be elucidated. Studies of antibody accessibility will help to clarify PrP^{Sc} structure (Kanyo et al., 1999). Our mAbs did not show intense reactivity to PK-treated PrP^{Sc}, which is associated with prion infectivity, although they strongly react with PrP^{Sc} after denaturation. This suggests none of the epitopes recognized by our mAb panel are accessible by mAbs on PrP^{Sc} aggregates. The epitope at the C-terminus of PrP^{Sc} is reported to be accessible to antibody (Peretz et al., 1997; Williamson et al., 1998). In that study, the authors used DLPC treatment, which can disperse the PrP^{Sc} aggregates into liposome and reduce particle size of PrP^{Sc} aggregates (Gabizon et al., 1987). Although we confirmed that DLPC treatment could reduce the PrP^{Sc} aggregate size, some mAbs including one recognizing the C-terminus showed a trace of reactivity even when we used DLPC-treated PrP^{Sc}. In contrast, denaturation of DLPC-treated PrP^{Sc} was required to expose the cryptic epitopes. This implies that DLPC treatment might not be sufficient to expose the cryptic epitope(s) on PrP^{Sc}. There are some differences in experimental conditions between our investigations and those of Peretz et al. that could explain the differences in our results. First, they used Sc237 hamster scrapie, while we used the Obihiro strain of mouse-adapted scrapie. Second, preparation of PrP^{Sc} for ELISA also varied. Peretz et al. treated PrP^{Sc} with PK and the resulting PrP^{27-30} was dispersed into liposomes. In contrast, we performed DLPC treatment first after which DLPC-treated PrP^{Sc} was digested with PK to eliminate PK-sensitive PrP, which is expected to possess some exposed epitopes. Third, they used streptavidin-coated plates to immobilize the PrP^{Sc} after biotinylation, while, in this study, we directly adsorbed PrP^{Sc} to the ELISA plate by possible hydrophobic interaction. Finally, the antibodies used in the two studies were different. Although pepspot analysis demonstrated that mAbs 39 and 147 recognize an extreme C-terminal part of PrP, we used purified IgG instead of a smaller single-strand Fab fragment. We do not know the reason for the difference in the mAb reactivity to the C-terminus in our results and those reported by Peretz et al., it is conceivable that these differences in the experimental conditions might influence the results.

Denaturation of PrP^{Sc} aggregates caused the exposure of cryptic epitopes (Serban et al., 1990; Williamson et al., 1996). Here we observed that the sensitivity to denaturant varies between the epitopes. In DLPC-treated PrP^{Sc}, the epitopes for mAb 118 and 43C5 appeared to be more resistant to denaturation as determined by the ratio of OD at 2 to 6 M GdnHCl treatment. This difference implies complexity in the inter- or intramolecular interactions involved in the formation of PrP^{Sc} aggregates. It is of particular interest to examine what kind of inter- or intramolecular interactions determine prion infectivity or if exposure of certain epitopes correlates to prion inactivation. Our data also showed that the DLPC-treated PrP^{Sc} is more sensitive to denaturant than DLPC-untreated PrP^{Sc}, indicating that prion inactivation methods are possibly influenced by the state of PrP^{Sc} aggregation and environment.

The epitopes for mAbs raised against rPrP seemed to be relatively restricted, and 9 of 14 mAbs recognized a discontinuous epitope within aa 155–231, indicating this epitope on rPrP was immunodominant in $PrP^{-/-}$ mice. In contrast, the epitopes for the mAbs raised against PrP^{Sc} broadly spanned the PrP molecules. Furthermore, 13 of 14 hybridomas from mice immunized with rPrP secreted IgG1, although hybridomas secreting IgG2b were predom-

inantly established from mice immunized with PrP^{Sc} (9 of 15). Therefore, the differences in the immunodominant regions and predominant immunoglobulin subtypes suggest that the two PrP preparations elicited different type of immune responses, although the two PrPs share primary structure and we used the same immunization procedure. Although PrP^{Sc}-specific antibodies are thought to be an attractive tool for analyzing properties of PrP^{Sc} as well as establishing new diagnostic methods, only one has been previously reported (Korth et al., 1997). Thus, the unique immune response against the PrP^{Sc} fraction suggests that the use of an infectivity-associated PrP^{Sc} fraction as an immunogen may help to generate PrP^{Sc}-specific antibodies. In addition, it is still possible that certain regions are located on the surface of PrP^{Sc} as either a linear epitope or as a PrP^{Sc}-specific discontinuous epitope. Actually, Paramithiotis et al. very recently reported that three amino acid residues, YYR, possibly located in the second β-strand, is not antibody accessible on PrP^{C}, although the region is exposed on the surface of PrP^{Sc} (Paramithiotis et al., 2003). Further generation of mAbs, especially those specific to PrP^{Sc}, will be required for determining the surface structure of PrP^{Sc}.

BSE and vCJD are now global concerns. Because therapeutics for prion diseases are not currently available, elimination of prion-contaminated foodstuff and biomedical materials is essential for preventing further spread of the disease. We have found that some of our mAbs possessed higher sensitivity for detecting bovine PrP^{Sc} than some commercial-based anti-PrP mAbs, including 6H4 (data not shown). Further generation of anti-PrP antibodies with higher affinity and avidity will contribute to enhance the sensitivity of PrP^{Sc} detection methods.

Materials and methods

Plasmid construction

The prokaryotic expression vectors pET22b(+) (Novagen) and pRSETB (Invitrogen) were used in these studies. For the construction of expression plasmids based on pET22b(+), cDNA encoding mouse (Mo) PrP codons 23–231 was amplified by PCR with primers MPrP2 and MPrP3, and genomic DNA encoding sheep (Sh) PrP codons 25–234 was amplified with primers SPrP102 and SPrP103. Amplified fragments were cloned into the *Eco*RV site of pBluescript KS(+) (Stratagene) to confirm nucleotide sequences. The cloned fragments were excised by *Msc*I and *Eco*RI digestion and ligated into the corresponding sites of pET22b(+).

For the construction of expression plasmids based on pRSETB, cDNA encoding MoPrP codons 23–231 was amplified with primers MPrP5 and MPrP3. To express deletion mutants of MoPrP aa 23–167, 23–214, 89–231, and 155–231, we used primer sets of MPrP5 and MPrP9, MPrP5 and MPrP11, MPrP10 and MPrP3, and MPrP12 and MPrP3, respectively, were used for PCR. Hamster (Ha) PrP cDNA encoding codons 23–231, ShPrP gene encoding codons 25–234, and bovine (Bo) PrP cDNA encoding codons 25–242 were amplified with primer sets of MPrP5 and MPrP9, SPrP101 and SPrP102, and BPrP101 and BPrP103, respectively. Amplified fragments were digested with *Bam*HI and *Eco*RI and cloned into the *Bam*HI and *Eco*RI sites of pRSETB. Nucleotide sequences of the cloned PrP gene fragments were confirmed before their expression. To generate the mutant HaPrP containing a single amino acid substitution at codon 179 (Cys to Ala) or 214 (Cys to Ala), we used the ExSite PCR-based site-directed mutagenesis kit (Stratagene) according to the supplier's instructions. Primer sets of HPrP1 and HPrP2, and HPrP3 and HPrP4 were used to introduce the nucleic acid substitution encoding codons 179 and 214, respectively. Primer sequences Primer sequences were as follows: MPrP2, 5′-AATGGCCA AAAAGCGGCCAAAGCCTGGA-3′; MPrP3, 5′-GAGAATTCAGCTGGATCTTCTCCCGTCGT-3′; MPrP5, 5′-AAGGATCC GAAAAAGCGGCCAAAGCCTGG-3′; MPrP9: 5′-GAGAATTC TACTGATCCACTGGCCTG-GTAG-3′; MPrP10, 5′-AAGGATCC GGGCCAAG-GAGGGGGTACCCATAATC-3′; MPrP11, 5′-GAGAAT **TC** AGACGCACATCTGCTCCACCAC-3′; MPrP12, 5′-AAGGATCC GCGCTACCCTAACCAAGTGTACT-3′; SPrP101, 5′-AAGGATCC GAAGAAGCGACCAA-AACCTGGCGG-3′; SPrP102, 5′-TTGAAT **TC** AACTTGCCCCCCTTTGGTAATAAG-3′; SPrP103, 5′-AATGGCCA AGAAGCGACCAAAACCTGGCGG-3′; BPrP101, 5′-AAGGATCC GAAGAAGCGAC-CAAAACCTGGAGG-3′; BPrP103, 5′-TTGAAT **TC**A ACTTGCCCCTCGTTGGTAATAAG-3′; HPrP1, 5′-CAC-GAT*GC*TGTCAACATCACCATCAAG-3′; HPrP2, 5′-CACAAAGTTGTTCTGGTTGTTGTACTG-3′; HPrP3, 5′-AGATG*GC*TACCACCCAGTATCAGAAGG-3′; HPrP4, 5′-GCTCCACCACGCGCTCCATTATCTTG-3′(underlines indicate restriction sites used for cloning, bold indicates stop codons, and italics indicate nucleotide substitutions for the mutation of Cys to Ala).

Expression and purification of recombinant PrP (rPrP)

The expression plasmids based on pRSETB and pET22b(+) were introduced into *E. coli* BL21(DE3)*LysS* and JM109(DE3), respectively. Protein expression was induced by adding isopropylthio-β-D-galactoside to a final concentration at 0.4 mM. Two to four hours after induction, bacterial cells were collected and inclusion bodies were prepared as described elsewhere (Sambrook et al., 1989). The inclusion bodies from BL21(DE3)*LysS* transformed with pRSETB-based expression plasmids were solubilized with 6 M GdnHCl in 20 mM phosphate buffer (pH 7.8). The rPrP was further purified by Ni^{2+}-immobilized metal affinity chromatography (IMAC) using Ni^{2+}-charged Chelating Sepharose Fast flow (Amersham Pharmacia) and a

stepwise elution gradient from pH 4.9 to 4.3 in the presence of 6 M GdnHCl. Inclusion bodies from JM109(DE3) transformed with pET22b(+)-based expression plasmids were solubilized with 8 M Urea and 20 mM Tris–HCl, pH 8.0. Next, the urea concentration was reduced to 6 M, and the mixture was applied to DEAE-Sepharose equilibrated with 6 M Urea and 20 mM Tris–HCl, pH 8.0. The unbound fraction was saved for further purification. The rPrP in the eluate from IMAC and the unbound fraction from DEAE-Sepharose were dialyzed against 10 mM acetate buffer (ranging from pH 4.4 to 3.6). After the dialysis, rPrP containing an intramolecular disulfide bond was purified by reverse-phase HPLC using TSKgel Phenyl-5PW RP (TOSOH) and a 30–50% linear gradient of acetonitrile with 0.05% trifluoroacetic acid. The purified rPrP was lyophilized and dissolved with Mili-Q water at 1 mg/ml and stored at −20 °C.

Purification of PrP^{Sc} and formation of detergent-lipid-protein complexes (DLPC)

A mouse-adapted scrapie Obihiro strain (Shinagawa et al., 1985) was used in this study. ICR/Slc female mice were inoculated intracerebrally with 20 μl of brain homogenate of Obihiro strain infected-mice and were sacrificed under anesthesia when they showed the clinical symptoms of the terminal stage of the disease. PrP^{Sc} was purified from the scrapie-affected mice brains without proteinase K treatment as described by Bolton et al. (1987) with minor modifications (Caughey et al., 1991). Protein concentration was determined by DC protein assay kit (Bio-Rad).

Ten micrograms of purified PrP^{Sc} was suspended in 1.6 ml of DLPC buffer containing 2% Sarkosyl, 0.4% phosphatidylcholine, 150 mM NaCl, and 50 mM Tris–HCl, pH 8.3. The suspension was sonicated for five cycles of 2 s with a Branson Sonifier Contamination-free Ultrasonic Sample Pre-processing System.

Production of monoclonal antibodies

Purified PrP^{Sc}, rMoPrP23–231, or rMoPrP89–231 was mixed with an equal volume of Freund's complete adjuvant and 200 μg of each PrP was inoculated subcutaneously into PrP gene-ablated mice (Yokoyama et al., 2001). After the first immunization, the mice received 100 μg of the same PrP preparation with Freund's incomplete adjuvant twice every 2 weeks. The booster shot was given intraperitoneally with 50 μg of each PrP preparation in PBS. Three days after the booster, splenocytes obtained from immunized mice were fused with P3U1 mouse myeloma cells using polyethylene glycol 1500 (Roche Diagnostic) according to the supplier's instruction, and hybridomas were selected in HAT medium. Hybridoma culture supernatants were screened by ELISA using purified PrP^{Sc} and rMoPrP as described below. The hybridomas secreting mAbs were cloned by limiting dilution. The isotypes of the mAbs were determined using the IsoStrip mouse monoclonal antibody isotyping kit (Roche Diagnostic). Large-scale preparations of mAbs were carried out in INTEGRA CELLine high density culture units (IBS Integra Biosciences). The supernatants harvested from the units were concentrated by precipitation with 50% saturated ammonium sulfate and then purified by size exclusion chromatography with Superdex-200 HR (Amersham Pharmacia Biotech).

ELISA

Ninety-six well plates (MaxiSorp, Nunc) were coated overnight at 4 °C with either 200 ng/well of purified PrP^{Sc} or 100 ng/well of rMoPrP in 50 μl of 20 mM phosphate buffer, pH 7.0. After adsorption, wells were blocked with 5% fetal bovine serum (FBS) in PBS containing 0.1% Tween 20 (PBST) for 2 h at room temperature (r.t.), and then incubated with culture supernatants or antibodies diluted with 1% FBS in PBST for 1 h. After washing with PBST, wells were incubated with 100 μl of 1:2500 diluted HRP-conjugated $F(ab')_2$ fragment anti-mouse Ig (Amersham Bioscience) for 1 h. Finally, antigen–antibody complexes were detected by adding a substrate solution of 100 μg/ml of 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid), 0.04% H_2O_2 in 50 mM citrate-phosphate buffer, pH 4.0, and the absorbance at 405 nm was measured with a microplate reader (Multiscan MS-UV, Labsystems). A ready to use 3,3′,5,5′-tetramethylbenzidine (TMB) was also used as a substrate, and the absorbance at 450 nm was measured for TMB.

Immunoblotting

The preparation of PrP^{Sc} and immunoblotting were carried out as described elsewhere (Grathwohl et al., 1997). The blots were developed with ECL Western blotting detection reagents (Amersham Pharmacia) and immunoreactive proteins were detected with X-ray film.

Pepspots analysis

In these studies, we used pepspots membrane to which an array of 99 overlapping synthetic peptides, corresponding to residues 23–231 of mouse PrP, was covalently attached to a cellulose support via carboxyl termini. Each peptide is 13 amino acid residues long, and there is a two amino acid shift along the mouse PrP amino acid sequence from one peptide to the next. The membrane was blocked with 5% skim milk and 5% sucrose in PBST, and then incubated with culture supernatants of hybridomas as primary antibodies. Bound antibodies were detected using a 1:2500 diluted HRP-conjugated $F(ab')_2$ fragment anti-mouse Ig and an ECL Western blotting detection reagent.

Acknowledgments

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NOTE *Clinical Pathology*

Unique Amino Acid Polymorphisms of PrP Genes in Mongolian Sheep Breeds

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ABSTRACT. To characterize amino acid polymorphisms of sheep prion protein (PrP) gene, DNA from 740 sheep of nine breeds raised in Mongolia was isolated and analyzed. A total of 16 genotypes and seven allelic variants of the PrP gene at codons 112, 136, 154, and 171 were found. The MARQ/MARQ genotype associated with susceptibility to scrapie was found in 82.6% of the sheep while the MARR/MARR genotype associated with resistance to scrapie was found in 1.8% of the sheep. The polymorphisms of valine and serine at codon 127, and leucine and arginine at codon 189 were detected in eight Mongolian sheep breeds, suggesting that these polymorphisms are a common feature among Mongolian sheep breeds.

KEY WORDS: PrP genotype, scrapie susceptibility.

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Scrapie in sheep and goats is a fatal and infectious neurodegenerative disease that has been categorized as a transmissible spongiform encephalopathy (TSE) or prion disease also found in humans and other animals. Prion diseases are characterized by the accumulation in the tissues of the central nervous system of an "infectious" abnormal protease-resistant isoform (PrP^{Sc}) of cellular prion proteins (PrP^{C}) encoded by the PrP gene [12]. Polymorphisms of the PrP gene have been linked to host susceptibility and the incubation period of the disease [11]. PrP allelic variant valine/arginine/glutamine (VRQ) at codons 136, 154, and 171 is associated with high susceptibility to scrapie for sheep breeds. While the allele VRQ is rare in Suffolk sheep, the wild-type PrP allele alanine/arginine/glutamine (ARQ) is associated with susceptibility to scrapie. It has been widely considered that the PrP allelic variant alanine/arginine/arginine (ARR) at codons 136, 154, and 171 is associated with resistance to scrapie in several breeds [1–5, 7–10, 13]. Links between DNA polymorpisms and scrapie susceptibility have been identified in outbreaks of scrapie in various breeds or flocks in different countries where scrapie has been diagnosed [1, 7–8, 10, 13]. However, in central Asian countries where many sheep are raised, the polymorpism of PrP genes associated with scrapie have not yet been characterized. Therefore, we examined the PrP genotypes of 740 Mongolian sheep, including 271 sheep previously reported [6].

DNA samples were collected from several breeds from different prefectures in Mongolia. A total of 271 sheep came from the central region of Mongolia: 112 Khalkh sheep (native breed) from Tuv prefecture (designated I in Fig. 1.); 60 Khalkh sheep from Uvurkhangai prefecture (J); and 35 Yeroo sheep, 35 Orkhon sheep, and 29 Khangai sheep from Selenge prefecture (K) [6]. From the western region, a total of 345 sheep were used: 70 Khalkh sheep and 36 Sartuul sheep from Zavhan prefecture (A); 35 Khalkh sheep and 35 Govi-altai sheep from Govi-Altai prefecture (B); 71 Khalkh sheep and 33 Bayad sheep from Uvs prefecture (C); and 34 Khalkh sheep and 31 Darhad sheep from Huvsgul prefecture (D). For the eastern region, a total of 124 sheep were used: 32 Sumber Karakul sheep from Govi-Sumber prefecture (H); 32 Khalkh sheep from Dornogovi prefecture (E); 31 Khalkh sheep from Suhbaatar prefecture (F); and 29 Khalkh sheep from Hentii prefecture (G). The Khalkh sheep comprised about 90% of 13.8 million Mongolian sheep. Crossbreeding between the Khalkh sheep and local sheep including imported sheep started in the 1930s, and the crossbreeding has led to develop several local crossbreeds in Mongolia [6].

The entire 794-bp open reading frame (ORF) of the PrP gene was amplified by polymerase chain reaction (PCR) in 50 μl reactions, using PrP primers (SPrP-1, SPrP-2, SPrP-3, and SPrP-5) as described by Gombojav *et al.* [6]. To confirm amplification, a portion of each reaction product was electrophoresed on a 0.7% agarose gel containing ethidium bromide (0.5 $\mu g/ml$), and visualized under ultraviolet radiation. Then, the primers were removed using a Centricon 100 micro-concentrator (Amicon, Bedford, MA), and 1 to 5 μl of the concentrated PCR product was used for direct sequencing [6].

In this study, the relative genotype frequencies of four codons (112, 136, 154, and 171) of the PrP gene are newly reported in five sheep breeds (Sartuul, Govi-altai, Bayad, Darhad, and Sumber Karakul), in addition to four previously reported sheep breeds (Khalkh, Yeroo, Orkhon, and Khangai) [6]. Table 1 shows the genotype frequencies of 16 different PrP genotypes in the 740 sheep examined. The PrP genotypes MARQ/MARQ was found in all nine breeds.

Among all the breeds studied, the Khalkh sheep showed the greatest variation with 11 PrP genotypes composed from 6 alleles, while three to nine PrP genotypes were found in

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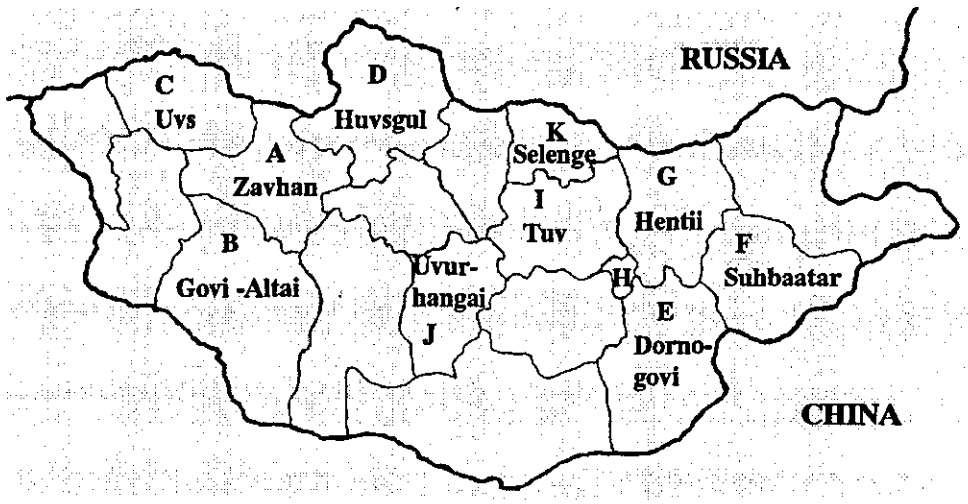

Fig. 1. Map of Mongolia. The blood samples were collected from different prefectures in Mongolia (A-K). H, Govi-Sumber prefecture.

Table 1. Frequency of PrP genotypes at codons 112, 136, 154 and 171 in Mongolian sheep breeds

Location[a]	A-G, I-J		A		B		C		D		H		K		K		K	
Breeds	Khalkh[b]		Sartuul		Govi-altai		Bayad		Darhad		Sumber		Yeroo[c]		Orkhon[c]		Khangai[c]	
PrP genotype	No	%	No	%	No	%	No	%	No	%	Karakul No	%	No	%	No	%	No	%
MARQ/MARQ	317	66.8	29	80.6	27	77.1	29	87.9	21	67.7	27	84.3	17	48.5	13	37.0	15	51.7
MARQ/TARQ	68	14.4	3	8.3	2	5.7	3	9.1	2	6.5			4	11.4	7	20.0	2	6.9
MARQ/MARH	31	6.6	2	5.5	2	5.7	1	3.0	5	16.2	3	9.4			1	2.9		
MARQ/MARR	13	2.7			2	5.7							8	22.8	8	22.8	9	31.0
MARH/MARH	7	1.5	1	2.8					1	3.2	2	6.3			1	2.9		
TARQ/TARQ	12	2.5							1	3.2			1	2.9				
TARQ/MARH	8	1.7													1	2.9		
TARQ/MARR	4	0.8			1	2.9									1	2.9		
MARQ/MAHQ	6	1.3																
MARR/MARR	7	1.5			1	2.9			1	3.2			2	5.7			2	6.9
MARK/MARK	1	0.2	1	2.8														
MARQ/MVRQ													1	2.9	2	5.7		
MARR/MVRQ													1	2.9				
MARH/MAHQ													1	2.9				
MARR/MAHQ															1	2.9		
TARQ/MAHQ																	1	3.5
Total	474		36		35		33		31		32		35		35		29	

a) Locations are shown in Fig. 1.
b) A total of 474 samples include 172 samples previously reported by Gombojav *et al.* [6].
c) Data from Gombojav *et al.* [6].

the other sheep breeds. The great variations in PrP genotypes suggest that Khalkh sheep originated from a mixture of several breeds from surrounding countries, while lesser variations in the other sheep breeds suggest that these breeds originated from a mixture of fewer numbers of breeds within Mongolia.

The allelic variant VRQ at codons 136, 154, and 171 is rare in Suffolk sheep [4, 9, 13] and this tendency was also found in the Mongolian sheep breeds. Valine at codon 136 (136V) associated with high susceptibility to scrapie was detected from two sheep breeds, Yeroo and Orkhon, but not in the other sheep breeds of Mongolia. Yeroo and Orkhon sheep have been raised in Selenge prefecture, which is in close proximity to Russia. The sheep raised in the prefecture had been genetically developed by crossbreeding with Russian sheep. Therefore, it is thought that the polymorphism of valine at codon 136 may have been introduced through the Russian sheep breeds, although little information about the PrP polymorphism of Russian sheep.

We found valine (V; nucleotides GTC) and serine (S;

Table 2. Frequency of PrP genotypes at codons 127 and 189 in Mongolian sheep breeds

Location[a]	A-G, I-J		A		B		C		D		H		K		K		K	
Breeds	Khalkh[b]		Sartuul		Govi-altai		Bayad		Darhad		Sumber Karakul		Yeroo[c]		Orkhon[c]		Khangai[c]	
PrP genotype	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
GQ/GQ[d]	354	74.6	26	72.2	17	48.6	22	66.8	20	64.5	23	71.9	28	80.0	32	91.4	29	100
GQ/SQ	68	14.4	5	13.9	4	11.4	6	18.2	8	25.8	5	15.6	7	20.0	1	2.9		
SQ/SQ	5	1.1									1	3.1						
GQ/VQ	19	4.0					1	3.0			2	6.3						
GQ/GL	20	4.2	4	11.1	11	31.4	2	6.0	3	9.7	1	3.1			2	5.7		
GL/GL	1	0.2																
GQ/GR	2	0.4			1	2.9												
GL/SQ	5	1.1	1	2.8	2	5.7	2	6.0										
Total	474		36		35		33		31		32		35		35		29	

a) Locations are shown in Fig. 1.
b) A total of 474 samples include 172 samples previously reported by Gombojav *et al.* [6]
c) Data from Gombojav *et al.* [6].
d)Wild type of sheep PrP gene.

AGC) at codon 127, and leucine (L; CTA) and arginine (R; CGA) at codon 189 in eight Mongolian sheep breeds (Table 2). These amino acid polymorphisms of PrP gene were widely observed in Mongolian sheep breeds but have not been reported in other sheep from European countries, suggesting that these are unique to indigenous sheep breeds including Mongolian sheep. However, it remains to be determined whether these polymorphisms have any correlation with susceptibility to scrapie.

Among the different sheep breeds raised in Mongolia, 66.9% had the MARQ/MARQ genotype and 1.8% had the MARR/MARR genotype, which are linked to susceptibility and resistance to scrapie, respectively (Table 1). In the previous study [6], we examined the scrapie form of the prion protein (PrP^{Sc}) in brain tissues from 10 sheep with neurological symptoms, but no PrP^{Sc} was obtained by Western blot analysis. Therefore, although there have been no reports of scrapie in Mongolia, these results suggest that the majority of Mongolian sheep are genetically susceptible to scrapie. However, since PrP genes linked to scrapie resistance were observed in five of the nine breeds, individual sheep carrying the scrapie-resistant genes can be identified and used in breeding programs to develop scrapie-resistant populations.

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Propagation of a protease-resistant form of prion protein in long-term cultured human glioblastoma cell line T98G

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Human prion diseases, such as Creutzfeldt–Jakob disease (CJD), a lethal, neurodegenerative condition, occur in sporadic, genetic and transmitted forms. CJD is associated with the conversion of normal cellular prion protein (PrP^{C}) into a protease-resistant isoform (PrP^{res}). The mechanism of the conversion has not been studied in human cell cultures, due to the lack of a model system. In this study, such a system has been developed by culturing cell lines. Human glioblastoma cell line T98G had no coding-region mutations of the prion protein gene, which was of the 129 M/V genotype, and expressed endogenous PrP^{C} constitutively. T98G cells produced a form of proteinase K (PK)-resistant prion protein fragment following long-term culture and high passage number; its deglycosylated form was approximately 18 kDa. The PK-treated PrP^{res} was detected by immunoblotting with the mAb 6H4, which recognizes residues 144–152, and a polyclonal anti-C-terminal antibody, but not by the mAb 3F4, which recognizes residues 109–112, or the anti-N-terminal mAb HUC2-13. These results suggest that PrP^{C} was converted into a proteinase-resistant form of PrP^{res} in T98G cells.

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INTRODUCTION

Fatal human prion diseases, including sporadic Creutzfeldt–Jakob disease (CJD), inherited prion diseases, iatrogenic CJD, kuru and variant CJD, are transmissible spongiform encephalopathies that are characterized by the formation and accumulation of an abnormal isoform of prion protein (PrP) in the brain (Prusiner, 2001). The PrP^{res} isoform is an insoluble aggregate that is resistant to proteinase K (PK) digestion. The conversion from cellular prion protein (PrP^{C}) into PrP^{res} could be a potential therapeutic target for prion diseases, but the mechanism of the conversion is unclear.

Several animal cell lines, including mouse neuroblastoma cells (Butler *et al.*, 1988; Race *et al.*, 1987), mouse hypothalamic neuronal cells (Nishida *et al.*, 2000; Schätzl *et al.*, 1997), mouse Schwann cells (Follet *et al.*, 2002) and rat pheochromocytoma cells (Rubenstein *et al.*, 1984), have been infected successfully with scrapie agents, and a human neuroblastoma cell line can also be infected with CJD agents (Ladogana *et al.*, 1995). These cells have been used to study the conversion mechanisms (Lehmann & Harris, 1997) and the subcellular localization (Naslavsky *et al.*, 1997; Vey *et al.*, 1996) of PrP^{res} and to evaluate therapeutic agents (Caughey & Raymond, 1993; Doh-Ura *et al.*, 2000). However, the efficiencies of infection and propagation of PrP^{res} are relatively low. The mouse cell line SMB was established from a scrapie-infected mouse brain (Clarke & Haig, 1970) and has been used to study the properties of PrP (Birkett *et al.*, 2001). Recently, stable cell lines were established from mouse peripheral neuroglial cells expressing ovine PrP and simian virus 40 T antigen. These cells were readily infectible by sheep PrP^{Sc}, a scrapie isoform of PrP (Archer *et al.*, 2004). However, there are currently no human cell lines that have been used to study the conversion mechanism from PrP^{C} into PrP^{res}.

PrP mRNA is expressed not only in neurons, but also in glia (Moser *et al.*, 1995) and PrP^{Sc} accumulates in the cytosol and cell-surface membrane of glial cells (van Keulen *et al.*, 1995). The role of glial cells in prion disease is not clear. Human glioblastoma T98G cells, like normal cells, become arrested in G_1 phase under stationary-phase conditions (Stein, 1979). In a previous study, we showed that T98G cells express PrP^{C} mRNA constitutively and produce a high level of endogenous PrP^{C} in G_1 phase (Kikuchi *et al.*, 2002). In the present study, we have investigated whether PrP^{C} is

converted into PrP^{res}, a marker for prion diseases, in cultured T98G cells under various conditions.

METHODS

Materials. A primer set for the human PrP coding sequence (CDS) (GenBank accession no. AL133396) [5′-CGAGGCAGAGCAGTCATT-3′, starting 18 nt before the ORF, and 5′-AGATGGTGAAAACGAGAAGAC-3′, ending 6 nt after the ORF (expected product size, 806 bp)] and an internal primer set (5′-GGCAGTGACTATGAGGACCGTTAC-3′ and 5′-GTAACGGTCCTCATAGTCACTGCC-3′, corresponding to nt 424–447 relative to the start site of the ORF) were synthesized chemically. Peptide *N*-glycosidase F (PNGase F) and *Bsa*AI were purchased from New England Biolabs and RPMI 1640 medium was purchased from Nissui Pharmaceutical. A BCA protein assay kit and SuperSignal West Femto Maximum Sensitivity substrate were from Pierce Biotechnology. Hybond-P PVDF membranes were purchased from Amersham Biosciences. Anti-human PrP mAb 3F4 was purchased from Signet Laboratories and 6H4 from Prionics AG. Fetal calf serum (FCS), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated rabbit anti-chicken IgG, aprotinin, leupeptin, PMSF, 4-methylumbelliferyl-β-D-galactoside (4-MUG) and mouse IgG were purchased from Sigma. PK was purchased from Merck and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) from Roche Diagnostics. SuperScript II reverse transcriptase and random primers were purchased from Invitrogen. β-Galactosidase-conjugated goat anti-mouse IgG was purchased from American Qualex, DNase I from Takara, KOD-Plus-DNA polymerase from Toyobo and 1,4-diazabicyclo[2.2.2]octane (DABCO) from Nacalai Tesque.

Preparation of antibodies. The preparation of chicken mAb HUC2-13 (IgG) against human PrP peptide residues 25–49 was reported previously (Matsuda *et al.*, 1999). The preparation of rabbit polyclonal antibody HPC2 (IgG) against human PrP peptide residues 214–230 was also reported previously (Kikuchi *et al.*, 2002).

Cell culture. Human glioblastoma cell line T98G (JCRB9041) at nominal passage level 433 was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). Human astrocytoma U373MG cells were kindly provided by Dr T. Kasahara (Kyoritsu College of Pharmacy, Tokyo, Japan). Cell cultures stored in liquid nitrogen were thawed as passage 0 (P0) and cultured at 37 °C in monolayers on a T75 plastic tissue-culture flask in RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated FCS, 60 μg kanamycin ml^{-1} and 10 mM HEPES/NaOH, pH 7·2. All cell lines were subcultivated routinely at a 1:5 or 1:10 split ratio once a week.

PCR direct sequencing and RFLP analysis. Extraction of total RNA from the cells and RT-PCR analysis were performed according to a published method (Kikuchi *et al.*, 2002) with slight modifications. Briefly, 5 μg total RNA was treated with DNase I for 15 min at room temperature. Random primers and SuperScript II reverse transcriptase were added to 20 μl (2·5 μg total RNA) and the mixture was incubated at 42 °C for 60 min to synthesize cDNA. Subsequently, 10 μl cDNA solution was subjected to PCR in a total volume of 50 μl, which included 0·2 mM dNTPs, 1 mM $MgSO_4$, 1 U KOD-Plus-DNA polymerase and 50 pmol sense and antisense primers. The amplification programme was as follows: denaturation at 94 °C for 20 s, annealing at 60 °C for 30 s and elongation at 68 °C for 60 s for 40 cycles. Final elongation was performed at 68 °C for 1 min. PCR was carried out in a GeneAmp PCR system 2400 (Applied Biosystems). PCR direct sequencing was performed with a CEQ 2000XL DNA Analysis system (Beckman Coulter) using the primer set for human PrP CDS and an internal primer. Codon 129 polymorphisms were detected by RFLP analysis; the PCR product (200 ng DNA) was digested with 5 U *Bsa*AI for 60 min at 37 °C; after incubation for 20 min at 80 °C, restriction fragments were separated by electrophoresis in 2 % agarose gels and visualized following ethidium bromide staining.

Preparation of whole-cell lysates. All cell lines were plated at $5·0 \times 10^5$ cells per 9 cm dish (55 cm^2) in 10 ml medium on day 0 (D0). The medium was changed every 4 days. At the indicated times, cells were washed twice with ice-cold PBS and scraped into lysis buffer [$1·8 \times 10^4$ cells μl^{-1}; 10 mM Tris/HCl (pH 7·5), 150 mM NaCl, 1 % sodium deoxycholate, 0·1 % SDS, 1 % NP-40, 10 mM NaF, 1 mM EDTA, 0·5 mM Na_3VO_3, 10 mM tetrasodium pyrophosphate] with protease inhibitor cocktail [0·06 trypsin inhibitor units (TIU) aprotinin ml^{-1}, 20 μM leupeptin and 1 mM PMSF]. After sonication, insoluble material was pelleted by centrifugation at 500 *g* for 15 min at 4 °C to yield whole-cell lysates. Protein concentration was determined by the BCA protein assay.

Subcellular fractionation. At the indicated times, cells were washed twice with ice-cold PBS and scraped into PBS/2·5 mM EDTA with the protease inhibitor cocktail. After sonication, insoluble material was pelleted by centrifugation at 500 *g* for 15 min at 4 °C to yield homogenates. The postnuclear fraction was centrifuged at 100 000 *g* for 60 min at 4 °C to obtain a cytosolic fraction and a membrane fraction. The membrane fraction was dissolved in PBS/2·5 mM EDTA with the protease inhibitor cocktail. Protein concentration was determined by the BCA protein assay.

Detergent solubility test. A detergent solubility test was carried out according to a described method (Capellari *et al.*, 2000) with slight modifications. Cells were washed twice with ice-cold PBS and scraped into PBS/2·5 mM EDTA with the protease inhibitor cocktail. After sonication, insoluble material was pelleted by centrifugation at 500 *g* for 15 min at 4 °C to yield homogenates. The postnuclear fraction was dissolved in 9 vols 0·5 % NP-40/0·5 % deoxycholate/PBS with the protease inhibitor cocktail and centrifuged at 100 000 *g* for 60 min at 4 °C to obtain a detergent-insoluble pellet fraction and a soluble supernatant fraction. The supernatant fraction was precipitated with 4 vols methanol for 16 h at −20 °C. Both fractions were resuspended in the same volume of lysis buffer.

Protease-resistant PrP assay. To generate material for the protease-resistant PrP assay, aliquots of the sample (50 μg protein) were precipitated with 4 vols methanol for 16 h at −20 °C to remove the protease inhibitor cocktail (Capellari *et al.*, 2000), centrifuged at 14 000 *g* for 15 min at 4 °C and the pellet was dissolved in 50 mM Tris/HCl (pH 7·2). Samples were treated with PK (at 10 μg ml^{-1} unless stated otherwise) at 37 °C for 30 min, according to a described method (Caughey *et al.*, 1999). After incubation, digestion was stopped by the addition of AEBSF to 4 mM. Samples were prepared with the protease inhibitor cocktail at a concentration that did not inhibit the activity of PK (Fig. 1a, lane 1).

Enzymic deglycosylation. For the removal of Asn-linked oligosaccharides, aliquots of whole-cell lysates were treated with PNGase F as follows (Kikuchi *et al.*, 2002): lysates (50 μg protein) were denatured by boiling for 10 min in 0·5 % SDS, 1 % 2-mercaptoethanol. After addition of NP-40 to 1 %, the lysates were incubated at 37 °C for 2 h with 0·77 IUB mU PNGase F in 50 mM phosphate buffer (pH 7·5).

Immunoblotting. Usually, 50 μg total protein (prepared from approximately $1·7 \times 10^5$ cells) was subjected to SDS gel electrophoresis. Briefly, aliquots of the samples were mixed with 2× electrophoresis sample buffer. After boiling for 10 min, the samples

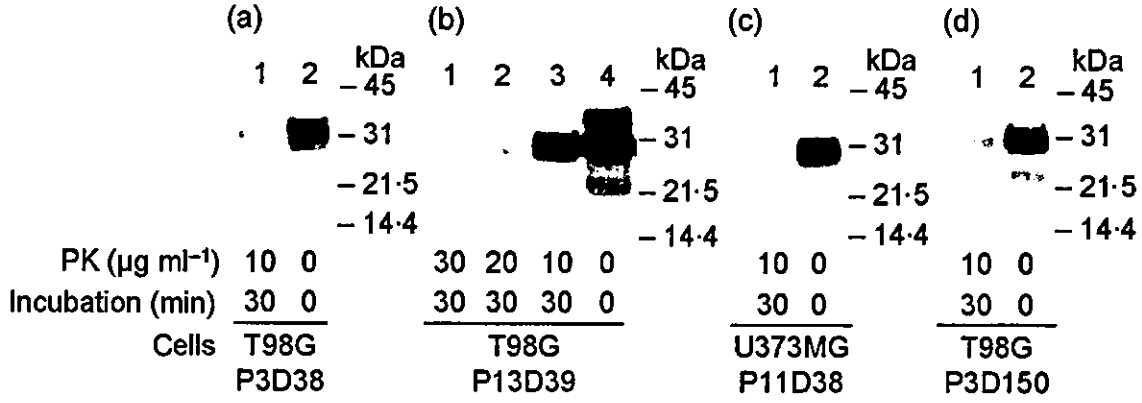

Fig. 1. Formation of a protease-resistant form of PrP in T98G cells is increased in a long-term incubation after repeated passages. T98G cells and U373MG cells were incubated under the following conditions with 10% FCS/RPMI 1640 and whole-cell, methanol-precipitated lysates (50 μg protein) were treated with PK (10 μg ml^{-1} unless stated otherwise) at for 30 min at 37 °C. (a) T98G cells were incubated for 38 days after 3 passages (P3D38); lysates were treated with PK (lane 1) or left undigested (lane 2). (b) T98G cells were incubated for 39 days after 13 passages (P13D39); lysates were treated with 10, 20 or 30 μg PK ml^{-1} (lanes 1–3) or left undigested (lane 4). (c) U373MG cells were incubated for 38 days after 11 passages (P11D38); lysates were treated with PK (lane 1) or left undigested (lane 2). (d) T98G cells were incubated for 150 days after 3 passages; lysates were treated with PK (lane 1) or left undigested (lane 2). PK-treated lysates were subjected to immunoblot with the 6H4 antibody as described in Methods.

were electrophoresed on 12·5% acrylamide gel and the proteins were transferred onto PVDF membranes. The membranes were blocked with 0·5% casein in PBS (casein/PBS) and incubated with anti-prion antibodies in casein/PBS. Immunoreactive bands were visualized with HRP-conjugated anti-IgG and SuperSignal West Femto Maximum Sensitivity substrate, according to the manufacturer's instructions (Pierce Biotechnology).

Indirect immunofluorescence staining. T98G cell monolayers grown on a 15 mm glass coverslip (Matsunami) in a 9 cm dish (55 cm^2) were maintained in 10 ml medium. At the indicated times, cells were washed twice with ice-cold PBS and then fixed with 3·7% formaldehyde in PBS for 30 min at 4 °C. The fixed cells were washed twice with PBS and then treated with 0·2% Triton X-100 in PBS for 15 min at room temperature. The cells were blocked with 10% normal goat serum in PBS (NGS/PBS) for 60 min and incubated with antibody (100 ng ml^{-1}) for 16 h at 4 °C. After extensive washing with 0·05% Tween 20/PBS, cells were treated with Alexa 594 goat anti-mouse IgG (H+L) conjugate (5 μg ml^{-1}) (Molecular Probes) in NGS/PBS for 1 h at 4 °C, washed with 0·05% Tween 20/PBS and mounted with 2·5% DABCO/90% glycerin/PBS. The stained cells were observed and photographed with the aid of a fluorescence microscope (Olympus).

Competitive ELISA. ELISA was carried out according to a method described previously (Kikuchi *et al.*, 1991). For a dilution buffer, casein/PBS was used throughout the present study. Briefly, the wells were coated with 100 ng recombinant bovine PrP (rBoPrP) (Takekida *et al.*, 2002) in PBS and left at 4 °C overnight. Appropriately diluted standard rBoPrP solutions or samples were added to the antigen-coated wells and incubated at room temperature for 60 min, in a total volume of 50 μl, with 6H4 antibody (460 pg). The wells were washed, incubated with β-galactosidase-conjugated goat anti-mouse IgG for 60 min, washed again and then incubated with 4-MUG as a substrate at 37 °C for 60 min. Enzyme activity was determined by fluorescence intensity measurements.

RESULTS

Production of protease-resistant isoform of PrP in T98G cells

We analysed whole-cell lysates of long-term cultured T98G cells by immunoblotting with anti-PrP antibodies. When we cultured the cells for 38 days after 3 passages [passage 3, day 38 (P3D38)], the lysates revealed two bands (35 and 31 kDa) that reacted with mouse anti-human PrP mAb 6H4 (Fig. 1a, lane 2) and were destroyed completely after digestion with PK (Fig. 1a, lane 1). When lysates from cells that were cultured for 39 days after 13 passages [passage 13, day 39 (P13D39)] were digested with PK (10, 20 or 30 μg ml^{-1}), the 35 kDa band, but not the 31 kDa band, was diminished (Fig. 1b), indicating the presence of PrP^{res}. We then attempted to detect PrP^{res} formation in long-term cultures of another human glial cell line, U373MG, an astrocytoma line that expresses consistently high levels of PrP^{C} mRNA (Satoh *et al.*, 1998). The lysates from P11D38 U373MG cells exhibited the 31 kDa band that reacted with the 6H4 antibody and disappeared after digestion with PK (Fig. 1c). Lysates from P3D150 T98G cells showed a faint 31 kDa band after PK treatment (Fig. 1d). In contrast, P13D39 T98G cells had produced highly PK-resistant PrP. These data indicated that PrP^{res} propagation in T98G cells required not only long-term culture, but also a high passage number.

Examination of phenotypic variants of PrP^{res}

We first asked whether an inherited or a sporadic CJD-like form of PrP^{res} was propagated in T98G cells. Inherited prion

diseases are determined by mutations in the 762 bp CDS of the prion protein gene (*PRNP*) (Kovács *et al.*, 2002). We performed PCR direct sequencing of the *PRNP* mRNA that was expressed in short- and long-term cultured T98G cells and found no mutations other than the presence of both adenine and guanine at the first position of codon 129 (the basis of the common M129V polymorphism) (data not shown). When digested by *Bsa*AI, the 806 bp PCR product from the M129V haplotype (Fig. 2a, lane 1) yielded products of 402 and 404 bp and also undigested wild-type product (Fig. 2a, lane 2), which we confirmed by RFLP analysis. These results indicated that T98G cells were heterozygotes, having both methionine and valine at codon 129 of *PRNP* with no coding-region mutation.

Next, to estimate the size of the deglycosylated PrP^{res}, we treated the lysates from P40D40 T98G cells with PK and/or PNGase F. PNGase F yields a full-length (25 kDa) and an N-terminally truncated (18 kDa) form of PrP^{C} (Kikuchi *et al.*, 2002). As shown in Fig. 2b, PNGase F treatment reduced the glycosylated 35 and 31 kDa bands (lane 4) to 25 and 18 kDa (lane 3), representing the deglycosylated full-length and N-terminally truncated forms. An additional PNGase F treatment changed fully glycosylated (31 kDa) and partially glycosylated (23 kDa) forms of PrP^{res}, detectable after digestion with PK (lane 2), to an unglycosylated form of 18 kDa (lane 1). These results established that the size of the deglycosylated PK-resistant fragment in T98G cells was approximately 18 kDa.

Confirming heterogeneity of PrP^{res} by immunoblotting with sets of anti-PrP antibodies

To further investigate the heterogeneity of PrP^{res} from long-term cultured T98G cells, we determined the antigenicity of PrP^{res}. By immunoblotting with sets of antibodies to PrP (Kikuchi *et al.*, 2002), we detected a full-length PrP (35 kDa) in lysates from P40D40 T98G cells that reacted with the anti-N terminus PrP antibody HUC2-13 (Fig. 3a, lane 2), as well as with the 6H4 antibody (Fig. 3c, lane 2). Following PK treatment of the lysates, the 31 kDa band was still detected by 6H4 antibody (Fig. 3c, lane 1), but not by HUC2-13 antibody (Fig. 3a, lane 1), indicating that PK treatment had cleaved the N terminus of PrP^{res}. The 31 kDa band was also detected by the anti-C terminus PrP antibody HPC2 (Fig. 3d, lane 1). HPC2 antibody, which reacts strongly with the deglycosylated form of PrP^{C}, but weakly with the glycosylated form (Kikuchi *et al.*, 2002), also recognized the N-terminally truncated form of PrP^{res}. Surprisingly, the 3F4 antibody, which recognizes residues 109–112, failed to detect the N-terminally truncated form of PrP^{res} (Fig. 3b), such as is seen with the HUC2-13 antibody (Fig. 3a). These experiments showed that the N-terminally truncated form of PrP^{res} in T98G cells lacks the epitope that is recognized by the 3F4 antibody.

Subcellular localization and detergent solubility of PrP^{res} in T98G cells

To determine the subcellular localization of PrP^{res}, we studied the distribution of PrP in P40D40 T98G cells

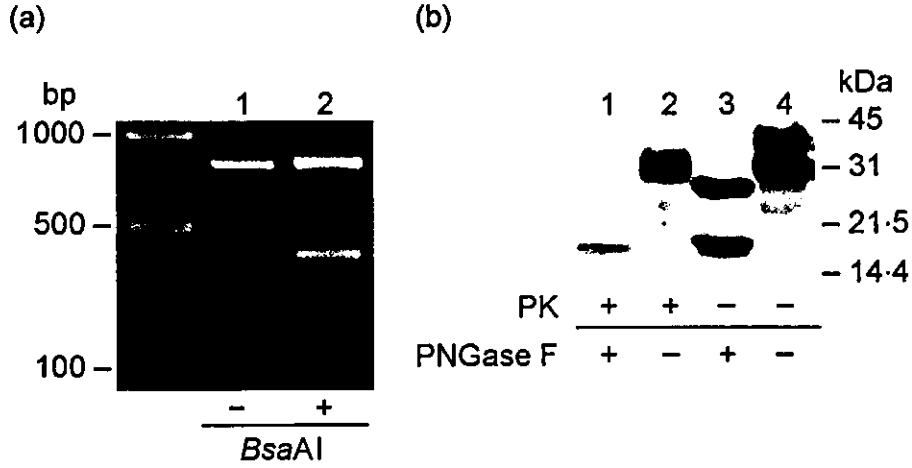

Fig. 2. Molecular analysis of PrP^{res} in T98G cells. (a) Detection of polymorphism at codon 129 on PrP mRNA in T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 for 5 days after 43 passages (P43D5) and total RNA was prepared, reverse-transcribed and PCR-amplified as described in Methods and digested with *Bsa*AI (lane 2) or left undigested (lane 1). A DNA size marker (100 bp ladder) is shown on the left. (b) Analysis of deglycosylated forms of PrP in T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 for 40 days after 40 passages (P40D40); whole-cell, methanol-precipitated lysates were treated with PK (lanes 1 and 2) or left undigested (lanes 3 and 4). All lysates were incubated with (lanes 1 and 3) or without (lanes 2 and 4) PNGase F for 120 min. PK-treated lysates were subjected to immunoblot with the 6H4 antibody as described in Methods.

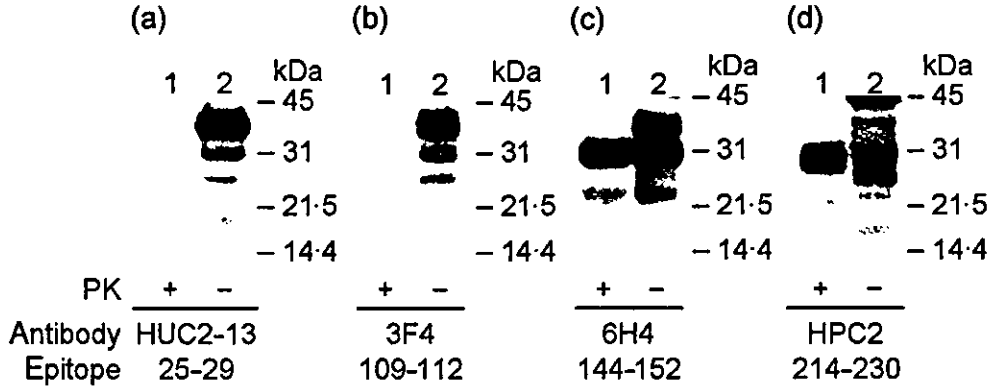

Fig. 3. Immunoblot analysis using anti-PrP antibodies for the protease-resistant form of PrP in T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 for 40 days after 40 passages (P40D40); whole-cell, methanol-precipitated lysates were treated with PK (lane 1) or left undigested (lane 2). PK-treated lysates were subjected to immunoblot with the HUC2-13 (a), 3F4 (b), 6H4 (c) or HPC2 (d) antibodies as described in Methods. Epitope recognition sites located within PrP are shown as amino acid numbers.

by indirect immunofluorescence staining. Immunoreactive PrP with 6H4 antibody was observed on the cell surface as a bright fluorescent signal (Fig. 4a), whereas little signal was observed with mouse IgG, a control antibody purified from normal mouse serum (data not shown). We next prepared membrane and cytosolic fractions from homogenates of P40D40 T98G cells and measured the amount of PrP by competitive ELISA using the 6H4 antibody. PrP was recovered predominantly in the membrane fraction (Table 1). As shown in Fig. 4b, the distribution of PrP^{res} in P40D40 T98G cells (left panel) was similar to that of PrP^{C} in P3D36 T98G cells (right panel); PrP^{res} was detected in the membrane fraction (left panel, lane 3), as well as in homogenates (left panel, lane 1), but no PrP was detected in the cytosolic fraction (left panel, lanes 5 and 6). These data indicated that most PrP^{res} was in the membrane fraction, probably on the plasma membrane. To test the detergent solubility of PrP, the homogenates of P40D40 T98G cells were centrifuged in non-ionic detergents. A large proportion of immunoreactive PrP was found in the supernatant fraction (Fig. 4c, lane 3), but no PrP was detected in the pellet fraction (Fig. 4c, lane 2). These

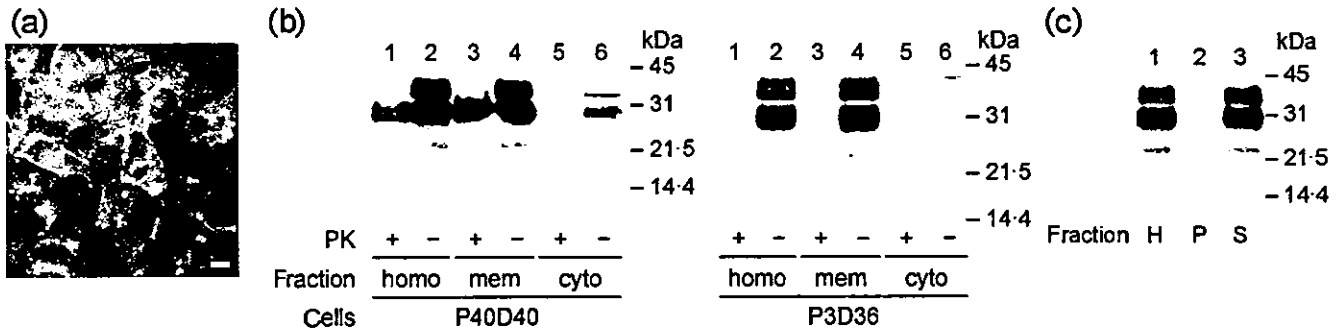

Fig. 4. Subcellular localization and detergent solubility of PrP^{res} in long-term cultured T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 in the long-term incubation after repeated passages. (a) T98G cells for 40 days after 40 passages (P40D40) on a 15 mm glass coverslip were subjected to indirect immunofluorescence staining with the 6H4 antibody as described in Methods. Bar, 10 μm. (b) T98G cells for 40 days after 40 passages (P40D40, left panel) and for 36 days after 3 passages (P3D36, right panel) were scraped into PBS/2·5 mM EDTA and sonicated. Homogenates (homo) were separated into a membrane fraction (mem) and a cytosolic fraction (cyto). Methanol-precipitated lysates were treated with PK (lanes 1, 3 and 5) or left undigested (lanes 2, 4 and 6). PK-treated samples were subjected to immunoblotting with the 6H4 antibody as described in Methods. (c) T98G cells for 40 days after 40 passages (P40D40) were scraped into PBS/2·5 mM EDTA and sonicated. Homogenates (H) of 50 μg protein were centrifuged as described in Methods to obtain a non-ionic detergent-insoluble pellet (P) and a soluble supernatant fraction (S). Homogenates, pellet and supernatant fractions (50 μg protein each) were subjected to immunoblot with the 6H4 antibody as described in Methods.

Table 1. Subcellular localization of PrP in long-term cultured T98G cells

The amount of PrP is expressed as recombinant bovine PrP equivalents per 10^7 cells. Values are means ± SEM ($n=4$).

Sample	PrP content	
	pmol	%
Homogenate	263·4±20·9	100·0
Membrane fraction	228·9±17·5	86·9
Cytosolic fraction	9·9±0·5	3·8

experiments indicated that PrP^{res} in T98G cells was non-ionic detergent-soluble.

DISCUSSION

The mechanism of the conversion of PrP has not been studied in human cell cultures, due to the lack of a model system. In the present study, we developed such a system by culturing human glioblastoma T98G cells, which express endogenous PrP^{C} constitutively. After reaching a high passage number, long-term cultured T98G cells converted PrP^{C} into PrP^{res}.

Direct sequencing of amplified *PRNP* mRNA and RFLP analysis indicated that the T98G cells were heterozygotes at codon 129 (129M/V) and that no new coding mutations were present in cells that had been subjected to long-term cultures. The deglycosylated form of PK-treated PrP^{res} in T98G cells migrated at approximately 18 kDa. In human prion diseases, two major types of PrP^{res} can be identified, based on electrophoretic migration; the relative molecular mass of the unglycosylated form is approximately 21 kDa (described as type 1) or 19 kDa (described as type 2) (Parchi *et al.*, 1997). Accordingly, PrP^{res} in T98G cells is similar to the previously described MV2 phenotypic variant (Parchi *et al.*, 1999a). However, the size of the deglycosylated PK-resistant fragment in T98G cells was smaller than that of the corresponding fragments observed in type 2 PrP^{res}. Most importantly, the 3F4 antibody, which is a well-characterized antibody known to target residues 109–112 as its epitope (Kascsak *et al.*, 1987; Matsunaga *et al.*, 2001), did not react with PK-digested PrP^{res} in T98G cells, suggesting that the N-terminal PrP region up to residue 109 might be absent in PK-treated PrP^{res} in T98G cells. Human PrP^{res} peptide is divided into three regions that are defined by their PK-cleavage patterns: an N-terminal region (residues 23–73) that is invariably PK-sensitive, a C-terminal region (residues 103–231) that is invariably PK-resistant and a variably digested region (residues 74–102), where the major cleavage sites are at G82 in type 1 and at S97 in type 2 (Parchi *et al.*, 2000). The 3F4 antibody was used to type PrP^{res} (Parchi *et al.*, 2000). Therefore, there are striking differences in the antigenicity, which reflect the PK-cleavage patterns, between type 2 PrP^{res} in sporadic CJD brain and in T98G cells. It is unlikely, but not impossible, that PK treatment generated conformational changes in the mid-region of PrP^{res} that interfered with epitope recognition by the 3F4 antibody. Further studies are needed to classify the type of PrP^{res} in lysates from long-term cultured T98G cells.

So far, human PrP^{Sc} has been analysed on immunoblots with the 3F4 antibody. Our finding may explain why previous studies have failed to detect PrP^{res} in cultured cells. Interestingly, an N-terminally truncated 18 kDa fragment of PrP (designated C1) in normal and sporadic CJD brains has similar properties except that it is PK-sensitive; it is recognized by the anti-C terminus antibody, but not by the 3F4 antibody, is cleaved around residue 111 and is associated with cell membranes (Chen *et al.*, 1995). PrP^{C} from human brain homogenates ($n=6$) originally displayed a partial PK resistance (20 μg ml^{-1} for 10 min) and has been detected by the antibody that recognizes residues 145–163, but not by the 3F4 antibody (Buschmann *et al.*, 1998). Taking the data from the various studies of PrP immunoreactivity into consideration, we believe that it would be better to incorporate an additional antibody that recognizes the C terminus of PrP into the standardly used protease resistance-dependent PrP^{Sc} assay.

Among the sets of antibodies used in this study, the anti-N-terminal portion antibodies (HUC2-13 and 3F4) reacted strongly with the fully glycosylated form and moderately with the partially glycosylated form. In contrast, the antibodies against the C-terminal portion of PrP (6H4 and HPC) reacted moderately with the fully glycosylated form and strongly with the partially glycosylated form. It is possible that PK digestion induces a conformational change of digested PrP and enhances its immunoreactivity to the anti-C-terminal antibodies. Recently, it has been reported that the amino acid motif Tyr-Tyr-Arg (YYR), located in a β-sheet, is exposed in PrP^{Sc}, whilst it is cryptic in PrP^{C}, and that antibodies recognize YYR in PrP^{Sc}, but not in PrP^{C} (Paramithiotis *et al.*, 2003). Another paper has reported that PK digestion enhances immunoreactivity to the anti-PrP antibody that recognizes the epitope YYR, located in a β-sheet (Brun *et al.*, 2004). These reports suggest that conformation of the C-terminal portion of PrP^{Sc} is essential for immunoreactivity of anti-YYR antibodies. The 6H4 antibody also recognizes residues 144–152 of PrP, including a YYR motif that is located in an α-helix, not in a β-sheet (Korth *et al.*, 1997). Further study is needed to clarify the immunoreactivity of anti-C-terminal PrP antibodies.

It has been proposed that PrP^{C} is converted into PrP^{res} either on the cell surface or in endocytic cellular compartments. PrP^{C} is a surface protein that contains a glycosylphosphatidylinositol anchor (Stahl *et al.*, 1987). A portion of PrP^{Sc} is also localized on the cell surface of scrapie-infected mouse neuroblastoma ScN2a cells (Naslavsky *et al.*, 1997; Vey *et al.*, 1996), although it is also found in lysosomes (Taraboulos *et al.*, 1990). Subcellular localization of PrP^{res} in long-term cultured T98G cells was similar to that of PrP^{Sc}-infected cells, being present on the cell surface.

PrP^{Sc} in ScN2a cells is sedimented by centrifugation in non-ionic detergents (Caughey *et al.*, 1991). Mutant PrP in stably transfected Chinese hamster ovary cells, which express murine homologues associated with human inherited prion diseases, is also non-ionic detergent-insoluble (Lehmann & Harris, 1996). However, the PrP^{res} in T98G cells is detergent-soluble. PrP^{res} in the human neuroblastoma cell line M-17 BE(2)C carrying the familial subtype CJD, the glutamic acid to lysine substitution at codon 200 (E200K), is also partially non-ionic detergent-insoluble (Capellari *et al.*, 2000). The present study indicates that not all PrP^{res} is non-ionic detergent-insoluble.

Many cultured cells that express PrP^{res} mutants carrying substitutions of inherited prion disease show considerably less protease resistance (up to 3·3 μg ml^{-1} for 10 min), compared with PrP^{res} mutants isolated from the human brain (Capellari *et al.*, 2000; Harris, 2001). In contrast, the PrP^{res} in T98G cells displayed a high resistance to digestion with PK (10 μg ml^{-1} for 30 min), but was less resistant than PrP^{res} in brain homogenates of sporadic CJD (up to 100 μg ml^{-1} for 24 h). Sporadic CJD is typically characterized by widespread spongiform degeneration with loss of neurons, gliosis and formation of amyloid plaques (Parchi *et al.*, 1999a). It has recently been reported that six cases of sporadic fatal insomnia, a prion disease mimicking fatal familial insomnia, had no coding-region mutation of *PRNP* with the 129 M/M genotype and an approximately 19 kDa deglycosylated PrP^{res}, the same as that of type 2 (Mastrianni *et al.*, 1999; Parchi *et al.*, 1999b). Familial progressive subcortical gliosis may also be a prion disease, characterized by astrogliosis at the cortex–white matter junction (Petersen *et al.*, 1995). All patients from two families with that disease showed no coding-region mutation of *PRNP*, the 129 M/M genotype and the 18·1–19·3 kDa form of deglycosylated PrP^{res} (Petersen *et al.*, 1995). T98G cells were grown out of human glioblastoma multiforma tumour tissue of a 61-year-old Caucasian man (Stein, 1979). We consider it possible that he also had a sporadic form of prion disease.

Conversion from PrP^{C} into PrP^{res} is an important process, because most prion diseases are characterized by presence of PrP^{res}. Some knowledge of the conversion mechanism is based on studies of scrapie-infected cells. Recently, it has been reported that several conditions can induce the formation of PrP^{res} in cultured cells. Proteasome inhibitors cause accumulation of the unglycosylated form of PrP^{res} in treated cells (Lehmann & Harris, 1997; Ma & Lindquist, 1999; Yedidia *et al.*, 2001). PrP that misfolds during maturation in the endoplasmic reticulum is delivered to the cytosol for degradation by proteasomes (Béranger *et al.*, 2002; Ma & Lindquist, 2001; Yedidia *et al.*, 2001). It has been hypothesized the conversion into PrP^{res} might occur when the number of PrP molecules exceeds the capacity of the cell to degrade them (Ma & Lindquist, 2002). Another study showed that manganese-treated mouse astrocytes express the glycosylated form of PrP^{res} (Brown *et al.*, 2000).

Here, we report for the first time the conversion of PrP^{C} into PrP^{res} in the widely used human glioblastoma cell line T98G; a large number of passages and prolonged incubation under routine cell-culture conditions are required. *In vitro*-generated PrP^{res} is reportedly not sufficient for the production of infectivity (Caughey *et al.*, 2001; Hill *et al.*, 1999) and further study is needed to clarify the infectivity of PrP^{res} in T98G cells (indeed, caution should be taken with T98G cells in the laboratory). Infectivity assays of PrP^{res} in T98G cells are now in progress in transgenic mice.

In conclusion, T98G cells should be a useful model for studying the mechanisms of PrP^{C} conversion into PrP^{res}.

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