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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 $5{\cdot}0 \times 10^5$ 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 [$1{\cdot}8 \times 10^4$ 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 $1{\cdot}7 \times 10^5$ 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

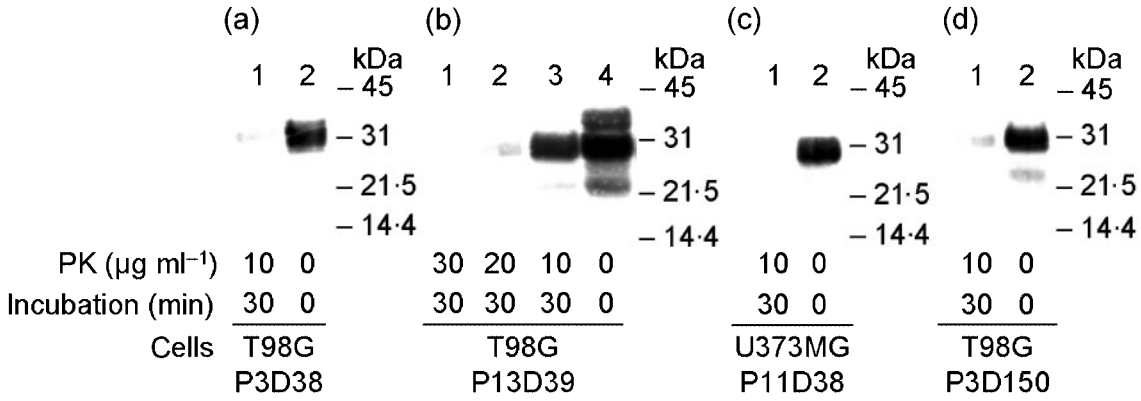

Fig. 1. Formation of a protease-resistant form of PrP in T98G cells is increased in a long-term incubation after repeated passages. T98G cells and U373MG cells were incubated under the following conditions with 10 % FCS/RPMI 1640 and whole-cell, methanol-precipitated lysates (50 μg protein) were treated 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

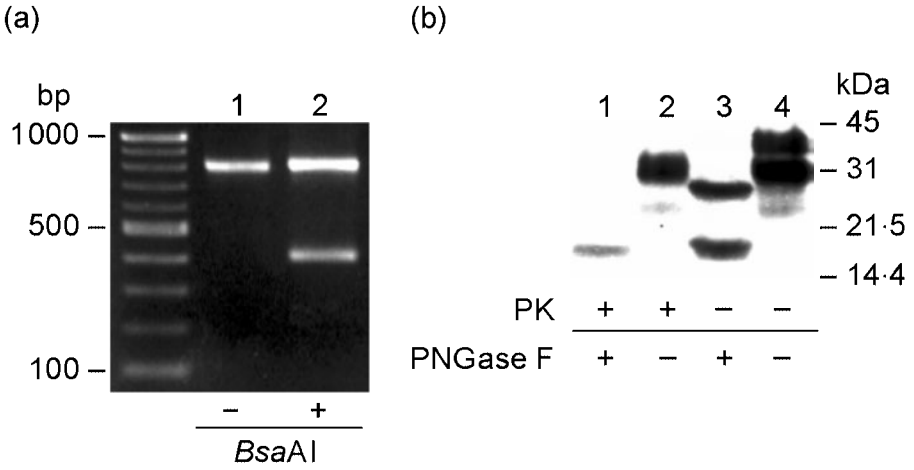

Fig. 2. Molecular analysis of PrP^{res} in T98G cells. (a) Detection of polymorphism at codon 129 on PrP mRNA in T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 for 5 days after 43 passages (P43D5) and total RNA was prepared, reverse-transcribed and PCR-amplified as described in Methods 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.

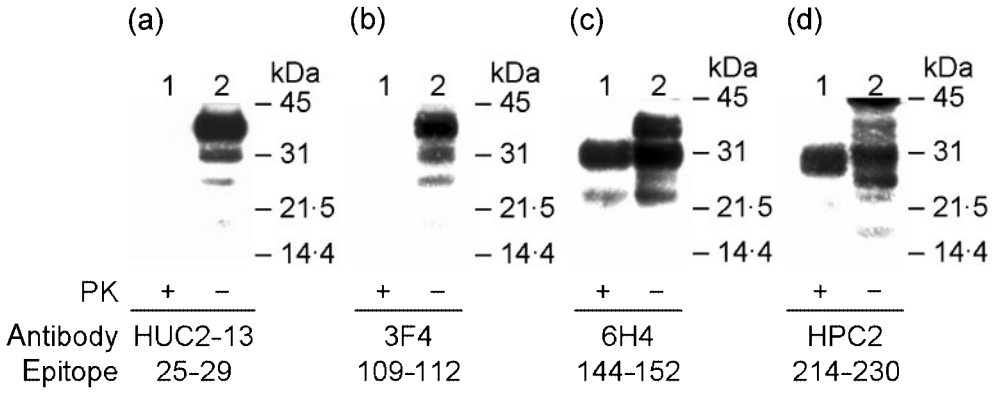

Fig. 3. Immunoblot analysis using anti-PrP antibodies for the protease-resistant form of PrP in T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 for 40 days after 40 passages (P40D40); whole-cell, methanol-precipitated lysates were treated with PK (lane 1) or left undigested (lane 2). PK-treated 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

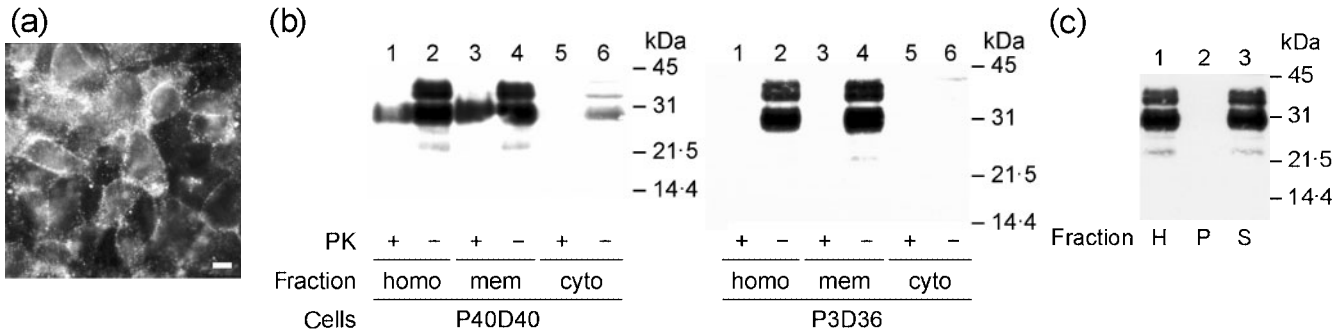

Fig. 4. Subcellular localization and detergent solubility of PrP^{res} in long-term cultured T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 in the long-term incubation after repeated passages. (a) T98G cells for 40 days after 40 passages (P40D40) on a 15 mm glass coverslip were subjected 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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