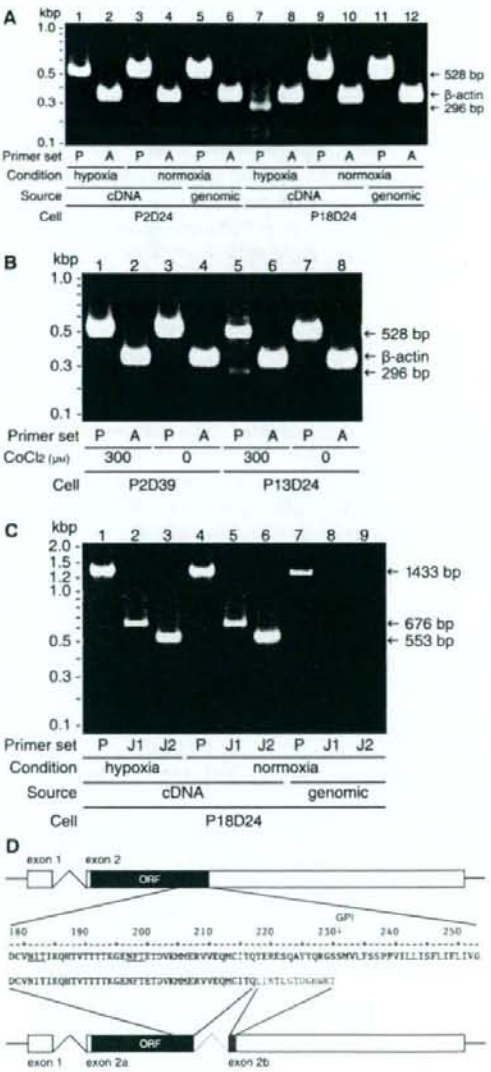

Fig. 1. Expression of a splice variant of PrP mRNA in T98G cells. (A) Exposure of T98G cells to hypoxia produces a splice variant of PrP mRNA. P2D24 and P18D24 cells were exposed to hypoxia (5% O_2) or normoxia for the last day in culture. First-strand cDNA from total RNA and genomic DNA were used for PCR using PrP (E2U0/E2L0, P) and β-actin (ACTBU1/ACTBL1, A) primer sets with KOD Plus DNA polymerase. The PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. (B) Cobalt chloride induced expression of a PrP mRNA splice variant in T98G cells. P2D39 and P13D24 cells were cultured with or without 300 μM $CoCl_2$ for the last day in culture. We used first-strand cDNA for PCR using human PrP primer sets (E2U0/E2L0, P) or β-actin (ACTBU1/ACTBL1, A) primer sets with KOD Plus DNA polymerase. The PCR products were analyzed as described above. (C) Detection of a splice variant of PrP mRNA using exon–exon junction primers. P18D24 cells were exposed to hypoxia or normoxia for the last day in culture. First-strand cDNA and genomic DNA were used for PCR using PrP (E2U1/E2L4, P) and exon–exon junction (E2U1/E2SV3, J1; E2SV4/E2L4, J2) primer sets with Ex Taq DNA polymerase. The PCR products were analyzed as described above. (D) Schematic representation of alternative splicing of *PRNP*. We confirmed the sequences of normally (upper panel) and alternatively (lower panel) spliced *PRNP*. Cryptic donor and acceptor sites are designated as exons 2a and 2b, respectively. The untranslated regions (white bars), open reading frames (blue bars), retained intron (turquoise line), additional open reading frame (magenta bar) and deduced amino acid sequences of normally (blue) and alternatively (magenta) spliced *PRNP* are indicated. The arrow indicates a GPI anchoring site. Asn-Xaa- (Ser/Thr) sequous for N-linked glycosylation.

an mAb, HPSV178, against the C-terminal portion of PrPSV (residues 214–230), and found that it reacted with recombinant PrPSV but not with recombinant PrP (supplementary Fig. S2). We determined PrPSV expression by immunoblotting homogenates of cells with various passage numbers. The mAb 6H4 against human PrP recognized di-, mono- and unglycosylated PrP (Fig. 2A, upper panel). No PrPSV was detected in the homogenates of P40D40 T98G cells (Fig. 2A, lower panel, lane 1), but HPSV178 did recognize a 25 kDa band in the homogenates of P52D40 and P77D40 T98G cells (Fig. 2A, lower panel, lanes 2 and 3). As we routinely sub-cultivated the cells once a week, these data indicated that detectable PrPSV first appeared after at least 1 year (i.e. after 52 passages). To assess the glycosylation status of PrPSV, we treated P77D40 cell homogenates with peptide *N*-glycosidase F (PNGase F), which yields a full-length (25 kDa) and an N-terminally truncated (18 kDa) form of PrP^C [3]. As shown in Fig. 2B, PNGase F treatment reduced the 35 and 31 kDa glycosylated bands to the deglycosylated full-length 25 kDa band and N-terminally truncated 18 kDa band, respectively (upper panel). The mobility of PrPSV was unaltered (lower panel), showing that PrPSV was mainly unglycosylated. To determine the subcellular location of PrPSV, we prepared membrane and cytosolic fractions from the homogenates. As shown in Fig. 2C, PrP was detected only in the membrane fraction, and PrPSV was detected only in the cytosolic fraction (lower panel), indicating that PrPSV lacks a GPI anchor. To test the detergent solubility of PrPSV, we centrifuged the homogenates in the presence of non-ionic detergents. A large proportion of immunoreactive PrP and PrPSV was found only in the soluble fraction (Fig. 2D), indicating that PrPSV, like PrP, is soluble in non-ionic detergents.

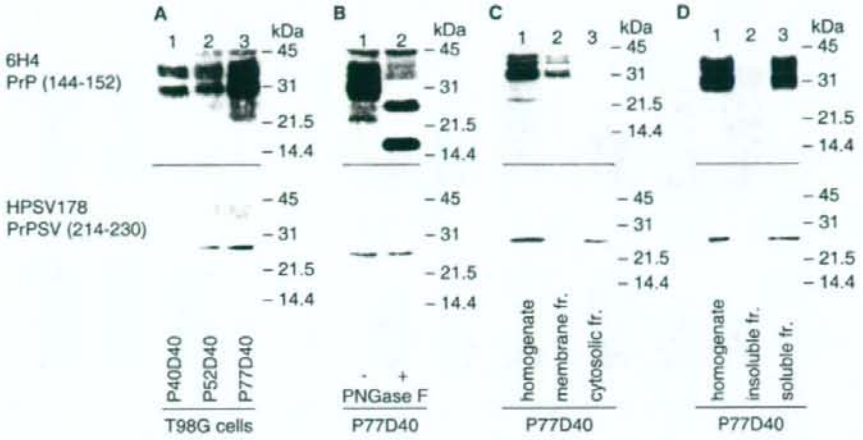

Fig. 2. Characterization of the GPI-anchorless splice variant of PrP in T98G cells. (A) Immunoblot analysis using mAb against GPI⁻ PrPSV in T98G cells. Homogenates (50 μg protein) of P40D40, P52D40 and P77D40 cells were subjected to immunoblotting with 6H4 (upper panel) and HPSV178 (lower panel) monoclonal antibodies as described in Experimental procedures. (B) Analysis of deglycosylated forms of GPI⁻ PrPSV. Homogenates (50 μg protein) of P77D40 cells were incubated with (+) or without (–) PNGase F and the products were subjected to immunoblotting as described above. (C) Subcellular localization of GPI⁻ PrPSV. Homogenates (50 μg protein) of P77D40 cells were separated into membrane and methanol-precipitated cytosolic fractions, resuspended in the same volume of NaCl/P_i containing 2.5 mM EDTA, and subjected to immunoblotting as described above. (D) Detergent solubility of GPI⁻ PrPSV. Homogenates (50 μg protein) of P77D40 cells were dissolved in nine volumes of 0.5% NP-40/0.5% deoxycholate/NaCl/P_i and centrifuged. The pellet fraction (insoluble fr.) and the methanol-precipitated supernatant fraction (soluble fr.) were resuspended in the same volume of NaCl/P_i containing 2.5 mM EDTA and subjected to immunoblotting as described above. Epitope sites located within PrP and PrPSV are shown on the left as residue numbers.

Thus, GPI-anchorless (GPI⁻) PrPSV is localized in the cytosol in unglycosylated form.

Induction of production of the GPI-anchorless splice variant of PrP by hypoxia

We then examined whether PrP^{res} is propagated under hypoxic conditions. For P40D40 cells, PrP mRNA levels were significantly lower when they were cultured for the last 1–4 days in 300 μM $CoCl_2$ rather than under normoxic conditions (Fig. 3A, upper panel), and immunoblotting with 6H4 mAb showed that PrP protein levels decreased (Fig. 3C, upper panel). After treatment with 300 μM $CoCl_2$ for the last 4 days, no PrP^{res} was detected, and PrPSV mRNA levels and their ratio to PrP mRNA levels increased (Fig. 3A, middle and lower panels). However, GPI⁻ PrPSV was not observed (Fig. 3C, middle panel). For P90D40 cells, PrP mRNA levels decreased significantly under hypoxic conditions (100 μM $CoCl_2$ or 2% O_2) for the last 4 days (Fig. 3B, upper panel), but PrP^{res} was still detected by 6H4 mAb (Fig. 3D, upper panel). PrPSV mRNA levels and their ratios to PrP mRNA levels were significantly higher (Fig. 3B, middle and lower panels), and GPI⁻ PrPSV was detected in the cell homogenates (Fig. 3D, middle panel), and is equivalent to the band shown in Fig. 2A–D (lower panels). No GPI⁻ PrPSV band was detected following PK treatment of the P90D40 cell homogenates (Fig. 3D, middle panel). Quantitative densitometric analysis of the 25 kDa bands (Fig. 3D, middle panel) revealed that the amounts of 100 μM $CoCl_2$- and 2% O_2-induced GPI⁻ PrPSV increased to almost 4.3 and 4.8 times their original levels, respectively (Fig. 3E, lower panel).

Detection of the splice variant of PrP mRNA in human brain

To investigate whether PrPSV mRNA is expressed in human brain, we subjected total RNA of adult and fetal brains to RT-PCR analysis using the exon–exon junction primers. As shown in Fig. 4A (lanes 1 and 5), 1433 bp products were detected in both samples using primers E2U1/E2L4 (primer set P). Surprisingly, two shorter RT-PCR products were detected when exon–exon junction primer sets were used – a 676 bp product with primers E2U1/E2SV3 (primer set J1) and a 553 bp product with primers E2SV4/E2L4 (primer set J2). These results indicate that very low levels of PrPSV mRNA are constitu-

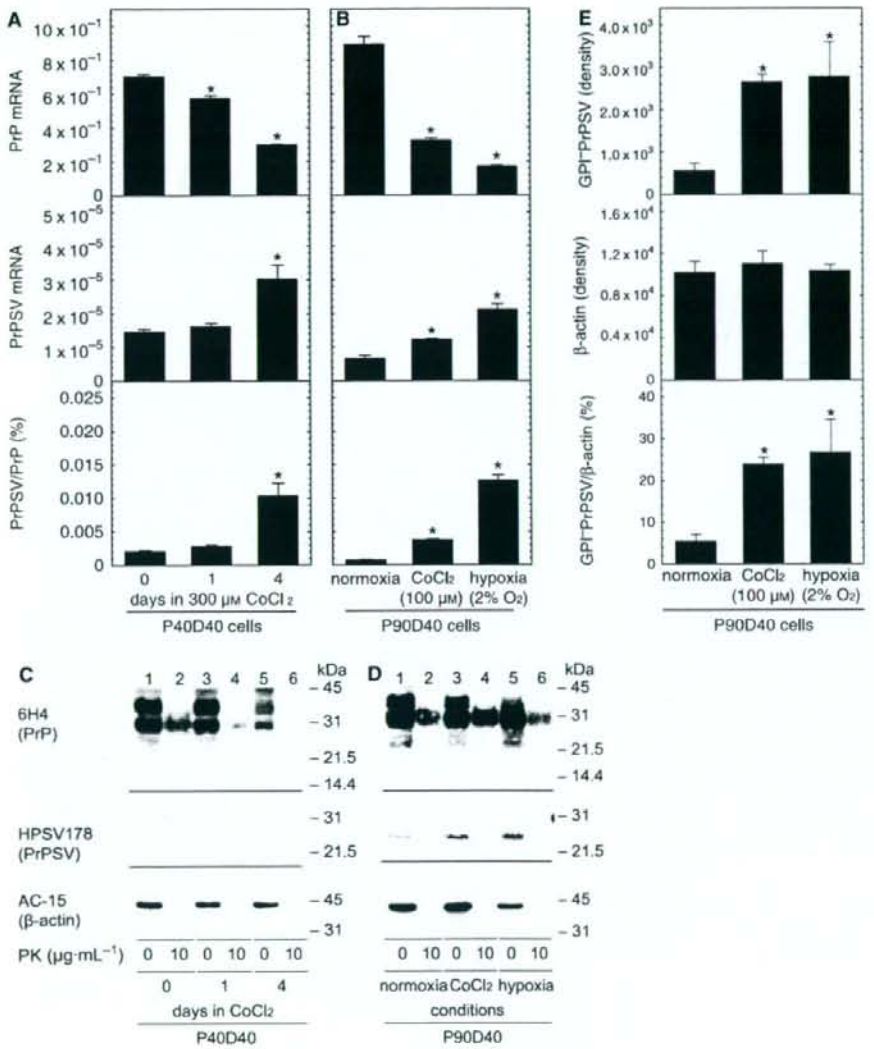

Fig. 3. Hypoxia-induced expression of the GPI-anchorless splice variant of PrP in T98G cells. (A,B) Quantification of PrPSV mRNA in T98G cells. P40D40 cells were exposed to $CoCl_2$ (300 μM) for the last 0, 1 or 4 days (A). P90D40 cells were exposed to hypoxia (2% O_2), $CoCl_2$ (100 μM) or normoxia for the last 4 days (B). The mRNA levels were analyzed by quantitative RT-PCR using PrP and exon–exon junction primer sets as described in Experimental procedures. The expression values of PrP and PrPSV and their ratios were normalized to those of β-actin. Values are the mean ± standard error of three independent cell samples. $^*P < 0.05$ compared with day 0 (A) or normoxia (B) (Dunnett test). (C,D) Proteinase K sensitivity of GPI^- PrPSV. Methanol-precipitated homogenates (50 μg protein) of P40D40 (A) and P90D40 (B) cells were treated with 0 or 10 $\mu g \cdot mL^{-1}$ PK at 37 °C for 30 min. The products were subjected to immunoblotting with 6H4 (upper panel), HPSV178 (middle panel) or AC-15 (lower panel) antibodies as described in Experimental procedures. (E) Densitometric quantification of GPI^- PrPSV. Quantitative analysis of GPI^- PrPSV and β-actin shown in (D) at 25 kDa (middle panel, odd-numbered lanes) and at 42 kDa (lower panel, odd-numbered lanes), respectively, were performed by computer-assisted densitometry. The density of GPI^- PrPSV (%) was normalized to that of β-actin (lower panel). Values are the means ± SD of triplicate measurements within one experiment. $^*P < 0.05$ compared with normoxia (Dunnett test).

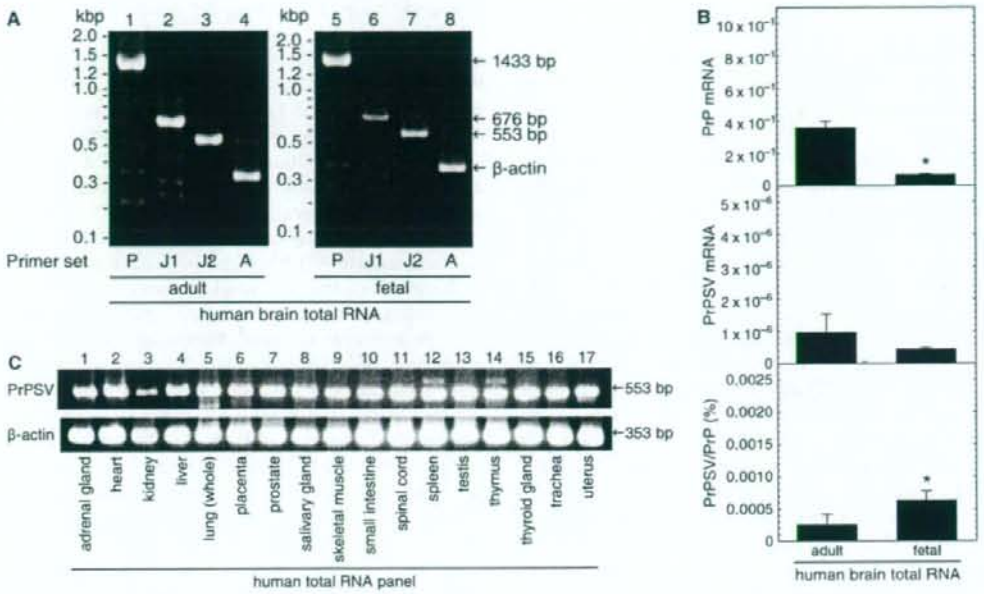

Fig. 4. Expression of the splice variant of PrP mRNA in human tissues. (A) Detection of the splice variant of PrP mRNA in total RNA from human brains. We prepared first-strand cDNA from total RNA from adult (43-year-old Caucasian male) and fetal human brains (5 μg each), and subjected it to PCR using *PRNP* exon 2 (E2U1/E2L4, P), exon–exon junction (E2U1/E2SV3, J1; E2SV4/E2L4, J2) and β-actin (ACTBU1/ACTBL1, A) primer sets using Ex Taq DNA polymerase. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. (B) Quantification of the splice variant of PrP mRNA in total RNA from human brain. We analyzed the human brain total RNA shown in (A) by quantitative RT-PCR using PrP primers and exon–exon junction primer sets as described in Experimental procedures. The expression values of PrP and PrPSV and their ratios were normalized to those of β-actin. Values are the means ± SD of triplicate measurements within one experiment. $^*P < 0.05$ compared with adult (Student's *t* test). (C) Expression of the splice variant of PrP mRNA in human tissues. RT-PCR showing the splice variant of PrP mRNA using exon–exon junction primers in various human tissues. We prepared total RNA from adult human tissues (5 μg each) and subjected it to PCR using exon–exon junction (E2SV4/E2L4, upper panel) and β-actin (ACTBU1/ACTBL1, lower panel) primer sets with Ex Taq DNA polymerase. Amplification of β-actin is shown as a quality check for total RNA (lower panel). The PCR products were analyzed as described above.

tively expressed in fetal and adult human brains. Relative quantitative RT-PCR analysis, which measures PrP mRNA in total RNA (Fig. 4B, upper panel), showed that the PrP mRNA level in adult brain was higher than that in fetal brain. PrPSV mRNA levels were not significantly different between the two samples (Fig. 4B, middle panel). We then used RT-PCR to determine PrPSV mRNA distribution in total RNA from a panel of adult human tissues (Fig. 4C). We found expression in all the tested samples, including non-neuronal samples, indicating that PrPSV mRNA is constitutively expressed in all major organs and tissues.

Discussion

At least two homologs have been mapped to the *PRNP* locus (20pter-p12): *PRND/Doppel* and *PRNT* [17]. Expression of the human PrP-like glycoprotein PrPLP/Dpl by these homologs has been confirmed in human adult testes [18] and astrocytomas [19]. The 176 amino acids of PrPLP/Dpl include an N-terminal signal sequence (residues 1–23) and a GPI anchor signal sequence (residues 153–176) [17]. PrPLP/Dpl (residues 24–152) has 25% sequence homology with PrP and has a truncated N-terminus. Despite their low homology, PrP and PrPLP/Dpl show similar structural features. The PrPLP/Dpl protein is expressed by intergenic splicing between *PRNP* and *PRND/Doppel*. GPI$^-$ PrPSV protein, on the other hand, is expressed by intragenic splicing within *PRNP* exon 2 and has 94% sequence homology with normally spliced PrP (residues 23–230). *PRNP* mRNA is ubiquitously expressed in brain and various tissues, including non-neuronal tissues [17], and, as discussed above, we demonstrated constitutive expression of PrPSV mRNA

in all major organs and tissues, including brain. Although expression levels were low, the ratio of PrPSV mRNA to PrP mRNA was 0.00026% in adult brain (Fig. 4B). We also detected PrPSV mRNA in human astrocytoma U373MG cells and human myelocytic leukemia HL-60 cells (supplementary Table S2).

We detected GPI$^-$ PrPSV production in T98G cells after 52 passages. The protein was mainly unglycosylated and appeared in the cytosolic fraction. This agrees with findings that mouse recombinant PrP lacking a GPI anchor signal sequence is unglycosylated in human neuroblastoma cell line SH-SY5Y [20]. Furthermore, GPI$^-$ PrP in the transgenic mouse brain is unglycosylated and is found in the cytosolic fraction [21,22]. Two N-glycosylation sites – Asn (181) and Asn (197) – are 73 and 57 residues from the C-terminus in PrP, and 50 and 34 residues from the C-terminus in PrPSV, respectively (Fig. 1D). N-glycosylation of mouse PrP was abolished when the first Asn-Xaa-(Ser/Thr) sequon for N-linked glycosylation was <65 residues from the C-terminus [20]. A similar effect has been reported for recombinant GPI-anchorless Thy-1 protein [23] and collagen XVII [24]. However, it has recently been shown that a GPI-anchorless PrP mutant is highly glycosylated and is tethered to cell membranes but does not assume transmembrane topology in Fisher rat thyroid cells [25]. Further study will be required to characterize the glycoforms of PrP seen in T98G cells.

Studies of adult human brain have revealed that PrP^C accumulates as a result of cerebral ischemia, and PrP mRNA is up-regulated during hypoxia [12]. In the present report, hypoxic conditions caused a dramatic decrease in the levels of PrP mRNA in T98G cells. In contrast, hypoxic conditions caused an increase in PrPSV mRNA levels. The ratio of PrPSV mRNA to PrP mRNA was 0.010% in P40D40 cells grown in 300 μM $CoCl_2$ for the last 4 days and 0.013% in P90D40 cells grown in 2% O_2 for the last 4 days. Furthermore, these ratios were almost 50 times higher than the ratios in adult brain. However, GPI$^-$ PrPSV was produced in P90D40 but not P40D40 cells. We detected GPI$^-$ PrPSV in T98G cells after frequent passages for more than 1 year. It is unclear why the increase in PrPSV mRNA level did not affect PrPSV protein production in P40D40 cells, unlike P90D40 cells. Several reports have suggested that the presence of PrP in the cytosol is linked to prion disease. When proteasome activity was blocked in mouse neuroblastoma cell line N2a [26] and human prostate cancer cell line PC3M [27], partially PK-resistant PrP^{res} was retrotransported to the cytosol, and the accumulation of cytosolic PrP is strongly neurotoxic in N2a cells and transgenic mice [26]. Transgenic mice that co-express GPI$^-$ PrP and PrP^C show accelerated clinical signs of scrapie, with vacuolation and amyloid plaque-like PrP^{res} deposition [21]. Additionally, transgenic mice that express cytosolic PrP, lacking background expression of PrP^C, display gliosis [22]. Our studies suggest that hypoxic conditions induce GPI$^-$ PrPSV production in the cytosol of T98G cells that have been cultured for over a year.

Alternative splicing of presenilin-2 mRNA has been shown in human AD brain and induced in human neuroblastoma cell line SK-N-SH by hypoxia, but not by other cellular stress [28]. Metal ions may contribute to neurodegenerative diseases. Aluminum ions cause the production of a splice variant of presenilin-2 mRNA [29], manganese-treated astrocytes from mouse brain produce partially PK-resistant PrP [30], and $CoCl_2$ induced the expression of PrPSV mRNA in T98G cells in the present study. Manganese ions can also mimic hypoxic conditions [13]. In T98G cells that underwent a number of passages, PrP^C was converted to the partially PK-resistant form of PrP^{res} and GPI$^-$ PrPSV after at least one year. These data suggest that alternative splicing of *PRNP* mRNA may contribute to the occurrence of prion diseases.

There is a link between PrP and plasminogen proteolytic systems. Human plasminogen precipitates disease-associated PrP^{CJD} from brain homogenates of patients with sporadic CJD [31]. In addition, tissue-type plasminogen activator-catalyzed plasminogen activation is enhanced by recombinant human PrP [32]. Interestingly, a FASTA biological sequence comparison search [33] revealed that a C-terminal portion of PrPSV (residues 210–230) has 42.9% identity (71.4% similar) to a 21 amino acid overlap in the plasminogen activator inhibitor-1 (accession number AAA60009) (supplementary Fig. S3), and the exon 2b-derived C-terminal portion (residues 218–230) has 53.8% identity (84.6% similar) to plasminogen activator inhibitor-1 (residues 188–200) while PrP (residues 218–230) has 7.7% identity to plasminogen activator inhibitor-1 (residues 188–200). Plasminogen activator inhibitor-1 is an inhibitor of tissue-type plasminogen activator, with which it reacts to form a complex [34]. These data imply an interaction between GPI$^-$ PrPSV and plasminogen proteolytic systems.

There are four major types of alternative splicing: exon inclusion or exclusion, alteration of 5′ or 3′ splicing sites, mutual exon exclusion, and intron retention [35]. Alternative splicing of PrP mRNAs where the coding region of the PrP exon is not involved has been described in cattle [36], mice [37] and sheep [38]. The ORF of human *PRNP* is included within

exon 2 [1]. Variant forms arising from alternative mRNA splicing within exon 2 have not been reported [1]. This study is the first report of an alternative splicing by intron retention within *PRNP* exon 2. Whether PrP or GPI$^-$ PrPSV is produced depends on whether splicing does not occur and the intron is retained under normoxic conditions, or splicing does occur and the intron is excised under hypoxic conditions. In conclusion, GPI$^-$ PrPSV induced under hypoxic conditions is present in the cytosolic isoform of PrP. It will be important to understand how oxygen tension regulates GPI$^-$ PrPSV expression, and its relationship with the expression level of GPI$^-$ PrPSV and prion diseases requires further investigation.

Experimental procedures

Materials

We purchased primer sets (supplementary Table S1) for the human PrP (*PRNP*) and β-actin (*ACTB*) genes and the C-terminal peptide of the splice variant of PrP (PrPSV) (residues 214–230, CITQLINTLGTDGHWKT) from Operon Biotechnologies (Tokyo, Japan). Hydrolyzed probes were obtained from Biosearch Technologies (Novato, CA, USA) (supplementary Table S1). The sequences for the E2U0/E2L0 primer set (supplementary Table S1) have been reported previously [39]. Adult human brain total RNA (normal whole brain from a 43-year-old Caucasian male; cause of death: sudden death), fetal human brain total RNA (normal fetal brains pooled from 21 spontaneously aborted male/female Caucasian fetuses aged 26–40 weeks), and human total RNA master panel II (male/female, aged 14–75 years) were obtained from BD Biosciences (Palo Alto, CA, USA). We purchased the mAb AC-15 against β-actin from Sigma (St Louis, MO, USA) and the mAb 6H4 against human PrP from Prionics AG (Zürich, Switzerland).

Preparation of antibodies

Preparation and purification of mouse monoclonal antibodies against GPI$^-$ PrPSV peptide residues 214–230 were carried out as previously described [40] with slight modifications. Briefly, peptide (3.2 μmol) was reacted with 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (3.2 μmol) and BSA (0.3 μmol), and used as an immunogen. BALB/c mice were immunized with five subcutaneous injections of 50 μg of the immunogen with complete Freund's adjuvant at 21–28-day intervals. Eleven days after the last injection, the mice were injected intraperitoneally with 50 μg of the immunogen in sterile saline. Three days later, the spleen cells were fused with P3/NS1/1-Ag4-1 (JCRB0009) cells using polyethylene glycol, and (hypoxanthine-aminopterin-thymidine)-selected. The hybridoma cells from ELISA-positive wells were cloned twice by limiting dilution. The cloned hybridoma cells were implanted intraperitoneally into BALB/c mice pretreated with incomplete Freund's adjuvant to obtain ascitic fluid. The antibody activity of the ascitic fluid was recovered in the IgG fraction when separated on an Affi-Gel Protein A column (Bio-Rad Laboratories, Hercules, CA, USA), and one mAb, HPSV178 (κ, γ2b), specific for PrPSV, was obtained. The chicken mAb HUC2-13 (IgY) against human PrP peptide residues 25–49 was prepared as reported previously [41], as was the mouse mAb 17H5 (IgG) against human PrP peptide residues 135–224 [42] and rabbit polyclonal antibody HPC2 (IgG) against human PrP peptide residues 214–230 [43]. All procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals by the Ethical Review Committee of National Institute of Health Sciences.

Cell culture

Human glioblastoma cell line T98G [44] (JCRB9041) at nominal passage level 433 was provided by the Japanese Cancer Research Resources Bank. Cell cultures stored in liquid nitrogen (passage level 435) were thawed as passage 0 (P0), and cultured at 37 °C in monolayers on a T75 plastic tissue culture flask in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% v/v heat-inactivated fetal calf serum, 60 μg·mL^{-1} kanamycin and 10 mM Hepes/NaOH, pH 7.2. Cell cultures were routinely subcultivated at a 1 : 5 or 1 : 10 split ratio once a week. The cells were re-plated at 5.0×10^5 cells per dish (55 cm^2) in 10 mL RPMI-1640 medium on day 0. The medium was changed every 4 days. The cells were confluent on days 12–16 [43]. Before use in low-oxygen experiments, cell lines were cultured in an incubator at 37 °C in 5% CO_2 in air. For low-oxygen conditions, a 2–5% O_2 environment was obtained in a controlled oxygen incubator with a residual gas mixture comprising 90–93% N_2 and 5% CO_2. The $CoCl_2$ low-oxygen environment was obtained in a standard incubator with 5% CO_2 in air using medium containing 100–300 μM $CoCl_2$. Human astrocytoma U373MG cells were kindly provided by T. Kasahara (Kyoritsu College of Pharmacy, Tokyo, Japan). Human myelocytic leukemia HL-60 cells were kindly provided by K. Suzuki (National Institute of Health Sciences, Tokyo, Japan).

Preparation of recombinant human PrP and the splice variant of PrP

PRNP exon 2 encoding human PrP was amplified from a *PRNP* gene fragment from T98G cells by means of PCR. PrPSV was constructed by assembly PCR. Briefly, the large 5′ portion was amplified from genomic DNA as described above, and the small 3′ portion was amplified from a synthetic oligonucleotide. The amplicons were subcloned into

pBluescript II KS(+) phagemid (Stratagene, La Jolla, CA, USA) and the nucleotide sequences were confirmed. The PrP genes and splice variants containing codon 129 for Met and Val were subcloned into pET-22b and transformed into *Escherichia coli* BL21(DE3)pLysS. Expression of recombinant PrP and PrPSV was performed using the Overnight Express Autoinduction System 1 (Novagen, San Diego, CA, USA).

RT-PCR analysis and direct sequencing

We extracted total RNA from cells and performed RT-PCR analysis as previously described [43], with slight modifications. Briefly, 5 μg of total RNA was treated with DNase I for 15 min at room temperature. Random primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) were added to the total RNA (5μg), and the mixture (20 μL) was incubated at 42 °C for 60 min for synthesis of cDNA. Subsequently, 2.5 μL of cDNA solution (first-strand cDNA or 2.5 ng of genomic DNA) was subjected to PCR in a total volume of 25 μL that contained 0.2 mM dNTPs, 1 mM $MgSO_4$, 1 unit DNA polymerase and 25 pmol sense and antisense primers. The amplification program was as follows: using KOD Plus DNA polymerase (Toyobo, Osaka, Japan), 40 cycles of denaturation at 94 °C for 20 s, annealing at 57 °C for 30 s, and elongation at 68 °C for 36 s (156 s for the last cycle); using Ex Taq DNA polymerase (Takara Bio, Shiga, Japan), 40 cycles of denaturation at 94 °C for 20 s, annealing at 57 °C for 30 s, and elongation at 72 °C for 60 s (180 s for the last cycle). PCR was carried out in a DNA Engine PTC-200 (Bio-Rad Laboratories). PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. PCR direct sequencing was performed using a CEQ 2000XL DNA Analysis System (Beckman Coulter, Fullerton, CA, USA).

Quantitative RT-PCR

DNase I-digested total RNA (5 μg) was subjected to reverse transcription with SuperScript III reverse transcriptase (Invitrogen). Briefly, random primers, RNase inhibitor and SuperScript III reverse transcriptase were added to 20 μL (5 μg total RNA), and the mixture was incubated at 50 °C for 60 min for synthesis of cDNA. Gene expression analysis using multiplex quantitative RT-PCR was performed using a Chromo4 real-time PCR detection system (Bio-Rad Laboratories). Subsequently, 1 μL of the first-strand cDNA solution was subjected to PCR in a total volume of 20 μL that included sense/antisense primers (200 nM each) and probes (100 nM each) (E2U1/E2L9 and E2P29 for PrP; E2SV5/E2L8 and E2P903 for PrPSV; ACTBU7/ACTBL7 and ACTB859 for β-actin), and 10 μL of FastStart TaqMan Probe Master (Rox) (Roche Diagnostics, Mannheim, Germany). The amplification program was as follows: initial denaturation at 95 °C for 10 min, then denaturation at 95 °C for 15 s and elongation at 60 °C for 35 s for 40 cycles. Plasmids harboring the PrP, PrPSV and β-actin coding sequences were used for generation of a standard curve. The relative quantities of PrP and PrPSV were normalized against β-actin for each sample by simultaneous amplification in the same reaction tubes.

Preparation of cell homogenates

At the indicated times, the cells were washed twice with ice-cold NaCl/P_i and were scraped into NaCl/P_i containing 2.5 mM EDTA and protease inhibitor cocktail (0.06 trypsin inhibitor units (TIU)·mL^{-1} aprotinin, 20 μM leupeptin and 1 mM phenylmethanesulfonyl fluoride). After sonication, insoluble material was pelleted by centrifugation at 500 ***g*** for 15 min at 4 °C to yield postnuclear fractions. The protein concentration was determined using the bicinchoninic acid protein assay.

Subcellular fractionation

The homogenates were centrifuged at 100 000 ***g*** for 60 min at 4 °C to separate the cytosol from the membranes. The cytosolic fraction was precipitated with four volumes of methanol for 16 h at −20 °C. Both fractions were resuspended in the same volume of NaCl/P_i containing 2.5 mM EDTA.

Detergent solubility test

The detergent solubility test was carried out according to a previously described method [45] with slight modifications. The homogenates were dissolved in nine volumes of 0.5% NP-40/0.5% deoxycholate/NaCl/P_i containing protease inhibitor cocktail and centrifuged at 100 000 ***g*** for 60 min at 4 °C to generate a detergent-insoluble pellet fraction and a soluble supernatant fraction. The supernatant fraction was precipitated with four volumes of methanol for 16 h at −20 °C. Both fractions were resuspended in the same volume of NaCl/P_i containing 2.5 mM EDTA.

Protease-resistant PrP assay

To generate material for the protease-resistant PrP assay, we precipitated aliquots of the sample (50 μg protein) with four volumes of methanol for 16 h at −20 °C to remove the protease inhibitor cocktail, centrifuged the mixture at 14 000 ***g*** for 15 min at 4 °C, and dissolved the pellet in 50 mM Tris/HCl, pH 7.2. The samples were treated with PK (Merck, Darmstadt, Germany) at various concentrations at 37 °C according to a previously described method [3]. After incubation, digestion was stopped by the addition of 4- (2-aminoethyl)-benzenesulfonyl fluoride hydrochloride to 4 mM. The samples were prepared with protease inhibitor cocktail at a concentration that did not inhibit the activity of PK (Fig. 3C, lane 6).

Enzymatic deglycosylation

For removal of Asn-linked oligosaccharides, aliquots of homogenates were treated with PNGase F (New England Biolabs, Beverly, MA, USA) as follows [43]: the homogenates (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 milliunits of PNGase F in 50 mM phosphate buffer, pH 7.5.

Immunoblotting

SDS gel electrophoresis was performed using 50 μg total protein. Briefly, aliquots of the samples were mixed with 2× electrophoresis sample buffer. The samples were boiled for 10 min and electrophoresed on 12.5% acrylamide gel, and the proteins were transferred onto poly(vinylidene difluoride) membranes. The membranes were blocked with 0.5% casein in NaCl/P_i and incubated with anti-prion or anti-β-actin serum in NaCl/P_i containing 0.5% casein Immunoreactive bands were visualized using horseradish peroxidase-conjugated antibody against chicken, mouse or rabbit IgG and SuperSignal West Femto maximum sensitivity substrate (Pierce, Rockford, IL, USA).

Nucleotide sequences

The nucleotide sequences of PrP and PrPSV determined in this study have been submitted to the DNA Data Bank of Japan (accession numbers AB300823–AB300826).

Acknowledgements

This work was supported in part by the Ministry of Health, Labour and Welfare, Japan (Research on Regulatory Science of Pharmaceuticals and Medical Devices H19-Iyaku-Ippan-011) and in part by the Ministry of Education, Culture, Sports, Sciences and Technology, Japan (Grant-in-Aid for Scientific Research C18602006, and the Budget for Nuclear Research Counseling by the Atomic Energy Commission). We thank Drs M. Honma and H. Akiyama (National Institute of Health Sciences) for helpful discussions, and Drs M. Yamada and K. Sugiyama (National Institute of Health Sciences) for preparation of plasmids.

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Supplementary material

The following supplementary material is available online:

Fig. S1. Comparison of open reading frames of PrP and the splice variant of PrP (PrPSV) within exon 2 of *PRNP* in T98G cells.

Fig. S2. Immunoblot analysis of recombinant human PrP and its splice variant.

Fig. S3. Alignment of amino acid sequences of plasminogen activator inhibitor-1 and GPI$^-$ PrPSV by FASTA searches.

Table S1. Sequences of primers and hydrolyzed probes.

Table S2. Expression of the splice variant of PrP mRNA in human cells.

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Table S1. Sequences of primers and hydrolyzed probes

name	nucleotide sequence*	position
PRNP (accession number AL133396)		
	exon 2 primers	
E2U0	GGCAGTGACTATGAGGACCGTTAC (sense)	70,037..70,060
E2U1	AGCAGTCATTATGGCGAACCTT (sense)	69,604..69,625
E2L0	GGCTTGACCAGCATCTCAGGTCTA (antisense)	70,564..70,541
E2L4	CTAGCCAGAGGTTCAGTGTTGTG (antisense)	71,036..71,014
E2L8	GGGCTTGACCAGCATCTCA (antisense)	70,565..70,547
E2L9	GAGGCCCAGGTCACTCCAT (antisense)	69,676..69,658
	exon-exon junction primers	
E2SV4	ATGTGTATCACCCAGCTAATCAAT (sense)	70,250..70,263∧70,496..70,505
E2SV5	CAGATGTGTATCACCCAGCTAATCA (sense)	70,247..70,263∧70,496..70,503
E2SV3	AAGGGTATTGATTAGCTGGGTGAT (antisense)	70,511..70,496∧70,263..70,256
	hydrolyzed probes†	
E2P29	5'-HEX-TGGATGCTGGTTCTCTTTGTGGCCA-BHQ-1-3'	69,632..69,656
E2P903	5'-FAM-ACCCTTGGCACTGATGGGCACTG-BHQ-1-3'	70,506..70,528
ACTB (accession number BC002409)		
	primers	
ACTBU1	GCTCGTCGTCGACAACGGCTC (sense)	92..112
ACTBU7	GCTGCCCTGAGGCACTCT (sense)	838..855
ACTBL1	CAAACATGATCTGGGTCATCTTCTC (antisense)	444..420
ACTBL7	CGGATGTCCACGTCACACTT (antisense)	940..921
	hydrolyzed probe†	
ACTB859	5'-Quasar 670-AGCCTTCCTTCCTGGGCATGGAGTC-BHQ-2-3'	859..883

* Oligonucleotides correspond to the PCR primers and probes.

† The hydrolyzed probes were labeled with the fluorophore (FAM, HEX, or Quasar 670) at the 5′ end and the quencher (BHQ-1 or BHQ-2) at the 3′ end.

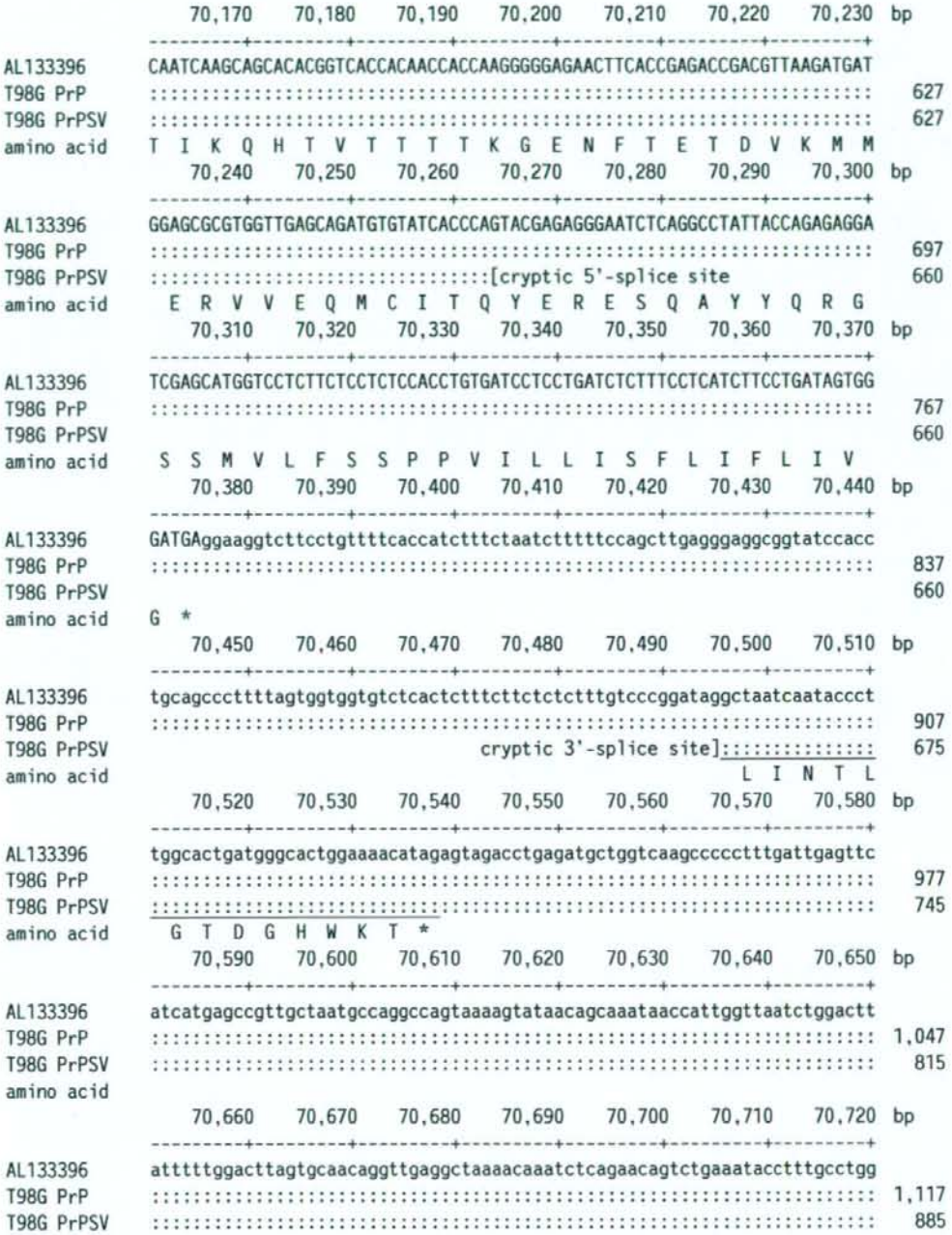
Table S2. Expression of the splice variant of PrP mRNA in human cells.
We analyzed total RNA from human cell lines by quantitative RT-PCR with PrP primer and exon-exon junction primer sets as described in Experimental procedures.

cells	mRNA [a]		
	PrP [b]	PrPSV [b]	PrPSV/PrP (%)
U373MG	$1.74 \times 10^{-1} \pm 5.29 \times 10^{-3}$	$1.43 \times 10^{-6} \pm 9.11 \times 10^{-8}$	0.0008
HL-60	$1.26 \times 10^{-2} \pm 1.87 \times 10^{-4}$	$1.44 \times 10^{-7} \pm 8.65 \times 10^{-8}$	0.0011

[a] Relative expression values of PrP and PrPSV and their ratios were normalized to β-actin.
[b] Values are the mean ± standard error of three independent cell samples.

Fig. S1. Comparison of open reading frames of PrP and the splice variant of PrP (PrPSV) within exon 2 of *PRNP* in T98G cells. The nucleotide sequence of the ORF within exon 2 of *PRNP* in T98G cells was determined and aligned with the reference sequence (accession no. AL133396). The 5' end of exon 2 of *PRNP* is shown with the intervening sequence in lower case and the coding sequence in upper case. Identical nucleotides are indicated by dots. The intron region in PrPSV is enclosed in brackets. Deduced amino acids are listed below the nucleotide sequences. An additional ORF of PrPSV is underlined. Stop codons are indicated by asterisks.

```
                 69,610    69,620    69,630    69,640    69,650    69,660    69,670  bp
                ---------+---------+---------+---------+---------+---------+---------+
AL133396        cagagcagtcattATGGCGAACCTTGGCTGCTGGATGCTGGTTCTCTTTGTGGCCACATGGAGTGACCTG
T98G PrP†          :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    67
T98G PrPSV‡        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    67
amino acid                   M  A  N  L  G  C  W  M  L  V  L  F  V  A  T  W  S  D  L
                 69,680    69,690    69,700    69,710    69,720    69,730    69,740  bp
                ---------+---------+---------+---------+---------+---------+---------+
AL133396        GGCCTCTGCAAGAAGCGCCCGAAGCCTGGAGGATGGAACACTGGGGGCAGCCGATACCCGGGGCAGGGCA
T98G PrP         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    137
T98G PrPSV       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    137
amino acid       G  L  C  K  K  R  P  K  P  G  G  W  N  T  G  G  S  R  Y  P  G  Q  G
                 69,750    69,760    69,770    69,780    69,790    69,800    69,810  bp
                ---------+---------+---------+---------+---------+---------+---------+
AL133396        GCCCTGGAGGCAACCGCTACCCACCTCAGGGCGGTGGTGGCTGGGGGCAGCCTCATGGTGGTGGCTGGGG
T98G PrP         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    207
T98G PrPSV       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    207
amino acid       S  P  G  G  N  R  Y  P  P  Q  G  G  G  G  W  G  Q  P  H  G  G  G  W  G
                 69,820    69,830    69,840    69,850    69,860    69,870    69,880  bp
                ---------+---------+---------+---------+---------+---------+---------+
AL133396        GCAGCCTCATGGTGGTGGCTGGGGGCAGCCCCATGGTGGTGGCTGGGGACAGCCTCATGGTGGTGGCTGG
T98G PrP         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    277
T98G PrPSV       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    277
amino acid         Q  P  H  G  G  G  W  G  Q  P  H  G  G  G  W  G  Q  P  H  G  G  G  W
                 69,890    69,900    69,910    69,920    69,930    69,940    69,950  bp
                ---------+---------+---------+---------+---------+---------+---------+
AL133396        GGTCAAGGAGGTGGCACCCACAGTCAGTGGAACAAGCCGAGTAAGCCAAAAACCAACATGAAGCACATGG
T98G PrP         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    347
T98G PrPSV       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    347
amino acid       G  Q  G  G  G  T  H  S  Q  W  N  K  P  S  K  P  K  T  N  M  K  H  M
                 69,960    69,970    69,980    69,990    70,000    70,010    70,020  bp
                ---------+---------+---------+---------+---------+---------+---------+
AL133396        CTGGTGCTGCAGCAGCTGGGGCAGTGGTGGGGGGCCTTGGCGGCTACATGCTGGGAAGTGCCATGAGCAG
T98G PrP         ::::::::::::::::::::::::::::::::::::::::::::::A:::::::::::::::::::::    417
T98G PrPSV       ::::::::::::::::::::::::::::::::::::::::::::::A:::::::::::::::::::::    417
amino acid       A  G  A  A  A  A  G  A  V  V  G  G  L  G  G  Y M/V L  G  S  A  M  S  R
                 70,030    70,040    70,050    70,060    70,070    70,080    70,090  bp
                ---------+---------+---------+---------+---------+---------+---------+
AL133396        GCCCATCATACATTTCGGCAGTGACTATGAGGACCGTTACTATCGTGAAAACATGCACCGTTACCCCAAC
T98G PrP         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    487
T98G PrPSV       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    487
amino acid         P  I  I  H  F  G  S  D  Y  E  D  R  Y  Y  R  E  N  M  H  R  Y  P  N
                 70,100    70,110    70,120    70,130    70,140    70,150    70,160  bp
                ---------+---------+---------+---------+---------+---------+---------+
AL133396        CAAGTGTACTACAGGCCCATGGATGAGTACAGCAACCAGAACAACTTTGTGCACGACTGCGTCAATATCA
T98G PrP         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    557
T98G PrPSV       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    557
amino acid       Q  V  Y  Y  R  P  M  D  E  Y  S  N  Q  N  N  F  V  H  D  C  V  N  I
```


70,170 70,180 70,190 70,200 70,210 70,220 70,230 bp
AL133396 CAATCAAGCAGCACACGGTCACCACAACCACCAAGGGGGAGAACTTCACCGAGACCGACGTTAAGATGAT
T98G PrP 627
T98G PrPSV 627
amino acid T I K Q H T V T T T T K G E N F T E T D V K M M
70,240 70,250 70,260 70,270 70,280 70,290 70,300 bp
AL133396 GGAGCGCGTGGTTGAGCAGATGTGTATCACCCAGTACGAGAGGGAATCTCAGGCCTATTACCAGAGAGGA
T98G PrP 697
T98G PrPSV [cryptic 5'-splice site 660
amino acid E R V V E Q M C I T Q Y E R E S Q A Y Y Q R G
70,310 70,320 70,330 70,340 70,350 70,360 70,370 bp
AL133396 TCGAGCATGGTCCTCTTCTCCTCTCCACCTGTGATCCTCCTGATCTCTTTCCTCATCTTCCTGATAGTGG
T98G PrP 767
T98G PrPSV 660
amino acid S S M V L F S S P P V I L L I S F L I F L I V
70,380 70,390 70,400 70,410 70,420 70,430 70,440 bp
AL133396 GATGAggaaggtcttcctgttttcaccatctttctaatctttttccagcttgagggaggcggtatccacc
T98G PrP 837
T98G PrPSV 660
amino acid G *
70,450 70,460 70,470 70,480 70,490 70,500 70,510 bp
AL133396 tgcagccctttagtggtggtgtctcactctttcttctctctttgtcccggataggctaatcaataccct
T98G PrP 907
T98G PrPSV cryptic 3'-splice site] 675
amino acid L I N T L
70,520 70,530 70,540 70,550 70,560 70,570 70,580 bp
AL133396 tggcactgatgggcactggaaaacatagagtagacctgagatgctggtcaagccccctttgattgagttc
T98G PrP 977
T98G PrPSV 745
amino acid G T D G H W K T *
70,590 70,600 70,610 70,620 70,630 70,640 70,650 bp
AL133396 atcatgagccgttgctaatgccaggccagtaaaagtataacagcaaataaccattggttaatctggactt
T98G PrP 1,047
T98G PrPSV 815
amino acid
70,660 70,670 70,680 70,690 70,700 70,710 70,720 bp
AL133396 atttttggacttagtgcaacaggttgaggctaaaacaaatctcagaacagtctgaaatacctttgcctgg
T98G PrP 1,117
T98G PrPSV 885
amino acid

```
                70,730    70,740    70,750    70,760    70,770    70,780    70,790  bp
            ----------+---------+---------+---------+---------+---------+---------+
AL133396    atacctctggctccttcagcagctagagctcagtatactaatgccctatcttagtagagatttcatagct
T98G PrP    :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::  1,187
T98G PrPSV  :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    955
amino acid
                70,800    70,810    70,820    70,830    70,840    70,850    70,860  bp
            ----------+---------+---------+---------+---------+---------+---------+
AL133396    atttagagatattttccattttaagaaaacccgacaacatttctgccaggtttgttaggaggccacatga
T98G PrP    :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::  1,257
T98G PrPSV  :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::  1,025
amino acid
                70,870    70,880    70,890    70,900    70,910    70,920    70,930  bp
            ----------+---------+---------+---------+---------+---------+---------+
AL133396    tacttattcaaaaaaatcctagagattcttagctcttgggatgcaggctcagcccgctggagcatgagct
T98G PrP    :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::  1,327
T98G PrPSV  :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::  1,095
amino acid
                70,940    70,950    70,960    70,970    70,980    70,990    71,000  bp
            ----------+---------+---------+---------+---------+---------+---------+
AL133396    ctgtgtgtaccgagaactggggtgatgttttacttttcacagtatgggctacacagcagctgttcaacaa
T98G PrP    :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::  1,397
T98G PrPSV  :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::  1,165
amino acid
                71,010    71,020    71,030    71,040    71,050    71,060    71,070  bp
            ----------+---------+---------+---------+---------+---------+---------+
AL133396    gagtaaatattgtcacaacactgaacctctggctagaggacatattcacagtgaacataactgtaacata
T98G PrP    ::::::::::::::::::::::::::::::::::::                                  1,433
T98G PrPSV  ::::::::::::::::::::::::::::::::::::                                  1,201
amino acid
```

'Accession nos. AB300823-4. 'Accession nos. AB300825-6.

Fig. S2. Immunoblot analysis of recombinant human PrP and its splice variant. Recombinant PrP [codon 129M (lane 1) and 129V (lane 2)], splice variant of PrP [codon 129M (lane 3) and 129V (lane 4)], and homogenates (lane 5) from P40D40 T98G cells were subjected to immunoblotting with HUC2-13 (A), 17H5 (B), HPC2 (C) or HPSV178 (D) antibodies as described in Methods. Epitope recognition sites located within PrP and PrPSV are shown as residues numbers. To detect PrPSV, we established an HPSV178 mAb against the carboxy-terminal portion of PrPSV (residues 214-230; CITQLINTLGTDGHWKT). We also prepared constructs of recombinant PrP (residues 23-230) and PrPSV (residues 23-230) with the 129 M and V genotypes to estimate the specificity of HPSV178. Anti-N-terminus PrP mAb HUC2-13 (A) and anti-C-terminus PrP mAb 17H5 (B) recognized both recombinant PrP and PrPSV as well as homogenates of P40D40 T98G cells at 25 kDa. HPC2 antibody against the carboxy-terminal portion of PrP (residues 214-230; CITQYERESQAYYQRGS) reacted with recombinant PrP but not with PrPSV (C). HPSV178, in contrast, reacted strongly with recombinant PrPSV but not with PrP (D).

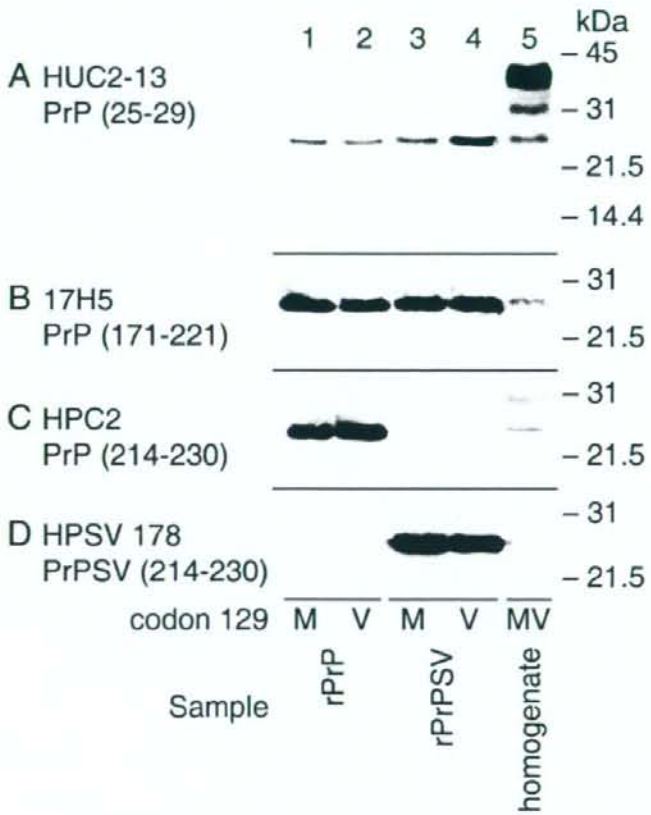


Fig. S3. Alignment of amino acid sequences of plasminogen activator inhibitor-1 and GPI¯PrPSV by FASTA searches. We compared the amino acid sequences of PrPSV (residues 210-230) and PAI-1 protein (AAA60009, 402 amino acids) using the FASTA program. The sequence of residues 168-210 are shown. Identical amino acids are indicated by dots. The newly added carboxy-terminal portion of PrPSV is underlined.

```
        170       180       190       200       210
        --+---------+---------+---------+---------+
PAI-1   KGMISNLLGKGAVDQLTRLVLVNALYFNGQWKTPFPDSSTHRR
                    :.:.    :.:.:  .:.:::
PrPSV               VEQMCITQLINTLGTDGHWKT
                    +---------+---------+
                   210       220       230
```