395 PrP^{Sc} (21, 33), whereas binding domains on PrP^{Sc} that are involved in binding to PrP^{C} still
396 remain undetermined. The N-terminally truncated PrP^{Sc} may be useful for the analysis of
397 the binding domain on the PrP^{Sc} molecule to PrP^{C}. Here, we showed an example of the
398 possible biochemical approach of PrP^{Sc} manipulation, in which we directly produced the
399 N-terminally truncated PrP^{Sc} from native PrP^{Sc}. It has been reported that some conditions
400 (*e.g.*, pH) of protease digestion affect the N-terminal truncation of the PK-resistant core of
401 PrP^{Sc} (24). Thus, further investigation of region-specific denaturation and proteolysis may
402 be useful not only for the analysis of prion strains but also for the manipulation of PrP^{Sc}.
403
404
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406
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FIGURE LEGENDS

Figure 1. Conformational stability of PrP^{Sc} of various prion strains. (A) Immunoblots for conformational-stability assay. Brain homogenates from prion-infected mice (indicated on the left) were treated with 0 - 4 M GdnHCl (indicated on the top) followed by PK digestion. PrP^{Sc} was detected by either pAb B103 (left colums) or mAb 44B1 (right column). Independent assays of each strain were carried out at least 3 times for mAb 44B1 (indicated in parentheses), and based on quantitative results of the blot probed with mAb 44B1, the denaturation curves were plotted using a non-linear least-squares fit. Half-maximum GdnHCl concentrations, $[GdnHCl]_{1/2}$, are indicated for each graph (mean ± SD). Numbers at the top-right corner in the blots probed with pAb B103 represent the $[GdnHCl]_{1/2}$ values (M). (B) Molecular weights of PrP^{Sc}. Brain homogenates from prion-infected mice (indicated on the top) were treated with PK and immunoblot was probed with pAb B103 (left). To compare the molecular weight of PK-resistant core of PrP^{Sc} more precisely, PK-treated samples were further treated with PNGasae F (right).

Figure 2. Region-dependent conformational stability of PrP^{Sc} of the Chandler strain. Brain homogenates from mice infected with the Chandler (left) and the Obihiro (right) strains were subjected to the conformational-stability assay and immunoblots were probed with various anti-PrP antibodies indicated on the left. Epitopes for antibodies are indicated in parentheses. Due to the relatively weak reactivity, 5-times sample volumes were loaded for mAb 118. Numbers at the top-right corner in each blot represent the $[GdnHCl]_{1/2}$ values (M).

Figure 3. Region-dependent conformational stability of PrP^{Sc} in cells persistsently

infected with the Chandler strain. PrP^{Sc}-enriched fractions obtained from ScN2a-5 cells were subjected to the conformational stability assay. Antibodies used are indicated on the left.

Figure 4. Region-dependent conformational stability of the Chandler PrP^{Sc} in mice with different genetic backgrounds. Brain homogenates from Jcl:ICR (*Prnp*$^{a/a}$), C57BL/6J (*Prnp*$^{a/a}$), and I/LnJ (*Prnp*$^{b/b}$) mice infected with the Chnadler strain were subjected to the conformational-stability assay. Antibodies used and their epitopes (in parentheses) are indicated. Numbers at the top-right corner in each blot represent the $[GdnHCl]_{1/2}$ values (M).

Figure 5. Region-specific denaturation or removal of PrP^{Sc} in inoculums for bioassay. (A) Confirmation of region-specific denaturation. Brains of mice infected with the Chandler strain were treated with various concentrations of GdnHCl (without PK treatment) and the fraction containing PrP^{Sc} was recovered by ultracentrigation. Small aliquots of the inoculums were treated with PK and analyzed by immunoblotting with mAb 44B1. (B) Confirmation of removal of the aa 81-137. Brain homogenates from mice infected with the Chandler and the Obihiro strains were treated with 0 or 3 M GdnHCl and followed by PK digestion. After terminating proteolysis, samples were ultracentrifuged to collect the fraction containing PrP^{Sc}. Small aliquots of the inoculums were analyzed by immunobloting with mAb 44B1. Equal brain tissues equivalent was loaded in each lane.

Figure 6. Schematic representation of region-specific denaturation of the Chandler PrP^{Sc}. PK-resistant core of the Chandler PrP^{Sc} (from ~aa 81 to 231) were depicted with the

592 locations of two β-strands (β1 and β2), three α-helices (α1 to α3), two N-glycosylation sites
593 (CHO), and an intramolecular disulfide bond (S-S). The locations of epitopes were
594 indicated with thick lines with aa numbers (in parentheses). The epitope for mAb 44B1 that
595 recognizes discontinous epitope was indicated with dashed line, while those for other
596 antibodies that recognize linear epitope were indicated with solid lines. The region I
597 indicated above (aa 81- 90) was denatured almost completely by up to 2 M GdnHCl treatment,
598 and the removal of this region generates the 1-2 kDa smaller PK-resistant PrP^{Sc}. The region
599 II (aa 90- 137) was denatured almost completely by up to 3 M GdnHCl treatment, and the
600 removal of the regions I and II consequently generates the 6-7 kDa smaller PK-resistant PrP^{Sc}
601 (region III, aa 137-C-terminus) that is highly resistant to denaturation but lacks prion
602 infectivity.

603 Table 1. Conformational stabilities and incubation periods of prion strains

Prions	Mouse strain for propagation	Number of serial passage[a]	$[GdnHCl]_{1/2}$ (M) pAb B103	$[GdnHCl]_{1/2}$ (M) mAbs 44B1[f] /31C6[g]	Incubation periods (days, mean ± SD)	Number of mice[h]
G1	slc:ICR	4[b]	2.1	2.1 ± 0.1[f]	326 ± 53	5
Obihioro	Jcl:ICR	>5	2.3	2.0 ± 0.0[f]	153 ± 7	24
Chandler	Jcl:ICR	>5	1.8	3.2 ± 0.2*[f]	150 ± 8	20
	I/LnJ	2[c]	2.2	>3.5[g]	227 ± 7	4
	C57BL/6J	3[c]	2.3	3.5[g]	153 ± 6	6
22L	Jcl:ICR	2[d]	1.5	1.7 ± 0.0**[f]	144 ± 3	5
Fukuoka-1	Jcl:ICR	2[d]	2.1	2.0 ± 0.0[f]	146 ± 8	8
KUS-m	RIII/J	3[e]	2.4	2.5 ± 0.2*[f]	165 ± 11	6
TE-m	C57BL/6J	3[e]	2.2	2.6 ± 0.2*[f]	168 ± 4	6

604 [a] History (number) of serial passage in mice listed on the left.

605 [b] Source of prion: experimental sheep scrapie G1.

606 [c] Chandler strain passaged in Jcl:ICR mice were then passaged in I/LnJ or C57BL/6J mice.

607 [d] Source of prions: the 22L and Fukuoka-1 strains passaged in mice carrying *Prnp*$^{a/a}$ but

608 different from Jcl:ICR mice.

609 [e] Source of prions: BSE field cases KUS and TE.

610 [f] The $[GdnHCl]_{1/2}$ values were estimated from the denaturation curves plotted by blots probed

611 with mAb 44B1 (at least three independent asssays). *, higher than G1 ($p < 0.05$); **, lower

612 than G1 ($p < 0.05$).

613 [g] The $[GdnHCl]_{1/2}$ values were estimated from the denaturation curves plotted by blots probed

614 with mAb 31C6.

615 [h] Number of mice used for the calculation of incubation period.

616 Table 2. Effect of GdnHCl treatment and PK digestion on prion infectivity

Strain	GdnHCl (M)	PK	Mice[a] (n/N)	Survival time (Mean ± SD) (dpi)
Chandler	0	–	4/4	159 ± 14
	1	–	5/5	150 ± 9
	1.5	–	7/7	165 ± 12
	2	–	4/4	176 ± 12
	3	–	5/5	207 ± 25
	0	+	6/6	170 ± 11
	3	+	2/5[b]	234, 236, >365
Obihiro	0	+	5/5	152 ± 7
	3	+	5/5	186 ± 11

617

618 [a] n, number of mice which showed typical clinical manifestations of scrapie and/or were
619 positive for PrP^{Sc} in immunoblotting; N, number of mice used in bioassay.

620 [b] Two mice showed typical clinical manifestations and were positive for PrP^{Sc} (at 234 and 236
621 dpi), one mouse was found died without any symptoms at 336 dpi and was negative for PrP^{Sc}.
622 Remaining two mice were still alive without any symptoms (>365 days).

623

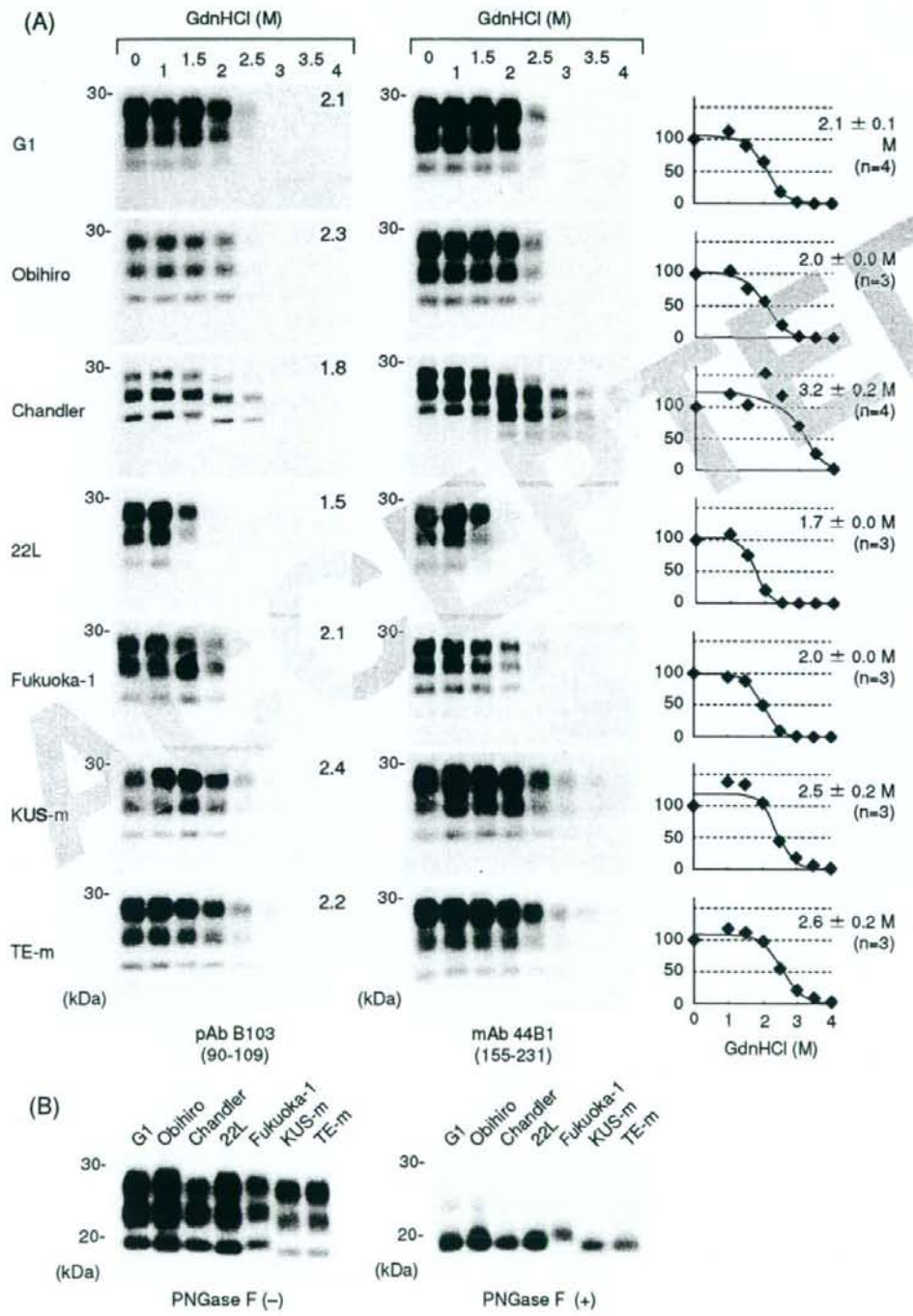


Fig. 1 Shindoh et al.,

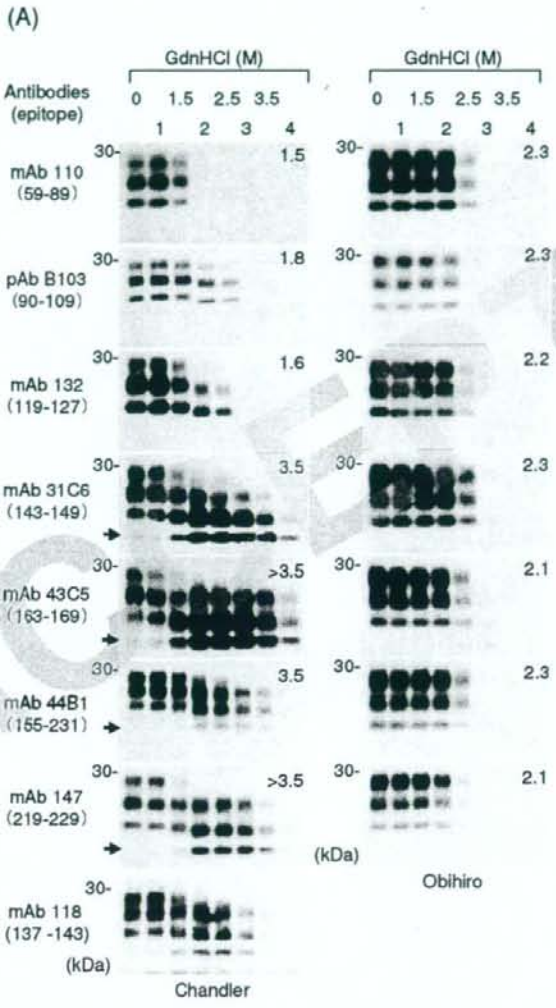
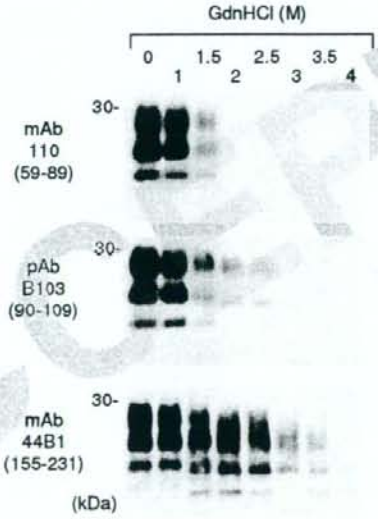


Fig. 2 Shindoh et al.,



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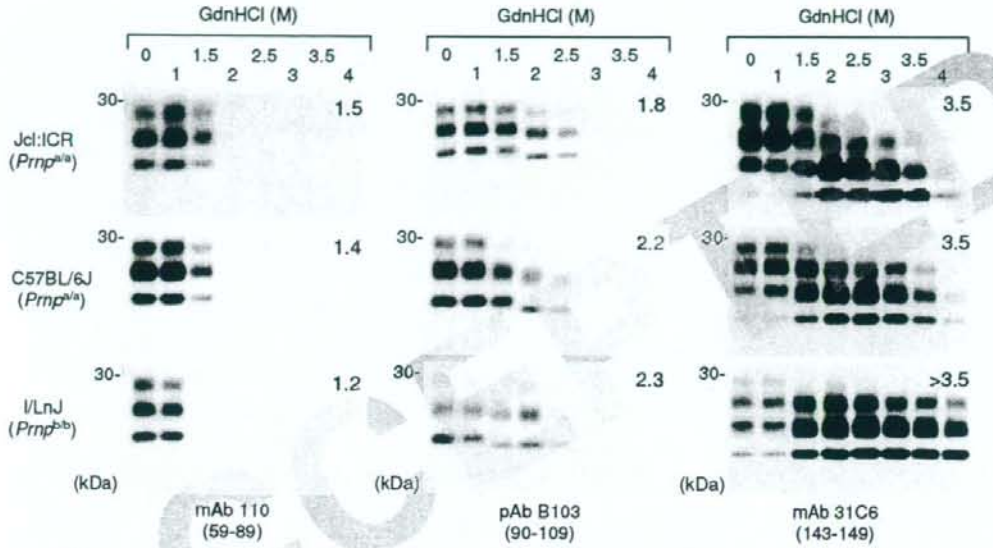

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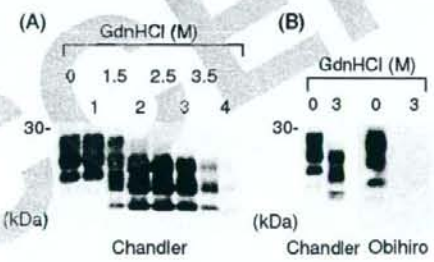

Fig. 5 Shindoh et al.,

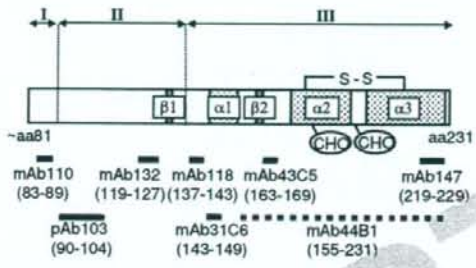

Fig. 6 Shindoh et al.,

the FEBS Journal

Hypoxia induces expression of a GPI-anchorless splice variant of the prion protein

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Keywords
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The human prion protein (PrP) is a glycoprotein with a glycosylphosphatidylinositol (GPI) anchor at its C-terminus. Here we report alternative splicing within exon 2 of the *PrP* gene (*PRNP*) in the human glioblastoma cell line T98G. The open reading frame of the alternatively spliced mRNA lacked the GPI anchor signal sequence and encoded a 230 amino acid polypeptide. Its product, GPI-anchorless PrP (GPI$^-$ PrPSV), was unglycosylated and soluble in non-ionic detergent, and was found in the cytosolic fraction. We also detected low levels of alternatively spliced mRNA in human brain and non-neuronal tissues. When long-term passaged T98G cells were placed in a low-oxygen environment, alternatively spliced mRNA expression increased and expression of normally spliced PrP mRNA decreased. These findings imply that oxygen tension regulates GPI$^-$ PrPSV expression in T98G cells.

Fatal human prion diseases, including sporadic, iatrogenic and variant Creudzfeldt–Jakob disease (CJD), inherited prion diseases and kuru, are transmissible spongiform encephalopathies characterized by the formation and accumulation of an abnormal isoform of prion protein (PrP) in the brain [1]. Cellular prion protein (PrP^C) is a glycoprotein that is anchored to the cell surface by a glycosylphosphatidylinositol (GPI) moiety [1]. CJD is associated with the conversion of PrP^C into a protease-resistant isoform (PrP^{res}), either on the cell surface or within its compartments [1].

Sporadic CJD is classified on the basis of the molecular mass of the unglycosylated fragment of PrP^{res} as type 1 (21 kDa) or type 2 (19 kDa), and on the basis of the genotype at the methionine/valine polymorphic codon 129, i.e. MM, MV or VV [2]. In a previous study, we showed that the prion protein gene (*PRNP*) in human glioblastoma cell line T98G, which is of the MV genotype and produces a form of PrP that is partially resistant to proteinase K (PK) following long-term culture and high passage number, has no mutation in the coding region [3]. The PrP^{res} fragment described here, which differed from corresponding fragments in typical sporadic CJD, had a mass of 18 kDa after deglycosylation and was detergent-soluble [3]. However, in one report, brain homogenates from dead patients with type 2 PrP and MV ($n = 5$) or VV ($n = 6$) genotypes also contained PrP fragments that migrated at 18 kDa after deglycosy-

Abbreviations
AD, Alzheimer's disease; CJD, Creudzfeldt–Jakob disease; GPI, glycosylphosphatidylinositol; GPI$^-$, GPI-anchorless; PK, proteinase K; PNGase F, peptide *N*-glycosidase F; *PRNP*, prion protein gene; PrP, prion protein; PrP^C, cellular PrP; PrP^{res}, protease-resistant isoform of PrP; PrPSV, splice valiant of PrP.

lation and were detergent-soluble [4]. These findings support the possibility that our findings in T98G cells may be relevant to PrP^{res} in sporadic CJD brain.

Alzheimer's disease (AD) and prion disease share a common feature – aggregation and deposition of abnormal proteins [5]. Intracerebral injection of post mortem brain extracts from AD patients induced deposition of amyloid β–peptides in the hippocampus of β-amyloid preursor protein transgenic mice [6]. Some cohort studies have indicated that cerebral ischemia and stroke significantly increase AD risk [7,8], and hypoxia seems to be an important contributor to the onset and progression of AD [9]. Recent magnetic resonance imaging studies have also suggested that changes in areas of the brain with the highest oxygen requirement are associated with sporadic CJD [10,11]. A retrospective study detected these changes in 39.1% of sporadic CJD patients (n = 1036) [10]. A study of human cerebral ischemia and perinatal hypoxic/ischemic injury confirmed the presence of PrP^{C} immunoreactivity within axons in the penumbra of white matter damage and within neuronal soma of gray matter damage [12]. We therefore speculated that oxidative stress is a causative factor in prion disease. To test this hypothesis, we investigated the effects of hypoxia on PrP expression using T98G cells as our model system.

Results

Detection of the splice variant form of PrP mRNA in T98G cells

First, we analyzed *PRNP* mRNA by RT-PCR. We used total RNA isolated from T98G cells to generate RT-PCR products from *PRNP* exon 2. We found that cells grown under normoxic conditions produced a 528 bp product (supplementary Fig. S1) when the cells were cultured for 24 days after two passages (P2D24) and for 24 days after 18 passages (P18D24) (Fig. 1A). In contrast, total RNA from P18D24 cells exposed to hypoxic conditions (5% O_2) for the last day expressed a shorter product, i.e. 296 bp, but the genomic DNA from P18D24 cells and total RNA from P2D24 cells did not express this product (Fig. 1A). Because addition of cobalt ion can mimic hypoxic conditions [13], we next studied its effects on RT-PCR products. Total RNA from P2D39 cells cultured with 0 or 300 μM $CoCl_2$ for the last day yielded the 528 bp RT-PCR product (Fig. 1B). Total RNA from P13D24 cells cultured the same way, however, expressed the 528 bp product and the shorter RT-PCR product (Fig. 1B), just as P18D24 cells exposed to hypoxic conditions (Fig. 1A). To amplify the shorter RT-PCR product, we decreased the elongation time to 36 s to avoid saturation by the longer PCR product [14]. With an elongation time of 60 s, the shorter RT-PCR product was not amplified and only the 528 bp product was produced (data not shown).

When we performed direct sequencing of the shorter product, we found that a 232 bp sequence was missing from the 528 bp sequence (supplementary Fig. S1). We identified an intronic sequence with the canonical dinucleotides for splicing (GT at the 5′ end and AG at the 3′ end) and a pyrimidine tract (16 pyrimidines/20 bases) 20 nucleotides upstream of the 3′ splice site [15,16]. Thus, our data indicate that alternative splicing occurred within *PRNP* exon 2.

To determine the cryptic splice sites, we designed exon–exon junction primers that annealed with the donor and acceptor sites (E2SV3, E2SV4 and E2SV5; supplementary Table S1), and used total RNA isolated from P18D24 cells to generate RT-PCR products. As expected, we detected a 1433 bp product from cells grown under hypoxic conditions. In addition, we detected two shorter RT-PCR products – a 676 bp product when we used E2SV3 and a 553 bp product when we used E2SV4 (Fig. 1C). Surprisingly, we also detected these products in total RNA from cells grown under normoxic condition (Fig. 1C). However, when using genomic DNA, we detected only full-length PCR products (Fig. 1C). Thus, the exon–exon junction primers were able to detect mRNA for the splice variant of PrP (PrPSV).

Direct sequence analysis revealed that the only mutation in the 1433 bp RT-PCR product was an adenine to guanine substitution in the first position of codon 129, i.e. the common M129V polymorphism (supplementary Fig. S1; T98G PrP, accession numbers AB300823 and AB300824); the alternatively spliced 1201 bp product also contained the polymorphism (supplementary Fig. S1; T98G PrPSV, accession numbers AB300825 and AB300826). *PRNP* encodes a 253 amino acid polypeptide, including an N-terminal signal sequence (residues 1–22) and a GPI anchor signal sequence (residues 231–253) (Fig. 1D, upper part). Alternative splicing resulted in use of exons 2a and 2b with a cryptic donor site and a cryptic acceptor site (Fig. 1D, lower part), with an open reading frame encoding a 230 amino acid polypeptide comprising the N-terminal portion (residues 1–217) of PrP from exon 2a and the C-terminal peptide (residues 218–230) from exon 2b (lower panel).

Expression of the GPI-anchorless splice variant of PrP in T98G cells

We next investigated the prion protein expressed by the alternatively spliced mRNA. To detect PrPSV, we raised