

Fig. 5. Intraventricular infusion of anti-PrP mAb prolongs survival. Intraventricular infusion of mAbs into mice inoculated with the Obihiro or Chandler strain was initiated at 60, 90 or 120 days p.i. using an Alzet Mini-Osmotic Pump model 2002 (antibody concentration 2 mg ml^{-1} , pumping rate $0.5 \mu\text{l h}^{-1}$, duration 14 days, volume $200 \mu\text{l}$). Mice were observed until they reached the terminal stage of the disease. The graphs show survival curves, and survival times (means \pm SD) in days are shown in Table 1, together with the numbers of mice in each group. \circ , Mice treated with mAb P2-284; \blacksquare , mice treated with mAb 31C6. The presence of PrP^{Sc} was confirmed by Western blotting in all experimental-group mice.

action between PrP^C and PrP^{Sc} (Enari *et al.*, 2001; Peretz *et al.*, 2001). It has also been suggested that a perturbation of the usual PrP^C trafficking by binding of the antibody to PrP^C on the cell surface, e.g. sequestration of PrP^C on the cell membrane, may be one of the mechanisms of inhibition (Kim *et al.*, 2004b; Feraudet *et al.*, 2005). Consistent with our previous observations (Kim *et al.*, 2004b), mAbs directed against the C-terminal domain (mAb 44B1) and the octapeptide repeat in the N-terminal region (mAb 110), as well as one directed against the first α -helix (mAb 31C6), antagonized PrP^{Sc} formation in the mouse brain (Fig. 2). Indeed, anti-PrP mAb infused into the lateral ventricle was still detectable in the hippocampus at 20 days after the termination of infusion, whereas only a low level of the negative-control mAb was detected in the same region, even during infusion (Fig. 1). These results suggest that the antibody-PrP^C complex remains in the brain parenchyma and therefore that sequestration of PrP^C

by the antibody is implicated in the inhibition of PrP^{Sc} formation *in vivo*.

Intraventricular infusion of anti-PrP mAbs at a late stage of infection (initiated at 120 days p.i.) reduced levels not only of PrP^{Sc} accumulation but also of microglial activation, astrogliosis and spongiform changes. Comparison of neurohistopathological changes observed at 127 versus 150 days p.i. in mice treated with anti-PrP mAbs and the negative-control mAb revealed an apparent reduction in gliosis observed at 150 days p.i., which may be due to a slowdown in the progression of gliosis (data not shown). Although the severity of microglial activation and astrogliosis differed in animals infected with the two different prion strains, the reduction observed was in accordance with the mAb distribution, and the levels of microglial activation and astrogliosis in the hippocampus and thalamus of mice infused with anti-PrP mAbs appeared

Table 1. Effect of intraventricular infusion of anti-PrP mAbs on survival of mice infected with the Obihiro or Chandler strain

Initiation of mAb infusion (days p.i.)	Survival time of mice [mean \pm SD (days)]			
	Obihiro strain		Chandler strain	
	P2-284 (n)	31C6 (n)	P2-284 (n)	31C6 (n)
60	153.4 \pm 9.0 (8)	163.5 \pm 8.1* (10)	150.7 \pm 10.2 (7)	161.0 \pm 8.3* (6)
90	152.8 \pm 4.3 (6)	153.3 \pm 3.5 (7)	151.7 \pm 9.8 (10)	165.0 \pm 10.9* (9)
120	154.4 \pm 9.4 (8)	157.4 \pm 7.0 (8)	152.9 \pm 7.4 (7)	164.9 \pm 9.3* (8)

*Statistically significant difference based on Student's *t*-test ($P < 0.05$).

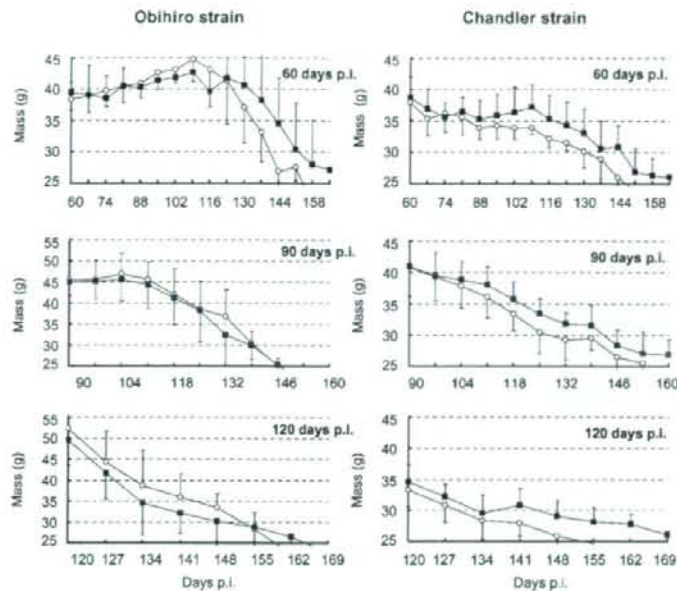


Fig. 6. Changes in body weight. After the initiation of mAb infusion, mice were weighed weekly up to the terminal stage of the disease. The groups of mice used in Fig. 5 were used to monitor body weight. ○, Mice treated with mAb P2-2B4; ■, mice treated with mAb 31C6.

to be milder than those observed in mice infused with the negative-control mAb. In contrast, no apparent reduction in microglial activation or astrogliosis by anti-PrP mAb was observed in the lateral cortex, where delivery of mAbs appeared to be inefficient (data not shown). It has been reported that the process of conversion of PrP^C to PrP^{Sc} on neurons rather than extracellular deposition of PrP^{Sc} is involved in neuronal degeneration (Brandner *et al.*, 1996; Mallucci *et al.*, 2003; Chesebro *et al.*, 2005). Although the mechanism by which PrP^{Sc} formation provokes microglial activation and astrogliosis remains to be elucidated, these results imply that arresting conversion of PrP^C to PrP^{Sc} via mAb infusion may contribute, at least to some extent, to the observed reduction in microglial activation and astrogliosis.

Previous results have shown that passive immunization with anti-PrP mAb via intraperitoneal injection does not have a protective effect after invasion of prion into the CNS or inoculation of prion via the intracerebral route (White *et al.*, 2003). In contrast, intraventricular infusion of anti-PrP mAb prolonged survival when mAb was infused at the time that PrP^{Sc} became detectable in the CNS (e.g. at 60 days p.i.). The difference seems to be explained by inadequate transfer of anti-PrP mAbs into the CNS across the blood-brain barrier when mAbs are administered peripherally. Several compounds, including amphotericin B, PPS, porphyrin derivatives and GN8, have been reported to prolong the survival of mice infected with prion when administered at the middle or late stage of infection, but the animals were still prior to clinical onset (Demaimay *et al.*, 1997; Doh-ura *et al.*, 2004; Kocisko *et al.*, 2006; Kuwata

et al., 2007). To the best of our knowledge, there has been only one report of a treatment that prolongs survival of animals already in the clinical phase. Specifically, intraperitoneal administration of MS-8209 was shown to prolong survival of mice infected with the C506M3 strain when treatment was carried out at the time of the appearance of neurological symptoms (Demaimay *et al.*, 1997). Human prion diseases are usually detected after clinical onset and thus the availability of treatments that are effective even after symptoms have begun to appear is highly important. Therefore, it is of interest that intraventricular infusion of anti-PrP mAb was effective for prolongation of survival, even when treatment was initiated after the appearance of early clinical signs (at 120 days p.i.) in mice infected with the Chandler strain. Although in this study mAb infusion for 14 days of duration achieved only 8% prolongation, the result should encourage further trials with mAbs that may be useful in the development of therapeutic treatment for prion diseases. For instance, continuing treatment over a longer duration, which may keep the effective concentration of mAbs in the brain higher over a longer period of time, may improve the effect on survival. In addition, anti-PrP mAb prolonged survival, despite the fact that the infused mAb was not evenly distributed in the brain but rather was primarily restricted to the hippocampus and thalamus. Thus, it is conceivable that improved delivery of mAb might enhance the effects of anti-PrP mAb on survival. Fab and single-chain antibody fragments have also been shown to inhibit PrP^{Sc} formation (Peretz *et al.*, 2001; Donofrio *et al.*, 2005), and the smaller size of these fragments may be beneficial for efficient delivery in tissues.

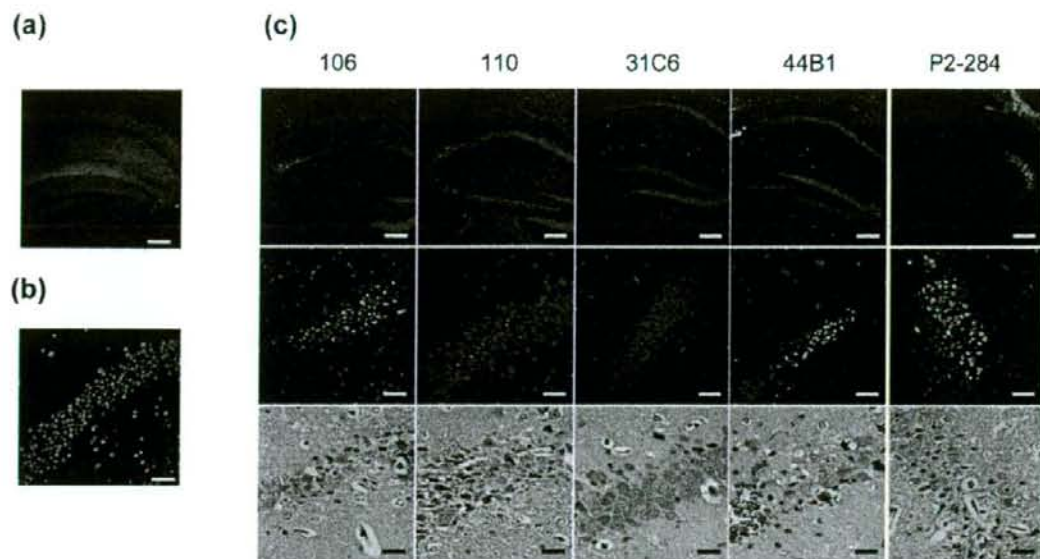


Fig. 7. Neuronal toxicity of anti-PrP mAbs. Anti-PrP mAbs (106, 110, 31C6 or 44B1) and the negative-control mAb P2-284 were injected into the left and right hippocampus, respectively, using stereotaxic apparatus. Seventy-two hours after injection, brains were obtained and fixed in 10% formalin. Paraffin sections were subjected to TUNEL and H&E staining. (a) Distribution of mAb. Alexa Fluor 488-conjugated mAb 31C6 was injected into the hippocampus and the distribution was analysed by confocal laser microscopy. (b) Positive control. Coronal sections were pre-treated with DNase I and then stained using the TUNEL procedure. The pyramidal cell layers of the hippocampus are shown. (c) TUNEL- and H&E-stained samples. Low-magnification (top panels) and high-magnification micrographs (middle panels) of TUNEL-stained samples and corresponding H&E-stained samples (bottom panels) are shown. The mAbs injected are indicated above the panels. Three to six mice were examined for each mAb. Bars, 200 μ m.

Although mice infected with either the Obihiro or Chandler strain succumbed to the disease at around 150 days p.i., the effect of mAb infusion on survival differed when administered to mice challenged with different prion strains. For example, mAb infusion of mice infected with the Chandler strain was effective when the mAb was administered at any of the three time points tested (60, 90 and 120 days p.i.), whereas no prolongation was observed in mice infected with the Obihiro strain when mAb infusion was initiated at 90 or 120 days p.i. (Fig. 6). At present, we do not have a precise explanation for this but can speculate on what might explain the difference. First, microglial activation in mice infected with the Obihiro strain was more severe than that in mice infected with the Chandler strain (Figs 3 and 4). Similar to the mouse model for Alzheimer's disease (El Khoury *et al.*, 2007), microglial recruitment is expected to have a protective role in prion disease; however, activated microglia also have neurotoxic effects via production of cytokines, chemokines and reactive oxygen species (Bate *et al.*, 2001, 2002; Marella *et al.*, 2005). Thus, even when mitigated by anti-PrP mAb, the severe microglial activation in mice infected with Obihiro strain may be sufficient for

progression of the disease. Secondly, differences in the distribution of PrP^{Sc} may account for the differences in the effect of anti-PrP mAb in one prion strain versus the other. For instance, PrP^{Sc} accumulation in the hypothalamus of mice infected with the Obihiro strain was more severe than that in mice infected with the Chandler strain (data not shown). Moreover, PrP^{Sc} formation in the hypothalamus might not be inhibited efficiently due to the uneven distribution of mAb in the hypothalamus (Fig. 1). These results may explain in part the lack of prolongation in the Obihiro strain-infected mice infused with mAb at 90 or 120 days p.i.

Immunotherapy has been of interest in the treatment of Alzheimer's disease; however, the fact that immunization of β -amyloid peptide caused meningoencephalitis in some patients in clinical trials warns of the adverse effects of autoimmune reactions *in vivo* (Check, 2002; Nicoll *et al.*, 2003; Orgogozo *et al.*, 2003). Additionally, cross-linking of PrP^C by an anti-PrP mAb that recognizes a specific epitope (aa 95–105) provoked degeneration of hippocampal and cerebellar neurons (Solforsio *et al.*, 2004). These adverse effects of antibodies on the CNS have prompted extreme

caution in the use of anti-PrP antibodies, in particular, their introduction into the CNS. However, passive immunization is less likely to initiate an autoimmune reaction (Schenk, 2002; Sadowski & Wisniewski, 2004). In this study, we observed no antibody-induced inflammation by intraventricular infusion. In addition, neuronal death in the hippocampus was not observed, even though mAbs 106 and 110, which recognize the region adjacent to aa 95–105, were injected directly into the hippocampus. Although the potential adverse effects, especially an inflammatory response, should be examined carefully, the fact that anti-PrP mAbs interfered with disease progression, even when administered after clinical onset, is particularly encouraging. Although the effect of anti-PrP mAb differed in prion strains, this implies that the immunotherapy might be effective in certain types of human prion disease, if not all. Therefore, the results of this study should promote further efforts to improve the effect of anti-PrP mAbs, such as the form of the antibody, the route of administration and an efficient way of delivering the antibodies.

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1 The region approximately between amino acids 81 and 137 of proteinase K-resistant PrP^{Sc} is
2 critical for the infectivity of the Chandler prion strain

3

4 Running title: Infectivity of N-terminal truncated PrP^{Sc}

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24 **Abstract**

25

26 Although the major component of prion is believed to be the oligomer of PrP^{Sc}, little
27 information is available concerning regions on the PrP^{Sc} molecule that affect prion infectivity.
28 During the analysis of PrP^{Sc} from various prion strains, we found that PrP^{Sc} of the Chandler
29 strain showed a unique property in the conformational-stability assay, and this property
30 appeared useful for studying the relation between regions of the PrP^{Sc} molecule and prion
31 infectivity. Thus, we analyzed PrP^{Sc} of the Chandler strain in detail and analyzed
32 infectivities of the N-terminally denatured and truncated proteinase K-resistant PrP. The
33 N-terminal region of PrP^{Sc} of the Chandler strain showed a region-dependent resistance to
34 guanidine hydrochloride (GdnHCl) treatment. The region approximately between amino
35 acids (aa) 81 and 137 began to be denatured by the treatment with 1.5 M GdnHCl. Within
36 this region, the region comprised of approximately aa 81-90 was denatured almost completely
37 with 2 M GdnHCl. Furthermore, the region approximately between aa 90 and 137 was
38 denatured completely with 3 M GdnHCl. However, the C-terminal region thereafter was
39 extremely resistant to the GdnHCl treatment. This property was not observed in PrP^{Sc} of
40 other prion strains. Denaturation of the aa 81-137 region by 3 M GdnHCl significantly
41 prolonged the incubation periods compared to the untreated control. More strikingly,
42 denaturation and removal of this region nearly abolished the infectivity. This suggests that
43 the conformation of the region between aa 81 and 137 of the PrP^{Sc} molecule of the Chandler
44 strain is directly associated with the prion infectivity.

45 **INTRODUCTION**

46

47 Prion diseases, such as scrapie, bovine spongiform encephalopathy (BSE) and
48 Creutzfeldt-Jakob disease, are fatal neurodegenerative disorders characterized by
49 accumulation of a disease-specific, abnormal isoform of the prion protein (PrP^{Sc}) in the
50 central nervous system, astrogliosis, neuronal vacuolation and neuronal cell death. PrP^{Sc} is
51 believed to generate from a cellular form of prion protein (PrP^C) by a post-translational
52 modification including conformational transformation. Although the entity of prion, the
53 causative agent of prion diseases, remains to be elucidated, PrP^{Sc} is believed to be a major
54 component of the prion.

55 Direct interaction between PrP^C and pre-existing PrP^{Sc} precedes the transformation of
56 PrP^C into newly generated PrP^{Sc}. Data on the regions of PrP^C that are indispensable for the
57 PrP^{Sc} formation and prion propagation have been accumulated using neuroblastoma cells
58 persistently infected with prion and transgenic mice expressing mutant PrPs. Although the
59 extreme N-terminal region from amino acid (aa) 23 to 32 modulates prion propagation (8, 9,
60 34), the region between aa 32 and around 90 is not essential for production of PrP^{Sc} and
61 propagation of the prion (9, 18, 22, 39). The residues 114-121, the most amyloidogenic
62 region of PrP, is essential for conversion of PrP^C into PrP^{Sc} (14, 23). A deletion mutant
63 lacking the residues 23-88 and 141-176 can convert into PrP^{Sc} and support prion propagation
64 in transgenic (Tg) mice, suggesting that the residues 141-176 is not essential for prion
65 propagation (22, 34). The cysteine residue at 178 that forms an intramolecular disulfide
66 bond with another cysteine residue at 213 is essential for PrP^{Sc} formation (22). Additionally,
67 amino acid substitutions at 167 and 218 prevent the PrP^{Sc} formation and showed
68 dominant-negative effect on prion propagation (15, 28). On the contrary, due to the
69 difficulty of direct manipulation of PrP^{Sc}, the regions of PrP^{Sc} that are important for the prion

70 infectivity have not been elucidated. It is well accepted that not the removal of the
71 protease-sensitive N-terminal domain (aa 23 to around 90) from PrP^{Sc} but the denaturation of
72 the remaining C-terminal domain diminishes the prion infectivity. However, the relationship
73 between prion infectivity and the region(s) of PrP^{Sc} is largely unclear.

74 From the analysis of biochemical properties of PrP^{Sc} of various prion strains, we found
75 that PrP^{Sc} of the Chandler strain has a region-dependent resistance to denaturation by
76 guanidine hydrochloride (GdnHCl). This property allows for the denaturation and removal
77 of specific regions of PrP^{Sc}. In this study, we describe the unique conformational stability of
78 PrP^{Sc} of the Chandler strain and the region approximately between aa 81 and 137 of PrP^{Sc} is
79 important for the infectivity of the Chandler prion strain.

80

81

82 MATERIALS AND METHODS

83

84 **Mice and prion strains.** Mouse-adapted prion strains 22L (7), Chandler (17),
85 Fukuoka-1 (35), G1 (unpublished), and Obihiro (32) were used in this study. These
86 mouse-adapted strains were propagated in female Jcl:ICR mice (CLEA Japan) except where
87 otherwise specified. In some cases, C56BL/6J (CLEA Japan), RIII/J and I/LnJ mice
88 (Jackson Laboratories) were used for prion propagation. In addition, BSE-derived
89 mouse-adapted prion strains, designated KUS-m and TE-m, which were obtained by a third
90 serial passage of Japanese BSE cases KUS and TE with RIII/J and C57BL/6J mice,
91 respectively, were also used. All procedures for animal experiments were carried out
92 according to protocols approved by the Institutional Committee for Animal Experiments.

93

94 **Antibodies.** Anti-PrP mAbs 110, 118, 147, 31C6, 43C5 and 44B1 (16) were used. In

95 addition, B103 rabbit polyclonal antibodies (pAb) raised against bovine PrP synthetic peptide
96 aa 103-121 that corresponds to the aa 90-109 of mouse PrP were also used (12).

97

98 **Conformational-stability assay.** Conformational-stability assays were carried out as
99 described by Legname et al. (19, 20) with some modifications. Brains of mice infected with
100 prion were homogenized in phosphate-buffered saline (PBS) to make 10% homogenates.
101 Aliquots of the homogenates were stored at -30°C until use. The 10% brain homogenates
102 (50 μl) were mixed with equal volumes of various concentrations of GdnHCl (0 to 8 M) and
103 incubated with 37°C for 1 h. Samples were then diluted by adding 850 μl of NTS buffer (10
104 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% Triton X-100 and 0.5% sodium deoxycholate).
105 To adjust the final GdnHCl concentration to 0.4 M, 50 μl of various concentrations of
106 GdnHCl (0 to 8 M) was added to each sample. The samples were then digested with
107 proteinase K (PK; Roche) at 20 $\mu\text{g}/\text{ml}$ for 30 min at 37°C . After terminating PK activity by
108 adding Pefabloc (Roche) to a final concentration at 2 mM, 500 μl of a 5:1 mixture of
109 2-butanol and methanol was added, mixed well, and kept for 10 min at ambient temperature.
110 PrP^{Sc} was pelleted by centrifugation at 20,000 \times g for 10 min at 20°C . The resulting pellet
111 was dissolved in 1x SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 5% glycerol, 3 mM
112 EDTA, 4% β -mercaptoethanol, 0.04% bromophenol blue, 5% SDS, 4 M Urea) by boiling for
113 5 min. SDS-PAGE and immunoblotting were carried out as described elsewhere (38). The
114 chemiluminescence intensities of bands of PrP^{Sc} were measured with a LAS-3000
115 chemiluminescence image analyzer (Fujifilm). Quantitative analyses of the blots were
116 carried out with Image Reader LAS-3000 version 1.11 (Fujifilm). The sigmoidal patterns of
117 denaturation curves were plotted using a non-linear least-squares fit. The concentrations of
118 GdnHCl required to denature 50% of PrP^{Sc} ($[\text{GdnHCl}]_{1/2}$) were estimated from the
119 denaturation curves and statistical analysis was carried out with one-way ANOVA followed

120 by a Newmann-Kuels test.

121

122 **Deglycosylation.** The 10% of brain homogenates (250 μ l) were mixed with equal
123 volumes of the NTS buffer and digested with PK at 20 μ g/ml for 1 h at 37 °C. Proteolysis
124 was terminated by addition of Pefabloc to a final concentration at 4 mM. Samples were
125 then mixed with 1/5 volume of 5x denaturation buffer (20 mM Tris-HCl [pH 7.5], 150 mM
126 NaCl, 2 mM EDTA, 5% SDS, 10% β -mercaptoethanol) and 5 units of N-Glycosidase F
127 (Roche), and incubated for 16 h at 37 °C. Proteins were precipitated by adding 1/2 volume
128 of a 5:1 mixture of 2-butanol and methanol followed by centrifugation at 20,000 x g for 10
129 min at 20 °C.

130

131 **Preparation of cell lysates.** Neuro2a subclone persistently infected with the Chandler
132 strain (ScN2a-5; 38) was used. ScN2a-5 cells grown in 10-cm dishes were collected by cell
133 scraper and pelleted by centrifugation at 300 x g for 5 min. The cells were washed once
134 with PBS and pelleted again by centrifugation. Resulting pellets were lysed with 1 ml of
135 lysis buffer (10 mM Tris-HCl [pH 7.5], 0.5% Triton X-100, 0.5% sodium deoxycholate, 150
136 mM NaCl, 5 mM EDTA) for 30 min on ice. Nuclei and cell debris were removed by low
137 speed centrifugation at 300 x g, supernatants were further centrifuged at 100,000 x g for 30
138 min at 4 °C. The resulting pellets were suspended with 50 μ l PBS and used for
139 conformational-stability assays as the PrP^{Sc}-enriched fraction.

140

141 **Bioassay.** The 10% brain homogenates (540 μ l) were mixed with equal volumes of
142 various concentrations of GdnHCl solution (0 to 6 M) and then incubated at 37 °C for 1 h.
143 Samples were then diluted by addition of 9.18 ml of NTS buffer and 540 μ l of various
144 concentrations of GdnHCl solution was added to adjust the final concentration of GdnHCl to

145 0.4 M. The mixtures were ultracentrifuged at 197,000 x g for 2.5 h at 4 °C, and the resulting
146 pellet was resuspended with 540 µl of PBS and used for the bioassay. The small aliquots of
147 the samples were digested with PK and analyzed by immunoblotting to confirm the existence
148 of PrP^{Sc}. To prepare the PK-treated inoculums for the bioassay, 540 µl of 10% brain
149 homogenates were treated with GdnHCl as described above. After the GdnHCl treatment,
150 samples were digested with 10 µg/ml of PK for 1 h at 37 °C, and digestion was stopped by
151 adding Pefabloc to a final concentration of 2 mM. Samples were ultracentrifuged and the
152 resulting pellets were resuspended with PBS as described above. Samples (20 µl) were
153 intracerebrally inoculated into 4-week-old female Jcl:ICR mice.

154

155

156 RESULTS

157

158 **Conformational stability of PrP^{Sc} of the mouse-adapted prion strains.** To examine
159 biochemical differences of PrP^{Sc} from various mouse-adapted prion strains, the
160 conformational-stability assays were carried out to assess the resistance of PrP^{Sc} to
161 denaturation by GdnHCl (Fig. 1A). When immunoblots were probed with pAb B103 and
162 mAb 44B1 that recognize aa 90-109 and aa 155-231 of mouse PrP, respectively, the amount of
163 PrP^{Sc} of the G1, Obihiro and Fukuoka-1 strains were nearly unchanged up to 2M GdnHCl
164 treatment. The treatment with 2.5 M GdnHCl led to the first decrease in the amount of PrP^{Sc},
165 and only a trace amount of PrP^{Sc} was detected after the treatment with 3 M GdnHCl. The
166 concentration of GdnHCl required to denature 50% of PrP^{Sc} ([GdnHCl]_{1/2}) was estimated
167 from the denaturation curve of each prion strain (Fig. 1A). The [GdnHCl]_{1/2} of the G1,
168 Obihiro and Fukuoka-1 strains from the results of mAb 44B1 ranged from 2.0 to 2.1 M and
169 there was no significant difference among them (Table 1). This indicates that these strains

170 have similar resistance to GdnHCl treatment. In contrast, $[\text{GdnHCl}]_{1/2}$ of the 22L strain was
171 significantly lower than those of the G1, Obihiro and Fukuoka-1 strains, indicating that PrP^{Sc}
172 of the 22L strains is less stable than that of other strains. Moreover, the $[\text{GdnHCl}]_{1/2}$ of
173 BSE-derived strains, KUS-m and TE-m, were higher than other mouse-adapted prion strains
174 except for the Chandler strain. The incubation periods of each prion strain and the
175 $[\text{GdnHCl}]_{1/2}$ values are summarized in Table 1. Although the $[\text{GdnHCl}]_{1/2}$ values are
176 comparable among the G1, Obihiro and Fukuoka strains, the G1 strain had an extremely long
177 incubation periods.

178 Among the prion strains used in this study, PrP^{Sc} of the Chandler strain showed a unique
179 alteration in molecular weight with the increase of GdnHCl concentration. When the blots
180 were probed with pAb B103, approximately 1-2 kDa smaller PrP^{Sc} bands were detected with
181 the 2.0 and 2.5 M GdnHCl treatments, and PrP^{Sc} was almost undetectable with the 3 M
182 GdnHCl treatment. When the blots were probed with mAb 44B1, approximately 6-7 kDa
183 smaller PrP^{Sc} bands were detected by the treatment with more than 2.0 M GdnHCl, and those
184 were still detected even after the treatment with 3.5 M GdnHCl. The $[\text{GdnHCl}]_{1/2}$ of PrP^{Sc} of
185 the Chandler strain was estimated as 3.2 M from the results of mAb 44B1.

186

187 **Further characterization of the GdnHCl resistance of PrP^{Sc} of the Chandler strain.**

188 The results of the conformational-stability assays suggested that the N- and C-terminal
189 regions of PK-resistant PrP^{Sc} of the Chandler strain have different resistance to GdnHCl
190 treatment. Thus, we analyzed PrP^{Sc} of the Chandler strain more precisely with six additional
191 mAbs (Fig. 2). Using mAb 110 recognizing repetitive amino acid sequences at 59-65 and
192 83-89, PrP^{Sc} was undetected with treatments of more than 2 M GdnHCl. The major
193 N-terminus of PK-resistant core of PrP^{Sc} (called as PrP27-30) of the ME7 and Obihiro strains
194 is reported to be a Gly at aa 81 (10, 11). Moreover, the molecular weight of de-glycosylated

195 Chandler PrP^{Sc} is identical to that of the Obihiro strain (Fig. 1B). Taken together, the major
196 N-terminus of PK-resistant core of the Chandler PrP^{Sc} is expected to be at aa 81. We
197 assumed therefore that the 1-2 kDa smaller PrP^{Sc} bands detected with pAb B103 with 2.0 and
198 2.5 M GdnHCl treatments resulted from the denaturation and removal of the region between
199 aa 81 and around 90 (hereafter referred to as aa 90) of mouse PrP^{Sc}. The PrP^{Sc} patterns
200 detected by mAb 132 appeared to be almost identical to those detected by pAb B103,
201 indicating the region between aa 90 and the epitope for mAb 132 (aa 119-127) were almost
202 denatured with treatment of more than 3 M GdnHCl. With more than 2 M GdnHCl
203 treatments, the presence of the approximately 6-7 kDa smaller PrP^{Sc} bands was evident on the
204 blots using mAb 31C6 (recognizing aa 143-149) and mAbs recognizing the C-terminal region
205 thereafter (mAbs 43C5, 44B1, and 147). With 2.0 and 2.5 M GdnHCl treatments, the 6-7
206 kDa smaller PrP^{Sc} bands are thought to overlap with the 1-2 kDa smaller PrP^{Sc} bands that
207 were detected with pAb B103 and mAb 132. Therefore, the presence of the 6-7 kDa smaller
208 PrP^{Sc} was more obvious with treatment of more than 3 M GdnHCl, at which the N-terminal
209 region of PK-resistant core of PrP^{Sc} between aa 81 and the epitope for mAb 132 was
210 denatured and undetectable after PK digestion. The mAb 118 that recognizes aa 137-143 of
211 mouse PrP also reacted with the 6-7 kDa smaller PrP^{Sc} bands (Fig. 2). This suggests that the
212 truncated PK-resistant PrP^{Sc} lacks the N-terminal region up to around aa 127-137, although
213 the exact N-terminus remains to be determined (hereafter referred to as aa 137). Taken
214 together, these results indicate that PK-resistant core of PrP^{Sc} (aa 81-231) of the Chandler
215 strain has a region-dependent conformational stability to GdnHCl treatment. The aa 81-90
216 of PrP^{Sc} is the most sensitive to GdnHCl and denatured almost completely with 2 M GdnHCl.
217 Secondly, the region between aa 90 and 137 is denatured almost completely by more than 3 M
218 GdnHCl, while the remaining C-terminal region of PrP^{Sc} is highly resistant to GdnHCl. The
219 N-terminally truncated non-glycosylated PrP^{Sc} was detectable with 1.5 M GdnHCl treatment

220 (Fig. 2, arrowheads in mAbs 31C6, 43C5, 44B1, and 147), suggesting that the region between
221 aa 81 and 137 begins to be denatured with 1.5 M GdnHCl treatment. In contrast to the
222 Chandler strain, PrP^{Sc} of the Obihiro strain was nearly undetected with 3 M GdnHCl
223 treatment independent of antibodies and the [GdnHCl]_{1/2} values estimated from each blot
224 were comparable (Fig. 2).

225 The 6-7 kDa smaller unglycosylated PrP^{Sc} was occasionally detected by mAbs
226 recognizing the C-terminal region of PrP without GdnHCl pretreatment, but usually very low
227 level. On the other hand, this band was not detected by antibodies recognizing the
228 N-terminal region of PrP (mAb 110 and 132, and pAb B103). These suggest that a
229 processing of region up to aa137 of the Chandler PrP^{Sc} occurs in the brain tissues albeit at
230 very low level. Alternatively, the processing may occur during the sample preparation or
231 autolysis.

232

233 **Conformational stability of PrP^{Sc} in cells infected with the Chandler strain.** Next,
234 we examined whether PrP^{Sc} in cells persistently infected with the Chandler strain shows the
235 region-dependent conformational stability. PrP^{Sc}-enriched fractions obtained from cell
236 lysates of ScN2a-5 were subjected to conformational-stability assays (Fig. 3). The mAb 110
237 detected the PK-resistant PrP^{Sc} bands with up to 1.5 M GdnHCl treatment, and the 1-2 kDa
238 smaller PrP^{Sc} bands were detected by pAb B103 with 2 and 2.5 M GdnHCl treatments.
239 Furthermore, the 6-7 kDa smaller N-terminally truncated PrP^{Sc} bands were detected by mAb
240 44B1 with even after 3 and 3.5 M GdnHCl treatment. These results were consistent with
241 those of PrP^{Sc} obtained from brains of mice infected with the Chandler strain, indicating that
242 the unique conformational stability was maintained in cultured cells.

243

244 **Conformational stability of the Chandler PrP^{Sc} in mice with different PrP genotypes.**

245 To examine whether the region-dependent conformational stability was maintained in mice
246 with different genotypes, assays were carried out using brains of C57BL/6J (*Prnp^{3/a}*) and
247 I/LnJ (*Prnp^{b/b}*) mice infected with the Chandler strain (Fig. 4). The patterns of PrP^{Sc} from
248 C57BL/6J mice were almost identical to those from Jcl:ICR mice. In contrast to PrP^{Sc} from
249 Jcl:ICR and C57BL/6J mice, the N-terminal region of PrP^{Sc} from I/LnJ mice was less
250 resistant to GdnHCl; the [GdnHCl]_{1/2} value of I/LnJ (1.2 M) was lower than those of Jcl:ICR
251 and C57BL/6J mice (1.5 and 1.4 M, respectively) and PrP^{Sc} was undetected after the 1.5 M
252 GdnHCl treatment by mAb 110. In addition, the C-terminus of PrP^{Sc} from I/LnJ mice
253 appeared to be more stable than those from Jcl:ICR and C57BL/6J mice. Although a slight
254 difference in the sensitivity to GdnHCl was observed, it should be emphasized that the
255 sequential shift in molecular weight with an increase of GdnHCl concentration was
256 reproduced in the Chandler PrP^{Sc} propagated in mice with *Prnp^{b/b}* genotype; the 1-2 kDa
257 smaller PrP^{Sc} bands were detected with pAB B103 at 1.5 and 2 M GdnHCl treatment, and the
258 intensity of the 6-7 kDa smaller unglycosylated PrP^{Sc} detected with mAb 31C6 increased
259 remarkably after 1.5 M or higher GdnHCl treatment. These results suggested that the
260 region-dependent conformational stability of the PrP^{Sc} from the Chandler strain was
261 maintained in mice with different PrP genotypes.

262

263 **Effect of denaturation and removal of the N-terminal region of PrP^{Sc} on prion**
264 **infectivity.** To examine whether denaturation of specific regions of PrP^{Sc} affects the prion
265 infectivity, brain homogenates from mice infected with the Chandler strain were treated with
266 GdnHCl and subjected to bioassays. Small aliquots were analyzed by immunoblotting to
267 confirm the region-specific denaturation of PrP^{Sc} in the inoculums (Fig. 5A). Survival times
268 of mice inoculated with samples treated with 1 and 1.5 M GdnHCl were equivalent to those of
269 the GdnHCl-untreated control (Table 2). Compared to the control (0 M, 159 ± 14 days),

270 survival time seemed to be prolonged by the 2 M GdnHCl treatment (176 ± 12 days); however,
271 the difference was not statistically significant ($p > 0.05$). In contrast, significant
272 prolongation was observed after the 3 M GdnHCl treatment (206 ± 25 days, $p < 0.01$).
273 These results suggest that denaturation of the aa 81-137 of PrP^{Sc} greatly influences the prion
274 infectivity. To confirm the involvement of the aa 81-137 in the prion infectivity more
275 precisely, this region was removed by treatment with 3 M GdnHCl followed by PK digestion.
276 The expected size shift of PrP^{Sc} in the inoculums was confirmed prior to the bioassay.
277 Furthermore, the intensities of the PrP^{Sc} bands in samples treated with 0 and 3 M GdnHCl
278 were relatively equivalent, indicating that an equal molar of PK-resistant PrP^{Sc} existed in the
279 inoculums (Fig. 5B). These samples were intracerebrally inoculated into mice to examine
280 the prion infectivity (Table 2). Compared to GdnHCl-untreated control (170 ± 11 days),
281 sample treated with 3 M GdnHCl revealed an attack rate of 40% and a mean survival time of
282 235 days ($n = 2$). Furthermore, 2 out of 5 mice were still alive at 365 days post inoculation
283 (dpi) (Table 2). These results suggest that the infectivity of the N-terminally truncated
284 PK-resistant PrP^{Sc} lacking the aa 81-137 was extremely low.

285 In contrast to the Chandler strain, the immunoreactivity of PK-resistant PrP^{Sc} of the
286 Obihiro strain decreased less than 1% of the original samples when the samples were treated
287 with 3 M GdnHCl and following PK digestion (Fig. 5B). Consistent with the decrease of the
288 amount of PrP^{Sc}, the survival time was prolonged for 34 days by treatment with 3 M GdnHCl
289 (Table 2). From the dose-survival time standard curve for the Obihiro strain in ICR mice,
290 the 34-day prolongation was estimated as more than a 2 Log reduction in infectivity.

291

292

293 DISCUSSION

294

295 Prion strains have been distinguished by their biological properties including incubation
296 periods and neuropathological lesion profiles in mice experimentally inoculated with test
297 samples (3, 4, 6, 7). However, these types of experiments are time-consuming and the
298 results are difficult to standardize among laboratories. Biochemical properties of PrP^{Sc}, such
299 as molecular weight, glycoforms, PK-resistance, and sensitivity to denaturants, often differ
300 among prion strains (2, 5, 13, 25-27, 29), although relationship between the biochemical and
301 biological properties are unclear. Elucidating the strain-specific biochemical properties as
302 well as direct relationship between biochemical and biological properties will facilitate the
303 distinction of prion strains without time-consuming bioassays and the understanding of the
304 mechanisms involved in prion strains. From our analyses of the stability of PrP^{Sc} to the
305 GdnHCl treatment with a panel of anti-PrP antibodies, we found that PrP^{Sc} of the Chandler
306 strain possesses a unique region-dependent conformational stability. The aa 81-137 of PrP^{Sc}
307 begins to be denatured by 1.5 M GdnHCl and is almost completely denatured and becomes
308 PK-sensitive by 3 M GdnHCl treatment. By contrast, the C-terminal region (after aa 137) is
309 extremely resistant to denaturation (Fig. 6).

310 When the blots in Fig. 2 were carefully examined, in the Chandler PrP^{Sc} treated with 2
311 and 2.5 M GdnHCl, the 1-2 kDa smaller di-glycosylated PrP^{Sc} was detected with mAbs 31C6
312 and 44B1, while the corresponding bands were unclear with mAbs 147 and 43C5. This
313 suggests that the C-terminal region is also truncated in certain fraction of PrP^{Sc}. However,
314 we think that the C-terminal truncation is not a major effect by the following reasons. First,
315 affinity of mAbs and the amount of the 1-2 kDa smaller PrP^{Sc} influenced the result. The
316 affinity of mAb 147 is lower than that of mAbs 31C6 and 44B1 (Sakata K. and Horiuchi M.,
317 in preparation), therefore, it is possible that mAb 147 could not visualize the relatively low
318 amount of the 1-2 kDa smaller PrP^{Sc} in the samples treated with 2 and 2.5 M GdnHCl.
319 Second, conformation of the particular region of PrP on the blot might influence the

320 interpretation of the results. The immunoreactivity of the 6-7 kDa smaller PrP^{Sc} increased
321 when mAbs recognizing middle part of PrP were used (mAbs 31C6 and 43C5), especially,
322 this tendency was obvious with mAb 43C5 (Fig. 2). We cannot explain the exact reason for
323 this at the moment. However, the results suggest that the epitope of mAb 43C5 on the 6-7
324 kDa smaller PrP^{Sc} on the blot may be more easily-accessible than that on the regular and the
325 1-2 kDa smaller PrP^{Sc}. If these two types of molecules exist on the limited area of the blot,
326 the reaction of mAb to the easily-accessible epitope will be pronounced. Although we do
327 not exclude the possibility of the C-terminal truncation, further fine experiments will be
328 required to address the C-terminal truncation.

329 The sequential size shift of PK-resistant PrP^{Sc} according to the denaturation was not
330 observed in our study of other mouse-adapted prion strains, natural and experimental sheep
331 scrapie and Japanese BSE cases (data not shown). Additionally, this property was
332 maintained in mice with different *Prnp* genotypes and in cells persistently infected with the
333 Chandler strain. Therefore, these results suggest that the region-dependent conformational
334 stability is specific to PrP^{Sc} of the Chandler strain. In contrast, the conformational-stability
335 assay of the RML prion, which is thought to be synonymous, or very close to the Chandler
336 strain, showed no region-dependent conformational stability (19, 36). One possibility that
337 explains this discrepancy is the use of different antibodies for PrP^{Sc} detection; Legname et al
338 (19) and Thackray et al (36) used the Fab HuM-D18 that recognizes the aa 132-156 and mAb
339 683 that recognizes the aa 168-172, respectively. Both antibodies recognize the C-terminal
340 region after the epitope for mAb 132, which should detect the molecular weight changes of
341 PrP^{Sc} that possesses region-dependent conformational stability as found in Chandler strain.
342 As these molecular weight changes were not detected in those studies, it is unlikely that the
343 difference in antibodies accounts for the discrepancy. Alternatively, genetic backgrounds of
344 mice used for prion propagation may cause the difference in the conformational stability. It

345 has been reported that the biochemical properties of PrP^{Sc} vary depending on the cell and
346 tissue types for prion propagation without changing biological properties (1). Indeed, mice
347 used for propagation of the RML prion in their study (CD-1 Swiss) were different from in this
348 study (Jcl:ICR and C57BL/6J). Thus, further analysis of the Chandler strain propagated in
349 various mice strains as well as analysis of other mouse-adapted prion strains, especially the
350 lineage of the Chandler strain such as 139A (6), will be required to conclude that the
351 region-dependent conformational stability is specific to the Chandler strain.

352 Legname et al (20) reported that a linear correlation between the [GdnHCl]_{1/2} values and
353 incubation periods. In contrast, no linear correlation was observed in our results (n = 9, r =
354 13, $\gamma^2 = 0.019$). We think that sample size in our study too small to make any conclusion.
355 Especially, few data are available for strains showing longer incubation periods or higher
356 [GdnHCl]_{1/2} values at present. Therefore, further accumulation of data will be required to
357 assess the correlation between incubation periods and conformational stabilities of PrP^{Sc}.

358 PrP^{Sc} is comprised of PK-sensitive and PK-resistant PrP^{Sc} (2, 29, 30, 37). Both types of
359 PrP^{Sc} are infectious and PK digestion alone decreases prion infectivity to some extent (2, 31).
360 However, it is well known that the PK-resistant core of PrP^{Sc}, called as PrP²⁷⁻³⁰, which is
361 produced by the removal of PK-sensitive N-terminal region of PrP^{Sc} (from aa 23 to around
362 90), possesses prion infectivity. Prions propagated in Tg mice expressing PrP that lacks the
363 aa 23-88 can propagate in mice expressing wild-type PrP (18). These previous results
364 indicate that this N-terminal region of PrP^{Sc} is not essential for the infectivity of prion.
365 However, analyzing the relationship between other regions of PrP^{Sc} and infectivity by making
366 deletions or mutations has been difficult. In this study, we utilized the region-dependent
367 conformational stability of the Chandler PrP^{Sc} and truncated the PrP^{Sc} directly at the
368 N-terminal region up to around aa 137 to produce the N-terminally truncated PK-resistant
369 PrP^{Sc}; this allowed us to then analyze the influence of this region on prion infectivity.