TABLE 1. Conformational stabilities and incubation periods of prion strains

Prion strain[a]	Mouse strain for propagation	No. of serial passages[b]	$[GdnHCl]_{1/2}$ (M) determined with:		Mean incubation period (days) ± SD	No. of mice[c]
			pAb B103	MAb 44B1 or 31C6[d]		
G1	slc:ICR	4	2.1	2.1 ± 0.1	326 ± 53	5
Obihiro	Jcl:ICR	>5	2.3	2.0 ± 0.0	153 ± 7	24
Chandler[e]	Jcl:ICR	>5	1.8	3.2 ± 0.2*	150 ± 2	15
	I/LnJ	2	2.2	>3.5†	227 ± 7	4
	C57BL/6J	3	2.3	3.5†	153 ± 6	6
22L	Jcl:ICR	2	1.5	1.7 ± 0.0**	144 ± 3	5
Fukuoka-1	Jcl:ICR	2	2.1	2.0 ± 0.0	146 ± 8	8
KUS-m	RIII/J	3	2.4	2.5 ± 0.2*	165 ± 11	6
TE-m	C57BL/6J	3	2.2	2.6 ± 0.2*	168 ± 4	6

[a] Strain G1 was obtained from sheep with experimental scrapie; the 22L and Fukuoka-1 strains were derived from prions passaged in mice carrying $Prnp^{a/a}$ but different from Jcl:ICR mice; and KUS-m and TE-m were obtained from BSE field cases KUS and TE.

[b] History (number) of serial passages in the mouse strain listed to the left.

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

[d] The $[GdnHCl]_{1/2}$ values were estimated from the denaturation curves plotted by using blots probed with MAb 44B1 (values shown are means ± SD of results from at least three independent assays) and MAb 31C6 (values determined with this MAb are indicated by †). *, higher than value for G1 ($P < 0.05$); **, lower than value for G1 ($P < 0.05$).

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

observations indicate the major N terminus of the PK-resistant core of the Chandler PrP^{Sc} to be at position 81. We assumed, therefore, that the 1- to 2-kDa-smaller PrP^{Sc} bands detected with pAb B103 after 2.0 and 2.5 M GdnHCl treatments resulted from the denaturation and removal of the region from aa 81 to a residue around aa 90 (herein referred to as aa 90) of mouse PrP^{Sc}. The PrP^{Sc} patterns detected by MAb 132 appeared to be almost identical to those detected by pAb B103, indicating that the region between aa 90 and the epitope for MAb 132 (aa 119 to 127) was almost denatured by treatment with more than 3 M GdnHCl. After treatments with more than 2 M GdnHCl, the presence of the approximately 6- to 7-kDa-smaller PrP^{Sc} bands was evident on the blots probed with MAb 31C6 (recognizing aa 143 to 149) and MAbs recognizing the C-terminal region thereafter (MAbs 43C5, 44B1, and 147). With 2.0 and 2.5 M GdnHCl treatments, the 6- to 7-kDa-smaller PrP^{Sc} bands are thought to overlap with the 1- to 2-kDa-smaller PrP^{Sc} bands that were detected with pAb B103 and MAb 132. Therefore, the presence of the 6- to 7-kDa-smaller PrP^{Sc} was more obvious after treatment with more than 3 M GdnHCl, at which the N-terminal region of the PK-resistant core of PrP^{Sc} between aa 81 and the epitope for MAb 132 was denatured and undetectable after PK digestion. MAb 118, which recognizes aa 137 to 143 of mouse PrP, also reacted with the 6- to 7-kDa-smaller PrP^{Sc} bands (Fig. 2). This result suggests that the truncated PK-resistant PrP^{Sc} lacks the N-terminal region up to around aa 127 to 137, although the exact N terminus (hereinafter referred to as aa 137) remains to be determined. Taken together, these results indicate that the PK-resistant core of PrP^{Sc} (aa 81 to 231) of the Chandler strain has region-dependent conformational stability under conditions of GdnHCl treatment. The region of aa 81 to 90 of PrP^{Sc} is the most sensitive to GdnHCl and is denatured almost completely by 2 M GdnHCl. The region between aa 90 and 137 is denatured almost completely by more than 3 M GdnHCl, while the remaining C-terminal region of PrP^{Sc} is highly resistant to GdnHCl. The N-terminally truncated nonglycosylated PrP^{Sc} was detectable after 1.5 M GdnHCl treatment (Fig. 2, blots probed with MAbs 31C6, 43C5, 44B1, and 147), suggesting that the region between aa 81 and 137 begins to be denatured with 1.5 M GdnHCl treatment. In contrast to PrP^{Sc} of the Chandler strain, PrP^{Sc} of the Obihiro strain was nearly undetectable after 3 M GdnHCl treatment, regardless of the antibodies used, and the $[GdnHCl]_{1/2}$ values estimated from the different blots were comparable (Fig. 2).

The 6- to 7-kDa-smaller unglycosylated PrP^{Sc} was occasionally detected by MAbs recognizing the C-terminal region of PrP without GdnHCl pretreatment, but usually at a very low level. On the other hand, this band was not detected by antibodies recognizing the N-terminal region of PrP (MAbs 110 and 132 and pAb B103). These findings suggest that processing of the region up to aa 137 of the Chandler PrP^{Sc} occurs in the brain tissues, albeit at a very low level. Alternatively, the processing may occur during the sample preparation or autolysis.

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

Conformational stability of the Chandler PrP^{Sc} in mice with different PrP genotypes. To examine whether the region-dependent conformational stability was maintained in mice with different genotypes, assays were carried out using brains of C57BL/6J ($Prnp^{a/a}$) and I/LnJ ($Prnp^{b/b}$) mice infected with the Chandler strain (Fig. 4). The patterns of PrP^{Sc} from C57BL/6J mice were almost identical to those of PrP^{Sc} from Jcl:ICR

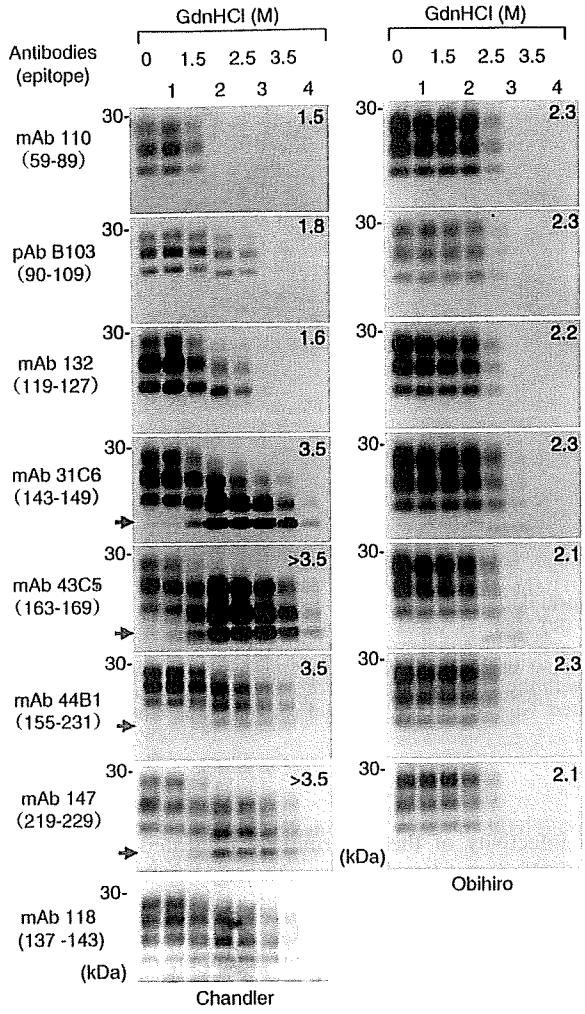

FIG. 2. Region-dependent conformational stability of PrP^{Sc} of the Chandler strain. Brain homogenates from mice infected with the Chandler (left) and Obihiro (right) strains were subjected to the conformational-stability assay, and immunoblots were probed with the various anti-PrP antibodies indicated to the left. Epitopes for antibodies are indicated in parentheses. Due to relatively weak reactivity, five times the tissue equivalents of those for the blots for the other MAbs were loaded for MAb 118. Numbers in the top right corners of the blots are the $[GdnHCl]_{1/2}$ values (in molars). Arrows, the N-terminally truncated nonglycosylated PrP^{Sc}.

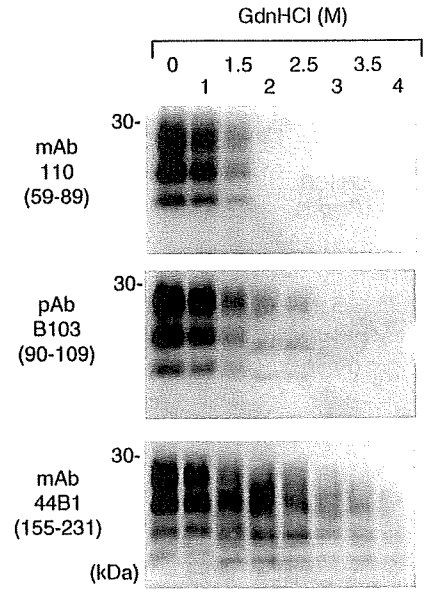

FIG. 3. Region-dependent conformational stability of PrP^{Sc} in cells persistently infected with the Chandler strain. PrP^{Sc}-enriched fractions obtained from ScN2a-5 cells were subjected to the conformational-stability assay. The antibodies used are listed to the left, and epitopes for antibodies are indicated in parentheses.

mice. In contrast, the N-terminal region of PrP^{Sc} from I/LnJ mice was less resistant to GdnHCl than those of PrP^{Sc} molecules from Jcl:ICR and C57BL/6J mice; the $[GdnHCl]_{1/2}$ value for PrP^{Sc} from I/LnJ mice (1.2 M) was lower than those for PrP^{Sc} molecules from Jcl:ICR and C57BL/6J mice (1.5 and 1.4 M, respectively), and PrP^{Sc} from I/LnJ mice was undetected by MAb 110 after the 1.5 M GdnHCl treatment. In addition, the C terminus of PrP^{Sc} from I/LnJ mice appeared to be more stable than those of PrP^{Sc} molecules from Jcl:ICR and C57BL/6J mice. Although a slight difference in the sensitivity to GdnHCl was observed, it should be emphasized that the sequential shift in molecular mass with an increase of GdnHCl concentration was reproduced for the Chandler PrP^{Sc} propagated in mice with the *Prnp*$^{b/b}$ genotype; the 1- to 2-kDa-smaller PrP^{Sc} bands were detected with pAB B103 after 1.5 and 2 M GdnHCl treatments, and the intensity of the 6- to 7-kDa-smaller unglycosylated PrP^{Sc} bands detected with MAb 31C6 increased remarkably after treatment with GdnHCl at 1.5 M or higher. These results suggested that the region-dependent conformational stability of the PrP^{Sc} from the Chandler strain was maintained in mice with different PrP genotypes.

Effect of denaturation and removal of the N-terminal region of PrP^{Sc} on prion infectivity. To examine whether the denaturation of specific regions of PrP^{Sc} affects the prion infectivity, brain homogenates from mice infected with the Chandler strain were treated with GdnHCl and subjected to bioassays. Small aliquots were analyzed by immunoblotting to confirm the region-specific denaturation of PrP^{Sc} in the inoculums (Fig. 5A). Survival times of mice inoculated with samples treated with 1 and 1.5 M GdnHCl were equivalent to those of mice inoculated with the non-GdnHCl-treated control (Table 2). Compared to the survival time of mice receiving the control (0 M; mean survival time ± standard deviation [SD], 159 ± 14 days), the survival time of mice receiving samples treated with 2 M GdnHCl seemed to be prolonged (176 ± 12 days); however, the difference was not statistically significant ($P > 0.05$). In contrast, significant prolongation of the survival time (206 ± 25 days; $P < 0.01$) after the 3 M GdnHCl treatment was observed. These results suggest that the denaturation of aa 81 to 137 of PrP^{Sc} greatly influences the prion infectivity. To confirm the involvement of aa 81 to 137 in the prion infectivity more precisely, this region was removed by treatment with 3 M GdnHCl followed by PK digestion. The expected size shift of PrP^{Sc} in the inoculums was confirmed prior to the bioassay. Furthermore, the intensities of the PrP^{Sc} bands in samples treated with 0 and 3 M GdnHCl were relatively equivalent,

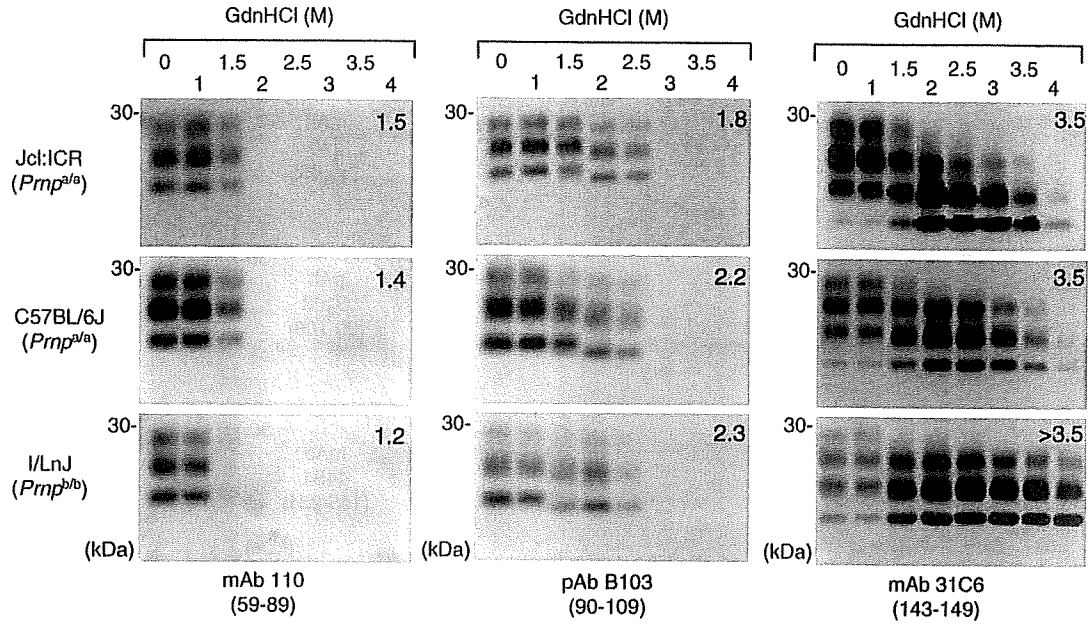

FIG. 4. Region-dependent conformational stability of the Chandler PrP^{Sc} in mice with different genetic backgrounds. Brain homogenates from Jcl:ICR ($Prnp^{a/a}$), C57BL/6J ($Prnp^{a/a}$), and I/LnJ ($Prnp^{b/b}$) mice infected with the Chandler strain were subjected to the conformational-stability assay. The antibodies used and their epitopes (in parentheses) are indicated. Numbers in the top right corners of the blots are the $[GdnHCl]_{1/2}$ values (in molars).

indicating that equal molar amounts of PK-resistant PrP^{Sc} existed in the inoculums (Fig. 5B). These samples were intracerebrally inoculated into mice to examine the prion infectivity (Table 2). In contrast to mice receiving the non-GdnHCl-treated control (survival time, 170 ± 11 days), mice inoculated with the sample treated with 3 M GdnHCl exhibited an attack rate of 40% and a mean survival time of 235 days (n = 2). Furthermore, two of five mice were still alive at 365 days postinoculation (dpi) (Table 2). These results suggest that the infectivity of the N-terminally truncated PK-resistant PrP^{Sc} lacking aa 81 to 137 was extremely low.

The immunoreactivity of PK-resistant PrP^{Sc} of the Obihiro strain, in contrast to that of PrP^{Sc} of the Chandler strain, decreased less than 1% from that in the original samples when the samples were treated with 3 M GdnHCl and subjected to PK digestion (Fig. 5B). Consistent with the decrease in the amount of PrP^{Sc}, the survival time was prolonged for 34 days by treatment with 3 M GdnHCl (Table 2). From the dose-survival time standard curve for the Obihiro strain in ICR

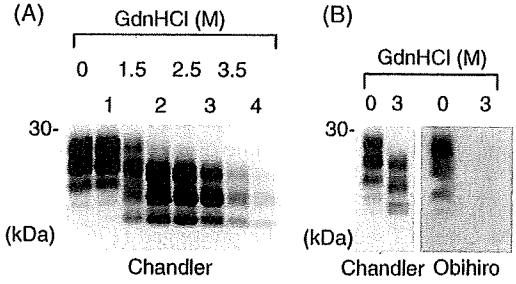

FIG. 5. Region-specific denaturation or removal of PrP^{Sc} in inoculums for the bioassay. (A) Confirmation of region-specific denaturation. Brains of mice infected with the Chandler strain were treated with various concentrations of GdnHCl (without PK treatment), and the fraction containing PrP^{Sc}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

TABLE 2. Effects of GdnHCl treatment and PK digestion on prion infectivity

Strain	GdnHCl concn (M)	PK digestion[a]	No. of infected mice/no. of mice in group[b]	Mean survival time (dpi) ± SD
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[c]	234, 236, >365
Obihiro	0	+	5/5	152 ± 7
	3	+	5/5	186 ± 11

[a] +, performed; −, not performed.

[b] Number of mice which showed typical clinical manifestations of scrapie and/or were positive for PrP^{Sc} by immunoblotting/number of mice used in the bioassay.

[c] Two mice showed typical clinical manifestations and were positive for PrP^{Sc} (at 234 and 236 dpi), and one mouse was found dead without having shown any symptoms at 336 dpi and was negative for PrP^{Sc}. The remaining two mice were still alive without any symptoms >365 dpi.

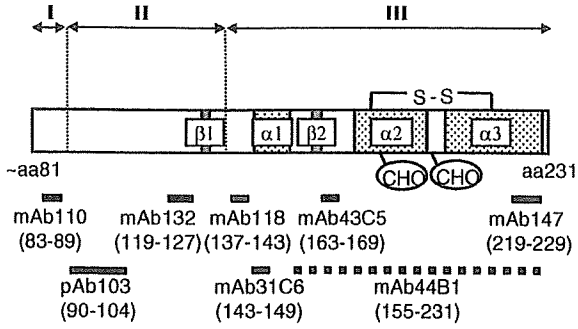

FIG. 6. Schematic representation of region-specific denaturation of the Chandler PrP^{Sc}. The PK-resistant core of the Chandler PrP^{Sc} (from aa ~81 to 231) is depicted, with the locations of two β-strands (β1 and β2), three α-helices (α1 to α3), two N glycosylation sites (CHO), and an intramolecular disulfide bond (S-S). The locations of epitopes are indicated by thick lines labeled with amino acid positions (in parentheses). The epitope for MAb 44B1, which recognizes a discontinuous epitope, is indicated by a dashed line, while those for other antibodies that recognize linear epitopes are indicated by solid lines. Region I (aa 81 to 90), indicated above, was denatured almost completely by treatment with up to 2 M GdnHCl, and the removal of this region generates the 1- to 2-kDa-smaller PK-resistant PrP^{Sc}. Region II (aa 90 to 137) was denatured almost completely by treatment with up to 3 M GdnHCl, and the removal of regions I and II consequently generates the 6- to 7-kDa-smaller PK-resistant PrP^{Sc} (region III, aa 137 to the C terminus) that is highly resistant to denaturation but lacks prion infectivity.

mice, the 34-day prolongation was estimated to represent more than a 2-log reduction in infectivity.

DISCUSSION

Prion strains have been distinguished by their biological properties, including incubation periods and neuropathological lesion profiles in mice experimentally inoculated with test samples (3, 4, 6, 7). However, these types of experiments are time-consuming, and the results are difficult to standardize among laboratories. Biochemical properties of PrP^{Sc}, such as molecular mass, glycoforms, PK resistance, and sensitivity to denaturants, often differ among prion strains (2, 5, 14, 25–27, 29), although relationships between the biochemical and biological properties are unclear. Elucidating the strain-specific biochemical properties as well as direct relationships between biochemical and biological properties will facilitate the distinction of prion strains without time-consuming bioassays and an understanding of the mechanisms involved in prion strains. From our analyses of the stabilities of PrP^{Sc} molecules to the treatment of GdnHCl with a panel of anti-PrP antibodies, we found that PrP^{Sc} of the Chandler strain possesses unique region-dependent conformational stability. The region of aa 81 to 137 of PrP^{Sc} begins to be denatured by 1.5 M GdnHCl and is almost completely denatured and becomes PK sensitive with 3 M GdnHCl treatment. In contrast, the C-terminal region (after aa 137) is extremely resistant to denaturation (Fig. 6).

When the blots in Fig. 2 were carefully examined, in the Chandler PrP^{Sc} sample treated with 2 and 2.5 M GdnHCl, the 1- to 2-kDa-smaller diglycosylated PrP^{Sc} was detected with MAbs 31C6 and 44B1 while the corresponding bands were unclear with MAbs 147 and 43C5. This result suggests that the C-terminal region is also truncated in certain fractions of PrP^{Sc}. However, we think that the C-terminal truncation is not a major effect for the following reasons. First, the affinity of the MAbs and the amount of the 1- to 2-kDa-smaller PrP^{Sc} influenced the result. The affinity of MAb 147 is lower than that of MAbs 31C6 and 44B1 (K. Sakata and M. Horiuchi, unpublished results); therefore, it is possible that MAb 147 could not visualize the relatively small amount of the 1- to 2-kDa-smaller PrP^{Sc} in the samples treated with 2 and 2.5 M GdnHCl. Second, the conformation of the particular region of PrP on the blot may influence the interpretation of the results. The immunoreactivity of the 6- to 7-kDa-smaller PrP^{Sc} increased when MAbs recognizing the middle part of PrP (MAbs 31C6 and 43C5) were used; this tendency was especially obvious with MAb 43C5 (Fig. 2). We cannot explain the exact reason for this effect at the moment. However, the results suggest that the epitope of MAb 43C5 on the 6- to 7-kDa-smaller PrP^{Sc} on the blot may be more easily accessible than that on the regular and the 1- to 2-kDa-smaller PrP^{Sc} molecules. If these two types of molecules exist in the limited area of the blot, the reaction of the MAb to the easily accessible epitope will be pronounced. Although we do not exclude the possibility of the C-terminal truncation, further fine-detail experiments will be required to address the C-terminal truncation.

The sequential size shift of PK-resistant PrP^{Sc} according to the denaturation profile was not observed in our study of other mouse-adapted prion strains, natural and experimental sheep scrapie and Japanese BSE cases (data not shown). Additionally, this property was maintained in mice with different *Prnp* genotypes and in cells persistently infected with the Chandler strain. Therefore, these results suggest that the region-dependent conformational stability is specific to PrP^{Sc} of the Chandler strain. In contrast, the conformational-stability assay of the RML prion, which is thought to be synonymous with or very close to the Chandler strain, showed no region-dependent conformational stability (19, 36). One possibility that explains this discrepancy is the use of different antibodies for PrP^{Sc} detection; Legname et al. (19) and Thackray et al. (36) used the Fab fragment HuM-D18, which recognizes aa 132 to 156, and MAb 683, which recognizes aa 168 to 172, respectively. Both antibodies recognize the C-terminal region after the epitope for MAb 132 and thus should detect the molecular size changes in PrP^{Sc} molecules that possess region-dependent conformational stability, as found in the Chandler strain. As these molecular size changes were not detected in those studies, it is unlikely that the difference in antibodies accounts for the discrepancy. Alternatively, genetic backgrounds of mice used for prion propagation may cause the difference in the conformational stability. It has been reported previously that the biochemical properties of PrP^{Sc} vary depending on the cell and tissue types for prion propagation without changing biological properties (1). Indeed, the mice used for the propagation of the RML prion in the previous study (CD-1 Swiss mice) were different from those used in this study (Jcl:ICR and C57BL/6J). Thus, further analysis of the Chandler strain propagated in various mouse strains, as well as analyses of other mouse-adapted prion strains, especially those of the lineage of the Chandler strain, such as 139A (6), will be required to conclude that the region-dependent conformational stability is specific to the Chandler strain.

Legname et al. (20) reported linear correlation between the $[GdnHCl]_{1/2}$ values and incubation periods. In contrast, no linear correlation was observed in our results ($n = 9$; $r = 0.007$). We think that the sample size in our study was too small to make any conclusion. In particular, few data are available for strains showing longer incubation periods or higher $[GdnHCl]_{1/2}$ values at present. Therefore, further accumulation of data will be required to assess the correlation between incubation periods and conformational stabilities of PrP^{Sc}.

PrP^{Sc} includes PK-sensitive and PK-resistant molecules (2, 29, 30, 37). Both types of PrP^{Sc} are infectious, and PK digestion alone decreases prion infectivity to some extent (2, 32). However, it is well known that the PK-resistant core of PrP^{Sc}, PrP27-30, which is produced by the removal of the PK-sensitive N-terminal region of PrP^{Sc} (from aa 23 to around aa 90), possesses prion infectivity. Prions propagated in Tg mice expressing PrP that lacks aa 23 to 88 can propagate in mice expressing wild-type PrP (18). These previous results indicate that this N-terminal region of PrP^{Sc} is not essential for the infectivity of the prion. However, analyzing the relationship between other regions of PrP^{Sc} and infectivity by making deletions or mutations has been difficult. In this study, we utilized the region-dependent conformational stability of the Chandler PrP^{Sc} and truncated the PrP^{Sc} directly at the N-terminal region up to around aa 137 to produce the N-terminally truncated PK-resistant PrP^{Sc}; this approach allowed us to then analyze the influence of this region on prion infectivity. Compared to the regular PK-resistant core of PrP^{Sc} that is produced by PK digestion without GdnHCl treatment, the N-terminally truncated PK-resistant PrP^{Sc} had extremely low infectivity despite the existence of the C-terminal region as PK-resistant fragments (Table 2). Since we have not produced a dose-incubation period standard curve for the Chandler strain in Jcl:ICR mice, we cannot estimate the exact reduction in infectivity. However, the attack rate and the survival time suggested that the infectivity decreased to nearly the detection limit in the bioassay. This result provides direct evidence that the region of aa 81 to 137 of PK-resistant PrP^{Sc} is critical for prion infectivity, although evidence for other prion strains remains to be elucidated. However, PK treatment alone reduced the infectivity of the Chandler strain (mean survival times, 159 and 170 days for mice receiving samples without and with PK treatment, respectively) (Table 2), indicating that the PK-sensitive PrP^{Sc} fraction possessing prion infectivity was present in the brain homogenates of the Chandler strain-infected mice. Our results clearly showed that the region of aa 81 to 137 of the PK-resistant core of the Chandler PrP^{Sc} is important for infectivity; however, it remains unclear whether the same conclusion is applicable to the infectivity of the PK-sensitive PrP^{Sc} fraction.

The denaturation of this region by 3 M GdnHCl treatment appeared to be less effective than the removal of this region in reducing prion infectivity. However, considering the effect of GdnHCl on PrP^{Sc} aggregates, the denaturation itself appears to result in a substantial loss of infectivity (Table 2). The GdnHCl treatment has two expected effects: the dissociation of large PrP^{Sc} aggregates into small aggregates and the denaturation of the PrP^{Sc} molecules. Hence, without PK digestion, small aggregates consisting of PrP^{Sc} with incompeletely denatured aa 81 to 137 may remain and infectivity may be observed. Such small PrP^{Sc} aggregates should be PK sensitive, and therefore, the infectivity should be diminished after PK digestion (32). Alternatively, this region may have been somewhat refolded after the GdnHCl treatment, which would lead to infectivity.

Several distinct domains of PrP^{C} are reported to be involved in the direct interaction with PrP^{Sc} (21, 33), whereas domains on PrP^{Sc} that are involved in binding to PrP^{C} remain undetermined. The N-terminally truncated PrP^{Sc} may be useful for the analysis of the PrP^{C} binding domain on the PrP^{Sc} molecule. Here, we showed an example of a possible biochemical approach to PrP^{Sc} manipulation, in which we directly produced the N-terminally truncated PrP^{Sc} from native PrP^{Sc}. It has been reported previously that some conditions (e.g., pH) in protease digestion affect the N-terminal truncation of the PK-resistant core of PrP^{Sc} (24). Thus, further investigation of region-specific denaturation and proteolysis may be useful not only for the analysis of prion strains but also for the manipulation of PrP^{Sc}.

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