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Octapeptide repeat region of prion protein (PrP) is required at an early stage for production of abnormal prion protein in PrP-deficient neuronal cell line

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Abstract

An abnormal isoform of prion protein (PrP^{Sc}), which is composed of the same amino acids as cellular PrP (PrP^{C}) and has proteinase K (PK)-resistance, hypothetically converts PrP^{C} into PrP^{Sc}. To investigate the region important for PrP^{Sc} production, we examined the levels of PrP^{Sc} in PrP gene-deficient cells (HpL3-4) expressing PrP^{C} deleted of various regions including the octapeptide repeat region (OR) or hydrophobic region (HR). After Chandler or Obihiro prion infection, PrP^{Sc} was produced in HpL3-4 cells expressing wild-type PrP^{C} or PrP^{C} deleted of HR at an early stage and further reduced to below the detectable level, whereas cells expressing PrP^{C} deleted of OR showed no PrP^{Sc} production. The results suggest that OR of PrP^{C} is required for the early step of efficient PrP^{Sc} production.

Keywords: Prion protein; PrP^{Sc}; PrP gene-deficient cell line

Transmissible spongiform encephalopathies (TSEs) are caused by an infectious agent, prion [1], and so are called prion diseases. They include scrapie in sheep, bovine spongiform encephalopathy in cattle, and Kuru and CJD in humans [1]. Prion is thought to be mainly composed of abnormal prion protein (PrP^{Sc}). A key event in the pathogenesis of prion diseases is the conversion of cellular PrP (PrP^{C}), which is expressed mainly in the brain and also in peripheral tissues [2], into PrP^{Sc} [1]. PrP^{Sc} contains more β-sheet and less α-helix than PrP^{C} [3]. This is why it is resistant to proteinase-K (PK). Therefore, resistance to digestion by PK is a specific feature distinguishing PrP^{Sc} from PrP^{C} [4]. Most current methods used for the diagnosis of prion infections rely on the presence of PrP^{Sc} [4]. PrP^{C} is completely degraded, whereas the C-terminal of fragment PrP^{Sc} which remains after PK digestion is detectable by methods such as Western blotting.

Transgenic mice have been used to analyze the specific amino acid residues and domains in PrP^{C} necessary for prion infections and the accumulation of PrP^{Sc} in organs/tissues [5–13]. Although results obtained from these experiments provide important information on prion biology, the events induced by prion infections in independent cells remain unclear, as results obtained *in vivo* may reflect a systemic process involving heterogeneous cell populations in the brain. Cell-culture models designed for the study of prion infections have improved understanding of the molecular mechanisms by which PrP^{Sc} forms as well as the role of the amino acid sequence and structural domains of PrP^{C} in the conversion of PrP^{C} to PrP^{Sc} in a cell-autonomous fashion [4]. Hitherto, such studies have been using persistently infected cell cultures [14–16], because *de novo* infections of cell cultures with prions are restricted by a relatively low infection efficiency [4,17]. Cell lines susceptible

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to prions co-expressed with exogenous PrP^C are used for some experiments [4]; however, interference by endogenous PrP^C in the pathogenicity of prion agents from other species has also been reported [18–20]. Moreover, the concomitant expression of heterogeneous species of PrP^C seems to inhibit prion infection, even if cell lines expressing undetectable levels of PrP^C (e.g., rabbit kidney epithelial RK13 cells) are used [21]. As such, PrP gene-deficient cell lines lacking endogenous PrP^C may serve as models for analysis of the domain of PrP^C required for the pathogenicity of prions without interference from endogenous PrP^C through the transfection of various deletion mutants of PrP^C following prion infection [22].

In this study, the requirement of specific regions of PrP^C for the production of PrP^{Sc} was examined by evaluating the PrP^{Sc} level in a PrP gene-deficient cell line expressing various deletion mutants of PrP^C after prion infection. Our findings indicate that full-length PrP^C leads to the production of PrP^{Sc} at an early stage, whereas deletion of the OR of PrP^C prevents production of PrP^{Sc}.

Materials and methods

Cell cultures. ScN2a I3/I5-9 cells [23] (kindly supplied by Professor Motohiro Horiuchi, Hokkaido University, Japan), an N2a cell line infected by the scrapie Chandler isolate and that persistently produces PrP^{Sc}, were grown in OptiMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum and standard antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). HpL3-4 cells [24] and the transfectants [HpL3-4-EM, HpL3-4-PrP, HpL3-4-Δ#1, and HpL3-4-Δ#2] [25–27] were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) and standard antibiotics at 37 °C in a humidified 5% CO_2 atmosphere. For the preparation of ScN2a lysates, two 10 cm dishes of ScN2a I3/I5-9 cells were solubilized in 100 μl of OptiMEM, freeze–thawed two times in −80 °C, and solubilized with a 22 G syringe. The prion infection was performed by plating HpL3-4-EM, HpL3-4-PrP, HpL3-4-Δ#1, and HpL3-4-Δ#2 cells at 5×10^4 cells/well in 24-well plates. After incubation for 24 h, cells were incubated for 4 h in the presence of 1 ml of OptiMEM containing 20 μl of ScN2a lysate or 200 μl of 1% brain homogenate of Chandler or Obihiro prion-infected mice (kindly supplied by Professor Motohiro Horiuchi), with 1 ml of 10% FCS–DMEM. After that, the medium was replaced with new 10% FCS–OptiMEM. The culture was moved to a 6 cm dish and then to a 10 cm dish. The lysate from the confluent culture of the 10 cm dish is designated passage 1 (P1). Cells were further passaged from P1 to P5. The levels of total PrP and PrP^{Sc} in the cell lysate were determined using Western blotting.

Preparation of HpL3-4 cell lysates. Lysates were made from the cell lysate of prion-infected HpL3-4 transfectants. Cells were detached with a scraper and washed twice with ice-cold phosphate-buffered saline (PBS). The washed cells were solubilized in radio-immunoprecipitation assay (RIPA) buffer containing 10 mM Tris–HCl (pH 7.4), 1% deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 150 mM NaCl and then sonicated at 4 °C for 10 min. The cellular debris was removed by centrifugation at 5000*g* for 1 min. The protein concentration of the supernatants was measured by Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). The sample (120 μg protein) was treated with PK at 20 μg/ml for 30 min at 37 °C. An equal volume of 2× SDS gel-loading buffer [90 mM Tris/HCl (pH 6.8), 10% mercaptoethanol, 2% SDS, 0.02% bromophenolblue, and 20% glycerol] was added and the samples were heated at 100 °C for 10 min to terminate the reaction before Western blotting. Cells treated as above except for the digestion by PK were also included.

Western blot analysis. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (12%) as described previously [28]. The proteins were further transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ) by using a semidry blotting system (Bio-Rad, Cambridge, MA). The membranes were blocked with 5% skim milk (Wako, Osaka, Japan) for 1 h at room temperature, and incubated for 1 h at room temperature with anti-PrP antibody SAF83 (SPI bio, Montigny le Bretonneux, France) which recognizes residues 126–164 of PrP [29] in PBS-Tween (0.1% Tween 20) containing 0.5% skim milk. Then, the membranes were washed three times for 10 min in PBS-Tween, incubated with horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin secondary antibody (Jackson Immunoresearch, West Grove, PA) in PBS-Tween containing 0.5% skim milk for 1 h at room temperature before being washed three times in PBS-Tween for 10 min. After development with an enhanced chemiluminescence (ECL) reagent (Amersham) for 5 min, blots were exposed to ECL Hypermax film (Amersham). Films were processed automatically in an X-ray film processor (Konica, Tokyo, Japan).

Results

To determine if the reintroduction of PrP gene into the PrP gene-deficient neuronal cell line restored the ability to produce PrP^{Sc} after prion infection, HpL3-4-PrP and HpL3-4-EM cells were treated with a mixture containing cell lysate of ScN2a or brain homogenate infected with Chandler or Obihiro prion. To detect PK-resistant PrP^{Sc} in extracts from the cell lysate, Western blotting with the anti-PrP antibody SAF83 was performed. The addition of lysate of ScN2a cells was able to induce PrP^{Sc} signals (18–27 kDa) in HpL3-4-PrP cells at P1 but not in HpL3-4-EM cells (Fig. 1A). Total PrP levels were also investigated by the detection of PrP in samples not treated with PK. The signals of total PrP were unchanged after several passages in HpL3-4-PrP cells. In addition, brain homogenate infected with Chandler prion (Fig. 1B) and Obihiro prion (Fig. 1C) also enabled HpL3-4-PrP cells to produce PrP^{Sc} at P2 (Chandler prion infected brain homogenate) or P1–P3 (Obihiro prion-infected brain homogenate). Time course experiments showed that PrP^{Sc} levels were reduced by P2 for ScN2a lysate, P3 for Chandler prion-infected brain homogenate, and P4 for Obihiro prion-infected brain homogenate and remained under the detectable limit up to P5 (Fig. 1A–C). Total PrP and PrP^{Sc} signals were not detected in HpL3-4-EM during passaging after the addition of ScN2a lysate (Fig. 1A), brain homogenate of Chandler prion (Fig. 1B), and Obihiro prion (Fig. 1C).

Western blotting with SAF83 recognizing the C-terminal of PrP showed that PrP exhibited broad signals with an approximate molecular weight of 20–37 kDa in PrP-expressing cells (HpL3-4-PrP) in the absence of PK after infection with ScN2a lysate but not in HpL3-4-EM cells (Fig. 2B). Comparable signals of PrP(Δ53–94, Q52H) and PrP(Δ95–132) proteins (Fig. 2B) were detected as well at slightly lower bands. ScN2a lysate-infected HpL3-4-PrP and HpL3-4-Δ#2 but not HpL3-4-Δ#1 produced PrP^{Sc} signals at P1 (Fig. 2B).

The HpL3-4-EM, HpL3-4-PrP, HpL3-4-Δ#1, and HpL3-4-Δ#2 cells were also infected with Chandler prion-infected brain homogenate to test whether the deletion of several regions in PrP^C with the OR or HR would

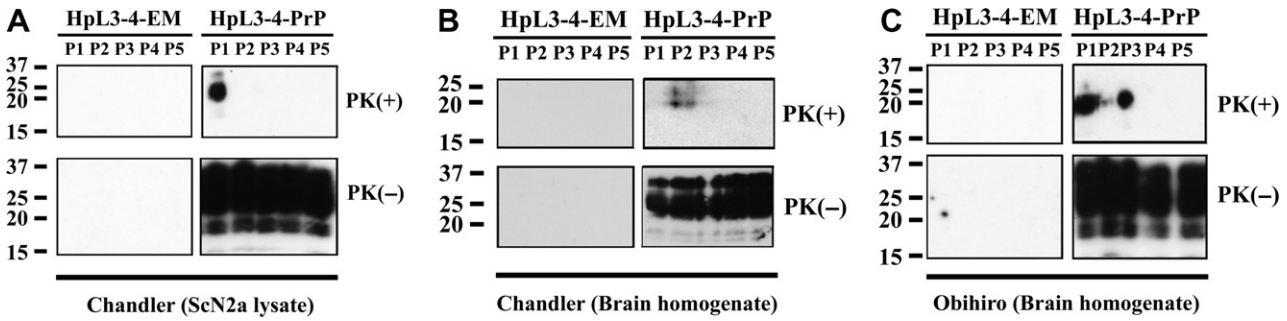

Fig. 1. The production of PrP^{Sc} in a PrP gene-deficient cell line transfected with PrP and infected by prion-infected cell lysate and brain homogenate. To measure production levels of PrP^{Sc}, HpL3-4-EM, and HpL3-4-PrP cells were plated at 5×10^4 cells/well in 24-well microtiter plates. Lysate of Chandler prion-infected ScN2a cells (A) or brain homogenate of Chandler prion-infected mice (B) or Obihiro prion-infected mice (C) was added to the culture of HpL3-4-EM and HpL3-4-PrP cells. Then, the cells were grown with changes of media. PrP^{Sc} production was evaluated after the spreading of cells on 10 cm dishes (designated as P1). Cells further passaged from P1 to P5 were also assayed as follows. The lysates from the passaged HpL3-4-EM and HpL3-4-PrP cells were treated with proteinase K [PK(+)] or not [PK(−)] and further subjected to Western blotting with anti-PrP antibody, SAF83, for PrP^{Sc} or total PrP, respectively. HpL3-4-PrP cells demonstrated broad PrP^{Sc} signals with an approximate molecular weight of 18–27 kDa after prion infection at P1 (Chandler prion-infected ScN2a lysate), P2 (Chandler prion-infected brain homogenate), or P1–P3 (Obihiro prion-infected brain homogenate), whereas PrP^{Sc} levels were rapidly reduced to below the detectable limit. The levels of total PrP showing broad signals with an approximate molecular weight of 20–37 kDa were not changed during passaging in HpL3-4-PrP cells. Both total PrP and PrP^{Sc} were not detected in passaged HpL3-4-EM cells.

produce PrP^{Sc} after prion infection. The total PrP level and PrP^{Sc} level were analyzed with Western blotting using SAF83 in the absence and presence of PK, respectively. The total PrP level was not changed during passaging after infection of HpL3-4-PrP, HpL3-4-Δ#1, and HpL3-4-Δ#2 cells. PrP^{Sc} was detected in HpL3-4-PrP and HpL3-4-Δ#2 cells at P2 and further reduced to below detectable levels within P3, but was not detected in HpL3-4-Δ#1 cells at P1–P5 (Fig. 2C). These results suggest that PrP and PrP(Δ95–132) retain the ability to produce PrP^{Sc} at an early stage, whereas PrP(Δ53–94, Q52H) does not.

Discussion

Cell lines are valuable in the analysis of mechanisms of PrP^{Sc} accumulation, but studies have been limited by difficulty in obtaining a prion-susceptible cell line [4]. If PrP gene-deficient cells into which PrP gene was reintroduced were susceptible to certain prions, they would offer profound advantages over previous prion-cell culture models. Furthermore, as the novel forms of PrP^{Sc} could be entirely derived from exogenous PrP, this system is appropriate for testing multiple artificial PrP molecules. The few studies of the mechanisms by which PrP^{C} is converted to PrP^{Sc}, all used the transfection of PrP mutants into PrP-expressing cell lines. Several studies have shown that the concomitant expression of heterogeneous species of PrP results in interaction and affects the conversion [22]. When using the PrP gene-deficient cell line to study prion infection, the absence of endogenous PrP is advantageous in terms of avoiding such effects. Taking advantage of this system, here we introduced artificial PrP molecules deleted of OR or HR into a PrP gene-deficient cell line to identify the region of PrP necessary for efficient production of PrP^{Sc}. PK-resistant PrP^{Sc} level was measured by Western blotting with anti-PrP antibody in cells after prion infection.

The several approaches to the knockdown of PrP have been used. RNAi technology reduced the amount of PrP by 50% in scrapie-infected neuroblastoma cells (N2aS12sc+) [33]. However, as it was reported that even an undetectable amount of PrP^{C} could influence the conversion [21], PrP gene-deficient cells established from PrP gene-knockout mice have been increasingly valuable for elucidating the mechanisms by which PrP^{C} is converted to PrP^{Sc}. The present study exploited structural/functional analyses of the N-terminal region of PrP^{C} to locate the specific domain of PrP^{C} responsible for its capability to retain PrP^{Sc} production at an early stage in PrP gene-deficient cells. To study the region(s) of the N-terminal domain of PrP^{C} affecting the production of PrP^{Sc} after prion infection, we transfected cells with plasmids containing several PrP cDNAs, rendering them capable of expressing full-length PrP [HpL3-4-PrP], expressing PrP deleted of the OR of PrP [HpL3-4-Δ#1], and expressing PrP deleted of the N-terminal half of HR [HpL3-4-Δ#2], suggesting that removal of the OR eliminates the ability to produce PrP^{Sc}. These results are not consistent with a previous study demonstrating the essential role of not only the OR but also the HR including amino acid residues 96, 132, 150, 167, 189, and 204 of mouse PrP for the efficient production of PrP^{Sc} after infection with the mouse-adapted scrapie strain 22L [31]. As 22L can easily induce a persistent infection in HpL3-4 cells [31], but Chandler and Obihiro prion cannot, it is suggested that the mechanism of persistent infection by 22L is different from that of the production of PrP^{Sc} at an early stage by Chandler and Obihiro prion. This also suggests that there are several steps including an early PrP^{Sc} production step and a late persistent PrP^{Sc} production step. The OR may be critical for both steps, but the HR may be only needed for the latter step. It also suggests that the ability to produce PrP^{Sc} is not only attributable copper-binding but to other factors as well. Moreover, Kim et al. have

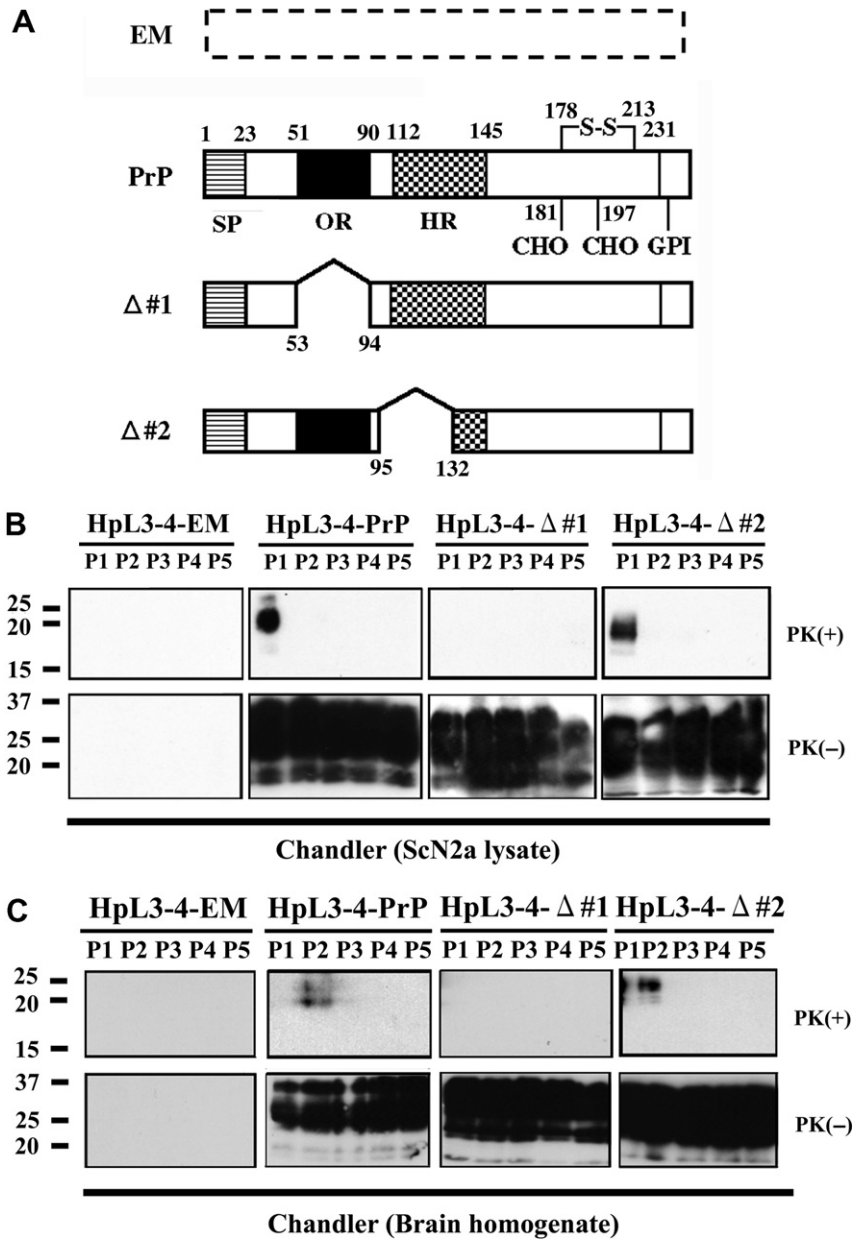

Fig. 2. Deletion of OR prevents PrP^{Sc} production after prion infection. (A) From a schematic comparison of PrP, and mutants [Δ#1: PrP(Δ53–94, Q52H), Δ#2: PrP(Δ95–132)], OR or HR was at least partially deleted in PrP(Δ53–94, Q52) or PrP(Δ95–132), respectively. The predicted glycosylphosphatidylinositol (GPI)-addition, disulfides (S-S) and Asn-linked glycosylation sites (CHO), and signal peptide sequences (SP) are also shown. Cell lysates of Chandler prion-infected ScN2a (B) or Chandler prion-infected brain homogenate (C) were added to cultures of HpL3-4 cells expressing wild-type PrP (PrP: HpL3-4-PrP), PrP(Δ53–94, Q52) (Δ#1: HpL3-4-Δ#1), PrP(Δ95–132) (Δ#2: HpL3-4-Δ#2), or empty vector *per se* (EM: HpL3-4-EM). Then, the cells were passaged from P1 to P5. The cell lysates from the passaged cells were treated with proteinase K [PK(+)] or not [PK(−)] for PrP^{Sc} or total PrP, respectively. The samples were subjected to Western blotting of anti-PrP antibody, SAF83. This assay showed that HpL3-4-Δ#1 cells did not exhibit any PrP^{Sc} signals similar to HpL3-4-EM cells after Chandler prion infection *via* the addition of ScN2a lysate and infected brain homogenate. HpL3-4-Δ#2 cells elicited PrP^{Sc} signals with slightly lower bands (17–25 kDa) than those with 18–27 kDa in HpL3-4-PrP cells after prion infection by Chandler prion-infected ScN2a lysate at P1 and Chandler prion-infected brain homogenate at P2, whereas PrP^{Sc} levels were rapidly reduced to below the detectable limit. These results showed that OR was responsible for PrP^{Sc} production in the early stage after prion infection. Total PrP signals with an approximate molecular weight of 20–37 kDa in HpL3-4-PrP cells and slightly lower bands (18–35 kDa) in HpL3-4-Δ#1 and HpL3-4-Δ#2 cells were not altered during passaging. Total PrP and PrP^{Sc} were not detected in HpL3-4-EM cells during passaging.

recently demonstrated that manganese, which binds to OR more weakly than copper [34], is required for the replication of PrP^{Sc} by protein misfolding cyclic amplification (PMCA), but copper did not facilitate amplification [35]. Therefore, further studies on the extent to which PrP^{Sc} production at an early stage attributable to the efficiency of PrP^{Sc} replication by copper binding are warranted to elucidate the mechanism(s) of PrP^{Sc} production.

PrP(106–126) corresponding to residues 106–126 of the human PrP sequence maintain most of the characteristics

of PrP^{Sc}, including the formation of aggregates [36] and partial resistance to proteolysis [37]; however, it is still a question whether the PrP(106–126) model reproduces the events occurring in prion diseases. For example, although PrP(106–126) induces cell death in neuronal cell lines such as SH-SY5Y [38] and PC12 cells [39], several neuronal cell lines are susceptible to prion infection but most show no cytotoxicity [40]. Our previous studies have shown that the HR but not OR of PrP was required for aged PrP(106–126) neurotoxicity [27], which also supports the dissimilarity between PrP^{Sc} infection and PrP(106–126) neurotoxicity. The mechanisms of PrP^{C} production after prion infection may be different from those of PrP(106–126) neurotoxicity.

Taken together, this study showed that the OR, which binds copper through histidine residues [32], regulates the ability to produce PrP^{Sc} at an early stage. This is because deletion mutagenesis indicated that the cellular capability for PrP^{Sc} production is nullified in PrP lacking the OR. Therefore, the OR seems to be a critical region in terms of PrP^{Sc} production. However, it remains unclear whether these activities of PrP^{C} are the result of copper-binding and which residues of the OR are relevant to PrP^{Sc} production at an early stage. Furthermore, this study also revealed that Chandler and Obihiro prion induced PrP^{Sc} production, which may be caused by a different mechanism from the previous study using 22L, because 22L achieved a persistent infection in HpL3-4 cells [31]. As PrP^{Sc} could be amplified by a recently developed method, PMCA [30], the combination of this cell line system and PMCA would further contribute to understanding the mechanisms of PrP^{C}'s conversion to PrP^{Sc}. As bioassays are the most sensitive method of detecting prion titers [4], use of the combination of a PrP gene-deficient cell line and a bioassay would also provide fruitful results.

Prion infection seems to be divided into several stages, including an early and reversible decision stage leading to persistent infection. Our data suggest that the OR of PrP^{C} is required at an early stage for PrP^{Sc} production. Further study is necessary to fully characterize the mechanisms of PrP^{Sc} production in early stages and to clarify its biological significance in prion infections. The cell model reported here would enhance our understanding of the treatment of prion diseases induced by the early step of PrP^{Sc} production.

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