遺伝子組換え医薬品等のプリオン安全性確保のための検出手法の標準化
及びプリオン除去工程評価への適用に関する研究
-異常型プリオンの新規検出法に関する試験研究-

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研究要旨

遺伝子組換え医薬品等の安全性を確保するため、その原材料に混入する恐れがあるウシ異常型プリオン蛋白質(PrP$^{Sc}$)の高感度な検出法の開発が望まれている。製造原材料の品質確保および種々の製造工程の安全性評価を目的としたPrP$^{Sc}$の検出法の開発に資する基礎研究として、競合的EIAによるウシプリオン蛋白質(PrP)測定法の構築、PrP$^{Sc}$易伝達性細胞を用いた検出法の確立を目的としたウシPrP高発現培養細胞株の樹立およびウシスプライス変異型PrP (PrPSV) mRNAの探索を行った。

競合的EIAでは、市販の抗PrPモノクローナル抗体6H4が認識するペプチドをβ-ガラクトシダーゼに結合させた酵素抗原複合体を作製し、阻害物質として組換えウシPrPを用いた測定系を構築した。本方法は従来の競合的ELISAと同様にウシPrPを測定可能で、測定時間の短縮への寄与が期待できる。

ウシPrP高発現細胞株にはPrP欠損マウス脳由来細胞株HpL3-4を用い、ウシPrP遺伝子を導入したクローンを樹立して、6H4抗体を用いたイムノブロット法でPrPの発現を確認した。本細胞株への遺伝子導入にはレトロウイルスベクターを用いたことから、PrPの安定的な産生が期待できる。

スプライス変異型PrP mRNAの探索にはウシ角膜細胞株BCE C/D-1bからtotal RNAを調製し、各種プライマーを用いて産生したPCR産物の塩基配列を解析し、ウシPrPのGPIアンカーシグナルペプチドを欠損したPrPSV mRNAをクローニングした。このmRNA由来fs-cDNAを特異的に増幅するエクソンジャンクションプライマーを用いたRT-PCRを構築したことから、BSE罹患ウシ試料を用いた解析等への応用が期待される。

## A. 研究目的

人のプリオン病には硬膜移植等によって発症した感染性CJD、プリオン蛋白質遺伝子(*PRNP*)にコードされた253残基のアミノ酸に変異がある遺伝型CJD及び*PRNP*に変異のない散発型CJDが知られ、約85-90%を散発性CJDが占めている。一方、1996年に英国で発症が確認された変異型CJDは、従来の散発型CJDとは異なって若年性の患者で発症し、異常型プリオン蛋白質(PrP$^{Sc}$)の生化学的研究及び英国で多発していた牛海綿状脳症(BSE)に関する疫学研究から、ウシPrP$^{Sc}$の人への伝達によって発症すると考えられている。また、輸血によって変異型CJDを発症したと推定される症例が4件報告されており、血液を介したCJDの伝達が注目を集めている。遺伝子組換え医薬品等の製造工程ではウシ胎児血清を用いた細胞培養を行うものが多く、医薬品にPrP$^{Sc}$が混入するおそれから、ウシ由来原材料中の原材料中のPrP$^{Sc}$測定法の確立が望まれている。

先の厚生労働科学研究費補助金で、遺伝子組換え医薬品等の製造原材料の品質確保および種々の製造工程の安全性評価を目的とし、PrP$^{Sc}$の検出法の開発に資する基礎研究として、PrP$^{C}$を高発現しているヒトグリオブラストーマ細胞株T98Gを対象とした研究を行ってきた。T98G細胞は継代を重ねた後の長期間培養で、プリオン蛋白質(PrP)のC末端とGPIアンカーシグナル配列が欠落したスプライス変異型PrP mRNAの発現することを確認し、そのC末端部位を認識するモノクローナル抗体HPSV178を用いてスプライス変異型GPI欠損プリオン蛋白質(GPI⁻PrPSV)を同定した(投稿中)。

以上の知見をもとに、平成19年度は異常型プリオン蛋白質の新規検出法として以下の研究を行った。ヒトGPI⁻PrPSVのC末端は通常のスプライシングによるPrPと異なり、特異的に認識する抗体によって異常型プリオン蛋白質検出法への応用が期待できることから、同様な手法を用いてウシスプライス変異型PrP mRNAの検索を行った。また、ELISAによるウシ異常プリオン蛋白質検出法の改良を目的とした競合的EIAの検討、異常プリオン蛋白質易伝達性細胞を用いた検出法の開発を目的としたウシPrP高発現細胞株の樹立を行った。

## B. 研究方法

### 1. 細胞培養

ウシ角膜細胞株BCE C/D-1b (JCRB9129)はT75組織培養用フラスコで培養し、1週間に1度の継代を行った。長期間の培養は9-cm組織培養用シャーレで行い、4日ごとに培地を交換した。

PrP欠損マウス脳由来細胞株HpL3-4は、東京大学東京大学大学院農学生命科学研究科応用免疫学 小野寺節教授より御供与して頂き、本研究

に用いた。
2. RT-PCR

BCE-C/D-1b細胞を培養後、DNase I消化した total RNAを調製し、スーパースクリプトIII RNase H-逆転写酵素(インビトロジェン株式会社)を用いてfs-cDNAを合成し、RT-PCRに用いた。同時にゲノムDNAを調製し、陽性対象として PCR に用いた。PCR はウシPrP遺伝子 (AJ298878)のエクソン3にコードされているオープンリーディングフレーム(ORF)のmRNAを検出する各種プライマーと、Pwo SuperYield DNA Polymerase (ロシュ・ダイアグノスティックス株式会社)又はEx taq polymerase (タカラバイオ株式会社)を用いて行った。

3. イムノブロット法

試料をSDS-PAGEで分離後にPVDF膜へ転写し、第1抗体として抗PrP抗体6H4 (ロシュ・ダイアグノスティックス株式会社)を、第2抗体にHRP標識抗IgG抗体を用いたイムノブロッティングを行い、化学発光法で検出した。

4. 競合的ELISA

固相抗原に組換えウシ・プリオン蛋白質 (rBoPrP)を、第1抗体に6H4抗体を用いて競合的ELISA を行い、阻害実験には標準物質として rBoPrPを用いた。

5. 競合的EIA

6H4抗体が認識するエピトープDYEDRYYRE を含むペプチドをβ-ガラクトシダーゼ (β-Gal)で標識し、酵素標識抗原β-Gal-hPrP (142-160)を調製した。抗体と酵素標識抗原による抗原-抗体複合体を形成させ、96穴プレートの固相に結合させた抗マウスIgG抗体で捕捉し、固相に結合した酵素の活性を基質として4-MUGを用いて蛍光強度を測定した。阻害実験には、標準物質としてrBoPrPを用いた。

6. ウシPrP産生細胞の樹立

モロニーマウス白血病ウイルス(MoMuLV)由来のレトロウイルスベクターにウシPrP遺伝子エクソン3のORFを組み込み、当ベクターを導入したヒトHEK細胞由来パッケージング細胞の培養上清からウイルス粒子を調製し、プリオンノックアウトマウス(prnp -/-)脳から樹立された培養細胞株HpL3-4に感染させ、ウシPrP産生細胞株を樹立した。

（倫理面への配慮)

本研究の遂行にあたり、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「国立医薬品食品衛生研究所研究倫理審査委員会規定」、「同病原体等安全管理規程」、「同動物実験に関する指針」及び「同遺伝子組換え実験安全管理規則」を遵守した。

C. 研究結果

1. マウス抗PrPモノクローナル抗体6H4を用いた EIAの構築

抗体として6H4を、酵素標識抗原としてβ-Gal-hPrP (142-160)を用いたEIA を構築した。50%最大結合量を示した抗体量で競合的EIAを行い、その結果をFig. 1に示した。rBoPrPは6H4抗体に対して$10^{-14}$-$10^{-11}$ moleで阻害活性を示し、そのIC50は6.5 x $10^{-13}$ moleであった。一方、従来の競合的ELISAでは$10^{-15}$-$10^{-11}$ moleで阻害活性を示し、そのIC50 (1.0 x $10^{-13}$ mole)は競合的EIAの1/6.5だった。
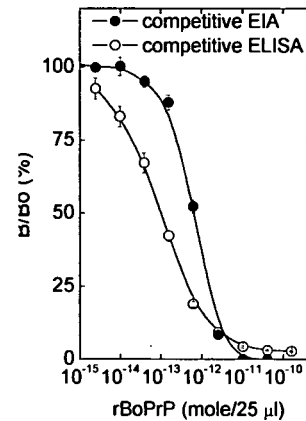


Fig. 1. **Standard curve for recombinant BoPrP.**
The ratio of the fluorescence intensity of the bound antibody in the presence of rBoPrP to that in the absence of rBoPrP (B/Bo) were plotted against the amounts of rBoPrP added. Bars are means ± SE ($n$=3).

2. ウシPrP産生細胞株の樹立

レトロウイルスベクターを用い、プリオンノックアウトマウス(prnp -/-)脳由来のPrP欠損細胞株HpL3-4にウシPrP遺伝子のORFを導入し、イムノブロット法でBoPrPの産生を調べた(Fig. 2)。
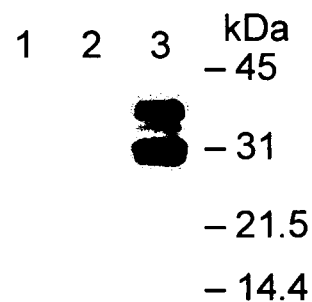


Fig. 2. **Expression of bovine PrP in *Prnp*-deficient neuronal HpL3-4 cells.**
Whole cell lysates from the parental HpL3-4 cells (lane 1), empty vector-transfected cells (lane 2), and vector coding for bovine PrP gene-transfected cells (lane 3) were subjected to immunoblotting with 6H4 mAb.

親株のHpL3-4細胞(lane 1)及びレトロウイルスベクターを導入した細胞(lane 2)から調製した全細

胞溶解液では、6H4抗体が認識するバンドは検出されなかった。一方、ウシPrP遺伝子のORFを導入した細胞から調製した全細胞溶解液では、6H4が認識する35及び31 kDaのバンドを示した(lane 3)。数回の継代後にもBoPrPの産生を確認し、樹立したBoPrP産生細胞株として凍結保存した。

3. スプライス変異型プリオン蛋白質mRNAの解析

ウシPrP mRNAの発現様式を調べるため、長期間培養したウシ角膜細胞株BCE C/D-1bから調製したtotal RNAを鋳型として、RT-PCRを行った。13回の継代後に40日間培養したBCE C/E-1b細胞(P13D40)のtotal RNAから合成したfs-cDNAを鋳型としたRT-PCRでは、1,347 bpのバンドのほかに、産生量は少ないが836 bpのバンドを検出した(Fig. 3, lane 3)。それぞれのバンドをゲルから切り出して塩基配列を解析したところ、1,347 bpの塩基配列はORFを含むウシPrPエクソン3をコードし、836 bpの塩基配列ではBoPrPのC末端に位置するGPIアンカーシグナル配列を含む511 bpが欠落していた(Fig. 4)。同時に調製したBCE C/E-1b細胞(P13D40)のゲノムDNAを鋳型としたPCRでは1,347 bpのバンドが検出され、その塩基配列はtotal RNA由来の1,347 bpのバンドと一致したが、836 bpのバンドは検出されなかった(Fig. 3, lane 7)。以上の結果から、BCE C/D-1b細胞はウシPrPエクソン3のスプライス変異型mRNAを発現することが示唆された。

次に、エクソン結合部位に結合するプライマー(exon-exon junction primer)を設計し、スプライス変異型PrPのmRNAを特異的に検出するRT-PCRを構築した。スプライシングで欠落する部位(511 bp)を挟み、その両端の配列を結合させたプライマーを用いたPCRは、fs-cDNAに130及び744 bpのバンドを示したが(Fig. 3, lanes 1-2)、genomic DNAではバンドが検出されなかった(Fig. 3, lanes 5-6)。また、切り出した130及び744 bpのバンドの塩基配列は、エクソン3のそれぞれ641及び1,255bpに相当する部位から511 bpの配列が欠失したものと一致した。以上の結果から、設計したexon-exon junction primerはエクソン結合部位と結合し、構築したRT-PCRでスプライス変異型PrPのmRNAを特異的に検出可能なことが示唆された。

D. 考察

プリオン病の検出法には、試料をマウス脳内に接種してプリオン病の発病を観察するバイオアッセイ、脳や脾臓の病理切片を用いた免疫染色法、脳乳液を試料としたイムノブロット法やELISA等がある。また、周期的に超音波処理をくり返すprotein misfolding cyclic amplification
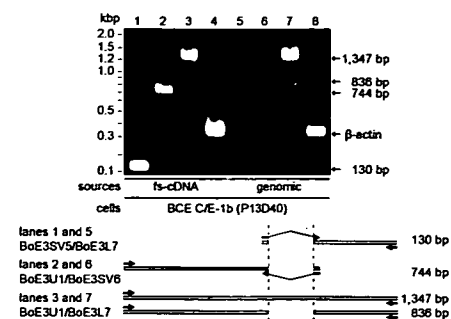


**Fig. 3. Detection of splice variant of bovine PrP mRNA using exon-exon junction primers.**
BCE C/E-1b cells for 40 days after 13 passages (P13D40) were cultured. First strand-cDNA from total RNA (5 μg) and genomic DNA (25 ng) were prepared, followed by PCR using bovine prp exon-exon junction primers (BoE3SV5/BoE3L7, lanes 1 and 5; BoE3U1/BoE3SV6, lanes 2 and 6), bovine prp primers (BoE3U1/BoE3L7, lanes 3 and 7) and β-actin primers with Ex taq polymerase. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide.
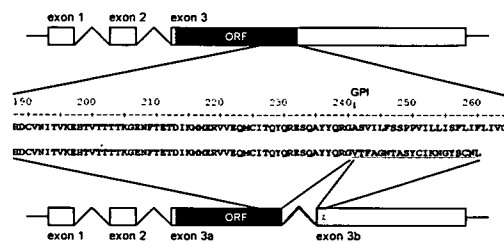


**Fig. 4. Schematic representation of alternative splicing model of bovine *prp* gene.**
We confirmed the sequences of ordinary (upper panel) and alternatively spliced bovine *prp* gene (lower panel). Cryptic donor and acceptor sites are designated as exon 3a and 3b, respectively. The untranslated regions (white bars), ORFs (black bars), retained intron (double line), additional ORF (gray bar), and deduced amino acid sequences of ordinary and alternatively spliced (underlined) *prp* are indicated. The arrow indicates a GPI anchoring site.

(PMCA)により、試料中の異常プリオン蛋白質を増幅して検出感度を向上させる試みがなされている。しかし、最も高感度な測定法は発症やPrPScの蓄積を検出するバイオアッセイで、検出には数か月間を要している。本研究では異常プリオン蛋白質の新規検出法の確立を目的とし、プリオン病のスクリーニングに利用されているELISAの改良、新たなバイオアッセイ系に用いるPrPSc易感染細胞株の樹立、プリオン病の新た

な代理バイオマーカー(sarrogate marker)の検索を行った。

市販の6H4抗体を用い、その認識するエピトープを含むペプチドを酵素標識した競合的EIAを構築した。本方法は主に低分子のハプテンの測定に利用されているが、本研究ではペプチドを酵素標識した測定系を構築した。競合的EIAは、通常の2抗体法による競合的ELISAに比較して抗原抗体反応に要する時間を短縮できる利点があり、本研究でも測定時間の大幅な短縮が可能となった。しかし、その検出感度は競合的ELISAに比較すると、若干低い値を示した。今回は酵素としてβ-Galを化学的にペプチドに結合させて用いたが、エピトープとして認識するアミノ酸配列をタグとしてβ-Galやルシフェラーゼ等の酵素に付加した組換え蛋白質発現系の利用によって酵素の比活性を上げることにより、検出感度の上昇が期待できる。この手法はPrPだけではなく、蛋白質の1次配列をエピトープとして認識する抗ペプチド抗体に適用が可能で、様々な蛋白質を検出する競合的EIAの構築への利用が見込まれる。

プリオン病のバイオアッセイにはマウスやハムスターへの脳内接種が用いられているが、発病までに長期間を要することから、培養細胞へPrP$^{Sc}$を伝達する迅速化が試みられている。PrP$^{Sc}$を持続的に維持する培養細胞はScN2a等があり、主にマウス神経系の細胞が利用されている。しかし、プリオン病には動物種のバリアが存在し、動物種が異なる脳乳液を接種したマウスでは発病が遅れる例が知られている。本研究ではウシ異常プリオン蛋白質の検出を行うことから、バイオアッセイにウシ型のPrPを発現する細胞の樹立を目的として、プリオン欠損マウス脳由来細胞株HpL3-4でウシPrP遺伝子の導入を試みた。また、安定した発現細胞を得るために、導入には持続的に蛋白質を発現させるレトロウイルスベクターを用いた。樹立した細胞株は6H4抗体が認識するウシ型のPrPを発現し、その発現は数回の継代後も維持されていた。今後は、この細胞株を用いたPrP$^{Sc}$の伝達実験を予定している。

プリオン病の診断には、脳内のPrP$^{Sc}$を直接測定する代わりに、罹患状態で特異的に増減するバイオマーカーを検出して生前診断に利用する試みとして、脳脊髄液中の14-3-3蛋白質の7種類アイソフォームやtau蛋白質の測定や、血液又は尿中の蛋白質の検索が行われいる。しかし、これらは代理マーカーで、すべてのプリオン病で共通の指標にするには注意が必要である。先の厚生労働科学研究費補助金で、我々は培養細胞及びヒト各種臓器由来のtotal RNA中でスプライス変異型PrP mRNAを検出し、抗体によりヒト培養細胞中でPrPSVの産生を確認した(投稿中)。

ヒトPrPSVはGPI欠損型のPrPで、細胞外への放出が予想される。本研究ではプリオン病のバイオマーカーとしてスプライス変異型PrPを利用することを目的とし、ウシ培養細胞から調製したtotal RNAを用いて検索を行った。ヒトPrPSVの検索と同様の手法を用い、ウシ角膜細胞株BCE C/D-1bからスプライス変異型PrP mRNAを同定し、その塩基配列から蛋白質のアミノ酸配列を推定した(Fig. 4)。ウシPrPは264残基のアミノ酸配列を有し、242-264残基はGPIアンカーシグナル配列と推定され、241残基のAlaを介して細胞膜上に結合している。一方、ウシPrPSV mRNAから推定されるアミノ酸配列は260残基からなり、1-240残基はPrPと共通だが、241-260残基は異なったアミノ酸配列を有している。予想されるウシPrPSVの構造は、ヒトと同様にそのC末端がPrPと異なっている。現在、241-260残基のアミノ酸を認識する抗体の作製を進めている。今後はウシPrPSVの産生を確認し、プリオン病のバイオマーカーとしての利用の可能性を調べる予定である。

E. 結論

本研究ではウシ異常プリオン蛋白質の新規検出法の確立を目的とし、6H4抗体とその認識するエピトープを含むペプチドをβ-Galに結合させた酵素抗原複合体を用いた競合的EIAの構築、PrP産生細胞株の樹立、スプライス変異型PrP mRNAを検出するRT-PCRを確立した。これらの結果は、抗体を用いたPrPScスクリーニング法の改良、新たなPrP$^{Sc}$バイオアッセイ系の構築、プリオン病の新たなバイオマーカー測定法開発への寄与が期待できる。

F. 健康危険情報
なし

G. 研究発表
1. 論文発表
なし
2. 学会発表
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なし

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# PrP$^{Sc}$ level and incubation time in a transgenic mouse model expressing Borna disease virus phosphoprotein after intracerebral prion infection

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## Abstract

Our previous studies have shown that the persistent expression of Borna disease virus phosphoprotein (BDV P) in mice leads to behavioral abnormalities resembling those in BDV-infected animals. In this study, we investigated whether the neurobehavioral abnormalities genetically induced by BDV P influence experimental prion disease. The effect of the phosphoprotein on prion diseases was evaluated based on the incubation time and survival curve, as well as the abnormal isoform of prion protein (PrP$^{Sc}$) levels in brains of BDV P Tg mice treated with proteinase K (PK) treatment and subjected to western blotting. Increased expression of the BDV P transgene had no effect on the PrP$^{Sc}$ level, incubation time, or survival curve. The abnormalities induced by BDV P are different from those induced by prion diseases, indicating that the signaling cascades induced by the phosphoprotein differ from those induced by prion diseases.
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*Keywords:* Borna disease virus; Prion; Scrapie; PrP; Psychiatric disease

Prion diseases are neurodegenerative disorders characterized by the accumulation of an abnormal isoform of prion protein (PrP$^{Sc}$) and astrocytosis in the central nervous system (CNS) [1]. The prion diseases include scrapie, bovine spongiform encephalopathy (BSE), Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), and fatal familial insomnia (FFI). The PrP$^{Sc}$ isoform shows increased β-sheet content and proteinase-K (PK) resistance compared to the cellular isoform of prion protein (PrP$^{C}$) [20]. The phenomena may be associated with the deposition of amyloid by scrapie-associated fibrils, which is specific to the prion diseases [15]. Therefore, resistance to proteinases such as PK is usually used as an index of prion infections in both clinical diagnosis and laboratory testing [26]. Furthermore, although the protein-only hypothesis has not been finally proved [19], it states that PrP$^{Sc}$ is replicated by autocatalytic conversion of PrP$^{C}$ into PrP$^{Sc}$ [20]. Recently, experiments with protein misfolding cyclic amplifica-

tion (PMCA) have come very close to proving this hypothesis by producing a prion disease in a natural host using a prion entirely generated *in vitro* [3,35].

Borna disease virus (BDV) is a noncytolytic and neurotrophic virus that belongs to *Mononegavirales* and can infect a broad range of vertebrates, including all warm-blooded animals and possibly also humans [7,21,31]. BDV infection leads to a variety of abnormal behaviors such as anxiety [22], aggression [22], hyperactivity [6,22], abnormal play behavior [18], chronic emotional overactivity [18], inhibition of responses to novel stimuli [6], and abnormal social behavior [18]. Various disorders could also be induced by BDV infection including cognitive deficits [5], reminiscent of autism [11], and schizophrenia [30]. Loss of Purkinje cells [4,6,33] and alterations in cytokine and chemokine gene expression [6,17,28] following neonatal BDV infection have been also demonstrated. Furthermore, epidemiological studies have demonstrated a higher prevalence of BDV infection in psychiatric patients such as schizophrenics than in controls [2,7,14]. Notably, as BDV phosphoprotein (P) is abundant in infected animal brains and interferes with a multifunctional protein, high-mobility group protein box 1 (HMGB1), in neuronal cells [10,16], it is thought to affect neuronal cells in the infected CNS. BDV P transgenic (Tg) mice develop behavioral abnor-

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malities resembling those in BDV-infected animals, and severe neurological disturbances are linked to neurobehavioral disorders. Therefore, the BDV P Tg mouse is thought to be a good experimental psychiatric animal model [29].

Interestingly, a rare mutation in the prion protein (PrP) gene has been discovered in a family with a strong history of psychiatric disease, and was linked to a complex phenotype including typical symptoms of schizophprenia like persecutory delusions and auditory hallucinations [27]. However, other studies found no association of single nucleotide polymorphisms (SNPs) in PrP gene with schizophrenia [23,32]. It is conceivable that psychiatric diseases and prion diseases share several signaling cascades and have some association. However, there was no appropriate experimental system for examining this issue. Here, we studied survival and the accumulation of PrP$^{Sc}$ in brains of scrapie (chandler)-infected BDV P Tg mice.

PrP$^{Sc}$ was intracerebrally (i.c.) inoculated into C57BL6J (wild-type, WT) mice and BDV P Tg mice [9] older than 8 weeks of age. The inoculation (i.c.) was conducted in P3 biohazard facilities as follows. Twenty microliters of inocula including 1% homogenate prepared from the brains of terminally diseased mice with mouse-adapted scrapie (chandler strain) was injected into the cerebral ventricular system of mice using a microsyringe as described previously [8]. The clinical symptoms (tremors and ataxia) were observed for the calculation of incubation time.

Semiquantitative reverse-transcription (RT)-polymerase chain reaction (PCR) and DNA-PCR was performed with the primers P1 (5'-TCA GAC CCA GAC CAG CGA A-3') and P2 (5'-AGC TGG GGA TAA ATG CGC G-3') for BDV P (p24), and G1 (5'-ACC ACA GTC CAT GCC ATC AC-3') and G2 (5'-TCC ACC ACC CTG TTG CTG TA-3') for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously [24]. The intensity of gel bands was analyzed with Scion Image software (Scion Corp., Frederick, MD).

To discriminate PrP$^{Sc}$ from PrP$^{C}$, 50 μg of protein per sample was treated in the absence and presence of 20 μg/ml of PK (Merck & Co. Inc., Whitehouse Station, NJ, USA) at 37 °C for 30 min. After termination of the reaction, the product was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and western blotting.

Brains were removed from dead mice within 24 h and frozen at −80 °C in a refrigerator. The samples were solubilized in radio-immunoprecipitation assay (RIPA) buffer and sonicated at 4 °C. The RIPA buffer was composed of 10 mM Tris–HCl (pH 7.4) containing 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS and 0.15 M sodium chloride. Then, the protein samples {20 μg/lane for PrP [PK(−)]; 50 μg/lane for PrP [PK(+)]} were dissolved in SDS-PAGE sample buffer [70 mM SDS, 10% (w/v) glycerol, 1% 2-mercaptoethanol, 15 mM bromophenol blue, and 62.5 mM Tris–HCl, pH 6.8] and incubated at 100 °C for 10 min before electrophoresis in a 15% polyacrylamide gel containing 0.1% SDS at a constant current of 30 mA for 1 h. After electrophoresis, the proteins in the gel were electrically transferred to a polyvinylidene difluoride (PVDF) membrane (see below).

Proteins separated by SDS-PAGE were electrically transferred onto PVDF membranes (Amersham Biosciences,

Piscataway, NJ), which were pretreated with methanol and transfer buffer [48 mM Tris, 39 mM glycine, 20% (v/v) methanol and 1.3 mM SDS] at a constant voltage of 12 V (ca. 100 mA) for 1 h. After the transfer, the membrane was blocked with 5% skim milk (Wako, Osaka, Japan) for 1 h at room temperature with gentle shaking before incubation with a mouse anti-PrP antibody, SAF83 (SPI bio, Montigny le Bretonneux, France) diluted with phosphate-buffered saline (PBS) containing 0.5% (v/v) skim milk and 0.1% (v/v) Tween 20. After 1 h of agitation at room temperature, the membrane was washed three times with PBS containing 1% (v/v) Tween 20 (PBS-T) for 5 min. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin antibody (Jackson Immunoresearch, West Grove, PA) for 1 h before being thoroughly washed with PBS-T. HRP was detected using an ECL$^{TM}$ kit (Amersham Biosciences) as described in the kit's instructions. The electrophoreogram was recorded on X-ray film (Amersham Biosciences). Each PrP$^{Sc}$ and/or total PrP band intensity was measured using Scion Image for Windows software (Scion Corp., Frederick, MD, USA) in western blotting, and the PrP$^{Sc}$ and/or total PrP level of each brain was estimated as a percentage (compared with the band intensity of cerebral PrP$^{C}$ of the uninfected mouse).

The survival curves were analyzed by the Logrank test using Prism 4 Software (GraphPad Software, San Diego, CA, USA). Data on incubation time was analyzed with Student's $t$-test. The band intensities in the western blotting and RT-PCR analysis were subjected to a non-repeated measure ANOVA followed by the Bonferroni correction test.

In this study, we intracerebrally injected chandler prion into brains of WT mice and BDV P Tg mice and compared the two groups in terms of survival rate, incubation time, and PrP$^{Sc}$ accumulation. First, the survival rate of the groups was compared (Table 1 and Fig. 1). All 11 prion-infected WT mice showed abnormal behavior including tremors and ataxia from 161 days and had died by 219 days. Similarly, all 5 prion-infected BDV P Tg mice showed clinical symptoms of prion disease from 182 days and died by 215 days. The Longman test showed that the survival rate of two groups was not significantly different (Fig. 1). The survival rate reflected a similar tendency in incubation time (Table 1). The incubation times of infected WT mice (197.3 ± 5.6) were not significantly different from those of infected BDV P Tg mice (194.8 ± 5.8) according to Student's $t$-test. As predicted, non-injected WT and BDV P Tg mice sur-

Table 1

Incubation time of wild-type (WT) and BDV P transgenic (Tg) mice infected with scrapie

| | Inoculum | Mean incubation time ± S.E.M.[a] (days) | $N/N_0$[b] |
|---|---|---|---|
| WT | Chandler prion | 197.3 ± 5.6 | 11/11 |
| | Normal brain homogenate | >280 | 0/10 |
| Tg | Chandler prion | 194.8 ± 5.8 | 5/5 |
| | Normal brain homogenate | >280 | 0/2 |

[a] S.E.M., standard error of the mean.

[b] $N$, number of dead animals; $N_0$, number of inoculated animals.
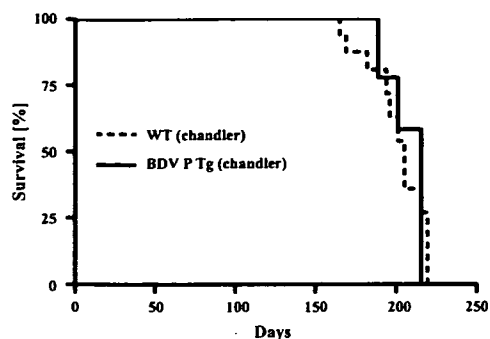
Fig. 1. Effect of Borna disease virus (BDV) phosphoprotein (P) on survival of scrapie-infected mice. Survival times of wild-type mice (WT, dotted line) and BDV P expressing transgenic mice (BDV P Tg, line) injected intracerebrally with chandler prion-infected brain homogenate. The survival curve of WT mice was not significantly different from that of BDV P Tg mice according to the Logrank test with $p < 0.05$ considered significant.

vived more than 280 days (Table 1). These results indicate that BDV P expression does not delay or accelerate the onset of clinical signs of disease and does not prolong survival.

Next, to investigate whether prion infection influences BDV p24 mRNA levels, a RT-PCR analysis of BDV p24 mRNA was performed using brains of prion-infected BDV P Tg mice at the terminal diseased stage as well as uninfected BDV P Tg mice (Fig. 2). All 5 infected and all 2 uninfected BDV P Tg mice expressed BDV p24 mRNA in the brain (Fig. 2, left panel).
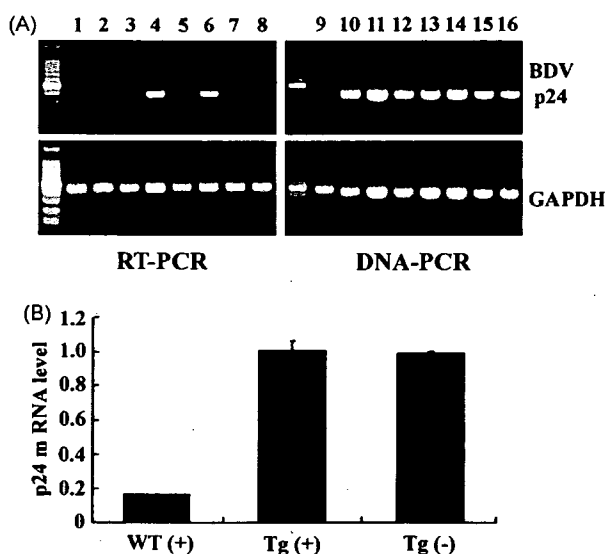


Fig. 2. Expression of BDV P transgene after scrapie infection in BDV P Tg mice. (A) The mRNA expression levels of BDV p24 in mouse brain were examined by reverse transcription (RT)-polymerase chain reaction (PCR) (left panel) and DNA-PCR (right panel). Brains of BDV P-expressing Tg mice (lanes 2–6, 10–14) and wild-type (WT) mice (lanes 1, 9) infected with chandler prion (+) were collected within 24 h of death. Non-infected Tg mice [Tg(−)] were also collected (lanes 7, 8, 15, 16). The presence of BDV p24 mRNA and DNA in the BDV P Tg mice was confirmed. (B) The expression levels of BDV p24 mRNA in BDV P Tg mice infected [Tg(+)] and non-infected [Tg(−)] with chandler prion were measured. As a control, WT mice infected with chandler prion [WT(+)] were also examined. The expression level of BDV p24 mRNA in Tg(+) was taken as 1. There is no significant difference in BDV p24 mRNA levels between infected and non-infected BDV P Tg mice. Error bars indicate standard deviations (S.D.).

Genomic DNA of these mice was confirmed to contain BDV p24 DNA by DNA-PCR (Fig. 2, right panel). The band intensities of GAPDH mRNA varied slightly among the mice. Therefore, the relative BDV p24 mRNA levels were normalized to the GAPDH band and compared based on band intensity in RT-PCR images (Fig. 2B). The results showed that BDV p24 mRNA levels were not significantly different between infected and uninfected mouse brains. This means that the proportion of cells expressing BDV p24 mRNA was not changed by prion infection, indicating that BDV p24 expression does not contribute to the onset of prion disease.

PrP$^{Sc}$ is characterized by a partial resistance to PK and can be distinguished from PrP$^{C}$ by western blotting after treatment with PK. The resistance is thought to reflect the protein's conformation and/or aggregation. Therefore, the level of PrP$^{Sc}$ is thought to correlate with the amount accumulated in the brain leading to the onset of prion disease. To investigate whether the expression of BDV P is able to affect the accumulation of PrP$^{Sc}$ in the brain, brain homogenates from prion-infected WT and BDV P Tg mice at the terminal of the disease as well as the corresponding uninfected mice were treated with PK and analyzed by western blotting using anti-PrP antibody, SAF83 (Fig. 3A). Without digestion, three broad bands of PrP were detected. The intensity of these three bands in BDV P Tg mice appeared to be similar to that in WT mice. Upon digestion with PK, the bands from uninfected WT and BDV P Tg mice disappeared, whereas the three bands in infected WT and BDV P Tg mice remained, albeit they shifted to a slightly lower position due to partial degradation of PrP$^{Sc}$, suggesting that PrP$^{C}$ was completely digested by PK whereas PrP$^{Sc}$ was only partially
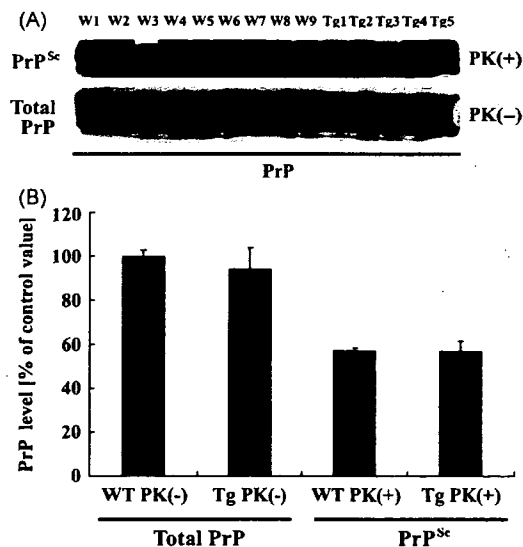


Fig. 3. PrP$^{Sc}$ and total PrP levels in brains of WT and BDV P Tg mice infected with chandler prions. (A) The relative concentrations of total PrP and PrP$^{Sc}$ in brains of WT mice (W1–W9) and BDV P Tg mice (Tg1–Tg5) infected with chandler prions via intracerebral inoculations were examined by western blotting before (−) and after (+) proteinase K (PK) treatment, respectively. (B) Densitometric measurements of the signal of PrP$^{Sc}$ and total PrP in the western blotting were performed. The level of total PrP in brains of uninfected WT mice in the absence of PK was taken as 100%. Error bars indicate standard deviations (S.D.).

degraded. No difference in levels of $PrP^{Sc}$ and total PrP was observed between WT and BDV P Tg mice after infection in the absence or presence of PK. To confirm the results, the relative PrP levels were statistically compared based on PrP band intensity in western blot images (Fig. 3B). The results showed that levels of $PrP^{Sc}$ and total PrP did not differ significantly between BDV P Tg and WT mice in the absence and presence of PK, indicating that BDV P expression did not affect $PrP^C$ and $PrP^{Sc}$ levels.

There are several reports investigating the relationship between prions and viruses. Transfection of human immunodeficiency virus (HIV) DNA-induced PK-resistant PrP in human 293T cells [12]. Moloney murine leukemia virus (MoMuLV) infection and transfection of MoMuLV Gag enhanced the release of $PrP^C$ and $PrP^{Sc}$ from NIH3T3 cells [13]. Furthermore, several reports have suggested that oxidative stress metabolism is related to the pathogenesis of diseases caused by prions and BDV infection [25,34]. Therefore, these diseases may be associated with signaling cascades.

In this study, we investigated the differences between WT mice and BDV P Tg mice after prion infection in terms of survival and $PrP^{Sc}$ accumulation using western blotting. This study design is important in the context of a possible search for relationships between prion diseases and other diseases. We approached whether psychiatric disease exerts an addictive effect on the onset of prion diseases using a psychiatric mouse model, BDV P Tg mice. This study shows that BDV P expression does not have any addictive effect on the onset or progression of prion diseases. This is consistent with the results of western blotting, which revealed no difference in $PrP^{Sc}$ and total PrP levels between BDV P Tg mice and WT mice at the terminal stage of prion diseases. This suggests that BDV P expression does not enhance the accumulation of $PrP^{Sc}$ and expression of $PrP^C$. Reduction in brain-derived neurotrophic factor (BDNF) expression, numbers of serotonin (5-HT) receptors, and the density of synapses have been reported in the hippocampus of BDV P Tg mice [9]. Therefore, it seems that these abnormalities do not have any association with the pathogenic mechanism of prion diseases. More importantly, this also suggests that the BDV P-induced psychiatric phenotype does not share signaling cascades with prion-induced neurodegeneration. In addition, although relationships between PrP polymorphisms and psychiatric disease were reported, the present results experimentally support that psychiatric disease is not associated with PrP polymorphisms [32]. Our experimental model is also consistent with the previous observation of no relationship between psychiatric disease and prion disease [1].

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**BBRC**

# 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.
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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 $5 \times 10^4$ 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 5000g 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 pro-

teins 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
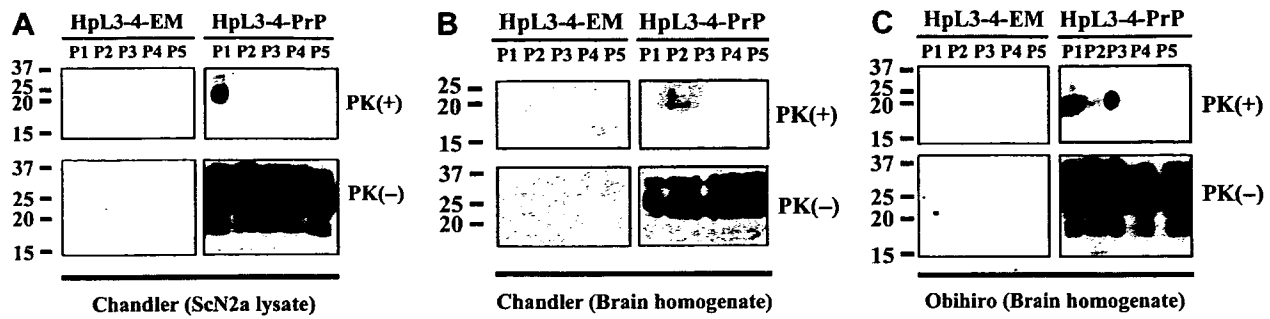
Fig. 1. The production of PrP$^{Sc}$ in a PrP gene-deficient cell line transfected with PrP and infected by prion-infected cell lysate and brain homogenate. To measure production levels of PrP$^{Sc}$, HpL3-4-EM, and HpL3-4-PrP cells were plated at $5 \times 10^4$ cells/well in 24-well microtiter plates. 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
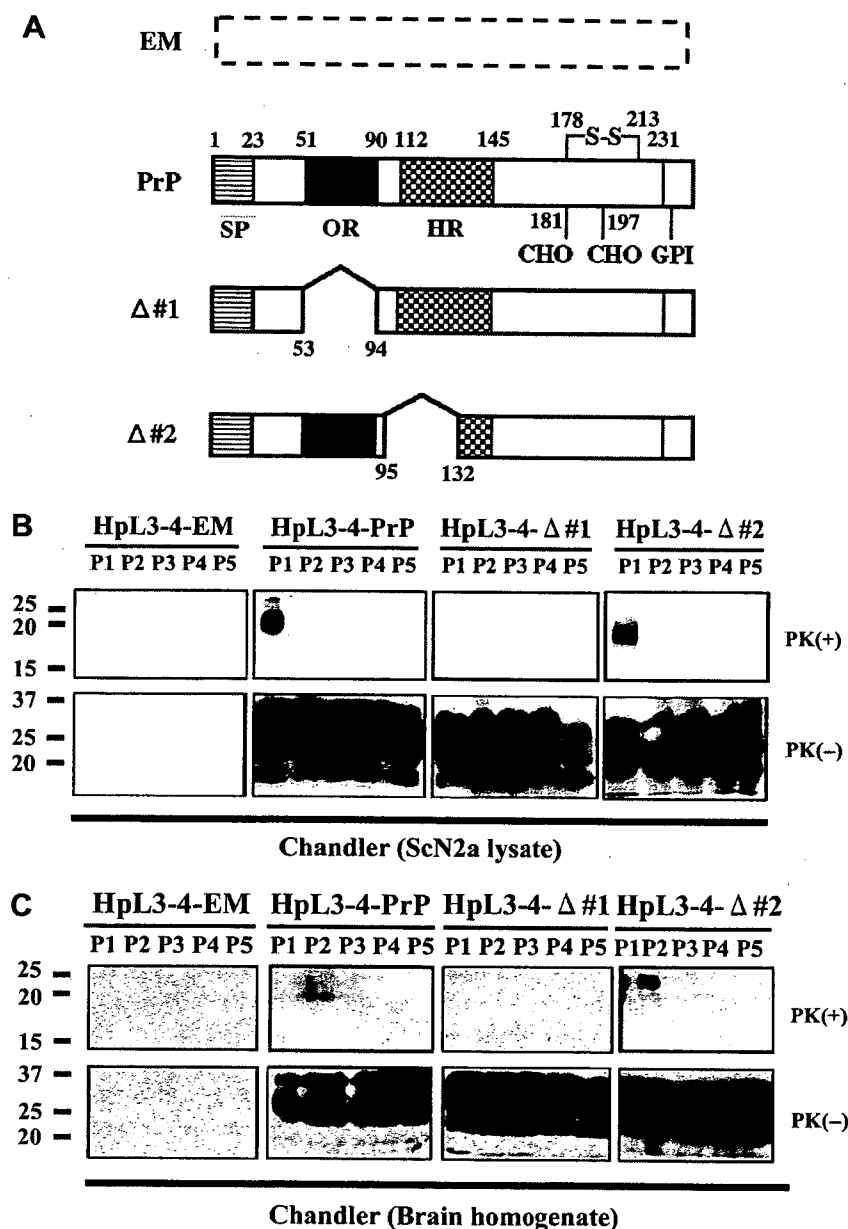
Fig. 2. Deletion of OR prevents PrP$^{Sc}$ production after prion infection. (A) From a schematic comparison of PrP, and mutants [Δ#1: PrP(Δ53–94, Q52H), Δ#2: PrP(Δ95–132)], OR or HR was at least partially deleted in PrP(Δ53–94, Q52) or PrP(Δ95–132), respectively. The predicted glycosylphosphatidylinositol (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$^{Sc}$ pro-

duction 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.

## Acknowledgment

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