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、継代を重ねた後に長期間培養してプロテナーゼ K（PK）処理抵抗性プリオン蛋白質（$PrP^{res}$）を産生する条件下における $PrP^{res}$ 産生様式の解析を行った。

平成 16 ～ 17 年度は、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（$GPI^-$ PrPSV）mRNA を T98G 細胞が発現することを確認し、その mRNA 配列から推定される $GPI^-$ PrPSV の C 末端部位を認識するモノクローナル抗体 HPSV178 を作製した。

平成 18 年度は、$GPI^-$ PrPSV の組換え蛋白質を調製した。次に、イムノブロット法で HPSV178 抗体の反応性を確認し、T98G 細胞が産生する $GPI^-$ PrPSV を同定した。

## B. 研究方法

### 1. 細胞培養

ヒトグリオブラストーマ細胞株 T98G 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。

### 2. 組換え蛋白質の調製

ヒト PrP の cDNA は T98G 細胞のゲノム DNA から調製した。$GPI^-$ PrPSV の cDNA は、PrP の cDNA との共通部位に、合成オリゴヌクレオチドをアッセンブリー PCR で結合させて作製した。それぞれの cDNA を pET-22b ベクターに組み込み、大腸菌 *E. coli* BL21(DE3)pLysS で発現させた。

### 3. イムノブロット法

試料を SDS-ポリアクリルアミドゲル電気泳動（SDS-PAGE）で分離後、ポリフッ化ビニリデン（PVDF）膜へ転写し、第 1 抗体として抗 PrP 抗体又は抗 $GPI^-$ PrPSV マウスモノクローナル抗体 HPSV178 を、第 2 抗体として西洋ワサビ由来ペルオキシダーゼ（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. 細胞分画法

ソニケーターで T98G 細胞を破砕後、遠心分離（500 × *g*、4°C、5 分間）で核画分を除いた細胞懸濁液を調製し、遠心分離後（100,000 × *g*、4°C、60 分間）に上清として細胞質画分を、沈殿物として膜画分を得た。

### 5. リアルタイム定量 PCR

T98G 細胞を培養後、DNase I で消化した総 RNA を調製し、SuperScript III RNase $H^-$ 逆転写酵素（インビトロジェン社）を用いて 1 本鎖 cDNA を合成した。これを PCR での鋳型 DNA として、*PRNP*（GenBank accession No. AL133396）のエキソン 2 にコードされている PrP オープンリーディングフレーム（ORF）の mRNA を検出するプライマー、スプライス変異を検出する exon-exon junction プライマー（図 1）及びハウスキーピング遺伝子である β-アクチンを検出するプライマー、並びにそれぞれに対応した TaqMan プローブとともに FastStart TaqMan Probe Master（ロッシュ・ダイアグノスティックス社）を用いてマルチプレックス PCR を行った。PCR の結果に基づく定量は、Chromo 4 リアルタイム PCR 解析システム（日本バイオ・ラッド・ラボラトリーズ社）を用いて相対定量法により実施した。

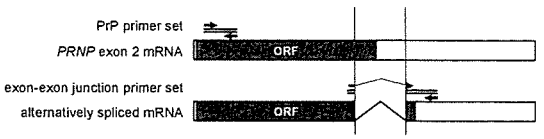


**図 1. Schematic representation of quantitative RT-PCR primer sets**

The arrowed regions represent the primer sets, and the doublets represent the expected products.

### 6. 蛋白質分解酵素消化

T98G 細胞の全細胞抽出液を PK で消化（10 μg/mL、37 ℃、30 分間）した後、イムノブロット法により PrP の蛋白質分解酵素抵抗性を調べた。

### （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及び同動物実験に関する指針を遵守した。

## C. 研究結果

### 1. 組換えヒトプリオン蛋白質及びスプライス変異型プリオン蛋白質の調製

ヒト PrP のアミノ酸配列 23 ～ 230 残基に相当する組換え蛋白質（rhPrP）及び T98G 細胞が発現する GPI$^-$ PrPSV mRNA の 23 ～ 230 残基に相当する組換え蛋白質（rhPrPSV）を調製した。T98G 細胞の *PRNP* がコードする PrP はコドン 129 に Met/Val をもっていることから、それぞれの多型を有する組換え蛋白質を大腸菌で発現させ、各種抗体を用いてその産生を確認した。PrP の N 末端を認識するニワトリモノクローナル抗体 HUC2-13（図 2（A））及び C 末端を広く認識するマウスモノクローナル抗体 17H5（図 2（B））は、T98G 細胞が産生する PrP を認識し、糖鎖がない PrP と同様の位置に組換え蛋白質に相当するバンドを示した。一方、PrP の C 末端側 214 ～ 230 残基を認識するウサギポリクローナル抗体 HPC2 は組換え hPrP を、GPI$^-$ PrPSV の C 末端側 214 ～ 230 残基に相当するペプチドを認識するマウスモノクローナル抗体 HPSV178 は組換え hPrPSV をそれぞれ認識した（図 2（C）・（D））。以上の結果から、HPSV178 は GPI$^-$ PrPSV を認識することが確認された。

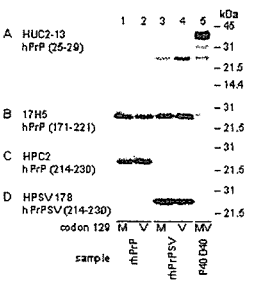


**図 2. Immunoblot analysis of recombinant human PrP and splice variant isoform of PrP**

Recombinant human PrP [codon 129M (lane 1) and 129V (lane 2)], splice variant isoform of PrP [codon 129M (lane 3) and 129V (lane 4)], and homogenates (lane 5) from T98G cells for 40 days after 40 passages (P40D40) were subjected to immunoblot with the HUC2-13 (**A**), 17H5 (B), HPC2 (**C**) or HPSV178 (**D**) antibodies. Epitope recognition sites located within PrP or PrPSV are shown as amino acid numbers.

### 2. T98G 細胞が産生するスプライス変異型 PrPSV の解析

T98G 細胞を長期間培養後に調製した細胞懸濁液を用い、PrPSV 産生の変化をイムノブロット法で調べた。継代 40 週間後に 40 日間培養した T98G 細胞（P40D40）では HPSV178 が認識するバンドは検出されないが（図 2（D）

レーン5、図3（A）レーン1)、52週間以降ではGPI⁻ PrPSVの産生が確認された（図3(A)レーン2・3)。

PrPは2本のアスパラギン酸結合型糖鎖を有する糖蛋白質で、継代77週間後に40日間培養したT98G細胞（P77D40）は糖鎖2本、1本及び糖鎖がないそれぞれ35、31及び25 kDaのバンドを示した（図3 (B)上段 レーン1)。*N*-グリコシド結合を切断するPNGase F処理によってマウスモノクローナル抗体6H4が認識する高分子量の2本のバンドは消失し、25 kDaのバンドが増大した（図3 (B)上段 レーン2)。一方、HPSV178が認識するPrPSVには糖鎖がなく、PNGase F処理で25 kDaのバンドに変化はなかった（図3(B)下段 レーン1・2)。

$PrP^C$はGPIアンカー型蛋白質で細胞膜上に存在することから、T98G細胞が産生するGPI⁻ PrPSVの細胞内での局在を調べた。T98G細胞（P77D44）の細胞懸濁液には6H4が認識するPrPは膜画分に局在し（図3 (C)上段)、HPSV178が認識するGPI⁻ PrPSVは細胞質画分に局在していた（図3 (C)下段)。

次に、T98G細胞が産生するPrPの非イオン性界面活性剤に対する溶解性を調べた。T98G細胞（P77D40）を破砕し、非イオン性界面活性剤に溶解後に遠心分離（100,000 × *g*、4°C、60分間）し、得られた上清と沈殿物のイムノブロット法を行った。細胞懸濁液(図3 (D)レーン1）と同様に、非イオン性界面活性剤可溶画分には6H4及びHPSV178がそれぞれ認識するPrP及びPrPSVが存在するが（図3 (D)レーン3)、不溶性画分では検出されなかった（図3(D)レーン2)。

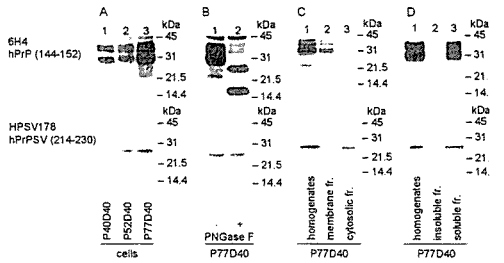


**図3. Characterization of splice variant form of GPI anchorless PrP in T98G cells**

(**A**) Detection of GPI⁻ PrPSV. T98G cells for 40 days after 40 passages (P40D40), 52 passages, and 77 passages were scraped into PBS − 2.5 mM EDTA and sonicated. The postnuclear fractions (50 μg protein each) were subjected to immunoblot with the 6H4 (upper panel) or HPSV178 (lower panel) antibodies.

(**B**) Analysis of deglycosylated forms of GPI⁻ PrPSV. T98G cells for 40 days after 77 passages (P77D40) were scraped into PBS − 2.5 mM EDTA and sonicated. The postnuclear fractions (50 μg protein each) were incubated with (lane 1) or without (lane 2) PNGase F for 120 min. The digested homogenates were boiled for 10 min and subjected to immunoblot with the 6H4 (upper panel) or HPSV178 (lower panel) antibodies.

(**C**) Subcellular localization of GPI⁻ PrPSV. T98G cells for 40 days after 77 passages (P77D40) were scraped into PBS − 2.5 mM EDTA and sonicated. The homogenates (50 μg protein each) were centrifuged at 100,000 × *g* for 60 min at 4°C to obtain a membrane fraction and a cytosolic fraction. The resultant fractions were subjected to immunoblot with the 6H4 (upper panel) or HPSV178 (lower panel) antibodies.

(**D**) Detergent solubility of GPI⁻ PrPSV. T98G cells for 40 days after 77 passages (P77D40) were scraped into PBS − 2.5 mM EDTA and sonicated. Homogenates (H) of 50 μg protein were dissolved in 9 volumes of 0.5 % NP-40 − 0.5 % deoxycholate − PBS and centrifuged at 100,000 × *g* for 60 min at 4°C to obtain a nonionic detergents-insoluble pellet and a soluble supernatant fraction. The pellet fraction (insoluble fr.) and the methanol-precipitated supernatant fraction (soluble fr.) were resuspended in the same volume of PBS − 2.5 mM EDTA. Homogenates (lane 1), pellet fraction (lane 2), and supernatant fraction (lane 3) (50 μg protein each) were subjected to immunoblot with the 6H4 (upper panel) or HPSV178 (lower panel) antibodies.

得られた上清と沈殿物のイムノブロット法を行った。細胞懸濁液（図3 (D) レーン 1）と同様に、非イオン性界面活性剤可溶画分には 6H4 及び HPSV178 がそれぞれ認識する PrP 及び $GPI^-$ PrPSV が存在するが（図3 (D) レーン 3)、不溶性画分では検出されなかった（図3 (D) レーン2)。

### 3. 低酸素濃度下で培養した T98G 細胞が産生する PrPSV の解析

低酸素濃度下で培養した T98G が発現する mRNA の解析を、リアルタイム定量 PCR で行った。90 回の継代後に 40 日間培養した T98G 細胞（P90D40）に比較して（図4 normoxia)、最後の4日間を低酸素濃度下（hypoxia ; 2 % $O_2$）で培養すると PrP mRNA の発現量は減少し、$GPI^-$ PrPSV mRNA は増加した（図4 hypoxia)。同様に、低酸素濃度下と類似した性状を引き起こすコバルト存在下（100 μmol/L $CoCl_2$）で T98G 細胞を4日間培養すると、PrP mRNA の発現量は減少し、$GPI^-$ PrPSV mRNA は増加した（図4 $CoCl_2$)。

次に、T98G 細胞（P90D40）の細胞懸濁液を用いたイムノブロット法で、PrPSV 産生の変化を調べた。低酸素濃度下（2 % $O_2$）又はコバルト存在下(100 μmol/L $CoCl_2$）で4日間培養すると、HPSV178 が認識する $GPI^-$ PrPSV の産生量が増加した（図5下段、レーン 1・3・5)。継代を重ねた T98G 細胞を長期間培養すると PrP は PK 処理抵抗性を示すが（図5上段)、$GPI^-$ PrPSV は PK 処理感受性だった（図5下段)。

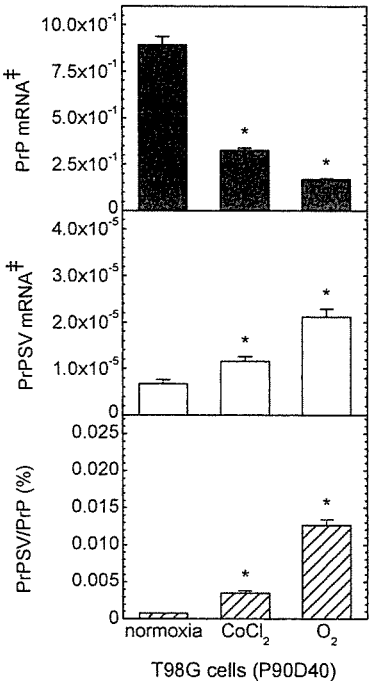


**図 4. Quantification of splice variant of PrP mRNA in T98G cells**

T98G cells for 40 days after 90 passages (P90D40) were exposed to hypoxia (2 % $O_2$), $CoCl_2$ (100 μmol/L), and normoxia for the last 4 days. The resulting total RNA (5 μg) were analyzed by real-time quantitative RT-PCR with PrP primer set (black bars) and exon-exon junction primer set (white bars). Splice variant of PrP mRNA were shown as average relative expression values normalized to PrP mRNA (hatched bars). Values are the mean ± standard error (SE) of three independent cell samples.

‡ The β-actin primer set was used as a control for the amount of RNA used in each reaction (data not shown).

* $p < 0.05$ compared with normoxia (Student $t$-test).

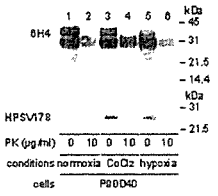


**図 5. Proteinase K sensitivity of splice variant form of GPI anchorless PrP in T98G cells**

T98G cells for 40 days after 90 passages (P90D40) were exposed to hypoxia (2 % $O_2$, lanes 5 and 6), $CoCl_2$ (100 μmol/L, lanes 3 and 4), and normoxia (lanes 1 and 2) for the last 4 days. Methanol-precipitated homogenates (50 μg protein) were treated with PK (10 μg/mL) at 37°C for 30 min (lanes 2, 4, and 6) or left undigested (lanes 1, 3, and 5). The resultants homogenates were boiled for 10 min and subjected to immunoblot with the 6H4 (upper panel) or HPSV178 (lower panel) antibodies.

## D. 考察

ヒトグリオーマ細胞株 T98G は、継代を重ねた後に長期間培養すると PK 処理抵抗性の $PrP^{res}$ を産生する（Kikuchi, Y., *et. al.*, *J. Gen. Virol.* 85: 3449-3457 (2004)）。T98G 細胞は、PrP の C 末端部位と GPI アンカーシグナル配列が欠落したスプライス変異型の mRNA を発現し、アスパラギン酸結合型の糖鎖がなく、非イオン性界面活性剤に可溶で、細胞質画分に局在するスプライス変異型 GPI アンカー欠損 PrP（$GPI^-$ PrPSV）を産生した。本研究で採用した HPSV178 を用いたイムノブロット法の検出感度では、52 週間以上継代しないと $GPI^-$ PrPSV の産生は確認できなかった。図には示していないが、2 週間継代後の T98G 細胞でもスプライス変異型 PrP mRNA が確認できたことから、T98G 細胞は微量ながら恒常的に $GPI^-$ PrPSV を発現している可能性がある。

プリオン蛋白質の 231 残基にストップコドンを挿入して GPI アンカーシグナルペプチドが欠損した組換え PrP 遺伝子を構築し、マウスニューロブラストーマ細胞株 ScN2a で発現させると、糖鎖がない PrP が細胞質中に産生されること（Rogers, *et. al.*, *Pros. Natl. Acad. Sci. USA* 90: 3182-3186 (1993)）、ヒトニューロブラストーマ細胞株 SH-SY5Y で発現させると糖鎖がない PrP が培養液中に放出されること（Walmsley, *et. al.*, *EMBO J.* 20: 703-712 (2001)）が報告されている。スプライシング変異によって生じた T98G 細胞が産生する $GPI^-$ PrPSV は、これらの人為的に構築された GPI アンカー欠損型 PrP と同様な性状を示していることから、培養上清へ放出されている可能性がある。

また、プリオン蛋白質の 231 基にストップコドンを挿入したトランスジェニックマウスでは、発現する PrP の大部分には糖鎖がなく、海馬のニューロンでは細胞内に局在し、スクレイピーの病変を促進することから、PrP の GPI アンカーはプリオン病の発症機構への関与が推定されている（Chesebro, *et al.*, *Science* 308: 1435-1439 (2005)）。継代を重ねた T98G 細胞は $GPI^-$ PrPSV を発現し、低酸素濃度下でその産生が誘導されることから、HIF1α 等の hypoxia に関連した因子とプリオン病の発症機構の関連を解明することが望まれる。

## E. 結論

本研究では、ヒト PrP 及び $GPI^-$ PrPSV の、コドン 129 Met/Val それぞれを発現するベクター及びそれらの組換え蛋白質、並びに $GPI^-$ PrPSV を特異的に認識

するモノクローナル抗体 HPSV178 を樹立した。組換え GPI⁻ PrPSV は C 末端 13 残基が PrP と異なり、その部位を認識する特異的な抗体 HPSV178 を得たことから、血液製剤への添加回収実験への応用が可能と考えられる。

今後は、プリオン病罹患者の脳や血液中の GPI⁻ PrPSV 測定など本研究で開発した新たな研究手法がプリオン病発症機構解明に寄与することが期待される。

**F. 健康危険情報**

なし。

**G. 研究発表**

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**H. 知的財産権の出願・登録状況** (予定を含む)

**1. 特許取得**

本年度は該当なし。

**2. 実用新案登録**

本年度は該当なし。

**3. その他**

本年度は該当なし。

# 研究成果の刊行に関する一覧表

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| 著者氏名 | 論文タイトル名 | 書籍全体の編集者名 | 書籍名 | 出版社名 | 出版年 | ページ |
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| 永田 龍二, 早川 堯夫 | 非臨床における安全性評価概論 | 早川 堯夫（監修） | バイオ医薬品の品質・安全性評価＜増補改訂版＞ | エル・アイ・シー | 出版準備中 | － |

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# 研究成果の刊行物・別刷

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# Inhibition of $PrP^{Sc}$ formation by synthetic *O*-sulfated glycopyranosides and their polymers

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**Abstract**

Sulfated glycosaminoglycans (GAGs) and sulfated glycans inhibit formation of the abnormal isoform of prion protein ($PrP^{Sc}$) in prion-infected cells and prolong the incubation time of scrapie-infected animals. Sulfation of GAGs is not tightly regulated and possible sites of sulfation are randomly modified, which complicates elucidation of the fundamental structures of GAGs that mediate the inhibition of $PrP^{Sc}$ formation. To address the structure–activity relationship of GAGs in the inhibition of $PrP^{Sc}$ formation, we screened the ability of various regioselectively *O*-sulfated glycopyranosides to inhibit $PrP^{Sc}$ formation in prion-infected cells. Among the glycopyranosides and their polymers examined, monomeric 4-sulfo-*N*-acetyl-glucosamine (4SGN), and two glycopolymers, poly-4SGN and poly-6-sulfo-*N*-acetyl-glucosamine (poly-6SGN), inhibited $PrP^{Sc}$ formation with 50% effective doses below 20 μg/ml, and their inhibitory effect became more evident with consecutive treatments. Structural comparisons suggested that a combination of an *N*-acetyl group at *C*-2 and an *O*-sulfate group at either *O*-4 or *O*-6 on glucopyranoside might be involved in the inhibition of $PrP^{Sc}$ formation. Furthermore, polymeric but not monomeric 6SGN inhibited $PrP^{Sc}$ formation, suggesting the importance of a polyvalent configuration in its effect. These results indicate that the synthetic sulfated glycosides are useful not only for the analysis of structure–activity relationship of GAGs but also for the development of therapeutics for prion diseases.


*Keywords:* Prion; Transmissible spongiform encephalopathy; Glycosaminoglycan; Sulfated glycosides

Transmissible spongiform encephalopathies (TSEs), so-called prion diseases, are neurodegenerative diseases with long incubation periods and invariably fatal outcomes. Prion diseases include Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträusser-Schinker syndrome (GSS) in human beings, scrapie in sheep and goats, and bovine spongiform encephalopathy. One of the characteristics of TSEs is an accumulation of a protease-resistant, abnormal isoform of prion protein ($PrP^{Sc}$) in the central nervous system. $PrP^{Sc}$ is posttranslationally generated from the host-encoded, protease-sensitive prion protein ($PrP^{C}$) [1]. A central event in the pathogenesis of TSEs is the conversion of $PrP^{C}$ to $PrP^{Sc}$ [1]; therefore, it is expected that inhibition of $PrP^{Sc}$ formation will be an effective way of treating prion diseases.

It is well known that sulfated glycosaminoglycans (GAGs) and sulfated glycans such as dextran sulfate 500 (DS500) and pentosan polysulfate (PPS) prevent prion infection via the peripheral route when administered prior

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to, simultaneously, or just after the inoculation with prion [2–5]. They also have been shown to inhibit $PrP^{Sc}$ formation in scrapie-infected murine neuroblastoma cells [6,7]. Based on these findings, sulfated GAGs and their analogues have been considered as candidates for the development of therapeutics for treating prion diseases. Recently, Doh-ura et al. reported that intraventricular infusion of PPS prolonged the survival time of prion-infected mice, antagonized $PrP^{Sc}$ accumulation and also reduced neuronal degeneration even when the infusion was given at the late stage of infection [8]. Clinical trials of intraventricular infusion of PPS to CJD and GSS patients have been started in some countries including the United Kingdom.

GAGs consist of a number of disaccharide repeating units, which are composed of uronic acid (glucuronic or iduronic acid) and an amino sugar (galactosamine or glucosamine). The uronic acid and the amino sugar have one to two and two to three possible sites of sulfation, respectively, although these sites are not always sulfated. Consequently, the various combinations of sulfations yield many different possible disaccharide units [9,10]. Although sulfated GAGs may be useful for treating prion diseases, core structures necessary for inhibition of $PrP^{Sc}$ formation are still unclear. The identification of the core structures should help in the development of compounds with enhanced therapeutic potential.

To examine the structure–activity relationship (SAR) of GAGs in the inhibition of $PrP^{Sc}$ formation, we screened various regioselectively *O*-sulfated glycopyranosides as mimics of GAGs and their components [11–13]. Here we show that some synthetic sulfated glycopyranosides and their polymers inhibit $PrP^{Sc}$ formation in prion-infected cells. The results presented here suggest that the locations of the *O*-sulfate and *N*-acetyl groups on glucopyranosides are important for the inhibition of $PrP^{Sc}$ formation.

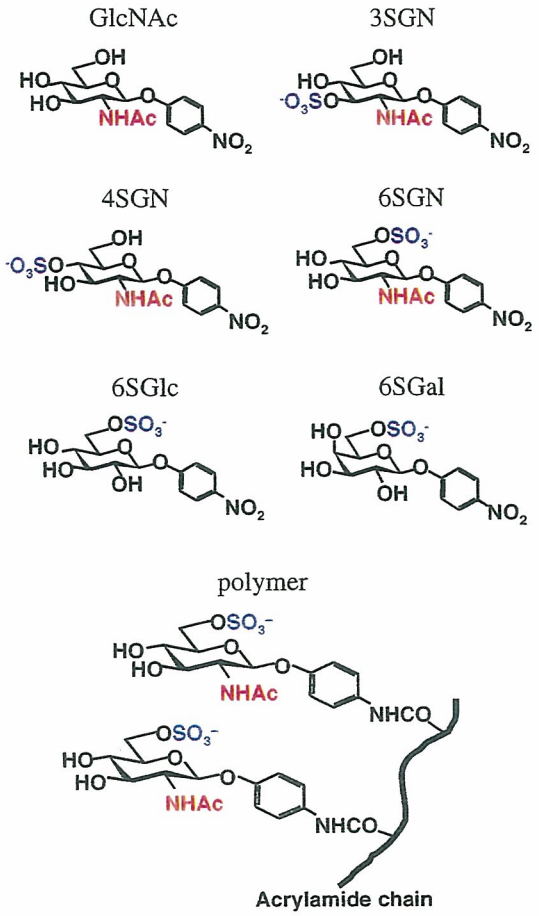


Fig. 1. Structures of sulfated glycopyranosides and their polymers. Structures of mono-glycopyranosides used in this study. 3SGN, 4SGN, 6SGN, 6SGal, 6SGlc, and GlcNAc were also used in polymeric form, in which the glycopyranosides were coupled to an acrylamide chain through their *p*NP residues [14].

## Materials and methods

*Glycopyranosides and their polymers.* The structures of glycopyranosides used in this study are shown in Fig. 1. Six monomeric (mono-) *p*-nitrophenyl (*p*NP) glycosides, *p*NP *N*-acetyl-glucosaminide (GlcNAc), *p*NP 3-sulfo-GlcNAc (3SGN), *p*NP 4-sulfo-GlcNAc (4SGN), *p*NP 6-sulfo-GlcNAc (6SGN), *p*NP 6-sulfo-glucopyranoside (6SGlc), and *p*NP 6-sulfo-galactopyranoside (6SGal) were used. In addition, we used polymers of the glycopyranosides, in which the mono-glycopyranosides were linked to acrylamide chains to mimic the oligosaccharide entity of GAGs [14]. Molar ratios of acrylamide to each glycopyranoside were approximately 9:1, indicating that each polymer has ca. 10% of glycopyranoside as residues. Average molecular weights of these polymers were estimated to be approximately $1.2–3.3 \times 10^5$. The compounds were dissolved in distilled water or dimethyl sulfoxide and filtered through a 0.45-μm Milex filter (Millipore). Heparan sulfate (HS) and heparin were purchased from Sigma. DS500 was purchased from Polysciences. Inc. PPS (Cartrophen Vet, Biopharm Australia Pty, Ltd.) was generously provided by Dr. Katsumi Doh-ura, Tohoku University.

*Cell culture.* Neuro2a mouse neuroblastoma cells (ATCC CCL-131) were cultured in Dulbecco's modified Eagle's medium (ICN Biomedicals) supplemented with 10% fetal bovine serum (FBS) and non-essential amino acids. Mouse neuroblastoma cells persistently infected with prion, which were originally established by Race et al. [15], were cloned by limiting dilution, and a resulting subclone (I3/I5-9) that possessed a high level of $PrP^{Sc}$ [16], was used in this study. The I3/I5-9 cells were maintained in Opti-MEM (Invitrogen) containing 10% FBS, and cells passaged fewer than 20 times were used for the experiments.

*Treatment of cells and sample preparation, SDS–PAGE, and immunoblotting.* The I3/I5-9 cells or Neuro2a cells nearly confluent in a 25 $cm^2$ flask were seeded in a 35 mm tissue culture dish with 1:10 dilution. On the second day, the medium was replaced with 3 ml of Opti-MEM containing 10% FBS (for I3/I5-9 cells) or 3 ml DMEM containing 10% FBS (for Neuro2a cells) and test compounds were added to the medium. The cells were cultured for 2 days in the presence of test compounds and examined for the presence of $PrP^{C}$ or $PrP^{Sc}$ by immunoblotting as described previously [16]. For quantitative analysis, we used one of the following: the Western-Star™ Protein Detection Kit (Tropix) for chemiluminescent detection and quantitation of immunoreactive bands using an LAS-1000 lumino image analyzer (Fujifilm) as described previously [16]; or an ECL Western Blotting detection kit (Amersham Biosciences) and quantitation with an LAS-3000 lumino image analyzer (Fujifilm).

*Indirect immunofluorescence assay (IFA).* Cells seeded on 8-well chamber slides (Nunc) were treated with test compounds for 2 days and then fixed with methanol for 20 min at −20 °C. After blocking for 30 min with PBS containing 5% FBS (FBS–PBS), the cells were incubated for 1 h at room temperature with mAb 31C6 [17] diluted in 1% FBS–PBS. After washing with PBS, the cells were incubated for 1 h with 1:1,000 diluted Alexa 488-labeled Fab fragment of goat anti-mouse IgG. Finally, the slide was mounted with PBS containing 50% glycerol, and 1% *n*-propyl gallate

and examined with an Olympus IX71 fluorescence microscope equipped with a cooled CCD unit (*Cool*SNAP™ HQ, Roper).

*Cell growth assay.* Cells were seeded in 96-well plates at $1 \times 10^4$ cells/well in 200 μl of medium. On day 2, test compounds were added and the cells were incubated for 48 h. All experiments were carried out in quadruplicate. Next, 20 μl of a mixture of 1 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1) and 0.2 mM 5-methylphenazinium methyl sulfate was added to each well and the plates were incubated for 3 h at 37 °C. Finally, the optical density at 450 nm was measured with a microplate reader. The relative growth ratio in the presence of test compounds was calculated by comparing the growth of cells with and without the test compounds.

## Results

### *Effect of O-sulfated glycopyranosides on $PrP^{Sc}$ formation in cells*

To investigate the SAR of GAGs in the inhibition of $PrP^{Sc}$ formation, we used regioselective *O*-sulfo glycopyranosides synthesized by a combination of chemical and enzymatic reactions (Fig. 1). Because GAGs are oligosaccharides consisting of uronic acids and amino sugars, we also used polymers of glycopyranosides. DS500, heparin, and PPS, which inhibit $PrP^{Sc}$ formation in cultured cells [6], were used as positive controls.

Fig. 2 shows representative results of immunoblot analysis for the inhibition of $PrP^{Sc}$ formation. A 2-day treatment with mono-4SGN, poly-4SGN, or poly-6SGN dose-dependently reduced $PrP^{Sc}$ formation. In agreement with previous reports, DS500, heparin, and PPS reduced $PrP^{Sc}$ formation, but HS did not [6,7]. Table 1 summarizes the effect of glycopyranosides on $PrP^{Sc}$ formation from at least three independent experiments. Three glycopyranosides, mono-4SGN, poly-4SGN, and poly-6SGN, reduced $PrP^{Sc}$ with low 50% effective dose ($ED_{50}$); $ED_{50}$ of them were 10, 4, and 9 μg/ml, respectively. However, they were less potent than PPS ($ED_{50} = 0.3$ μg/ml) and DS500 ($ED_{50} = 0.5$ μg/ml), which are known to be the most effective GAG analogues for inhibiting $PrP^{Sc}$ formation (Fig. 2 and Table 1). Other three glycopyranosides, mono-6SGal, poly-3SGN, and poly-6SGal, showed weak inhibitory activities ($ED_{50} > 20$ μg/ml; Table 1). We mainly focused on mono-4SGN, poly-4SGN, and poly-6SGN in the subse-

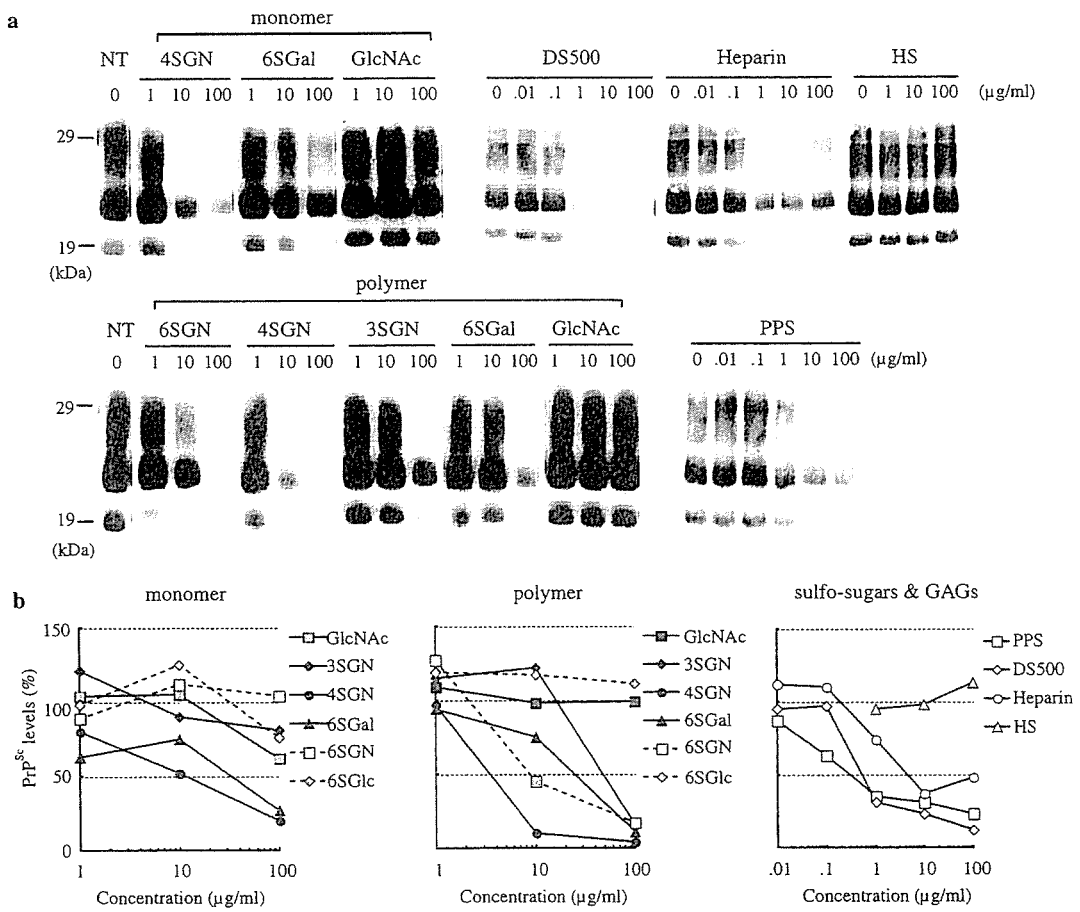


Fig. 2. Inhibition of $PrP^{Sc}$ formation in prion-infected cells by sulfated glycopyranosides. (a) Representative results of $PrP^{Sc}$ detection. I3/I5-9 prion-infected cells were treated for 2 days with various monomeric glycopyranosides and their polymers or GAGs (PPS, DS500, heparin, and HS) at the indicated concentrations. GlcNAc was included as a representative non-inhibitory glycoside. Western blots for the samples containing PPS, DS500, heparin, and HS were visualized with a LAS-3000 lumino image analyzer, whereas the samples containing the other compounds were detected with X-ray film. Molecular mass markers are indicated on the left. NT, untreated cells. (b) Quantitative analysis. Quantitative analyses were carried out using an LAS-1000 or an LAS-3000 lumino image analyzer. Results represent the average of at least three independent experiments.

Table 1
Effect of glycopyranosides and their polymers on $PrP^{Sc}$ formation

| Compound | $ED_{50}$[a] (μg/ml) |
|---|---|
| GlcNAc | >50 |
| 3SGN | >50 |
| 4SGN | 10 |
| 6SGal | 31 |
| 6SGN | >50 |
| 6SGlc | >50 |
| Poly-GlcNAc | >50 |
| Poly-3SGN | 50 |
| Poly-4SGN | 4 |
| Poly-6SGal | 21 |
| Poly-6SGN | 9 |
| Poly-6SGlc | >50 |
| PPS | 0.3 |
| DS500 | 0.5 |
| Heparin | 4 |
| HS | >50 |

[a] The $ED_{50}$ values were estimated from the graphs shown in Fig. 2.

quent experiments because they were relatively strong inhibitors of $PrP^{Sc}$ formation.

Fig. 3 shows the results of long-term treatment with glycopyranosides. Mono-4SGN, and two polymers, poly-4SGN and poly-6SGN, which reduced the level of $PrP^{Sc}$ for two-day treatment, decreased $PrP^{Sc}$ to undetectable level during the serial passage in the presence of the compounds. In contrast, poly-6SGlc, which did not affect the $PrP^{Sc}$ formation in 2-day treatment, did not reduce the level of $PrP^{Sc}$ even when used long-term.

### *Effect of O-sulfated glycopyranosides on the expression of $PrP^C$*

GAGs bind to N-terminal region of PrP, which contains basic amino acid residues [18,19]. In addition, GAGs are known to accelerate $PrP^C$ endocytosis and to reduce the total $PrP^C$ level and cell surface expression of $PrP^C$ [20]. These facts suggest that direct interaction of GAGs with $PrP^C$ is involved in the inhibition of $PrP^{Sc}$ formation, although the mechanism of inhibition remains unclear. Thus, we investigated the effect of glycopyranosides on the expression of $PrP^C$. Neuro2a cells were treated with test compounds at 50 μg/ml for 2 days, and expression of $PrP^C$ was analyzed by immunoblot and IFA. The positive controls DS500 and PPS clearly reduced the total level of $PrP^C$ (Fig. 4). Similar to the positive controls, mono-4SGN, poly-4SGN, and poly-6SGN, which inhibited $PrP^{Sc}$ formation, significantly reduced the total $PrP^C$ level (Fig. 4). In contrast, test compounds that did not inhibit $PrP^{Sc}$ formation (mono-6SGN, mono-6SGlc, poly-6SGlc, and poly-GlcNAc) did not reduce the total level of $PrP^C$. In agreement with the immunoblot analysis, fluorescence intensities in Neuro2a cells treated with DS500, mono-4SGN, poly-4SGN, and poly-6SGN appeared to be lower than that of untreated control cells (Fig. 5). Although the total $PrP^C$ level was reduced by the glycopyranosides, we did not observe a marked difference in localization of $PrP^C$.

### *Effect on cell growth*

The inhibition of $PrP^{Sc}$ formation by *O*-sulfated glycopyranosides suggests that they may be useful for treating prion diseases, however, the reduction of $PrP^C$ level might

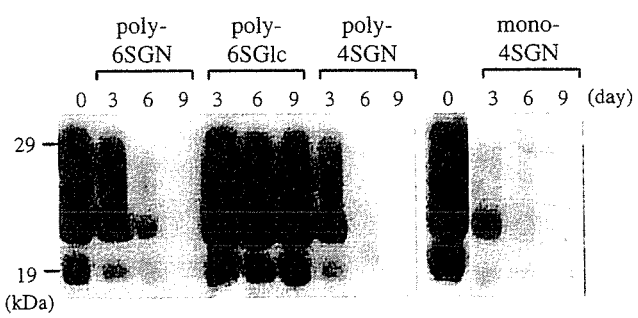


Fig. 3. Long-term effect of sulfated glycopyranosides on $PrP^{Sc}$ biosynthesis. I3/I5-9 cells were cultured in the presence of 20 μg/ml of glycopyranosides for 3, 6, or 9 days. Day 0 indicates the untreated control.

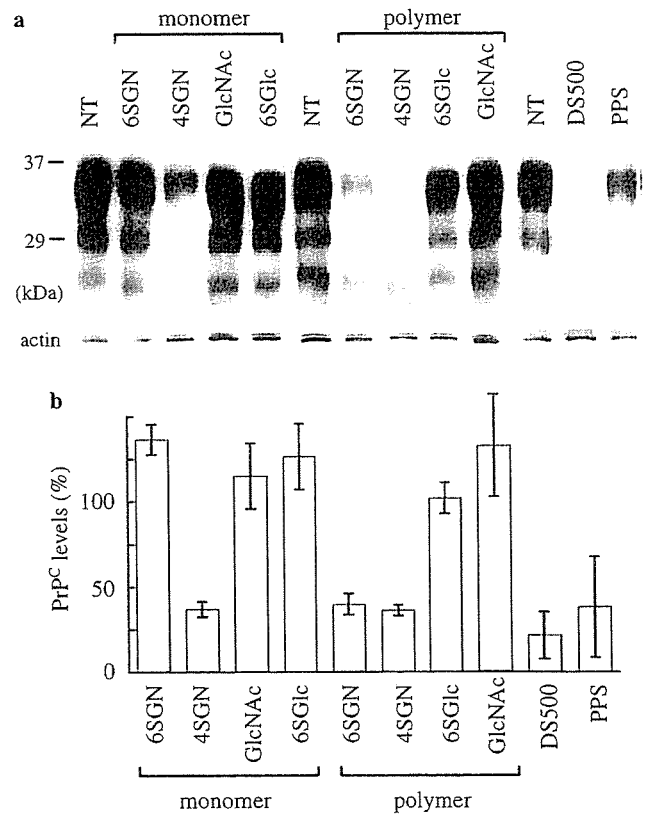


Fig. 4. Effect of sulfated glycopyranosides on $PrP^C$ level. (a) Representative results for $PrP^C$ detection. Neuro2a cells were treated for 2 days with various glycosides at 50 μg/ml. α-Sarcomeric actin was used as an internal loading control. $PrP^C$ was detected with mAb 31C6. NT, untreated control. (b) Quantitative analysis of the effect of sulfated glycosides on $PrP^C$ level. The experiment in (a) was repeated at least three times, and the graph in (b) indicates level of $PrP^C$ relative to the untreated control.

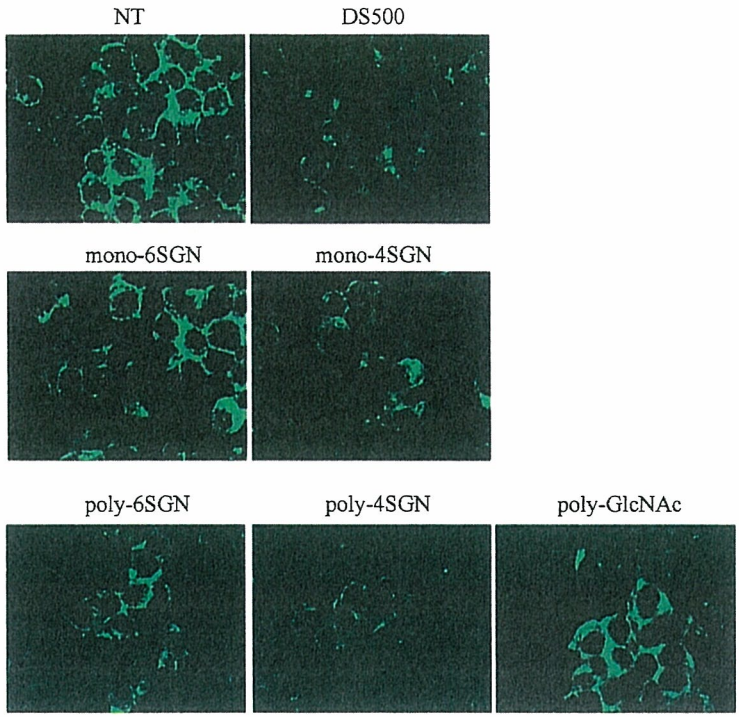


Fig. 5. Localization of $PrP^C$ in Neuro2a cells treated with sulfated glycopyranosides. Neuro2a cells were cultured for 2 days in the presence of the indicated glycosides at 50 μg/ml. $PrP^C$ was detected by IFA using mAb 31C6 and Alexa-488-conjugated secondary antibody. NT, untreated cells.

produce side-effects. To examine whether the synthetic glycopyranosides influence cell growth or have cytotoxicity, we performed WST-1 and lactate dehydrogenase-release assay. We found that mono-4SGN, poly-4SGN, and poly-6SGN had no effect on cell growth (Fig. 6) and were not cytotoxic (data not shown) at any of the concentrations examined.

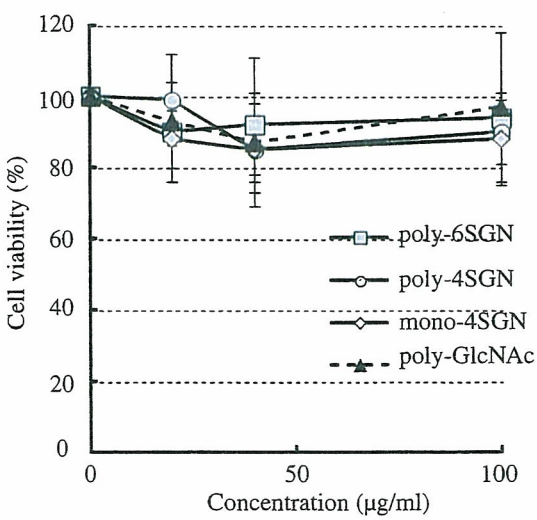


Fig. 6. Effect of sulfated glycopyranosides on cell growth. Cell growth in the presence of sulfated glycosides was determined by WST-1 assay as described in Materials and methods. Values represent means ± SD ($n = 4$) relative cell growth compared to the untreated control.

## Discussion

Sulfated glycans inhibit $PrP^{Sc}$ formation in prion-infected cells [6,7], prevent scrapie infection by peripheral challenge [2–5], and reduce the level of $PrP^{Sc}$ in the brain of prion-infected mice [8]. The degree of sulfation appears to be one of the factors affecting anti-prion activity of sulfated glycans [21,22], although other properties, such as the chain length, repeating unit of glycans, the location of sulfate groups, and the type of glycan chains, will also be involved in the inhibition of $PrP^{Sc}$ formation [6,23]. Selective desulfation is one way to address the SAR of sulfated GAGs [24], however, in the current studies, to examine the SAR of sulfated glycans for inhibition of $PrP^{Sc}$ formation, we used synthetic sulfated glycopyranosides and their polymers, in which the position of sulfation was controlled by chemical and enzymatic reactions [11–13].

Among the compounds tested, mono-4SGN, poly-4SGN, and poly-6SGN inhibited $PrP^{Sc}$ formation with $ED_{50}$ below 20 μg/ml. This suggests that a combination of an *N*-acetyl group at *C*-2 and an *O*-sulfate group at either *O*-4 or *O*-6 on glucopyranoside is important for the inhibition of $PrP^{Sc}$ formation. In fact, the monomeric and polymeric forms of GlcNAc and 6SGlc did not inhibit $PrP^{Sc}$ formation, emphasizing the importance of both the *N*-acetyl group at *C*-2 and the *O*-sulfate group at *O*-6 in the inhibition by 6SGN. However, mono-6SGN did not inhibit $PrP^{Sc}$ formation, suggesting that polyvalent or cluster effects are also important for the inhibitory effect of poly-6SGN.

Heparin is a sulfated GAG that inhibits $PrP^{Sc}$ formation in cells [6,7]. Major constituents of heparin are disaccharide units consisting of 2-*O*-sulfate-L-iduronic acid and 2-*N*-, 6-*O*-disulfate D-glucosamine, although the sulfation sites are not always sulfated [25]. Thus, the inhibitory effect of heparin on *O*-sulfate at *O*-6 and an *N*-acetyl group at *C*-2 participate in the anti-prion effect of heparin. Preliminary experiments showed that 2-*N*-, 6-*O*-disulfate glucosamine and its polymer inhibited $PrP^{Sc}$ formation (data not shown), supporting the role of an *N*- or *O*-sulfate group at *C*-2 and *C*-6 in the anti-prion effect of heparin.

Here, we showed that mono-4SGN inhibited $PrP^{Sc}$ formation with an $ED_{50}$ below 20 μg/ml. To our knowledge, this is the first report that a monomeric glycoside antagonizes $PrP^{Sc}$ formation. The GlcNAc did not inhibit $PrP^{Sc}$ formation, suggesting a combination of *O*-sulfate group at *C*-4 and *N*-acetyl group at *C*-2 is important for the effect of mono-4SGN. However, further analyses of other glucopyranosides such as 4SGlc will be required to address the importance of *O*-sulfate group at *C*-4 more precisely. Although poly-4SGN was more effective than the monomer, monomeric glycosides have an advantage with respect to understanding the SAR of GAGs and for the development of new therapeutic compounds against prion diseases. The purpose of this study was mainly focused to analyze the effect of sulfated glucopyranosides on $PrP^{Sc}$ formation as constituents of GAGs. However, we also found that mono-6SGal showed weak inhibitory effect ($ED_{50} = 31$ μg/ml) and the effect was enhanced in its polymer form ($ED_{50} = 21$ μg/ml). Thus this finding will prompt to analyze the effect of other sulfated galactopyranosides.

Treatment of cells with sulfated glycans such as PPS and DS500 stimulated endocytosis of $PrP^{C}$ and reduced the total and cell surface level of $PrP^{C}$ [16,20]. Reduction of the amount of $PrP^{C}$, i.e., reduction of the amount of substrate available for $PrP^{Sc}$ biosynthesis, may be linked to the inhibition of $PrP^{Sc}$ formation. In this study, mono-4SGN, poly-4SGN, and poly-6SGN reduced the $PrP^{C}$ level to about 50% of that in untreated cells, suggesting that the mechanism of the inhibition is similar to that of PPS and DS500. In contrast, a chemically modified dextran, heparan mimetics HM 2062, was reported to inhibit $PrP^{Sc}$ formation without altering the level of $PrP^{C}$ [21,22]. Thus, there may be several mechanisms for the inhibition of $PrP^{Sc}$ formation by sulfated glycans. HS binds to $PrP^{C}$ possibly via the N-terminal portion of $PrP^{C}$ and this interaction is enhanced by Cu(II) [18,19]. The interaction between $PrP^{C}$ and HS is thought to be involved in the biosynthesis of $PrP^{C}$ and possibly in the conversion of $PrP^{C}$ into $PrP^{Sc}$ [26,27]. Thus, exogenous sulfated glycans may compete with endogenous GAG in binding to $PrP^{C}$ or other molecules such as laminin receptor precursor/laminin receptor. As a consequence, blocking the interaction of $PrP^{C}$ with an endogenous GAG may inhibit $PrP^{Sc}$ formation.

This study provided new information on the SAR of GAGs in the inhibition of $PrP^{Sc}$ formation. In addition, the inhibition of $PrP^{Sc}$ formation by the monoglycoside, mono-4SGN, showed that studies of synthetic sulfated glycosides can aid in the development of compounds for treating prion diseases. Although it is unlikely that sulfated polyanions can pass through blood-brain barrier (BBB), small molecule such as mono-glycosides may be able to pass. The aglycons of glycopyranosides, *p*NP residue, can be modified to hydrophobic moieties [28]. Such modification may facilitate the delivery of the glycopyranosides to the brain through BBB. Further studies using synthetic sulfated glycosides may provide lead structures for the development of new compounds for the treatment of prion diseases.

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# Identification of pH-sensitive regions in the mouse prion by the cysteine-scanning spin-labeling ESR technique

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**Abstract**

We analyzed the pH-induced mobility changes in moPrP$^C$ α-helix and β-sheets by cysteine-scanning site-directed spin labeling (SDSL) with ESR. Nine amino acid residues of α-helix1 (H1, codon 143–151), four amino acid residues of β-sheet1 (S1, codon 127–130), and four amino acid residues of β-sheet2 (S2, codon 160–163) were substituted for by cysteine residues. These recombinant mouse PrP$^C$ (moPrP$^C$) mutants were reacted with a methane thiosulfonate sulfhydryl-specific spin labeling reagent (MTSSL). The 1/δH of the central ($^{14}$N hyperfine) component ($M_I = 0$) in the ESR spectrum of spin-labeled moPrP$^C$ was measured as a mobility parameter of nitroxide residues (R1). The mobilities of E145R1 and Y149R1 at pH 7.4, which was identified as a tertiary contact site by a previous NMR study of moPrP, were lower than those of D143R1, R147R1, and R150R1 reported on the helix surface. Thus, the mobility in the H1 region in the neutral solution was observed with the periodicity associated with a helical structure. On the other hand, the values in the S2 region, known to be located in the buried side, were lower than those in the S1 region located in the surface side. These results indicated that the mobility parameter of the nitroxide label was well correlated with the 3D structure of moPrP. Furthermore, the present study clearly demonstrated three pH-sensitive sites in moPrP, i.e., (1) the N-terminal tertiary contact site of H1, (2) the C-terminal end of H1, and (3) the S2 region. In particular, among these pH-sensitive sites, the N-terminal tertiary contact region of H1 was found to be the most pH-sensitive one and was easily converted to a flexible structure by a slight decrease of pH in the solution. These data provided molecular evidence to explain the cellular mechanism for conversion from PrP$^C$ to PrP$^{Sc}$ in acidic organelles such as the endosome.


*Keywords:* SDSL; ESR; Prion; Domain mobility; pH-sensitive region

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of fatal neurodegenerative disorders including Creutzfeldt–Jacob disease, Gerstmann–Sträusler–Scheinker syndrome, fatal familial insomnia, and kuru in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle [1,2]. According to the "prion-only hypothesis" [1,3,4], the abnormal (scrapie-like and β-sheet-rich) form of prion protein (PrP$^{Sc}$) converted from the normal cellular prion protein (PrP$^C$) is recognized as the only pathogenic component of TSEs. Mammalian PrP$^C$ is a ubiquitous glycoprotein attached to the plasma membrane via a glycosyl phosphatidylinositol (GPI) anchor [1]. As illustrated in Fig. 1A, mouse PrP (moPrP) consists of 208 amino acids (residues 23–231). The carboxy-terminal domain of moPrP (121–231) is defined as a tertiary structure and contains three α-helices

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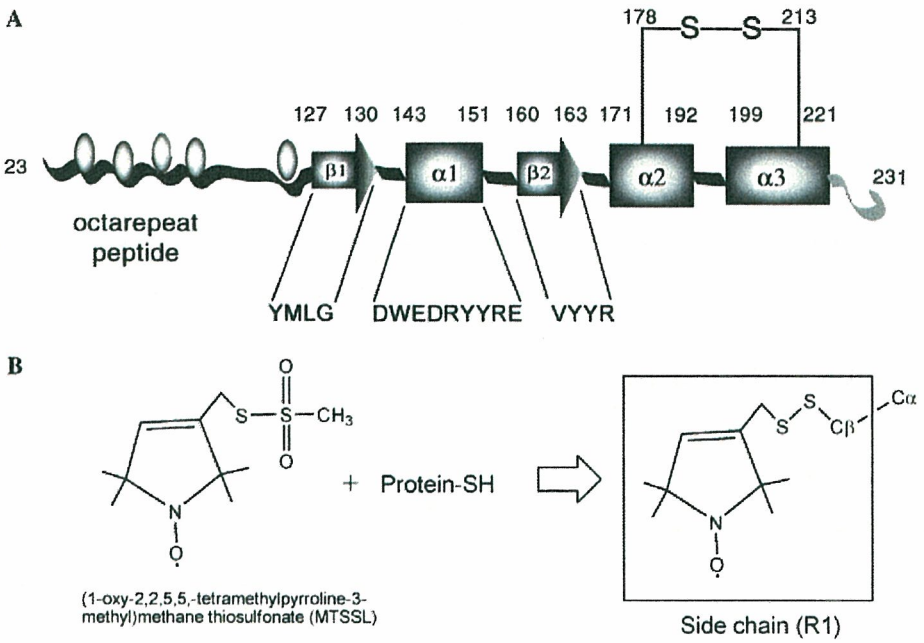


Fig. 1. A schematic diagram of the full-length moPrP and the site-directed spin labeling (SDSL) technique. (A) The full-length moPrP and the target region for SDSL–ESR. The full-length moPrP consisted of 208 amino acids (residues 23–231). The N-terminal domain is largely flexible and has four octapeptide repeats. The C-terminal domain is comprised of three α-helices (H1, H2, and H3) and two β-sheets (S1 and S2). moPrP contains five $Cu^{2+}$-binding sites, two cysteines (codons 178 and 213) forming one disulfide bond, two N-glycosylation sites (codons 180 and 196), and one GPI anchor (C-terminal end). The targets of cysteine mutation are 17 amino acids at H1, S1, and S2. (B) The reaction of the methanethiosulfonate spin-labeling reagent with the cysteine residue generates the nitroxide side chain (R1) on moPrP.

(Helix1, Helix2, and Helix3) and two short anti-parallel β-sheets (Sheet1 and Sheet2) [1,5,6].

Though the precise mechanism of conversion from $PrP^C$ to $PrP^{Sc}$ is still unknown, the accumulation of $PrP^{Sc}$ in endosomes, the main intracellular acidic organelles, indicates that the process of conversion from $PrP^C$ to $PrP^{Sc}$ requires physiological acidic pH conditions [7–9]. Recent circular dichroism (CD) spectroscopic studies showed that acidic conditions in the presence of a denatured agent induce a β-sheet-rich intermediate in human (90–231) and mouse PrP (121–231) *in vitro* [7,10,11]. The study, which used antibodies to probe the structure of recombinant Sylian hamster PrP (residues 90–231), indicated that the conformation of epitopes localized in the C-terminus was insensitive to pH, whereas that of the N-terminus was sensitive [12]. NMR measurement of the full-length human PrP showed that the octapeptide repeats in the N-terminal domain constituted pH-dependent PrP oligomerization; however, this was not detectable around pH-sensitive regions in the C-terminal domain [13]. In contrast, studies using molecular dynamics (MD) simulations proposed the presence of a pH-sensitive region in the C-terminal globular domain on Sylian hamster PrP 109–219, human PrP 125–228, and bovine PrP 124–227 [14,15]. In fact, high resolution NMR and the thermal stability of the globular domain of truncated prion protein (hPrP 121–230) suggested that the residues at the C-terminal end of helix1 and residues 161–164 of β-strand2 were candidates for the "starting point" of pH-induced unfolding and implicated in endosomic $PrP^C$ to $PrP^{Sc}$ conformational transition resulting in TSEs [16]. However, for the full-length $PrP^C$, there is no experimental evidence that low pH induces a conformational change in the globular region of PrP.

Recently, site-directed spin labeling (SDSL) combined with electron spin resonance spectroscopy (ESR) has proven to be a useful technique for protein structural and motional analyses, such as determination of the secondary structure and its orientation, areas of tertiary interactions, and domain mobility [17–20]. The data of SDSL–ESR are applicable for conformational analysis of high molecular weight proteins, whereas NMR and X-ray crystallographic methods are impossible to use for such analysis [17]. In SDSL, the nitroxide side chain (R1) derived from a sulfhydryl-specific nitroxide agent such as a methane thiosulfonate spin label (MTSSL) is introduced into the target codon in the protein sequences by using site-directed mutagenesis (Fig. 1B). Recently, by using this cysteine-scanning spin labeling method to obtain structural information on erythroid α and β spectrin peptides, which are not easily studied by either NMR or X-ray methods, a new amphipathic nature of the helical regions, which is critical in αβ spectrin association at the tetramerization site, was reported by Mehboob et al. [21], indicating that this technique is a powerful tool for monitoring the structure and dynamics of proteins. We have also applied this method to obtain biophysical information on moPrP and reported the thermal stability and pH-dependent mobility changes in three recombinant moPrP mutations (N96C, D143C, and T189C) labeled with MTSSL on the full-length prion protein [22].