Cell-surface prion protein interacts with glycosaminoglycans

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We used ELISA and flow cytometry to study the binding of prion protein PrP to glycosaminoglycans (GAGs). We found that recombinant human PrP (rPrP) binds GAGs including chondroitin sulphate A, chondroitin sulphate B, hyaluronic acid, and heparin. rPrP binding to GAGs occurs via the N-terminus, a region known to bind divalent cations. Additionally, rPrP binding to GAGs is enhanced in the presence of Cu²⁺ and Zn²⁺, but not Ca²⁺ and Mn²⁺. rPrP binds heparin strongest, and the binding is inhibited by certain heparin analogues, including heparin disaccharide and sulphate-containing monosaccharides, but not by acetylated heparin. Full-length normal cellular prion protein (PrP^C), but not N-terminally truncated PrP^C species, from human brain bind GAGs in a similar Cu²⁺/Zn²⁺-enhanced

fashion. We found that GAGs specifically bind to a synthetic peptide corresponding to amino acid residues 23–35 in the N-terminus of rPrP. We further demonstrated that while both wild-type PrP^C and an octapeptide-repeat-deleted mutant PrP produced by transfected cells bound heparin at the cell surface, the PrP N-terminal deletion mutant and non-transfectant control failed to bind heparin. Binding of heparin to wild-type PrP^C on the cell surface results in a reduction of the level of cell-surface PrP^C. These results provide strong evidence that PrP^C is a surface receptor for GAGs.

Key words: ELISA, glycosaminoglycan (GAG), heparin.

INTRODUCTION

According to the ‘protein only’ hypothesis, transmissible spongiform encephalopathies are caused by a conformational conversion of the α -helix-rich, protease K-sensitive normal cellular prion protein (PrP^C) to a β -sheet-rich, relatively protease K-resistant and infectious form commonly known as scrapie PrP (PrP^{Sc}) [1–4]. PrP^C is a glycosylphosphatidylinositol-anchored cell-surface membrane protein encoded by the *Prnp* gene [5–7]. Mature, full-length human PrP^C spans from residues 23 to 231 of the predicted polypeptide with two highly conserved potential N-linked glycosylation sites [5–7]. NMR studies have revealed that the N-terminus of PrP^C is very flexible and lacks any regular secondary structures, while the C-terminal region contains a well-defined folded domain [8–10].

Despite earlier reports of neurological deficiencies in PrP^C–/– mice [11], more recent studies reveal that PrP^C–/– mice are normal [12]. Thus the normal function(s) of PrP^C remain unclear. Accumulated evidence suggests that PrP^C is a metal-binding protein, and may function as a metal transporter [13,14]. Binding of Cu²⁺ also enables PrP^C to acquire a superoxide dismutase (SOD)-like antioxidant activity [15], suggesting that PrP^C may also participate in the regulation of oxidative stress [16]. Other ligands that have been reported to bind PrP^C include laminin [17], laminin receptor [18], nucleic acid [19,20], plasminogen [21] and neural cell-adhesion molecule (N-CAM) [22].

Earlier studies suggested that brain-derived PrP^C binds polyanions such as glycosaminoglycans (GAGs) [23,23a]. This is supported by the finding that injection of some GAGs prolongs the incubation period in experimentally infected animals [24,25]. In addition, immunohistochemical staining of brain tissues from infected animals and Creutzfeldt–Jakob disease patients shows co-localization of GAGs and PrP^{Sc} [26]. Cell culture studies demonstrated that some polyanions could induce the internalization of cell-surface PrP^C [27]. There are also studies

suggesting that heparan sulphate greatly increased the infectivity of protease-resistant PrP [28], and stimulated PrP^{Sc} formation [29]. In contrast, other studies have shown that polyanions inhibit PrP^{Sc} replication *in vitro* [30,31]. The inhibitory mechanism postulated is that the polyanions block PrP^{Sc} replication by directly inhibiting the PrP^{Sc} binding to PrP^C. There is evidence that proteoglycans such as heparan are involved in PrP interaction with one of its potential receptors (ligands), the laminin receptor [32]. More recently, it has been reported that recombinant mouse PrP produced in mammalian cells also bind GAGs and this binding was enhanced by divalent cations [33]. Another study with recombinant hamster PrP and small human PrP fragments has identified three heparin-binding sites at the N-terminal region of PrP. In this study, it was reported that copper reduced rather than enhanced the binding of PrP to GAG [34]. The reasons for this discrepancy are not known. Collectively, these results suggest intimate and complex relationships between PrP^C, PrP^{Sc}, metals and GAGs.

In this study, we used recombinant human PrP (rPrP) proteins, including full-length rPrP_{23–231}, N-terminally truncated rPrP_{90–231}, C-terminally truncated rPrP_{23–145} and affinity-purified brain-derived native PrP^C species, to study PrP^C binding to GAGs in greater detail. We also studied the effects of divalent cations on the binding of different PrP^C species to GAGs. In addition, we identified a motif on PrP^C that is responsible for GAG binding. Finally, since PrP^C is normally a cell-surface protein, we also investigated whether cell-surface PrP^C binds GAGs.

MATERIALS AND METHODS

rPrP and cell lines

The generation, purification and characterization of full-length rPrP_{23–231}, N-terminally truncated rPrP_{90–231} and C-terminally truncated rPrP_{23–145} have been described in detail [35]. All the

Abbreviations used: ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); GAG, glycosaminoglycan; HRP, horseradish peroxidase; mAb, monoclonal antibody; PrP^C, normal cellular prion protein; PrP^{Sc}, scrapie PrP; rPrP, recombinant human PrP; SOD, superoxide dismutase.

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M17 is the parental neuroblastoma cell line that does not express PrP^C [36]. Stable M17 transfectants, with the episomal vector CEP4 β containing different PrP coding sequences, were also used in this study. They were designated WV, VT and δ 8. WV contains the full-length human PrP coding sequence with Val at codon 129, VT contains a mutated PrP coding sequence with a deletion of codons 23–90, and δ 8 contains a PrP mutant with a deletion of codons 51–90. Both deletion mutants were constructed using the Bio-Rad Muta-Gene phagemid *in vitro* mutagenesis kit. The primer used for VT is 5'-GGAGTGACCTGGGCCTCTGC*GGTCAAGGAGGTGGC-3', and the primer for δ 8 is 5'-GGCAACCGCTACCCA*CAAGGAGGTGGCACC-3'. The * indicates the site of the loop out.

Preparation of full-length and N-terminally truncated PrP^C

Human brain tissues of the front cortex were obtained from autopsies of non-prion-disease cases. Brain homogenate was prepared in 10 vol. of lysis buffer (10 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate and 5 mM EDTA, pH 8.0) in the presence of 1 mM PMSF. After centrifugation at 11 000 *g* for 10 min with a microcentrifuge, the supernatants were collected and stored at –80 °C. The PrP^C present in brain homogenate was separated into full-length and N-terminally truncated fractions with anti-PrP monoclonal antibody (mAb) coupled Protein G immunoaffinity columns.

The anti-PrP mAb-coupled columns were prepared with the Seize X Protein G Immunoprecipitation kit (Pierce, Rockford, IL, U.S.A.) as described by the manufacturer. Two anti-PrP^C mAbs, 8B4 (N-terminus specific) and 8H4 (C-terminus specific), were used for the antibody coupling [37]. To enrich the full-length PrP^C, the 8B4-coupled Protein G column was incubated with brain homogenate at 4 °C overnight: after washing three times with PBS the bound full-length PrP^C was eluted with 0.1 M glycine, pH 2.7. The flow-through, which contained only N-terminally truncated PrP^C, was further incubated with 8H4-coupled affinity column; the bound N-terminally truncated PrP^C was also eluted with 0.1 M glycine, pH 2.7. The column-enriched full-length and N-terminally truncated PrP^Cs were quantified with immunoblots and ELISA [37].

SDS/PAGE and immunoblotting

For SDS/PAGE, enriched full-length or N-terminally truncated human brain PrP^C species were mixed with 2 $\times$ SDS loading buffer (160 mM Tris, 4% SDS, 4% 2-mercaptoethanol, 20% glycerol and 0.04% Bromophenol Blue, pH 6.8), heated for 10 min at 95 °C, then loaded on to an SDS/PAGE gel (15%; Bio-Rad, Hercules, CA, U.S.A.). After electrophoresis, SDS/PAGE slabs were electrotransferred on to nitrocellulose membranes and immunoblotted as described in [38]. Transferred PrP^C was detected using mAb 8H4 in combination with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG Fc region-specific antibody as the secondary antibody. PrP visualization was performed using the chemiluminescence blotting system (Roche, Indianapolis, IN, U.S.A.).

Detection of PrP binding to GAGs by ELISA

We established an ELISA to study PrP binding to GAGs. Chondroitin sulphate A (from bovine trachea), chondroitin sulphate B (from porcine intestinal mucosa), hyaluronic acid

(from rooster comb) and heparin (from porcine intestinal mucosa; all from Sigma, St. Louis, MO, U.S.A.) were coated on to flat-bottomed, 96-well Costar plates (Corning, Corning, NY, U.S.A.) at 2 μ g/well at 4 °C overnight, and blocked with 3% BSA in PBS at room temperature for 2 h. Plates coated with BSA (Sigma) were used as controls. Pre-determined amounts of recombinant rPrP, including rPrP^{23–231} (full-length), rPrP^{23–145} (C-terminally truncated) and rPrP^{90–231} (N-terminally truncated) or brain PrP^C (in 10 mM Hepes/NaOH/100 mM NaCl, pH 7.5), were incubated with the coated plate for 1–2 h. After three washes with PBS containing 0.05% Tween-20, the bound rPrP was detected with mAbs 8H4 or 8B4. Bound mAbs were detected with an HRP-conjugated goat anti-mouse IgG (Chemicon, Temecula, CA, U.S.A.). A_{405} values were measured for 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; Roche).

For the GAG-binding competition studies, the rPrP (0.1 μ g/ml) was incubated with GAG-coated plates in the presence of various amounts of free GAGs or heparin analogues (Sigma) as indicated in the individual experiments.

To study the effect of divalent cations on GAG binding, prior to binding to the GAG-coated ELISA plates, different concentrations of divalent cation solutions, including CuCl₂, ZnCl₂, CaCl₂ and MnCl₂ (Sigma), were incubated with full-length or truncated PrP (0.1 μ g/ml, 0.1 ml/well) for at least 2 h, as indicated in individual experiments. All experiments were carried out in duplicate and repeated a minimum of three times.

Binding of biotinylated heparin to PrP synthetic peptides

To identify the amino acid sequence in PrP that binds heparin, we established an ELISA using biotinylated heparin and synthetic PrP peptides. Heparin was biotinylated using the EZ-link Sulpho-NHS-Biotin kit (Pierce). Each PrP peptide has 13 amino acid residues, and overlaps with the next peptide by three amino acids. The panel of peptides covers the N-terminus of PrP from residue 23 to 146 (Princeton Biomolecules, Langhorne, PA, U.S.A.). The peptides used are listed here: peptide^{23–35}, KKRPKPGGWNTGG; peptide^{33–45}, TGGSRYPGQGSPG; peptide^{43–55}, SPGGNRYPPQGGG; peptide^{53–65}, GGGGWWGP-HGGGW; peptide^{73–85}, GGWWGQPHGGGWWGQ; peptide^{93–105}, GGTHSQWNKPSKP; peptide^{103–115}, SKPKTNMKHMAGA; peptide^{113–125}, AGAAAAGAVVGGL; peptide^{123–135}, GGLGG-YMLGSAMS and peptide^{133–145}, AMSRPIHFGSDY. Peptide^{53–65} and peptide^{73–85} each contain one of the octapeptide repeats (as indicated by an underscore). We did not use the two overlapping peptide^{63–75} and peptide^{83–95}.

Each PrP peptide was coated at 1 μ g/well on to a 96-well plate, and incubated at 4 °C overnight. The coated plate was blocked with 3% BSA in PBS at room temperature for 2 h. Different amounts of biotinylated heparin were added to the wells, followed by incubation at room temperature for 1 h. With extensive washing, the bound biotinylated heparin was detected by HRP-conjugated streptavidin. A values at 405 nm were measured for ABTS (Roche). The plate included duplicate wells, and the experiment was repeated at least three times.

Immunofluorescent staining and flow cytometry analysis

To detect heparin binding to various cell lines, 1 $\times$ 10⁶ cells were washed and suspended in a washing buffer (PBS with 5% calf serum and 0.1% NaN₃, pH 7.5), followed by incubation with 1–2 μ g of biotinylated heparin on ice for 1 h. After extensive washing, the bound biotinylated heparin was detected by phycoerythrin-conjugated streptavidin. The stained cells were analysed with FACScan (Becton Dickinson, Mountain View,

CA, U.S.A.). To demonstrate the binding specificity, biotinylated heparin was also used to stain WV cells in the presence of different concentrations of unlabelled heparin.

To detect the binding of hyaluronic acid to WV cells, WV cells were stained with FITC-conjugated hyaluronic acid, and analysed with FACSscan similarly. FITC-conjugated hyaluronic acid was prepared as described in [39].

For the detection of cell-surface PrP^C, various M17-PrP and M17 cells were stained with mAb 8H4 or 8B4 followed by FITC-conjugated goat anti-mouse IgG Fc region-specific antibody (Chemicon) before analysis by FACSscan. All experiments were repeated at least three times with comparable results.

Binding of heparin reduces the levels of cell-surface PrP^C

The parental M17 cell line and various M17-PrP transfectants were incubated with heparin at 0, 1 or 10 $\mu\text{g/ml}$ in RPMI media for 12 h. After fixation in 3% paraformaldehyde, cell-surface PrP^C was measured by immunofluorescent staining with mAb 8H4 as described earlier.

RESULTS

Binding of rPrP to GAGs is mediated by the N-terminus of PrP

We used a full-length recombinant rPrP₂₃₋₂₃₁, an N-terminally truncated rPrP₉₀₋₂₃₁ and a C-terminally truncated rPrP₂₃₋₁₄₅ to study the binding of rPrP to GAGs by ELISA. In the first series of experiments, ELISA plates were coated with heparin; different amounts of rPrP₂₃₋₂₃₁ or rPrP₉₀₋₂₃₁ were then added on to the

plate. After extensive washing, bound rPrP was detected with mAb 8H4, which reacts with an epitope located in the C-terminus between PrP amino acid residues 176 and 185. As a control, BSA was used. We found that full-length rPrP₂₃₋₂₃₁ bound heparin (Figure 1A) much more strongly than N-terminally truncated rPrP₉₀₋₂₃₁ (Figure 1B). The N-terminally truncated rPrP₉₀₋₂₃₁ only exhibits binding at the highest concentration and the binding is much weaker. Neither rPrP₂₃₋₂₃₁ nor rPrP₉₀₋₂₃₁ bind BSA (result not shown). Therefore, rPrP binding to heparin is specific.

Next we compared rPrP₂₃₋₂₃₁ binding (Figure 1C) and C-terminally truncated rPrP₂₃₋₁₄₅ binding (Figure 1D) to heparin. We detected the bound rPrP with mAb 8B4, which reacts with an epitope located between residues 34 and 45 in the N-terminus. We found that both rPrP₂₃₋₂₃₁ and rPrP₂₃₋₁₄₅ bind heparin (Figure 1D). We also examined the ability of rPrP to bind three other GAGs: chondroitin sulphate A, chondroitin sulphate B and hyaluronic acid, and the results were similar to those for heparin. Binding of GAGs to rPrP under our experimental conditions is not due to the charge properties of GAGs. rPrP does not bind ELISA plates similarly coated with polyglutamic acid (results not shown). Collectively, these results show that the N-terminus is the region of the rPrP molecule that binds GAGs.

Inhibition of heparin binding with GAGs, heparin polymers, disaccharides and monosaccharides

We next determined whether binding of full-length rPrP to heparin could be inhibited by other GAGs as well as certain heparin analogues (Figure 2). Free chondroitin sulphate A,

Figure 1 Binding of rPrP to heparin

We compared the abilities of various forms of bacterially produced rPrP to bind heparin in ELISA. ELISA plates were first coated with heparin. The binding of full-length rPrP₂₃₋₂₃₁ (A) and an N-terminally truncated form, rPrP₉₀₋₂₃₁ (B), to heparin was compared and measured by mAb 8H4 and HRP-conjugated goat anti-mouse IgG1, while the binding of full-length (C) versus C-terminally truncated rPrP₂₃₋₁₄₅ (D) was compared using mAb 8B4 and HRP-conjugated goat anti-mouse IgG1. In both cases, plates coated with BSA were used as controls. Only full-length rPrP₂₃₋₂₃₁ and C-terminally truncated rPrP₂₃₋₁₄₅ bind strongly to heparin, while none of the rPrPs bind significant levels of BSA or polyglutamic acids (results not shown). O.D. 405 represents A_{405} .

Figure 2 Inhibition of PrP binding to heparin by various GAGs, heparin polymers, heparin di- and mono-saccharides

Full-length rPrP₂₃₋₂₃₁ (0.1 μg/ml) was incubated with plate-coated heparin in the presence of various amounts of soluble inhibitors as listed below, and the bound PrP was detected with mAb 8H4 and HRP-conjugated goat anti-mouse IgG-Fc. Results are presented as the percentages of inhibition, which were calculated as $(A_{405} \text{ with inhibitor} / A_{405} \text{ without inhibitor}) \times 100$. Top panel: PrP binding to immobilized heparin was inhibited by various soluble GAGs: chondroitin A, ■; chondroitin sulphate B, ▲; hyaluronic acid, ○; heparin, ×. Middle panel: PrP binding to immobilized heparin was inhibited by various heparin polymers: acetylated heparin, ○; low-molecular-mass heparin, ■; heparin, ▲. Bottom panel: PrP binding to immobilized heparin was inhibited by various heparin di- or mono-saccharides: D-glucosamine, ■; D-glucose, ○; D-glucuronic acid, ▲; D-glucosamine 2-sulphate, +; D-glucosamine 6-sulphate, ×; D-glucosamine 2,6-disulphate, △; heparin disaccharide, □.

chondroitin sulphate B and hyaluronic acid all inhibited the binding of full-length PrP to immobilized heparin. However, compared with soluble heparin itself, the other three GAGs are much weaker inhibitors (Figure 2, top panel). These results suggested that the interaction between heparin and PrP is stronger than the interaction between PrP and other GAGs. Besides heparin itself, low-molecular-mass heparin, heparin disaccharides and several types of monosaccharide (such as D-glucosamine 2,6-disulphate, D-glucosamine 2-sulphate and D-glucosamine 6-sul-

A. rPrP₂₃₋₂₃₁

B. rPrP₂₃₋₁₄₅

Figure 3 Effects of divalent metal ions on rPrP binding to heparin

To study the role that divalent metal ions play in PrP binding to GAGs, full-length rPrP₂₃₋₂₃₁ (A) and C-terminally truncated rPrP₂₃₋₁₄₅ (B) were incubated with plates coated with heparin. Before the binding, the rPrP was incubated in the presence or absence of various metal ions: Ca^{2+} , Mn^{2+} , Zn^{2+} and Cu^{2+} . mAb 8B4 was used to detect heparin-bound PrP. Of the four divalent cations tested, only Cu^{2+} and Zn^{2+} were able to enhance the rPrP binding to heparin. Neither Ca^{2+} nor Mn^{2+} were able to enhance rPrP binding to heparin. O.D. 405 represents A_{405} .

phate) also inhibited PrP binding to immobilized heparin (Figure 2, middle and bottom panels). Chemical modification of amine groups on heparin by acetylation significantly reduces its inhibitory activity, as compared with other heparin polymers, which may be due to the decreased negative charges (Figure 2, middle panel). Monosaccharides such as glucose, glucosamine and glucuronic acid do not inhibit binding (Figure 2, bottom panel). Therefore, the presence of the sulphate group is important in GAG binding to rPrP.

Enhancement of heparin binding by divalent cations

The N-terminus of PrP binds metals [13]. We investigated the effects of divalent cations such as Cu^{2+} , Zn^{2+} , Ca^{2+} and Mn^{2+} on

Figure 4 Binding of human brain-derived PrP^C species to heparin

The entire human brain PrP^C population (lanes 1 in **A** and **B**) was separated into full-length and N-terminally truncated PrP^C by mAb affinity columns. The purified full-length PrP^C (**A**, lane 2) and N-terminally truncated PrP^C (**B**, lane 2) were incubated with plates coated with heparin. The bound PrP were detected by mAb 8H4 and HRP-conjugated goat anti-mouse IgG1. Only full-length PrP^C species bound significant levels of heparin. The N-terminally truncated PrP^C species did not bind at all. Molecular-mass markers (in kDa; kD) are indicated on the left. O.D. 405 represents A_{405} .

the binding of rPrP_{23–231} (Figure 3A) or rPrP_{23–145} (Figure 3B) to heparin. We found that Cu²⁺ and Zn²⁺, but not Ca²⁺ or Mn²⁺, enhance the binding of rPrP_{23–231} and rPrP_{23–145} to heparin. Enhanced binding is cation-concentration-dependent, and can be detected at concentrations as low as 40 μM CuCl₂ or ZnCl₂. None of the metals has any effect on rPrP_{90–231} binding to GAGs (results not shown). Therefore, the enhancement seen with Cu²⁺ or Zn²⁺ is specific for rPrP_{23–231}. Cu²⁺ and Zn²⁺, but not Mn²⁺, also enhance the binding of rPrP to other GAGs (results not shown).

Brain-derived full-length PrP^C species bind heparin and binding is enhanced by divalent cations

We investigated whether the observations made using rPrP are applicable to native PrP^C isolated from human brain. We used an N-terminus-specific mAb, 8B4, and a C-terminus-specific mAb, 8H4, to purify full-length human brain PrP^C species and the N-terminally truncated PrP^C species [38]. After affinity purification, we confirmed the identities of the PrP species using immunoblots. On SDS/PAGE, the full-length brain PrP^C species appeared as a single 35 kDa band (Figure 4A, lane 2) while N-terminally truncated PrP^C species contained multiple bands with molecular masses ranging from 18 to 30 kDa (Figure 4B, lane 2).

We next determined the ability of the purified full-length PrP^C species and the N-terminally truncated PrP^C species to bind GAGs in the ELISA. Consistent with the results using rPrP, we found that only the full-length PrP^C bound heparin (Figure 4A). The N-terminally truncated PrP^C species did not bind heparin at

Figure 5 Effects of divalent metal ions on native PrP^C binding to heparin

To assess the effects of divalent metal ions on GAGs binding, 20 ng of purified full-length human brain PrP^C was incubated with plates coated with heparin. Binding was measured in the presence or absence of various amounts of divalent metal ions, Cu²⁺ (**A**) and Zn²⁺ (**B**). The bound PrP^C was detected by mAb 8H4 and HRP-conjugated goat anti-mouse IgG1. Similar to results with rPrP, both Cu²⁺ and Zn²⁺ were able to enhance the binding of native PrP^C to heparin. O.D. 405 represents A_{405} .

all (Figure 4B). Similar to full-length rPrP_{23–231} and C-terminally truncated rPrP_{23–145}, the binding of full-length native PrP^C species from human brain is also enhanced by divalent metal ions such as Cu²⁺ (Figure 5A) and Zn²⁺ (Figure 5B), but not Mn²⁺ (results not shown). The enhanced binding is also dependent on the concentration of the cations (Figure 5).

Identification of the GAG-binding motif on rPrP

We investigated whether GAGs bind to a panel of synthetic peptides corresponding to amino acids 23–146 of PrP. Each synthetic peptide is comprised of 13 amino acids and overlaps with the neighbouring peptide by three residues. We found that heparin bound high levels of synthetic peptides that contain amino acids 23–35 (Figure 6A). The binding of heparin to this peptide is concentration-dependent (Figure 6B). This peptide has a stretch of four basic amino acid residues spanning positions 23–27, KKRPK (the basic residues are underlined), which is very similar to the consensus sequence for heparin binding [40]. Weaker binding was detected with synthetic peptides that contain residues 33–45. Much weaker binding was detected with synthetic peptides corresponding to residues 43–55, 53–65 and 104–116. Even though there is weak binding between heparin and peptide_{53–65}, no binding was detected between heparin and the other octapeptide sequences, such as peptide_{73–85}.

Heparin binding to cell-surface PrP^C

PrP^C is normally present at the cell surface as a glycosyl-phosphatidylinositol-anchored protein. To establish the biological significance of the *in vitro* results, we next investigated whether heparin could bind cell-surface PrP^C. We used

Figure 6 Binding of biotinylated heparin to synthetic PrP peptides

To identify the amino acid sequence responsible for heparin binding in PrP, we coated ELISA plates with a panel of synthetic PrP peptides corresponding to amino acids 23–146. Each peptide had 13 amino acid residues. We determined whether biotinylated heparin was able to bind any of these synthetic peptides. High level of binding was detected with peptide_{23–35}. Lower levels of binding were also detected with four other peptides (A). Binding to peptide_{23–35} is specific and dependent on heparin dosage (B). These results provide conclusive evidence that the extreme N-terminus of rPrP binds GAGs. O.D. 405 represents A_{405} .

biotinylated heparin to study heparin binding to a panel of cell lines that have been transfected to express either human wild-type PrP^C or mutant PrP^C: (i) M17 is the parental cell line that does not express PrP^C; (ii) WV is a cell line that expresses wild-type human PrP^C with a Val at position 129; (iii) δ 8 is a cell line that expresses a mutated human PrP^C in which the octapeptide repeats have been deleted; and (iv) VT is another cell line that expresses a human PrP^C, in which the N-terminal domain at residues 23–90 has been deleted.

The PrP expression levels in the cell lines WV, VT and δ 8 are similar, as demonstrated by mAb 8H4 staining (Figure 7A). Only WV and δ 8 contain the N-terminus, as demonstrated by the N-terminus-specific mAb 8B4 (Figure 7B). As expected, WV and δ 8 can bind biotinylated heparin intensively (Figure 7C), while the parental M17 cell line, which lacks PrP^C, binds heparin much more weakly. These results confirmed our earlier findings on rPrP that the octapeptide sequence is not essential for GAG binding. Binding of heparin to WV cells is specific, for neither polyglutamic acids nor BSA could inhibit the binding of biotinylated heparin (results not shown). On the other hand, unlabelled heparin inhibited the binding of biotinylated heparin in a concentration-dependent manner (Figure 8A). The inability of polyglutamic acids to block binding of heparin provides additional support for our conclusion that binding of PrP^C to GAGs is not solely mediated by the charge properties of GAGs.

Another GAG, FITC-conjugated hyaluronic acid, also binds WV cells, but not the parental M17 cells (Figure 8B).

GAGs have been demonstrated to induce the endocytosis of chicken PrP^C [27]. We next investigated whether incubation with heparin reduces the levels of wild-type PrP^C on the cell surface. We found that incubation of WV cells with heparin significantly reduced the immunoreactivity of PrP^C on the cell surface, as detected by mAb 8H4, which reacts with an epitope in the C-terminus of PrP^C (Figure 8C). On the other hand, addition of heparin to VT cells, which do not bind heparin, has no effect on the immunoreactivity of cell-surface PrP^C (Figure 8D). From these experiments, we concluded that binding of heparin to cell-surface PrP^C reduces the level of surface PrP^C on WV cells.

DISCUSSION

In this study, we demonstrated that binding of PrP to GAGs is mediated by a sequence in its N-terminus, a region that has also been implicated in metal binding [13,41]. The observation that Cu²⁺ and Zn²⁺ enhance PrP binding to GAGs, but that Mn²⁺ does not, raises several interesting points about the binding of metals to PrP. Although it is known that the N-terminus of PrP binds metals, the specificity of that binding is still a matter of debate. Some studies showed that PrP binds Cu²⁺, Zn²⁺ and Mn²⁺ [42], whereas others showed PrP only binds Cu²⁺ [41]. In this study, we demonstrate that Cu²⁺ and Zn²⁺ enhance PrP binding of GAGs, but not Mn²⁺. If PrP binds Mn²⁺, as has been reported [42], why is Mn²⁺ unable to enhance the binding of PrP to GAGs? One possibility is that the affinity of PrP for Mn²⁺ may be too low, as shown by others [42]. Alternatively, Mn²⁺ may be unable to bind to our PrP, since binding of Mn²⁺ to PrP is known to be dependent on the conformation of PrP [43]. Lastly, PrP may bind Cu²⁺, Zn²⁺ and Mn²⁺, but the conformation of Cu²⁺-bound PrP and Mn²⁺-bound PrP may be different. Only the conformations adopted by Cu²⁺- and Zn²⁺-bound PrP have an increased capacity to bind GAGs.

As we have demonstrated, the metal-binding site and the dominant GAG-binding sites on PrP are different. We identified the GAG-binding motif on PrP using a panel of overlapping synthetic peptides. Of all the synthetic peptides tested, including those from octapeptide repeat regions, only one was able to bind high levels of GAGs. This peptide contains a sequence that includes amino acid residues 23–35. This region contained a motif of four basic residues, KKRPK, which we hypothesize is the site where GAGs bind. This motif is very similar to an identified consensus heparin-binding motif, which contains six amino acids that are basic, basic, basic, non-basic, non-basic and basic residues [40]. Much weaker bindings were observed with synthetic peptides of residues 33–65 and 103–115. The consensus heparin-binding motif is absent in the octapeptide repeat region.

Although the exact mechanism by which Cu²⁺ and Zn²⁺ enhance the binding of GAG is not known, it is possible that the binding of metals may increase or even stabilize the exposure of the GAG-binding motif on the N-terminus. This interpretation is supported by our earlier findings, which indicates that refolding of rPrP_{23–231} in the presence of Cu²⁺ increases the binding of an anti-PrP mAb 5B2 [44]. The epitope of mAb 5B2 is located between residues 35 and 45, adjacent to the GAG-binding motif. Alternatively, Cu²⁺ is known to cause intermolecular interactions between rPrP molecules [45]. These interactions may promote binding of GAGs.

Earlier studies suggested that Cu²⁺-bound PrP has SOD-like activity [15]. It is believed that binding of Cu²⁺ to PrP at the octapeptide repeats results in a conformational change in the

Figure 7 Binding of biotinylated heparin to cell-surface PrP^C

Three M17 neuroblastoma cell lines, WV, VT and $\delta 8$, were transfected with wild-type, N-terminally truncated and octapeptide-repeat-deleted PrP expression constructs, respectively. The PrP expression in these cell lines was examined by flow cytometry with mAbs 8H4 (A) and 8B4 (B), and FITC-goat anti-mouse IgG Fc. Both WV and $\delta 8$ express N-terminus-intact cell-surface PrP, while VT does not. Biotinylated heparin (Bio-HE) was used to stain these cells (C); WV and $\delta 8$ bind heparin intensively, but VT does not. In all the histograms, the shaded peaks represent M17 cells, the non-transfected parental cells.

C-terminus, which is the domain that mediates the SOD-like activity. The results presented here suggest that binding of metals to PrP can have multiple effects on PrP, resulting in both conformational and functional changes in the N-terminus as well as the C-terminus of PrP. The enhanced binding of PrP to GAGs by Cu²⁺ can be observed at 40–500 μ M, which is within the physiological levels of Cu²⁺ in the brain [41]. We also observed that while full-length PrP^C species derived from human brain bind all GAGs tested, the N-terminally truncated PrP^C species did not. Binding of GAGs is also increased in the presence of either Cu²⁺ or Zn²⁺ but not Mn²⁺. However, maximum enhancement of GAG binding of full-length, brain-derived PrP^C appears to require a higher concentration of Cu²⁺ and Zn²⁺. Although we observed low levels of rPrP_{90–231} binding to GAGs, no physical association between GAGs and N-terminally truncated PrP^C species derived from human brain was detected. As most of the N-terminally truncated PrP^C species in human brain are truncated at around residue 110 [46], it is conceivable that rPrP_{90–231} may contain additional GAG-binding sites. Interestingly, the synthetic peptide that spans amino acid residues 103–115 binds weakly to GAGs. We recently found that in human brain most of the PrP^C species are N-terminally truncated [47]. We further suggest that truncation may be a process by which PrP^C function is regulated. Based on the results presented here, we speculate that one of the consequences of truncation would be to render the PrP^C molecule unable to bind GAGs.

Some polyanions have been reported to prolong the incubation period in experimentally infected mice [24,25], presumably by

interfering with the binding of PrP^{Sc} to PrP^C. We report here that binding of GAGs to PrP^C is inhibited by heparin disaccharide- and sulphate-containing monosaccharides such as D-glucosamine 6-sulphate and D-glucosamine 2,6-disulphate. In contrast, monosaccharides such as D-glucose, D-glucuronic acid or D-glucosamine, which lack sulphate, were ineffective. These results suggest that the negatively charged sulphate group is important in the binding of GAG to PrP^C. Presumably the negatively charged sulphate group interacts with the four basic residues KKRPK in the N-terminus. However, PrP^C also binds hyaluronic acid, which lacks sulphate groups. The fact that acetylated heparin is a less effective inhibitor than heparin and low-molecular-mass heparin may also be due to the reduction in sulphate groups present.

Polyanions have been used therapeutically to delay the onset of prion diseases. We hypothesize that *in vivo* injection of sulphate-containing monosaccharide may provide significant therapeutic advantages; monosaccharides tend to have a longer *in vivo* half-life, have fewer side effects and are much more likely to cross the blood–brain barrier. Experiments are now in progress to determine whether these sulphate-containing monosaccharides can prolong the latency period in experimentally infected mice. While monosaccharide can compete for binding of GAG to rPrP, the synthetic peptide that contains residues 23–35 cannot compete with rPrP for binding to GAGs (results not shown). This is most likely due to the fact that in each GAG molecule there are multiple PrP-binding sites. On the other hand, each PrP molecule has only one dominant GAG-binding site.

Figure 8 Heparin–PrP interaction induced cell-surface PrP down-regulation

The WV cells strongly bound biotinylated heparin (**A**, trace 1), while control cells did not (**A**, shaded peak). This binding can be specifically inhibited by the addition of un-conjugated heparin at 10 $\mu\text{g}/\text{ml}$ (**A**, trace 3) or 1 $\mu\text{g}/\text{ml}$ (**A**, trace 2). WV cells also bound FITC-conjugated hyaluronic acid (**B**, thick trace), while the parental M17 cell line did not (**B**, shaded peak). These results provide direct evidence that cell-surface PrP^C is a receptor for heparin and hyaluronic acid. The level of cell-surface PrP decreased in WV cells (**C**, trace 1) but not in VT (**D**), when incubated with heparin at 10 mg/ml (**C**, trace 3) and 1 mg/ml (**C**, trace 2) for 12 h. The shaded peaks in the histograms are non-transfected controls. The PrP was detected by mAb 8H4 staining.

We demonstrated that cell-surface PrP^C binds heparin and hyaluronic acid. The fact that WV and 88 cell lines bind heparin is consistent with our findings with rPrP and human brain-derived PrP^C. We believe that it is the N-terminus of cell-surface PrP^C that binds heparin and hyaluronic acid. Attempts to block binding of GAG with many anti-PrP mAbs were unsuccessful. However, mAb 8B4 slightly decreased the binding of heparin (results not shown). mAb 8B4 reacts with an epitope between residues 33 and 44, an area adjacent to the GAG-binding motif [37]. A mAb that reacts with amino acid residues from 23 to 35, which we do not have, should be able to block GAG binding. Another cell-surface molecule known to bind hyaluronic acid is CD44 [39]. However, the M17 neuroblastoma cells do not express CD44 (results not shown). Therefore, binding of hyaluronic acid to WV cells is not mediated by CD44 but is PrP^C-dependent.

It is known that heparin and hyaluronic acid participate in cell signalling. Binding of heparin is essential for the functions of some growth factors, such as fibroblast growth factor [48]. GAGs may cross-link cell-surface PrP^C, and may result in the downstream signalling events. We showed that the cross-linking of PrP^C on human lymphocytes by anti-PrP mAb could indeed

influence cell signalling [49]. PrP^C has also been shown to participate in signalling in neurons [50]. High levels of GAGs are present in the extracellular matrix in the central nervous system. GAG-containing proteoglycans are also present on the cell surface. We hypothesize that interactions between PrP^C on one cell and GAGs in the extracellular matrix, or on the surface of another cell, may be important in cell–cell adhesion and signalling. While GAGs bind PrP^C on the cell surface, the mechanism by which binding of GAGs reduces the cell-surface levels of PrP^C is not known. We were unable to demonstrate consistently the internalization of GAG–PrP^C complex from the cell surface (results not shown). Alternatively, binding of GAGs may also result in the shedding of the complex from the cell surface.

Finally, our findings may also have relevance to the pathogenesis of prion diseases. Recently, we reported that PrP species isolated from Creutzfeldt–Jakob disease patients have increased Mn²⁺, decreased Cu²⁺ and reduced antioxidant activity [43]. Therefore, we hypothesize that an alteration in the metal content of PrP can not only cause the loss of antioxidant functions, but it can also influence PrP binding to GAGs. Both of these events may contribute to the pathogenesis of prion diseases.

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