Identification of the Heparan Sulfate Binding Sites in the Cellular Prion Protein

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Summary

Data from cell culture and animal models of prion disease support the separate involvement of both heparan sulfate proteoglycans and copper (II) ions in prion (PrP) metabolism. Though direct interactions between prion protein and heparin have been recorded, little is known of the structural features implicit in this interaction or of the involvement of copper (II) ions. Using biosensor and ELISA methodology we report direct heparin and heparan sulfate-binding activity in recombinant PrPc. We also demonstrate that the interaction of recombinant PrPc with heparin is weakened in the presence of Cu (II) ions and is particularly sensitive to competition with dextran sulfate. Competitive inhibition experiments with chemically modified heparins also indicate that 2-O-sulfate groups (but not 6-O-sulfate groups) are essential for heparin recognition. We have also identified three regions of the prion protein capable of independent binding to heparin and heparan sulfate: residues 23-52, 53-93 and 110-128. Interestingly, the interaction of an octapeptide-spanning peptide motif aa53-93 with heparin is enhanced by Cu (II) ions. Significantly, a peptide of this sequence is able to inhibit the binding of full-length prion molecule to heparin, suggesting a direct role in heparin recognition within the intact protein. The collective data suggest a complex interaction between prion protein and heparin/ heparan sulfate and has implications for the cellular and pathological functions of prion proteins.
Introduction

Considerable effort is being devoted to the identification of natural receptors for prion protein (PrP) both to refine our understanding of prion metabolism and to reveal potential targets for therapeutic intervention in the transmissible spongiform encephalopathies (TSEs). One group of potential co-factors are the glycosaminoglycans (GAGs) and in particular membrane proteins and extracellular matrix components elaborated with heparan sulfate (HS) sugar chains. Heparan sulfates (of which heparin is a heavily sulfated variant) are expressed on a wide variety of cell types, including those of neural origin, and modulate the activity of a wealth of cell-surface and extracellular signaling molecules such as growth factors and cytokines (1, 2). In the TSE field it is known that HS proteoglycans (HSPGs) co-localize with the insoluble aggregates of the prion (PrPSc) that accumulate in the brain tissue of TSE affected animals (3-5). They also promote the formation of amyloid structures typical of Alzheimer’s disease when co-injected into the brains of rats with Aβ-protein (6). Furthermore, administration of select anionic compounds can restrict both tissue-specific accumulation of PrPSc and the onset of neurodegenerative features in experimental models of prion disease. In this respect pentosan sulfate and DS500 (dextran sulfate with av. MW 500,000) are particularly effective (7-9). The basis for the anti-prion activity of these compounds is uncertain; one suggestion is that they compete with endogenous HSPGs for prion, another that they stabilise conformations of PrPSc which are not favoured as templates for PrPSc-PrPc conversion (10). Sulfated GAGs and lipopolyamines (a group of molecules that reverse many of the actions of heparin) modulate the expression of PrPSc in cultured cells (11-13) and several investigators have described direct interactions between cellular or recombinant prion and GAGs (14, 15). Of particular significance is the detection of a glucose polysaccharide in prion rods purified from scrapie-infected hamster brains (16). Two recent papers hint at the critical role HS may play in the molecular events leading PrPSc propagation and the acquisition of infectivity. Wong and colleagues have demonstrated that the cell-free conversion of PrP to a protease-
resistant folding-variant can be stimulated by the addition of HS (17) and an heparitinase-sensitive fraction of a cell extract will promote the reconstitution of infectivity to DMSO-dispersed prion rods (18). Additionally, the 37 kDa/67 kDa laminin receptor (LRP/LR), originally proposed as a potential binding partner for PrP (19) has now been confirmed as the cell surface receptor (20). Heparan sulfate proteoglycans (HSPGs) have been identified as co-receptors in this interaction via HS-binding domains on both proteins (21).

A notable feature of prion biology is the chelation of divalent cations such as copper (22-24) and there is considerable interest in possible connections between metal-binding status and conformation (25-30). Intriguingly manganese and copper have quite distinct effects on the adoption of proteinase resistance by PrP (29). Metal binding resides primarily in an octapeptide repeat motif between residues 53-93 of mammalian PrP though more distal binding sites have also been proposed (24, 30). The role of the octapeptide repeat in prion disease progression is poorly understood. Though PrP null-mutant mice can be restored to scrapie susceptibility by introduction of PrP transgenes lacking this motif (31, 32), scrapie incubation times are still extended compared to wild type (33).

Combined heparin-binding activity and divalent cation chelation have been demonstrated for several other proteins, notably superoxide dismutase (34) and the APP protein of Alzheimer’s disease (35). An interaction between the heparin- and copper-binding functions of PrP would be very interesting and there is certainly evidence that the two functions map to the same region of the molecule. Shyng and colleagues have reported that the region between residues 25 and 91 of PrP (incorporating the octapeptide repeat region) is sufficient for PrP binding to HSPG moieties on N2a cells. They also demonstrated that the surface expression of a mutant PrP lacking this N-terminal segment, unlike intact PrP<sup>C</sup>, is not subject to down-regulation induced by soluble pentosan polysulfate (36). Elsewhere, it has been shown that a naturally truncated PrP present in
significant amounts in the human brain and lacking residues amino-terminal to 111 or 112 (including the octapeptide-repeat), has no heparin-binding activity (37). Lastly, the PrP sequence aa53-93 is directly involved in an HSPG-dependent interaction between recombinant PrP and the 37kDa/67kDa laminin receptor precursor (LRP/LR) (21). Significantly, whereas Caughey reported heparin binding by PrP that was independent of divalent ion concentration (14) a study by Brimacombe et al. describes heparin-sensitive binding of recombinant PrP to experimental nickel surfaces (15).

The only other region of PrP with supporting evidence for a role in GAG binding is a central, hydrophobic and amyloidogenic sequence between residues 106-126 (38, 39). This sequence undoubtedly plays a major role in the biosynthesis of the protease-resistant form of PrP (PrPSc). Cells expressing engineered variants of PrP deleted for residues in this region of the molecule do not support the propagation of homologous PrPSc, and PrP molecules deleted for this sequence cannot be converted to protease-resistant forms in vitro (40). Several groups have investigated the cytotoxic properties of the central region (41, 42) and significantly a neurotoxic activity associated with a peptide corresponding to residues 106-126 is abrogated by soluble heparin and related GAGs (43). Though copper has been shown to affect the aggregation and neurotoxic properties of this region (24), the influence of copper ions on any interaction between this sequence and GAGs has yet to be reported.

Recombinant PrP has now been expressed and purified by several groups (44-46). These efforts have yielded proteins that resemble PrPSc in that they fold into alpha-helical and beta-sheeted structures (20, 46). They are also proteinase K sensitive (47), suggesting a lack of infectivity. Crucially for this study, N-terminally-tagged PrP retains propagating activity (48) and PrP tagged with glutathione sulfotransferase (GST) retains Hsp60 chaperone binding activity (49).
In this study we confirm direct interactions between recombinant PrP\(^c\) and both heparin and HS and show that the binding of full-length recombinant GST::PrP\(^c\) to heparin is weakened significantly in the presence of copper (II) ions. By competitive inhibition studies we reveal that dextran sulfate is a highly potent inhibitor of the PrP\(^c\)-heparin interaction and show that 2-O sulfates of heparin are an essential component of the PrP\(^c\) binding site(s). In a second series of experiments biosensor and ELISA analysis of GST-tagged recombinant peptides (covering the whole sequence of hamster PrP; 21, 49) and synthetic peptides have enabled us to identify three sequences in PrP with independent heparin / HS binding activity: amino acids 23-52, 53-93 and 110-128. At a higher concentration than that which affects heparin binding by intact PrP protein, copper (II) enhances the heparin-binding ability of isolated peptide 53-93, and in the presence of Cu (II) this peptide competes with full-length haPrP\(^c\) for heparin binding, suggesting a central role in this interaction. The divergent actions of copper (II) ions on full-length PrP and on the peptide 53-93 are discussed.

**Experimental procedures**

**Materials**

Bovine lung heparin (BLH; H-4898), porcine intestinal heparin (PIH; H-9399), low molecular weight heparin from porcine intestinal mucosa (LMW PIH; H-5284, average mol. weight approx. 6000), pentosan polysulfate (PPS), dextran sulfate (DS8; D-4911, average mol. weight approx. 8000) and chondroitin sulfate (CS; C-8254) were obtained from Sigma. Porcine intestinal heparan sulfate fraction II (PMHS) was obtained from Organon and bovine kidney HS (BKHS, H-7640) was from Sigma. Both types of HS were pre-treated with chondroitin ABC lyase to remove potential chondroitin sulfate contaminants. Stock solution of 2.2 mM Cu (II), Zn (II), Ni (II), Mg (II) and Mn (II) were prepared in glycine (4.5 mM in distilled water) after the method of Brown
et al. (23). Recombinant streptavidin from *streptomyces avidinii* was purchased from Sigma (S-0677). The biotin donors biocytin hydrazide (B-9014), biotin amidocaproate-NHS (B-3295) and biotin amidocaproate 3-sulfo-NHS (B-1022) were also from Sigma. Modified heparins: persulfated (oversulfated), and 2O- and 6-O-desulfated BLH, were prepared and sulfation status confirmed as previously reported (50, 51).

**Expression and purification of recombinant hamster GST::PrP fusion protein and peptides**

GST::PrP (23-231) of Syrian golden hamster and human sequence and the GST:: haPrP fragments GST::P23-52 (P1), GST::P53-93 (P2), GST::P90-109 (P3), GST::P110-128 (P4n), GST::P129-175 (P4), GST::P180-210 (Px) and GST::P218-231 (P5) were expressed from baculovirus infected Sf9 insect cells (GST::PrP) and from *E. coli* (peptides) as previously described (21, 44). Protein and peptides were dialysed into 20mM HEPES pH 7.4 and stored at 4°C. Heparin/ HS binding activity was examined within 2 months of preparation.

**Preparation of synthetic PrP peptides with human sequence**

Peptides corresponding to residues 23-52 (P1), 53-93 (P2), 90-109 (P3), 110-128 (P4n), 129-175 (P4), 180-210 (Px) and 218-231 (P5) of the normal human PrP sequence were synthesized on an AMS 422 multiple peptide synthesizer (Abimed) using FMOC chemistry (preloaded HMP (Wang) resin, tBu/Trt protection, Arg-Pmc; Trp-Boc, PyBOP activation). After completion of the synthesis the peptides were cleaved using 92.5% TFA / 2.5 % H2O / 5% tri-Isopropylsilane, precipitated and washed with tert-Butyl-methyl-ether. The deprotected crude peptides were purified by reversed-phase HPLC (Sykam HPLC-system, GROM C18 column, 20 x 250 mm, 5 μ). The identity of the purified peptides was confirmed by MALDI-TOF mass spectrometry (Bruker Reflex III).
Biotinylation of heparin and HS for immobilisation

Three methods were chosen for biotin labeling of the heparins and HS used in this study: a.) GAGs were labeled by reaction of their aldehydic reducing groups using a method based on Nadkarni et al. (52) Briefly 50 nmol of saccharide was dissolved in 50 µl formamide containing 50 mM biocytin hydrazide and heated at 37°C for 24 h. Heparin/ HS labeled in this way was used as substrates in occasional biosensor analysis of GST::haPrPc binding (e.g. Fig. 2b.) The second procedure is a modification of that described by Rahmoune et al. (53) and labels the free amino groups reported to occur occasionally along the length of heparin and HS. 30 µl of a 50 mM solution of biotin amidocaproate-NHS in DMSO was added to 50 nmol heparin / HS in dH2O. The mixture was briefly mixed and left for 3 days at room temperature. This method was used to biotinylate heparin and HS for most biosensor analyses of GST::haPrPc and peptides. c.) A third procedure, used in ELISA studies of PrP and peptide binding is a modification of that of Lee and Conrad and also labels internally (54): 5 µmol heparin or HS was dissolved in 0.5 ml of sodium carbonate buffer pH 8.6 containing 15 µmol biotin amidocaproate 3-sulfo-NHS. The mixtures were shaken briefly to mix and left to stand at room temperature for 3 days. Free label and solvent was removed from all labelling reactions as follows: 5 volumes of pre-chilled ethanol were added to each tube and the sample stored at −20°C for 30 minutes. The sample was next centrifuged (5 minutes, 13,000 rpm) and the ethanol decanted, chilled for a second time and re-centrifuged. The precipitate from both stages was combined in 400µl dH2O and fractionated by gel filtration on three Hi-Trap columns (Pharmacia) arranged in series. Biotin-containing fractions were detected at 232 nm and the labeled GAG eluting in the void volume was retained.

Biosensor analysis of GST::PrPc and PrP peptide binding to heparin and HS

Biosensor analysis was performed on a Bia2000 instrument (BiaCore). Two channels of a Pioneer-C1 biosensor chip (BiaCore, planar surface) were activated according to manufacturer’s
recommendations (BiaCore). Briefly, the surface was treated with 50mM N-ethyl-N'-(3- 
dimethylaminopropyl)-carbodiimide/200mM N-hydroxysuccinimide and reacted with 
streptavidin (injection of 50 µl solution of streptavidin, 0.2 mg/ml in 10 mM sodium acetate 
buffer, pH 4.5). Unreacted sites were blocked by treatment with with 1M ethanolamine, pH 8.5. 
One streptavidin-conditioned channel was incubated with biotinylated heparin or HS (10 µl 
injection, 2 mg/ml in HBS-10mM HEPES pH 7.4, 0.15 M NaCl, BiaCore), and the second 
surface left unmodified to control for non-GAG specific binding events. The mobile phase 
between sample injections was pre-prepared HBS-P (HEPES buffered saline, 10mM HEPES pH 
7.4, 0.15 M NaCl, 0.005% polysorbate 20, BiaCore). GST::haPrP\(^c\) and GST::haPrP\(^c\) partial 
peptides were obtained as dilute (ca. 10-100 ng/µl) solutions in 20mM HEPES buffer, pH 7.4. 
Proteins were injected at stock concentrations or pre-diluted in HBS-N (HEPES buffered saline 
with no detergent) and centrifuged for 5 minutes at 10000 rpm prior to analysis to remove 
particulates. Samples were injected (KINJECT command, 30 µl) onto both biosensor surfaces at a 
flow-rate of 10-20 µl/min, at a temperature of 25 °C. A dissociation period of 120 seconds was 
selected. The following solutions were routinely used to regenerate the chip surfaces between PrP 
samples (volume): 2M NaCl (10 µl), 10mM HCl (5 µl), 10 mM NaOH (5 µl) and 2 mg/ml BLH 
(10 µl). To resolve heparin/HS specific binding events the pattern of mass changes (response 
units: RU) recorded at the non-derivatised surface of the biosensor chip was subtracted from the 
signal recorded at the heparin/ HS-derivatised surface.

**ELISA analysis of binding of GST:: PrP peptides and protein to heparin**

Maxisorb and Polysorb (Nunc) 96-well plates were used for analysis of heparin /HS binding by 
full-length and peptidic GST fusions respectively. Plates were pre-coated with 3 µg/ml 
streptavidin in 0.2 M bicarbonate buffer, 0.15 M NaCl, pH 9.3 and left to stand overnight at 4°C. 
Plates were then washed briefly in PBST (0.05% Tween-20 in phosphate buffered saline, pH 7.4)
and blocked for 2 hours with 10% Seablock blocking reagent (Pierce), 0.5% Tween-20 in phosphate buffered saline. Biotin-conjugated bovine lung heparin, porcine intestinal heparin and porcine mucosal HS were then applied to the plates (3 columns each x 8 rows) at a concentration of 75 µg/ml, diluted in PBST. A separate array of control wells (3 columns x 8 rows) was incubated with PBST alone. Biotinylated heparin / HS / PBST was left in contact with the wells for a minimum of three hours at room temperature after which period the wells were washed with PBST. Then GST-tagged proteins were applied, diluted in 10% Seablock (Pierce) in PBS to minimise non-specific interactions. Normally one row of 12 wells (3 wells each per biotinylated GAG and control) were devoted to each GST:: PrPc or GST:: peptide preparation, 35 µl of sample dispensed in each well. In certain experiments PrP preparations were mixed with non-biotinylated GAGs and synthetic PrP peptides prior to incubation. Divalent cations ions were also added in certain experiments from stock solutions containing a 1:2 (mol/mol) ratio of metal (II) ions with glycine. Following the incubation period (minimum 2 hour duration) GST::PrPc / peptide was decanted and the wells washed thoroughly with PBST. All wells were then treated for 1hr (room temperature) with either monoclonal anti-GST (Clone GST-2, G-1160, Sigma) or polyclonal rabbit anti-GST (G-7781, Sigma), 35 µl of a 1:200 dilution in PBST. In a final incubation phase, following another round of washing, wells were incubated (1 hour, room temperature) with 35 µl sheep anti-mouse IgG (F(ab’)2)-peroxidase conjugate (NA 9310, Amersham, for assays employing the monoclonal anti-GST) or anti-rabbit Ig (F(ab’)2)-peroxidase conjugate (NA-9340, Amersham, for assays employing the polyclonal anti-GST) diluted 1:1000 in PBST. Colour was developed by addition of 100 µl per well 3-phenylene-diamine substrate solution (1 x 30mg OPD tablet (Sigma P-8412) per 75 µl phosphate-citrate buffer (0.05M) pH 5.0 containing 0.03% sodium perborate (Sigma P-4922)). Absorbance was measured at 490nm after quenching the wells with 50 µl 0.5M H2SO4. Results with monoclonal and polyclonal anti-GST antibodies were essentially identical.
ELISA analysis of binding of GST::haPrPc to immobilised synthetic PrP peptides.

An ELISA was established to examine the potential for PrP-PrP interactions. Peptides P1 (23-52), P2 (53-93) and P4n (110-128) (50 µg/ml in 0.2 M sodium bicarbonate buffer, pH 9.3 containing 0.15 M NaCl) were coated onto wells of a Maxisorb microtitre plate (Nunc). A fourth block of three columns was left uncoated. After coating (overnight, 4°C) all wells were blocked with 3% bovine serum albumin in PBS containing 0.2% tween-20 (2 hours at room temperature). GST::haPrPc was subsequently applied at 1µg/ml in PBS containing 3% BSA. After 2 hours incubation the plate was thoroughly washed (6 x in PBS/ 0.05% tween-20) and bound GST::haPrPc detected with polyclonal rabbit anti-GST/ anti-rabbit Ig (F(ab’)2)-peroxidase conjugate as described above (conventional heparin-binding ELISA). In inhibition studies a selection of GAGs were included (100 µg/ml) during the GST::haPrPc incubation phase.

Results

Recombinant hamster PrP binds to immobilised heparin and HS

Surface plasmon resonance instruments are able to detect the mass changes that accompany the binding of soluble analyte to ligand immobilised on a detector surface. Initial experiments in this study investigated the binding of the GST::haPrPc fusion protein to immobilised heparin and HS. Figures 1a and 1b show the resonance profiles or sensograms recorded when short pulses of recombinant hamster GST::PrPc were injected over bovine lung heparin and porcine intestinal HS surfaces. The sensograms indicate the net binding response on the derivatised surfaces after subtraction of signal generated on an underderivatised control surface. Also shown are the resonance patterns generated by injection of an identical volume of carrier buffer alone (“buffer”). During
the period of contact between sample and surface (phase A, 180 secs), a positive binding signal which was considerably higher than that of buffer alone (60-80 RU vs. 10 RU) was observed on both GAG-derivatised surfaces. Since the size of the signal achieved is in part dependent on the density of immobilised GAGs (unknown for these surfaces) it is not appropriate to estimate relative binding avidity of heparin and HS from a simple comparison of signal strengths. Under identical conditions glutathione sulfotransferase alone yielded no net binding to heparin or HS-derivatised surfaces (equivalent to buffer control). The two surfaces differed considerably in the shape of the response curve obtained during the dissociative period (D, 120 secs). Approximately half of the GST::haPrPc which had accumulated on the heparin surface during the contact phase (A) showed very rapid dissociation at the onset of the dissociative phase (D) to be followed by a stable period when very little further movement was recorded. In contrast, dissociation of GST::haPrPc from the HS surface was more progressive, though the total proportion of RU lost over this period was similar to heparin at 50 percent. Both surfaces were washed with short pulses of strong saline (2M NaCl) and weak acid (10mM HCl) at identical times after PrP application. This treatment would be expected to have a strongly disruptive effect on heparin and HS directed binding and accounted for approximately 50% and 70% removal of residual GST::haPrPc from heparin and HS respectively.

Prion protein is known to bind copper (II) ions in vitro, and a linkage between divalent cation and polyanion binding activities has been demonstrated previously (15). This prompted us to undertake a second series of biosensor experiments in which GST::haPrPc was mixed with 10 µM copper (II) before injection onto heparin (Fig. 2a). This had three consequences: the absolute signal strength due to binding of PrP to heparin was reduced, the binding curve tended to plateau more quickly (i.e. tended towards equilibrium more rapidly) and bound protein was more resistant to removal with a strong salt solution. A physiological concentration of copper (II) at synaptic terminals (a tissue rich in PrP) is of the order of 10-20 µM (23). Fig. 2b displays the
biosensor responses recorded on a heparin surface during sequential injections of GST::haPrPc mixed with increasing concentrations (0-50 μM) of copper (II) ions. The curve shapes altered dramatically in the range 0-10 μM Cu (II) ions. As in Fig. 2a the curves assumed a more flattened shape toward the end of the contact phase and the high salt washes had a progressively smaller effect. Thus although the total accumulation of PrP in the presence of copper (II) ions is reduced, bound protein is more difficult to elute. Note that the transient elevation of RU at the start of the dissociative period (indicated by the arrows “D”) was encountered occasionally in experiments with full-length GST::haPrPc and is difficult to explain in terms of movement of the protein (which should show net dissociation during this phase). A difference in the interaction between the running buffer and the derivatised and coated surfaces in such experiments may account for this effect though buffer-only injections were essentially without signal. In parallel experiments GST::haPrPc was applied to an HS-derivatised surface in the presence or absence of Cu (II). In this case Cu (II) had no observable effect on the extent of binding (data not shown).

An ELISA for heparin / HS binding by GST-tagged PrPc proteins and peptides was developed to confirm the findings of biosensor analysis. Fig. 3a shows the result of titration of GST::haPrPc on two types of heparin (bovine lung and porcine intestinal heparin) and porcine mucosal HS. Copper (II) was not added in these experiments. Binding to the two heparins was comparable and much more extensive than that achieved on HS-coated wells, but for both GAGs (heparin and HS) was dose dependent. Fig. 3b displays the results of a related ELISA in which GST::huPrPc (at 1 μg/ml) was applied to porcine intestinal heparin-coated wells in the presence of increasing concentrations of four divalent cations: Cu (II), Zn (II), Ni (II) and Mn (II). Copper (II) produced the most considerable inhibition of binding (maximal inhibition between 2-4 μM Cu (II)), whilst manganese (II) had no effect. An intermediate level of inhibition was associated with zinc and nickel addition. The ability of copper (II) to disrupt binding of GST::PrPc to heparin supports data
from biosensor experiments and both methodologies show that high levels (50µM) of copper (II) fails to abolish heparin binding completely. A primary purpose of the ELISA was to grade sulfated polysaccharides on their ability to disrupt the interaction between GST:: haPrPc and heparin. Fig. 3c and d show the results of incubation of GST::haPrPc onto PIH coated wells in the presence of increasing concentrations of sulfated GAGs, with and without added Cu (II). Similar data were obtained for binding of PrP to immobilised BLH (data not shown) and soluble BLH and bovine kidney HS (BKHS) produced similar inhibitory profiles to PIH and PMHS respectively (data not shown). Pentosan polysulfate (PPS) and dextran sulfate (MW8000, DS8) were the most potent inhibitors of heparin binding (on a weight / volume basis). The heparins were substantially weaker and both the low molecular weight porcine intestinal heparin (LMW PIH) and two sources of HS were without effect. When copper (II) was added, the inhibitory activity of the GAGs/ polysaccharides was generally increased. Intact PIH (and also BLH, data not shown) approached DS and PPS in inhibitory effect and the binding of GST::haPrP to PIH could be weakly (but incompletely) disrupted by LMW PIH. These findings support the earlier evidence that Cu (II) reduces the interaction between full-length hamster PrP and heparin.

In a separate experiment human recombinant PrPc was allowed to bind to PI-heparin in the presence of a number of GAGs and modified heparins, added at 10 and 100 µg/ml (Fig. 4). Again BLH was a good inhibitor of heparin-binding, whereas PMHS had essentially no effect. Both persulfated (oversulfated) heparin and selectively de-6-O-sulfated heparin were less effective inhibitors than unmodified BLH. Selectively de-2-O-sulfated heparin lacked inhibitory activity completely at 10 µg/ml and inhibited only weakly at 100 µg/ml. These results indicate an important role for 2-O-sulfate groups in the prion-heparin interaction. Interestingly, low concentrations of 2-O-desulfated heparin, like CS (Fig. 3c) appeared to promote PrPc binding to immobilised heparin.
Binding of GST:: hamster PrP peptide fusions and synthetic PrP peptides (of human sequence) to immobilised heparin / HS

To identify regions of the PrP molecule with independent heparin / HS binding activity, recombinant GST::fusions of partial hamster PrP sequences collectively spanning the entire hamster prion sequence (21, 44) were injected over a heparin-derivatised biosensor surface. When several independent batches of peptides were tested (in each case no more than 2 months after expression-purification), P2 (53-93) and P4n (110-128) consistently gave the most significant binding to heparin (Fig. 5a). P1, P3, P4, P5 and Px generally yielded weak biosensor responses, although significant binding of P1 and P5 to heparin was recorded in occasional batches. Note that the increase in signal resulting from P4n injection was not reversed by high salt, weak acid, alkaline or soluble heparin washes: procedures which should have disrupted all but the strongest electrostatic interactions. Peptides were tested in succession (as in Fig. 5a) and individually with no evidence of sample order-related enhancement or reduction of signal strength for any peptide. In some experiments FGF-receptor (a known heparin-binding protein) was injected before and after a series of the PrP peptides and no change was observed in the extent of binding of this protein (data not shown).

Subsequent experiments revealed that the heparin-binding activity of both GST:: P2 (53-93) and GST:: P4n (110-128) were sensitive to copper (II) addition, elevating and suppressing biosensor response respectively. GST:: haPrP P2 (53-93) produced the most significant response when applied to the HS-derivatised biosensor surface but in contrast to its binding of heparin, recognition of HS was not influenced by Cu (II) (data not shown).

Although GST itself displays no heparin-binding activity in the buffer conditions chosen for GST:: haPrP/peptides (data not shown), it is conceivable that the GST portion of the fusion
peptides could influence heparin binding activities of the attached PrP sequence. Indeed it may explain the inconsistent binding observed for some GST-linked peptides (e.g. GST::P5 (218-231). For this reason synthetic PrP peptides containing no tag were also tested for binding to bovine lung heparin (Fig. 5b). In biosensor analysis low molecular weight entities such as small peptides tend to produce relatively weak signals per mol of bound analyte. By working at high sample concentrations however (e.g. 0.1-1.0 mg/ml) the signal strength can be maximised, so long as surface binding sites are non-limiting. Such data should be interpreted with caution however as high analyte concentrations can produce rate-limiting diffusional artefacts, re-binding phenomena, and increase the tendency for self-self interactions. Of the seven synthetic peptides only P1 (23-52), P2 (53-93) and P4n (110-128) yielded significant biosensor responses indicating that these three sequences are the strongest candidate heparin/ HS-binding regions in the prion molecule. As with the GSTtagged peptides the synthetic peptides were injected singly and in different order with no effect on the patterns of binding.

All GST-fusion peptides were tested for heparin binding by ELISA (synthetic peptides with no GST were not detectable in this method). Only GST:: P1 (23-52) bound heparin reproducibly, providing a signal that could be selectively inhibited (Fig. 6a). Note that the heparin-binding exhibited by P1 (23-52), like full-length PrP, was not competed by chondroitin sulfate. In order to explore the potential for intermolecular PrP-PrP interactions via heparin-binding sequences, GST::haPrPc was incubated in microtitre wells pre-coated with each of the synthetic peptides P1 (23-52), P2 (53-93) and P4n (110-128) (Fig. 6b). A clear interaction was observed between the full-length PrP and immobilised P1 (23-52). This binding was not significantly inhibited by addition of soluble heparin, pentosan polysulfate or dextran sulfate suggesting a predominantly hydrophobic nature.
Additional biosensor experiments showed that synthetic peptides P1 (23-52), P2 (53-93) and P4n (110-128) were capable of binding HS (data not shown), however only P2 (53-93) bound both GAGs in a concentration dependent manner (Fig. 7a, b). Heparin recognition was successfully competed with soluble heparin (data not shown). Porcine intestinal HS, though a weak inhibitor of the binding of synthetic P2 to immobilised heparin (data not shown) was an effective competitor for the binding of P2 to an HS-derivatised surface (Fig. 7c). It was noted that P2 (53-93) bound to the HS-derivatised surface in the presence of soluble PMHS was more readily eluted with the wash sequence (BLH, NaCl, NaOH) than P2 bound in the absence of competitor.

**Copper (II) ions substantially enhance the heparin binding activity of synthetic peptide P2 (53-93)**

As P2 (53-93) encompasses a region of PrP containing motifs for copper binding, biosensor experiments were conducted to assess any influence of copper availability on the heparin binding activity of this peptide. The biosensor response produced by 1 mg/mL P2 (53-93) was substantially enhanced (~7-fold) by the addition of 10µM Cu (II) (Fig. 8a). Intriguingly a second injection of P2 peptide (without added copper) applied immediately after one containing Cu (II) generated a higher signal (ca. 2-3 fold) than the initial injection of P2 onto a washed heparin surface. This result strongly suggested that a proportion of the enhancing activity of Cu (II) might be exercised as a complex with the heparin substrate. To investigate this further we compared the accumulation of synthetic P2 on the heparin surface after two pre-treatments: i.) after injection of Cu (II) alone and ii.) after injection of Cu (II) followed by EGTA (a potent chelator of copper) (Fig. 8b). Injection of a brief pulse of EGTA between injections of Cu (II) and P2 greatly reduced the enhancement possible by pre-treatment with Cu (II) alone. In a demonstration of the selectivity of this effect no such enhancement was recorded when Cu (II) was applied in advance P1 peptide (Fig. 8b) or P4n peptide (data not shown). To assess the ability of alternative metals to enhance heparin-binding by P2 (53-93), P2 (53-93) the peptide was injected in the absence of
additional cation and also in the presence of 50µM copper (II), magnesium (II), nickel (II) or manganese (II) (Fig. 8c). None of the alternative cations replicated precisely the action of copper. Neither magnesium (II) or manganese (II) affected binding significantly and although nickel (II) induced a larger absolute response, the rapid return of signal to baseline at completion of the contact period was indicative of a highly weak interaction. Since copper (II) had an inhibitory influence on binding of PrP to heparin in the range 0-2 µM (Fig. 3b), we decided to apply P2 peptide in a range of Cu (II) concentrations (Fig. 8d). The greatest enhancing activity was afforded at relatively high concentrations (>10 µM) whereas in the concentration range that inhibits full-length PrP binding to heparin, Cu (II) had no clear influence on synthetic P2 (53-93) peptide.

Competitive ELISA to determine the relevance of P1, P2 and P4n sequences in the binding of full-length GST::haPrP to heparin

To access which sequences, singly or in combination, might contact heparin within the fully folded recombinant GST:: haPrP molecule a competition ELISA was developed in which GST:: haPrP was incubated on heparin in the presence of synthetic peptides P1, P2 and P4n (Fig. 9). As the 2° antibody in this ELISA recognises GST, only heparin-bound GST:: haPrP was detectable. It was anticipated that sequences contributing to heparin-binding in native PrP would compete with PrP for heparin when presented as peptides in solution. In this experiment synthetic peptides were either pre-incubated on heparin in advance of GST:: haPrP or mixed directly with GST:: haPrP. Experiments were also conducted both in the presence and absence of added Cu (II). Without Cu (II), none of the peptides affected the binding of GST:: haPrP to heparin (Fig. 9a) but when copper (II) was supplemented at 50 µM (which predictably reduced the binding signal due to GST:: haPrP) co-incubation of GST:: haPrP with synthetic peptide P2 (53-93)
resulted in a significantly lower binding signal on both BLH and PIH substrates than GST::haPrP\textsuperscript{c} applied with no competitor (Fig. 9b).

**Discussion**

Prion proteins are placed firmly in the heparin-binding category of proteins. Not only have direct interactions been demonstrated with PrP (14, 15), GAGs have been shown to influence PrP\textsuperscript{sc} accumulation in both cell-culture and *in vitro* converting experiments (12-13, 17, 18) and to modulate PrP\textsuperscript{sc} propagation and disease onset in animal models for scrapie (8, 9). This study has addressed three areas of current interest: the structural features of GAGs that engender PrP-binding ability, the influence of metal ions on GAG-binding and the location of GAG binding domains within PrP.

By both biosensor and ELISA techniques we were able to show direct binding of PrP to heparin, thus confirming earlier work (14, 15). We were also able to demonstrate a direct interaction between PrP and purified heparan sulfate. This complements previous studies in which PrP-HS binding was strongly implicated but not directly demonstrated (eg. 17, 18, 21). Two aspects of the interaction with heparin were of special interest. The ability of copper (II) ions to disrupt PrP binding was observed in several experiments (e.g. Figs. 2a\&b, 3b-d, Fig. 9). The direct ELISA (Fig. 3b) proved that this inhibition was only partial i.e. that a significant proportion of heparin affinity still remains even at high concentrations of copper. This suggests that copper alters the conformation of PrP rather than simply shielding binding sites in the heparin substrate. Another explanation which we cannot rule out is that the main effect of copper ions is disrupt oligomers/aggregates of PrP bound to heparin rather than inhibition of the PrP-heparin interaction directly, hence the incomplete inhibition. Nickel (II) and zinc (II), but not manganese (II) ions
were also inhibitory, though not as strongly as copper. A related effect was first described by Brimacombe et al., who reported that recombinant PrP could be displaced from an experimental nickel biosensor surface by pulses of heparin (15). PrP loaded with copper and manganese assumes distinct conformations with differing levels of protease-resistance (25-30). Our data suggests that one of the first consequences of metal-induced conformational changes might be an altered affinity for endogenous HSPGs. The reverse situation, that bioactive HSPGs might influence the uptake of metal ions by PrP is also a possibility.

The specificity of the PrP-heparin interaction was also investigated. The interaction of PrP with heparin (as detected by the ELISA) could be disrupted by soluble heparin, HS and by other sulfated polysaccharides (Fig. 3c, d and Fig. 4) but was refractory to inhibition by CS, confirming earlier work by others (14). This last observation on the non-activity of CS has a special significance as it suggests that presentation of sulfates is not in itself sufficient for prion-binding activity. A particularly interesting finding was the potent inhibitory activity of a fraction of dextran sulfate of average MW 8000 (DS8). On a weight / volume basis this preparation was found to be superior to heparin and at least as effective as pentosan polysulfate (PPS), a polyanion with well-documented anti-prion activities in tissue culture and animal models (36, 7). It would be of great interest to compare the inhibitory activity of DS8 with fractions of higher MW such as DS500 (average MW 500,000) which is a particularly effective inhibitor of PrP\textsuperscript{sc} propagation in cell culture (13) and a potent anti-prion agent in animal studies of prion disease (8, 9). Using this competition ELISA we also explored the contribution to prion binding of 2- and 6-\textit{O} sulfate groupings, two of the three types of sulfate which elaborate heparin and HS (N-sulfates were not examined in this study). Removal of 2-\textit{O} sulfates was found to significantly reduce the inhibitory activity of BLH in the competitive ELISA (Fig. 4), suggesting an important role in prion-ligation. FGF-2 and hepatocyte growth factor are other heparin-binding proteins with a particular requirement for 2-\textit{O} sulfate (56).
The location of heparin-binding sites in the prion protein has not previously been determined with certainty. This study revealed three sites in recombinant PrP with independent affinity for GAGs: amino acids 23-52, 53-93 and 110-128. Whilst the first sequence (23-52) has no previously reported affinity for GAGs, there is some evidence for GAG binding activity at the other two sites (21, 36, 37, 43). The biosensor response generated by P2 (53-93) on both heparin and HS surfaces was concentration-dependent and selectively inhibited and so is least likely to be artefactual. In contrast to the intact molecule, accumulation of P2 (53-93) on heparin was clearly enhanced by Cu (II) addition. The enhancing effect was not dependent on co-mixing of Cu (II) and P2 (53-93) as it could be replicated by conditioning the heparin surface with copper in advance of P2 (53-93). Certainly it is possible to form complexes between GAGs and copper (II) in vitro and this is the basis of a sensitive detection method for heparin (57). This observation supports a model in which copper ions can exist not only directly bound to the P2 sequence, but also after transfer from the heparin surface, or as a complex of all three components (P2 (53-93) or PrP, Cu (II) and heparin. It is difficult to understand why the influence of copper on intact PrP does not reflect its effect on isolated P2 (53-93). Either the enhancing influence via P2 is not manifest in the native protein, or if present, is masked by another copper-modulated heparin-binding activity elsewhere in the sequence. A candidate for such a site is P4n (110-128) and preliminary observations in our laboratory, not presented here, indicate that heparin-binding of P4n is abrogated by Cu (II). As with the complete molecule alternative metals had divergent effects on the binding of P2 (53-93) to heparin and it may be significant that manganese afforded no enhancing effect. Given its strong heparin-binding behaviour in biosensor studies, it was surprising that GST::P2 (53-93) was not identified as heparin-binding in the direct ELISA and the reason for this is not clear. Nevertheless in the presence of Cu (II) ions synthetic P2 (53-93) appeared to reduce heparin binding by GST::haPrPc. This result supports previous evidence for a heparin-binding function for P2 (53-
93) and suggests that the sequence is the predominant location of heparin binding in the full-length protein.

P1(23-52) demands particular attention as it proved positive in all tests of direct heparin and HS binding, including in the ELISA where concentration-dependence and target specificity were established (Fig. 6a). However, since we were unable to demonstrate competition between this peptide and full-length GST::haPrPc for binding to heparin (Fig. 9a, b) we propose that this sequence is not a major heparin-binding site in intact PrPc. A more important association may be with sequences within PrP itself for we were able to demonstrate affinity for GST::haPrPc (Fig. 6b). Such an interaction may be a means by which the putative acceptor molecules for this sequence such as nucleic acids (55) and Hsp60-like chaperonins might influence PrP function.

In biosensor analysis P4n (110-128) bound heparin and HS both as GST::fusion and free peptide, but was not positive in the ELISA. This peptide particularly yielded biosensor signals that could not be reversed by salt washing or extremes of pH. A possible explanation for this unusual behaviour is extensive aggregate formation either in solution or in situ at the heparin surface especially as a related sequence (106-126) shows a well described propensity for fibril formation (38, 39). Significantly the β–sheeted structures characteristic of peptide 106-126 in weakly acidic ionic buffers (conditions in which fibrils are also favoured) are evidently highly stable (resistant to 5% SDS or alkali to pH 12) (39). That P4n should show an affinity for heparin/HS was of interest as it has shown elsewhere that the cytotoxic properties of peptide 106-126 may be abrogated by GAGs (43). Again, as an excess of P4n (110-128) was not seen to interfere with the binding of full-length PrP to heparin this peptide is considered a weak candidate for a true physiological role in heparin/HS binding by PrP.
The findings in this study support a prominent role for P2 (53-93) in direct HS binding by PrP predicted from recent work demonstrating the strictly HSPG dependent binding of this peptide to mammalian cells (21). Importantly, the differing influence of Cu (II) ions on the heparin-binding properties of this peptide and of the intact PrP molecule serve to illustrate the potential hazards of over-reliance on peptide-derived data alone. The sensitivity of PrPc-heparin binding to disruption with dextran sulfate and pentosan polysulfate, both of which have been proposed as prophylactic agents, and the demonstration of intimate role of 2-O sulfate in heparin recognition was also significant. Our data suggests that further investigation of the identified heparin-binding domains, and the potential specificity of carbohydrate binding sites in heparan sulfate, will lead to further insights into the role of HS in the function of prion proteins.

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Figure legends

**Fig. 1: Biosensor analysis of binding of full-length GST::haPrPc to heparin and HS-derivatised surfaces.** Sensorgrams produced by injection of recombinant GST::haPrPc (30 µl, 15 µg/ml, 10µl/min flow rate) onto bovine lung heparin (A) and porcine intestinal heparan sulfate (B) biosensor surfaces (immobilised GAGs biotinylated mid-chain according to (53)). The sensorgram displays the net response (in RU) for the GAG-derivatised surface after subtraction of non-specific events as recorded on the non-derivatised control surface. The response changes recorded after injection of buffer alone have been superimposed (“buffer”). After a short period during which the sample makes contact with the control and heparin-derivatised surfaces (period A, 180 secs) the sample was exchanged for running buffer (period D, 120 secs). During this period the the major movement of GST::haPrPc is dissociation from the chip surface. After each injection of GST::haPrPc the surfaces were washed with 2M NaCl and 10mM HCl. The amount of residual GST::haPrPc removed from the heparin and HS surfaces by these washes is marked $R_{\text{HEP}}$ and $R_{\text{HS}}$.

**Fig. 2: Cu (II) ions suppress the biosensor response generated when GST::haPrPc is injected onto heparin.** (A) GST::haPrP (40 µl, 16 µg/ml) was injected over heparin (BLH, biotinylated at reducing termini according to Nadkarni (52) ) in the absence of added Cu(II) ions, and subsequently in the presence of 10µM Cu (II). Initiation of dissociation is marked with the letter D. (B) GST::haPrPc (30 µl, 10 µg/ml) mixed with successively higher concentrations of Cu (II) (final concentration in µM shown in boxes)
was injected over heparin. Initiation of injections marked by black arrows. Initiation of
dissociation phase for each sample indicated by arrow with adjacent letter D. Between
injections the chip surface was washed with 2 M NaCl (solid arrows) and 10mM HCl
(open arrows). Note that neither buffer itself nor buffer containing 50 µM Cu (II) yielded
a biosensor response on the heparin surface (data not shown).

**Fig. 3:** ELISA analysis of PrPc binding to heparin/ HS. Concentration and divalent
cation dependency and inhibition with soluble GAGs. (A) ELISA signals generated by
the application of increasing concentrations of GST::haPrPc to immobilised BLH
(triangles), PIH (boxes) or PMHS (circles). (B) Signals generated by incubation of
1µg/ml GST::huPrPc onto PIH coated wells in the presence of increasing concentrations
(0-16µM) of Mn (II), Zn (II), Ni (II) and Cu (II). (C, D) ELISA experiments in which
GST::haPrPc was incubated on immobilised PIH in the presence of increasing
concentrations of sulfated polysaccharides and GAGs. The experiment was conducted in
the absence (B) or presence (C) of 50 µM Cu (II). Inhibitors are as follows: PIH: solid
line, open square; LMW PIH: solid line, open diamond; PMHS: solid line, closed circle;
PPS: dashed line, open triangle; DS8: dashed line, open circle; CS: dashed line, open
square. In all cases data points represent mean ± standard deviation of net absorbances
(background subtracted) achieved from three experiments.

**Fig. 4:** Contribution of 2-O and 6-O sulfation to heparin recognition by PrPc. Data
from an ELISA in which GST:: huPrPc (2.5 µg/ml) was incubated in PIH-coated wells
alone or in the presence of 10 and 100 µg/ml of selected GAGs (BLH, PMHS, CS) or
chemically modified heparins. PER-S = oversulfated bovine lung heparin; 2-\(O\)-DeS = de-2-\(O\)-sulfated BLH; 6-\(O\)-DeS: de-6-\(O\)-sulfated BLH.

**Fig. 5: Biosensor analysis of heparin-binding by GST-tagged and synthetic peptides.** (A) GST::haPrP peptides were applied to a heparin surface (BLH modified with biotin at mid-chain sites according to (53)). Concentrations were as follows (µg/ml): P1(23-52), 50; P2 (53-93), 50; P3 (90-109), 100; P4 (129-175), 80; P4n (110-128), 110; P5 (218-231), 80; Px (180-210), 120. Injected volumes were 30 µl. Asterisks mark the sample applications (GST::P2 and GST::P4n ) that yielded significant biosensor responses. The surfaces were washed with 10 µl 2 M NaCl, 5 µl 10 mM HCl and 5 µl 10 mM NaOH following the injection of GST:: P1 and washed with with 10 µl 2 M NaCl following injection of GST:: P4n . (B) Synthetic peptides P1 (23-52), P2 (53-93), P3 (90-109), P4 (129-175), P4n (110-128), Px (180-210) and P5 (218-231) of human sequence were applied at a concentration of 1.0 mg/ml (volume 30 µl) to the same bovine lung heparin-derivatised surface.

**Fig. 6: Heparin binding by P1 (23-52) is inhibited by soluble heparin but not PMHS or CS and immobilised P1 peptide, but not P2 or P4n binds PrP\(^{c}\).** (A) Histogram showing the results of an ELISA to determine the inhibitory effect of soluble bovine lung heparin (BLH), chondroitin sulfate (CS) and porcine mucosa heparan sulfate (PMHS) all at 20 µg/ml, on the binding of GST:: P1 (23-52) (5 µg/ml) to immobilised heparins. Target surfaces are as follows: un-modified control surface (white column); BLH (black column) and PIH (shaded column). The right-hand side of the histogram displays the
absorbances achieved when a dilution series of GST:: P1 (23-52) is applied to the same surfaces in the absence of competitor GAGs (single measurements, no error bars). (B) Histogram showing the signals produced in an ELISA to detect binding of GST:: haPrPc to immobilised peptides P1, P2 and P4n. The following GAGs were co-incubated with GST:: haPrPc at 100 µg/ml to assess the GAG-dependency of any interaction: column 1: buffer only (no haPrPc, no GAG); column 2: GST: haPrPc only (no GAG), column 3-8: haPrPc + BLH, PMHS, PPS, DS, CS and LMW PIH respectively.

**Fig. 7:** Biosensor response due to binding of synthetic P2 (53-93) peptide to heparin and HS is concentration dependent. HS binding is sensitive to competition with soluble HS. Synthetic P2 (53-93) was applied to BLH (A) and PMHS (B)–derivatised surfaces across a range of concentrations (values in mg/ml shown above the respective sensorgram, overlaid on a common axis). (C): injection of 1 mg/ml synthetic P2 (53-93) on an HS surface (HS biotinylated mid-chain according to Rahmoune method (53)) in the absence (injection at t = 500 s) or presence of 20 µg/ml soluble PMHS (injection at t = 1500 s). At position (N) the baseline response on HS- and control channels has been normalised to facilitate comparison of the extent of binding. After each injection surfaces were washed with 10 µl BLH (2 mg/ml), 10 µl 2 M NaCl and 5 µl 10 mM NaOH.

**Fig. 8:** The influence of Cu (II) on the biosensor response generated upon injection of P2 (53-93) over heparin. (A) Synthetic peptide P2 (53-93) was applied to a heparin surface at 1.0 mg/ml without addition of copper (1st injection) or in buffer containing 10 µM Cu (II) ions (2nd injection). A third injection of peptide (2nd Cu-free application)
bound more strongly than the first. 10 μM Cu (II) alone (injection # 4) produces no signal. (B) Synthetic P2 (53-93) peptide (0.25 mg/ml) was applied to the heparin surface following a pulse (10 μl, 2 mM) of EGTA (1st injection); a pulse (30 μl, 50 μM) of Cu (II) (2nd injection); a pulse of Cu (II) followed by a pulse of EGTA (3rd injection). Closed arrows (other than P2 applications) indicate applications of EGTA. Open arrows indicate applications of Cu (II). A triple wash regime of NaCl (2M), HCl (10mM) and NaOH (10mM) was applied immediately after each injection of P2 (53-93). At t= 3000s: synthetic P1 (23-52) peptide (0.25 mg/ml) applied alone or supplemented with 50 μM Cu (II). (C) Synthetic P2 (53-93) peptide (0.25 mg/ml) was injected with no added divalent cation, or mixed with 50 μM of each of Cu (II), Mg (II), Ni (II) or Mn (II) ions (total injected volume 30 μl). After each application of peptide the heparin (and control) surfaces were washed (W) with NaCl (2M), HCl (10mM) and NaOH (10mM) and also with EGTA (2 mM, 10 μl) (arrows marked E). Injections of buffer and copper (II) ions (50 μM) are identified by the first pair of arrowed boxes. Both provide no response. (D) Sensorgram displaying the biosensor responses on the BLH surface after application of synthetic P2 (53-93) peptide (33 μg/mL in HBS) containing increasing concentrations of Cu (II) (final Cu (II) concentration in μM shown in boxes). Between injections the heparin surface was washed with 5μl each of 2M NaCl, 10mM HCl, 10mM NaOH and 5mM EGTA.

**Fig. 9:** Synthetic P2 (53-93) peptide interferes with the binding of GST::haPrPc to immobilised heparin. (A) Histogram depicting the results of a two-phase ELISA designed to test the effect of pre-application or co-incubation of synthetic peptides P1, P2
and P4n on the binding of GST::haPrPc to heparin. In the first phase of the ELISA each of the synthetic peptides (at 50 µg/ml) or buffer alone were incubated in wells of a microtitre plate pre-coated with heparin. In the second phase two combinations were tested: 1. GST::haPrPc (1 µg/ml) or buffer alone was added to each of the wells (columns marked “PRE”). 2. a mixture of GST::haPrPc (at 1 µg/ml) and each of the synthetic peptides (50 µg/ml) was added to wells pre-incubated with buffer only (columns marked “CO”). (B) The experiment was repeated in the presence of 50µM Cu (II) ions. Target substrates were as follows: none (white column), BLH (black column) and PIH (shaded column). Error bars indicate the standard deviations of the net (background subtracted) absorbances from three wells. Control wells receiving only buffer (no GST:: haPrPc) presented a minimal background signal as did control wells challenged with buffer alone or synthetic peptides followed by buffer (i.e. no GST::haPrPc) (data not shown). The asterisk marks the data point representing GST:: haPrPc co-incubated with P2 (53-93) peptide. A students t-test was used to compare the absorbance intensity from this co-incubation with that of GST:: haPrPc alone (no added peptide). A resulting probability value of P<0.01 indicates that the reduction in ELISA signal as a result of co-incubation with P2 (53-93) is statistically significant.
Figure 1

A

B

Response (RU)

Response (RU)

Time (secs)

Time (secs)

GST::haPrPc

GST::haPrPc

buffer

buffer

wash 1: 2M NaCl

wash 1: 2M NaCl

wash 2: 10mM HCl

wash 2: 10mM HCl

R HEP

R HS

10mM HCl

10mM HCl
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

A

B

C

D
Figure 9

A

B

Condition

Condition

Absorbance (490 nm)

Absorbance (490 nm)

+ 50 µM Cu(II)

+ 50 µM Cu(II)

no added Cu (II)

no added Cu (II)

+ P1

+ P1

+ P2

+ P2

+ P4n

+ P4n

Absorbance (490 nm)

Absorbance (490 nm)

blank no added peptide

blank no added peptide

PRE CO PRE CO PRE CO PRE CO

PRE CO PRE CO PRE CO

PRE CO PRE CO PRE CO

PRE CO PRE CO PRE CO

* P< 0.01

* P< 0.01

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