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Binding of the Protease-Sensitive Form of Prion Protein PrP to Sulfated Glycosaminoglycan and Congo Red

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Congo red and certain sulfated glycans are potent inhibitors of protease-resistant PrP accumulation in scrapie-infected cells. One hypothesis is that these inhibitors act by blocking the association between protease-resistant PrP and sulfated glycosaminoglycans or proteoglycans (e.g., heparan sulfate proteoglycan) that is observed in amyloid plaques of scrapie-infected brain tissue. Accordingly, we have investigated whether the apparent precursor of protease-resistant PrP, protease-sensitive PrP, binds to Congo red and heparin, a highly sulfated glycosaminoglycan with an inhibitory potency like that of heparan sulfate. Protease-sensitive PrP released from the surface of mouse neuroblastoma cells bound to heparin-agarose and Congo red-glass beads. Sucrose density gradient fractionation provided evidence that at least some of the PrP capable of binding heparin-agarose was monomeric. Free Congo red blocked PrP binding to heparin and vice versa, suggesting that these ligands share a common binding site. The relative efficacies of pentosan polysulfate, Congo red, heparin, and chondroitin sulfate in blocking PrP binding to heparin-agarose corresponded with their previously demonstrated potencies in inhibiting protease-resistant PrP accumulation. These results are consistent with the idea that sulfated glycans and Congo red inhibit protease-resistant PrP accumulation by interfering with the interaction of PrP with an endogenous glycosaminoglycan or proteoglycan.

The accumulation in the brain of an abnormally protease-resistant isoform of the host protein PrP is specific to scrapie and related transmissible spongiform encephalopathies and important in the pathogenesis of these neurodegenerative diseases. The protease-resistant PrP is a component of extracellular amyloid plaques in the central nervous system (1) and can be isolated in the form of amyloid fibrils or rods (11, 24) that are specific for scrapie-infected tissues (21). The association of high levels of scrapie infectivity with the isolated preparations of protease-resistant PrP led to the proposal (23) that it is the infectious protein agent postulated by Griffith (16) and others, but this proposal remains speculative. Studies in scrapie-infected cell cultures have indicated that the protease-resistant PrP is formed from an apparently normal, protease- and phospholipase-sensitive PrP precursor (PrP-sen) (2, 7). The scrapie-specific conversion to protease-resistant PrP occurs on the plasma membrane or along an endocytic pathway to the lysosomes (7, 9).

Inhibitors of protease-resistant PrP accumulation should be useful as potential therapeutic agents and as reagents in studies of the fundamental mechanism of protease-resistant PrP formation and its putative involvement in scrapie agent replication. Recently, Congo red, a dye that has long been used as a diagnostic stain for amyloids (15), was identified as an inhibitor of protease-resistant PrP accumulation and scrapie agent replication in scrapie-infected mouse neuroblastoma (sc^+-MNB) cells (4, 6, 8). The inhibition by Congo red is selective in that it occurs without apparent effects on PrP-sen metabolism or protein biosynthesis in general. The effect of Congo red is due primarily to prevention of new accumulation rather than destabilization of preexisting protease-resistant PrP. The accumulation of protease-resistant PrP remains depressed in the cultures after removal of the Congo red, indicating that the effect is not reversible.

The mechanism for the inhibition of protease-resistant PrP accumulation by Congo red has not been established. However, since Congo red binds to amyloid fibrils of protease-resistant PrP (24), it may directly block protease-resistant PrP formation or destabilize the structure formed (6). Congo red is a sulfonated compound and in that respect resembles the sulfated glycans which also inhibit protease-resistant PrP accumulation in sc^+-MNBs (8) and have prophylactic value against scrapie in mice and hamsters (10, 12–14, 19, 20). All of these inhibitors can be viewed as sulfated glycosaminoglycan (GAG) analogs, leading us to propose that their mechanism of action may be to prevent the association between protease-resistant PrP and endogenous sulfated GAG or proteoglycan (i.e., heparan sulfate proteoglycan) that is observed in protease-resistant PrP amyloid plaques in vivo (3, 8). For instance, the inhibitors might competitively block an interaction between PrP-sen and an endogenous GAG that could be required for the conversion to protease-resistant PrP. In the study reported here, we provide evidence for this proposed mechanism of inhibition by showing that PrP-sen from scrapie-competent cells binds to sulfated GAG (heparin) and Congo red and that the binding to one can be blocked by the other. We chose heparin as a model sulfated GAG for these binding studies for several reasons: (i) heparin is closely related structurally to heparan sulfate; (ii) it is an inhibitor of protease-resistant PrP accumulation in sc^+-MNB cells (8) and, as shown here, is nearly as potent as heparan sulfate; and (iii)

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heparin is commercially available in multiple free and immobilized forms.

MATERIALS AND METHODS

Labeling and release of PrP-sen from sc$^+$-MNB cells. PrP-sen was labeled with [^{35}S]methionine and [^{35}S]cysteine (Expre^{35}S^{35}S; New England Nuclear) and released from intact sc$^+$-MNB cells into phosphate-buffered balanced salts solution (PBBS) by using phosphatidylinositol-specific phospholipase C (PI-PLC) as described previously (6). The resulting PI-PLC medium containing labeled PrP-sen was centrifuged at 1,000 $\times$ *g* for 2 min, and the supernatant was collected and supplemented with 1 mg of bovine serum albumin per ml and protease inhibitors to give final concentrations of 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg of pepstatin per ml, 0.5 μg of leupeptin per ml, and 1 μg of aprotinin per ml.

Heparin-agarose (HA) binding assay. Aliquots of the PI-PLC medium (0.2 to 0.3 ml) that in some experiments had been supplemented with the designated potential inhibitors of HA binding were incubated at room temperature for 30 min with aliquots of various HAs or control beads (Sepharose CL-4B). Twenty to thirty microliters of a 50% bed volume suspension of the beads in 0.5 M NaCl was used except in the case of type III HA. Since type III HA has approximately half the heparin density per unit of bed volume of the other HAs, 60 μl of a 50% bed volume suspension was used. The beads were pelleted, and both the supernatants and the beads were analyzed for labeled PrP-sen as follows. The beads were washed, and the bound PrP was eluted directly or indirectly (after boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] sample buffer) into 0.5% Triton X-100–0.5% sodium deoxycholate–5 mM Tris-HCl (pH 7.4)–150 mM NaCl–5 mM EDTA–0.7 μg of pepstatin per ml–0.5 μg of leupeptin per ml–0.5 mM phenylmethylsulfonyl fluoride (TDSE) as described for the individual experiments prior to immunoprecipitation. The supernatants were mixed with 0.5 volume of 3$\times$ TDSE prior to immunoprecipitation. The bead eluates and TDSE-treated supernatants were incubated at 4°C for 16 to 20 h with 2 μl of rabbit antiserum raised against a synthetic peptide corresponding to PrP residues 89 to 103 (9). Immune complexes were bound to protein A-Sepharose beads, washed three times with TDSE and once with water, and eluted by boiling in SDS-PAGE sample buffer. The labeled proteins in the samples were analyzed by SDS-PAGE–fluorography by using the PhastSystem (Pharmacia) as described previously (6). Within individual experiments, identical PI-PLC medium equivalents were applied to the lanes corresponding to HA-bound and unbound fractions.

Sucrose gradients. PI-PLC medium (0.4 ml) prepared as described above from sc$^+$-MNB cells labeled with 1 mCi of Expre^{35}S^{35}S per 25-cm^2 flask was layered over 4.6-ml 5 to 20% linear sucrose gradients in PBBS with a 0.35-ml saturated sucrose pad at the bottom in Ultraclear tubes (Beckman). Duplicate gradients were centrifuged for 17 h at 300,000 $\times$ g_{max} at 4°C together with a third similar gradient loaded with protein molecular weight standards in PBBS. Ten 0.5-ml fractions were collected from the bottom of each gradient through a needle puncturing the base of the tube. Following the fractionation, any pelleted material and liquid remaining in the bottom of the centrifuge tube were collected with a 0.25-ml TDSE wash and designated the bottom fraction. The corresponding fractions from the duplicate PI-PLC medium gradients were mixed and then redivided into two equal parts. One half was given 0.5 volume of 3$\times$ TDSE and directly immunoprecipitated as described above. The other half of each fraction was assayed for HA binding as described above. The resulting immunoprecipitates were analyzed by SDS-PAGE–fluorography on 12% polyacrylamide gels (Novex). Aliquots of the molecular weight standard gradient fractions were mixed directly with SDS-PAGE sample buffer, electrophoresed on 20% polyacrylamide PhastSystem gels, and visualized by a protein silver staining procedure.

Coupling of Congo red to glass beads. Congo red was covalently attached to glass beads by a modification of the method described by Janolino and Swaisgood (18). Aminopropyl-glass beads were prepared by heating 1 g of clean, dry glass (either Sigma no. G-4649 nonporous, ≤106-μm diameter, or 1,000-Å (100-nm)-pore-diameter, 200/400-mesh silica) in 10% (vol/vol) 3-aminopropyltriethoxysilane (Sigma no. A-3648) in toluene to 70°C for 4 h, swirling every 15 to 30 min. The beads were transferred to a sintered glass funnel and washed with 1 liter of acetone. Succinamidopropyl-glass beads were prepared by swirling 1 g of aminopropyl-glass in a 10% aqueous solution of succinic anhydride. Triethylamine was added in 1-ml aliquots until the pH became slightly alkaline. The suspension was mixed by bubbling with N_2 for 20 min at room temperature. The beads were then washed with 1 liter of acetone. The succinamidopropyl-glass was activated by mixing (by inversion) with 0.01 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodimide (Sigma no. e-6383) in pH 7.0 phosphate buffer for 20 min. A concentrated solution of Congo red was added to a final concentration of 1 mM, and the suspension was mixed by inversion overnight. The Congo red-glass beads were washed sequentially with 1 liter of 8 M urea, 1 liter of 2 M NaCl, and 1 liter of acetone and stored dry until used.

Congo red-glass bead binding assay. The Congo red-glass bead binding assay was identical to the HA binding assay except that 500 μg of Congo red beads was substituted for the HA beads.

RESULTS

Binding of PrP-sen to HA beads. To test the ability of PrP-sen from scrapie-competent cells to bind to heparin under near-physiological conditions, i.e., in the absence of detergent, we first released ^{35}S-labeled PrP-sen from the surface of live sc$^+$-MNB cells with PI-PLC in PBBS. Previous studies have shown that PI-PLC selectively releases PrP-sen, and not protease-resistant PrP, from these cells (5). The PI-PLC medium containing labeled PrP-sen was incubated with HA beads (Sigma type II). Because numerous other labeled proteins were present in the bound (Fig. 1B) and unbound (data not shown) fractions, the PrP content of the fractions was compared by radioimmunoprecipitation with an anti-PrP antiserum (Fig. 1A). Nearly equivalent amounts of PrP-sen were detected in the bound (lanes 3 and 4) and unbound (lane 1) fractions. When the PI-PLC medium was preincubated with excess free heparin, no PrP-sen was detected in the bound fraction (lane 5) and the PrP-sen in the unbound fraction was increased (lane 2), providing evidence that the binding of PrP-sen to the HA beads was specific for the heparin component of the beads.

HA beads with bound PrP-sen were washed with different solutions to gain insight into the chemical nature of the interaction. Neither washing the beads with phosphate-buffered saline (PBS)–1 mM EDTA (Fig. 1A, lane 6) nor pretreatment of the PI-PLC medium with 10 mM EDTA prior to the binding reaction (as in Fig. 2) disrupted the binding of the PrP-sen, providing evidence that the binding was not dependent on divalent cations. Buffered saline containing 0.05% Tween 20 (Fig. 1A, lane 9) also failed to elute the PrP-sen,

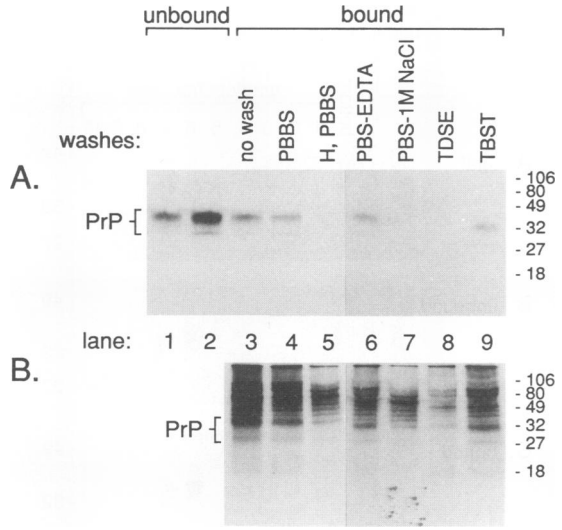

FIG. 1. Binding and elution of PrP-sen from HA. PI-PLC medium from sc$^+$-MNB cells labeled with [^{35}S]methionine and [^{35}S]cysteine was treated with 1 mg of heparin per ml (lanes 2 and 5) or left untreated (lanes 1, 3, 4, and 6 through 9) and incubated with Sigma type II HA beads. (A) The proportion of PrP-sen bound to HA after various washes (bound) was compared with that remaining in the PI-PLC medium (unbound). The unbound PrP-sen was immunoprecipitated directly from the PI-PLC medium supernatants after the beads were pelleted and analyzed by SDS-PAGE–fluorography as described in Materials and Methods. The HA beads were either not washed (lane 3) or washed with PBBS (lanes 4 and 5), PBS–1 mM EDTA (lane 6), PBS–1 M NaCl (lane 7), TDSE (lane 8), or 0.05% Tween 20–150 mM NaCl–10 mM Tris-HCl, pH 8.0 (TBST, lane 9). After an additional wash with PBBS for all but the unwashed beads, the bound proteins were eluted by heating the beads in boiling water in 20 μl of 2× SDS-PAGE sample buffer (10% SDS, 0.13 M Tris-HCl [pH 6.8], 6 mM EDTA, 0.1% [wt/vol] bromophenol blue, 8% [vol/vol] 2-mercaptoethanol). A 10-μl aliquot was diluted into 0.75 ml of TDSE, and the PrP was immunoprecipitated and analyzed by the method used for the unbound samples. (B) Another aliquot of the SDS-PAGE buffer bead eluate was analyzed directly by SDS-PAGE–fluorography to visualize all labeled proteins bound to the HA beads after the various washes described above. The positions of prestained molecular weight markers are designated in kilodaltons on the right.

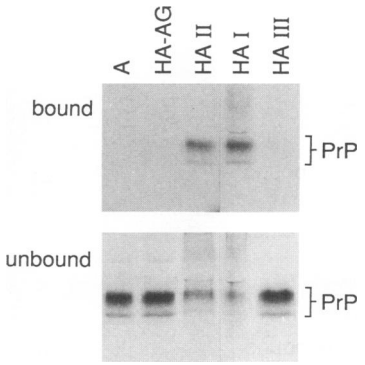

FIG. 2. Binding of PrP-sen to different HAs. Blank agarose beads (A), Affi-Gel heparin (HA-AG), and Sigma HA types II (HA II), I (HA I), and III (HA III) were incubated with labeled PI-PLC medium supplemented additionally with 10 mM EDTA. The PrP-sen levels in the bound (top) and unbound (bottom) fractions were compared as described in the legend to Fig. 1 and Materials and Methods except that the bound PrP was eluted directly with TDSE rather than SDS-PAGE sample buffer.

indicating that the binding was stable in the presence of a very mild detergent solution. However, washing the beads with PBS–1 M NaCl eluted the PrP-sen (lane 7), indicating that ionic interactions are important, as would be expected since heparin is a highly charged glycan. A stronger detergent solution containing 0.5% Triton X-100 and 0.5% deoxycholate (TDSE) also removed the PrP-sen from the beads (lane 8), suggesting that hydrophobic interactions or a detergent-sensitive protein conformation plays a role in the binding. In other experiments, e.g., in Fig. 2, it was shown that the PrP-sen eluted with TDSE could be recovered from the eluate.

The selectivity of the PrP interaction with HA beads was tested by comparing the binding of PrP to four different types of HA beads and blank agarose beads. All the beads were made with 4% cross-linked agarose. Sigma's type I and II beads contain distinct classes of heparin that are coupled to the beads via primary amino groups on heparin. Bio-Rad's Affi-Gel heparin and Sigma's type III beads are coupled to carboxylate and hydroxyl groups, respectively, of the same heparin preparation used in Sigma type II HA. As shown in Fig. 2, PrP-sen bound to Sigma type I and II HAs but did not bind to the blank beads, Bio-Rad Affi-Gel heparin, or Sigma type III HA. Accordingly, less PrP was detected in the unbound fractions from the Sigma type I and II HA incubations than in fractions from the incubations with the other beads. The lack of binding to the blank Sepharose beads provided additional evidence that the PrP binding was dependent on the presence of heparin. The lack of binding to Affi-Gel and Sigma type III HA provides evidence that the coupling of agarose to the carboxylate or hydroxyl groups of heparin can prevent PrP-sen binding.

A PrP-sen population resistant to heparin binding. Since a substantial proportion of the PrP molecules remained in the PI-PLC medium after incubation with the active HAs (Sigma types I and II; Fig. 2), we tested whether an additional round of fresh HA (type II) would bind more of the PrP released from sc$^+$-MNB cells. This would be the case if the first round of HA beads were saturated with PrP. However, little additional PrP was bound to the second aliquot of HA or removed from the unbound fraction, indicating that most of the unbound PrP remaining from the first round of HA treatment was resistant to HA binding (Fig. 3).

This approximate 1:1 ratio of HA-bound to unbound PrP-sen was consistently observed for the sc$^+$-MNB clone used above; however, more divergent ratios, i.e., from ~30 to 90% bound, were observed for other scrapie-infected and uninfected MNB clones, with no apparent correlation with scrapie infection (data not shown).

Sucrose gradient fractionation of PrP-sen prior to heparin binding. Sedimentation analysis of the PrP in the PI-PLC medium was performed to analyze the aggregation state of the heparin-binding and -nonbinding PrP molecules. PrP in the PI-PLC medium was centrifuged through a sucrose gradient, and the gradient fractions were analyzed for heparin binding. Although the yields of PrP detected in the sucrose gradients were 10 to 20% of that loaded (see Addendum in Proof for an explanation), the PrP that was recovered sedimented as an apparent monomer, i.e., with marker proteins of 20 and 30 kDa rather than those of ≥43 kDa (Fig. 4). The low recovery of PrP from the gradient was not due to interference by sucrose in the immunoprecipitation, because the addition of 20% sucrose to the PI-PLC medium did not affect the PrP-sen signal (Fig. 4A). When the fractions were assayed for HA binding, the ratio of

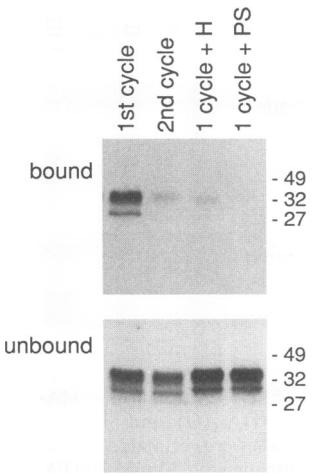

FIG. 3. Effect of multiple cycles of HA treatment and inhibition of binding with pentosan polysulfate. Control (untreated) labeled PI-PLC medium or PI-PLC medium pretreated with excess free heparin (1 mg/ml) (H) or pentosan polysulfate (1 mg/ml) (PS) was incubated with one or two sequential cycles of type II HA beads. The unbound PrP-sen remaining in the PI-PLC medium was compared with that bound to the beads as described in the legend to Fig. 1 and Materials and Methods. The positions of prestained molecular weight markers are designated in kilodaltons on the right.

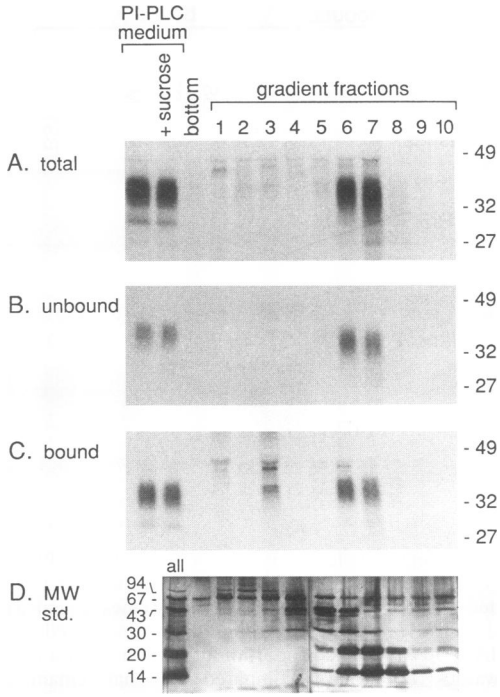

FIG. 4. Sedimentation analysis of PrP-sen prior to HA binding. PI-PLC medium was fractionated on 5 to 20% sucrose gradients, and the fractions were analyzed by direct immunoprecipitation of PrP (A) or by the HA binding assay (B and C) as described in Materials and Methods. Protein molecular weight standards (phosphorylase *b* [M_r = 94,000], bovine serum albumin [67,000], ovalbumin [43,000], carbonic anhydrase [30,000], soybean trypsin inhibitor [20,100], and alpha-lactalbumin [14,400]) were fractionated simultaneously on an identical gradient and analyzed directly by SDS-PAGE and silver staining (D). The numbering of the fractions begins at the dense end of the gradient. The PI-PLC medium lanes contained aliquots of original PI-PLC medium (6% of the total loaded onto the gradients) that were diluted in 0.75 ml of TDSE or TDSE containing 20% sucrose and directly immunoprecipitated (A) or assayed for HA binding (B and C). The PrP bands in the gradient fractions are those comigrating with those prominent in the PI-PLC medium lanes. The positions of protein molecular weight standards are designated in kilodaltons on the right. The difference in the vertical scale of panel D relative to that of panels A to C is due to the fact that different types of gels were used as described in Materials and Methods.

bound to unbound PrP in the apparent monomer band (fractions 6 and 7) was nearly as high as that observed for the original PI-PLC medium (compare Fig. 4B and C). This provided evidence that PrP can bind to heparin as a monomer. The nonbinding PrP population was not resolved from the binding population by sedimentation, indicating that its lack of binding was not due to its prior association with another molecule of sufficient size to detectably increase its sedimentation rate.

Immunoprecipitation of PrP-sen under conditions of heparin binding. As another approach to detecting other proteins that might be associated with PrP-sen and involved in HA binding, we immunoprecipitated PrP from the PI-PLC medium under the conditions used for HA binding, i.e., in PBBS with no added detergent. Figure 5 shows that no other detectable ^{35}S-labeled proteins were specifically immunoprecipitated along with PrP-sen, providing evidence that PrP-sen was not strongly associated with other proteins in the PI-PLC medium prior to HA binding.

Inhibition of PrP-sen binding to HA beads with other inhibitors of protease-resistant PrP accumulation. Since our hypothesis is that sulfated glycans and Congo red inhibit protease-resistant PrP accumulation in scrapie-infected cells by competitively blocking an interaction of PrP with a cellular GAG (3, 8), we tested whether, like free heparin, these compounds could block PrP-sen binding to HA. Pentosan polysulfate, the most potent inhibitor of protease-resistant PrP accumulation (8), blocked PrP-sen binding to HA (Fig. 3). The concentration of free heparin (Sigma grade I) giving half-maximal inhibition of binding to HA (IC_{50}) was ~60 μg/ml (Fig. 6 and 7), and virtually identical results were obtained with Sigma grade II heparin (data not shown). Similar experiments indicated that pentosan polysulfate and Congo red have IC_{50}s of ~6 μg/ml (Fig. 7). These results indicated that pentosan polysulfate and Congo red can block heparin binding by PrP and may thus bind to the heparin binding site. However, chondroitin sulfate, a GAG which is a poor inhibitor of protease-resistant PrP accumulation (8), showed no effect on PrP-sen binding at 100 μg/ml (Fig. 6) and had an IC_{50} of >1 mg/ml (Fig. 7).

Binding of PrP-sen to Congo red-glass beads. To directly test for binding of Congo red to PrP, we incubated PI-PLC medium with glass beads cross-linked to Congo red and compared the bound and unbound fractions (Fig. 8A). The amount of PrP bound to both porous and nonporous Congo red beads was similar to that bound to HA beads. No PrP was bound to blank glass beads, indicating that PrP binding was dependent on the presence of Congo red. Furthermore, the binding to the Congo red beads could be blocked by preincubating the PI-PLC medium with ≥10 μM (7 μg/ml) free Congo red or 1 mg of heparin per ml (Fig. 8B).

Inhibition of protease-resistant PrP accumulation by heparan sulfate. One of the premises of these heparin binding studies is that, in addition to being an inhibitor of protease-resistant PrP accumulation, heparin is likely to be a reasonable functional substitute for heparan sulfate, the closely related

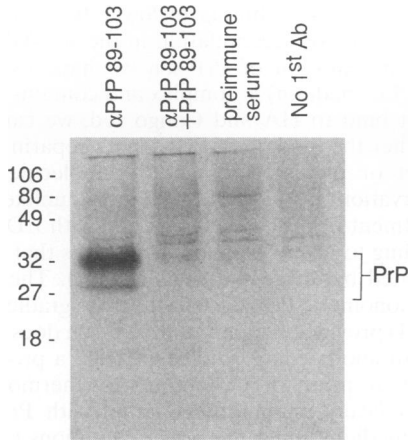

FIG. 5. Immunoprecipitation of PrP-sen under conditions of heparin binding. Aliquots of PI-PLC medium (0.5 ml) prepared from labeled sc$^+$-MNB cells as described in Materials and Methods were incubated for 16 h at 4°C with 2 μl of anti-PrP 89-103 (R27), 2 μl of anti-PrP 89-103 preincubated with 0.2 μg of PrP peptide 89-103, 2 μl of preimmune R27 serum, or no antiserum. The samples were then incubated with protein A-Sepharose beads which were then washed four times with PBS–1 mM EDTA. Bound proteins were eluted by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE–fluorography on 20% acrylamide PhastSystem gels. The positions of prestained molecular weight markers are designated in kilodaltons on the left.

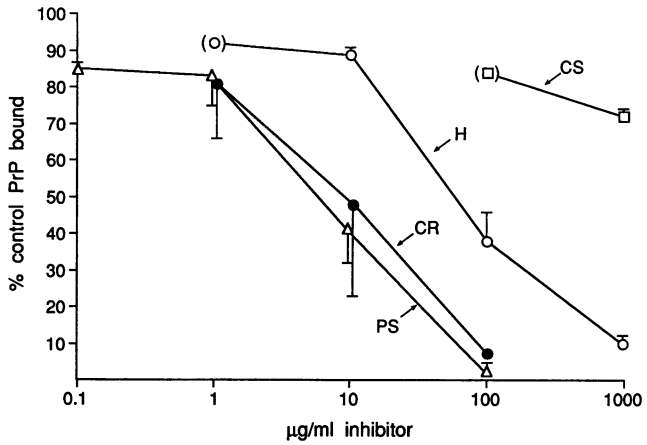

FIG. 7. Inhibition of PrP-sen binding to HA by pentosan polysulfate, Congo red, heparin, and chondroitin sulfate. Labeled PI-PLC medium was pretreated with the designated concentrations of soluble Congo red (CR), pentosan polysulfate (PS), heparin (Sigma grade I) (H), or chondroitin sulfates (CS) prior to the addition of type II HA beads. The PrP-sen levels in the bound fractions were compared by immunoblotting as described in the legend to Figure 2. Laser densitometry of the fluorography films was performed on the PrP bands, and the areas under the optical density traces were quantitated and plotted as a percentage of the control (no inhibitor) value. The datum points represent averages from at least two independent determinations ± standard errors of the means, except for the datum points enclosed in parentheses, which are results from single determinations.

GAG that has been identified in the form of proteoglycan in protease-resistant PrP plaques in vivo. We sought an indication of the validity of this premise by comparing the relative potencies of these GAGs in inhibiting protease-resistant PrP accumulation in sc$^+$-MNB cells by the long-term protease-resistant PrP accumulation assay that has been described in detail elsewhere (8). Briefly, the cells were seeded at low density in the presence of various concentrations of heparan sulfate (fast-moving fraction, Sigma H5393), grown to confluence, and assayed for protease-resistant PrP content by semi-quantitative immunoblotting. The concentration dependence of inhibition by heparan sulfate was similar to that previously

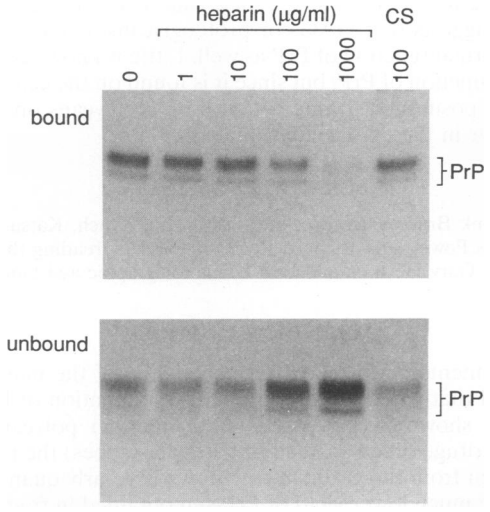

FIG. 6. Inhibition of PrP binding to HA by free heparin. Labeled PI-PLC medium was pretreated with the designated concentrations of soluble heparin or an equimolar mixture of chondroitin sulfates A, B, and C (CS) prior to the addition of type II HA beads. The PrP-sen levels in the bound (top) and unbound (bottom) fractions were compared as described in the legend to Fig. 2.

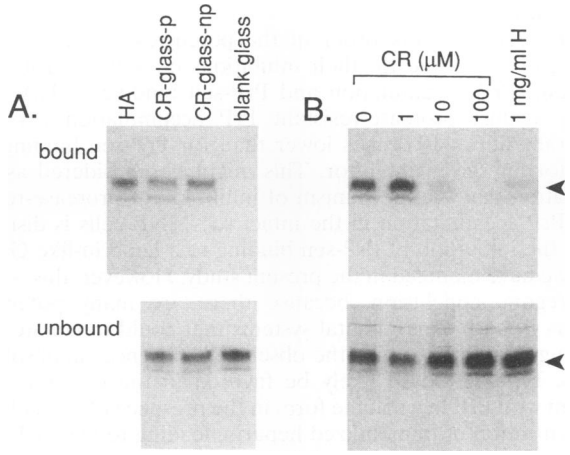

FIG. 8. Binding of PrP to Congo red coupled to glass beads. (A) Labeled PI-PLC medium was incubated with type II HA, porous glass beads coupled with Congo red (CR-glass-p), nonporous glass beads coupled with Congo red (CR-glass-np), or blank porous glass beads. The PrP-sen levels in the bound and unbound fractions were compared as described in the legend to Fig. 1. (B) Labeled PI-PLC medium was pretreated with the designated concentrations of free Congo red prior to incubation with porous Congo red-glass beads. The bound and unbound PrP-sen was analyzed as in panel A. The arrowheads designate the positions of the major PrP-sen band.

observed with heparin (data not shown), giving half-maximal inhibition of protease-resistant PrP accumulation (IC_{50} ± standard deviation) of 55 ± 21 ng/ml as opposed to 100 ± 30 ng/ml for heparin (8). Thus, in terms of inhibiting protease-resistant PrP accumulation, heparin and heparan sulfate are functionally similar.

DISCUSSION

The observation that naturally derived amyloids contain associated sulfated proteoglycans raises the question of what physiological role, if any, GAGs or proteoglycans have in amyloid accumulation (17, 22, 25). Although protease-resistant PrP is not always observed in the form of classic amyloid fibrils, its formation can be viewed as a model of the metabolic stabilization of precursor proteins that must occur in all amyloidoses in order for amyloid to accumulate. Our previous studies showing that GAGs and GAG analogs can potently inhibit protease-resistant PrP accumulation in scrapie-infected cells have provided support for the notion that endogenous GAG-PrP interactions are functionally important in this process (3, 8). A further question is whether, in forming the final GAG–protease-resistant PrP complex, it is PrP-sen or preformed protease-resistant PrP that binds first to the endogenous GAGs or proteoglycans. The results presented here indicate that PrP-sen has the capability to bind to a heparin-like GAG.

Furthermore, we have shown that PrP-sen binds to Congo red as well as to heparin and that the binding to one can be blocked by the other, suggesting that Congo red and heparin bind to the same site. The relative efficacies of Congo red and the sulfated glycans in blocking PrP-sen binding to HA (pentosan polysulfate ≥ Congo red > heparin > chondroitin sulfate) correspond with their previously demonstrated relative potencies in inhibiting protease-resistant PrP accumulation (8). This is consistent with the hypothesis that these inhibitors act by binding to the GAG binding site, thus blocking the linkage of PrP to an endogenous heparin-like GAG or proteoglycan that is necessary for protease-resistant PrP formation or stabilization.

Although the rank order of the potencies of these GAG analogs is the same for their inhibitions of cellular protease-resistant PrP accumulation and PrP-sen binding to HA, the IC_{50}s against protease-resistant PrP accumulation (8) are generally about 10^3 times lower than for PrP-sen binding to HA for any given inhibitor. This might be considered as an indication that the mechanism of inhibition of protease-resistant PrP accumulation in the intact sc^+-MNB cells is distinct from the inhibition of PrP-sen binding to a heparin-like GAG that we have modeled in the present study. However, this is not a foregone conclusion, because there are many potential factors in both experimental systems that could modulate the binding equilibria to give the observed differences in absolute IC_{50}s. Binding would likely be favored in the cell-free HA system with PrP in a soluble form in the presence of a very high concentration of immobilized heparin, leading to higher IC_{50}s for the competitive inhibitors. Other factors, such as (i) a low effective endogenous GAG concentration in the site where the PrP binding reaction occurs, (ii) a lower PrP affinity for the endogenous GAG than for heparin, or (iii) the immobility of membrane-bound PrP, could be unfavorable to the binding of PrP to an endogenous GAG in the MNB cells. Furthermore, the cells may be able to sequester the inhibitors from the medium to create higher local concentrations of inhibitors at the site of PrP-GAG interactions. All these factors would make it easier for exogenous compounds to block PrP binding to the endogenous GAG, contributing to lower IC_{50}s for inhibiting protease-resistant PrP accumulation in the sc^+-MNB cells.

Since the preparation of PrP-sen we have used in these studies (PI-PLC medium) is complex and contains some other proteins that bind to HA and Congo red, we cannot yet be certain whether the binding of PrP-sen to heparin and Congo red is direct or mediated by another molecule. However, several observations indicate that the latter is unlikely. The fact that pretreatment of the PI-PLC medium with EDTA did not prevent binding to HA or Congo red suggests that the binding is not mediated by divalent cations (Fig. 2). The binding of apparently monomeric PrP-sen from sucrose gradient fractions to HA (Fig. 4) provides evidence that PrP-sen does not need to associate with another large molecule (e.g., a protein) in the PI-PLC medium prior to HA binding. Furthermore, no such proteins specifically immunoprecipitated with PrP-sen from the PI-PLC medium under the buffer conditions used for HA and Congo red-glass binding (Fig. 5). However, a PrP-sen-bound protein might not be detected in this immunoprecipitation assay if it were not metabolically labeled efficiently. Alternatively, such a protein might require heparin to potentiate PrP-sen binding. Thus, the ultimate answer to this question of whether PrP-sen binds directly to heparin and Congo red will likely await the availability of purified, native PrP-sen.

It is also not clear whether PrP-sen associates with a GAG or proteoglycan in the living cell. We suspect, however, that the population of PrP-sen molecules that is resistant to HA binding may have cellular GAG bound to the putative GAG binding site. However, since the nonbinding PrP can sediment in sucrose gradients like the HA-binding PrP (Fig. 4), it is likely that if a GAG is bound to the latter, it is too small to detectably alter the sedimentation velocity. As noted above, the relative proportion of the total PrP-sen in the PI-PLC medium that bound to HA was somewhat variable, and this could be due to variable content of endogenous GAG released into the PI-PLC medium along with the PrP-sen. Alternatively, the observed differences in heparin binding by PrP-sen populations could be due to structural heterogeneity in PrP-sen itself.

Although our primary focus has been on the apparent role of PrP-GAG interactions in protease-resistant PrP accumulation in scrapie-infected cells, the fact that PrP-sen can bind to GAGs suggests that GAGs or proteoglycans may be involved in the normal function of PrP as well. Little is known about the normal function of PrP, but since it is found on the cell surface, it is well positioned to interact with proteoglycans on the cell surface or in the extracellular matrix.

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ADDENDUM IN PROOF

Experiments performed after submission of the manuscript indicated that when sucrose gradient fractionation of PrP-sen like that shown in Fig. 4 was performed in polycarbonate ultracentrifuge tubes (instead of Ultraclear tubes) the recovery of PrP-sen from the gradient fractions was nearly quantitative. Thus, the much lower yield of PrP-sen obtained in fractions of the sucrose gradients of Fig. 4 was likely due to the binding of PrP-sen to the Ultraclear centrifuge tubes. The sedimentation velocity of PrP-sen in the gradients in polycarbonate tubes was similar to that shown in Fig. 4, providing evidence that the vast majority of PrP-sen in PI-PLC medium sedimented as monomers rather than oligomers.

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