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Interactions of neural glycosaminoglycans and proteoglycans with protein ligands: assessment of selectivity, heterogeneity and the participation of core proteins in binding

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The method of affinity coelectrophoresis was used to study the binding of nine representative glycosaminoglycan (GAG)-binding proteins, all thought to play roles in nervous system development, to GAGs and proteoglycans isolated from developing rat brain. Binding to heparin and non-neural heparan and chondroitin sulfates was also measured. All nine proteins—laminin-1, fibronectin, thrombospondin-1, NCAM, L1, protease nexin-1, urokinase plasminogen activator, thrombin, and fibroblast growth factor-2—bound brain heparan sulfate less strongly than heparin, but the degree of difference in affinity varied considerably. Protease nexin-1 bound brain heparan sulfate only 1.8-fold less tightly than heparin (K_d values of 35 vs. 20 nM, respectively), whereas NCAM and L1 bound heparin well (K_d ~140 nM) but failed to bind detectably to brain heparan sulfate (K_d > 3 μM). Four proteins bound brain chondroitin sulfate, with affinities equal to or a few fold stronger than the same proteins displayed toward cartilage chondroitin sulfate. Overall, the highest affinities were observed with intact heparan sulfate proteoglycans: laminin-1's affinities for the proteoglycans cerebroglycan (glypican-2), glypican-1 and syndecan-3 were 300- to 1800-fold stronger than its affinity for brain heparan sulfate. In contrast, the affinities of fibroblast growth factor-2 for cerebroglycan and for brain heparan sulfate were similar. Interestingly, partial proteolysis of cerebroglycan resulted in a >400-fold loss of laminin affinity. These data support the views that (1) GAG-binding proteins can be differentially sensitive to variations in GAG structure, and (2) core proteins can have dramatic, ligand-specific influences on protein–proteoglycan interactions.

Key words: cerebroglycan/chondroitin sulfate/extracellular matrix/glypican/heparan sulfate

Introduction

Several types of proteins bind, under physiological conditions, to glycosaminoglycans (GAGs), the polysaccharides that define proteoglycans (PGs). These include a subset of polypeptide growth factors, many secreted proteases and anti-proteases, most proteins of the extracellular matrix, and several cell-surface proteins involved in cell adhesion (Jackson *et al*., 1991; Lander, 1994). Recent studies suggest that the interaction of these proteins with GAGs is critically important in regulating their functions. Examples include a requirement for GAGs in the interactions of fibroblast growth factors (FGFs), hepatocyte growth factor, and wnts with their tyrosine kinase receptors (Rapraeger *et al*., 1991; Yayon *et al*., 1991; Kan *et al*., 1993; Zioncheck *et al*., 1995; Reichsman *et al*., 1996; Forsten *et al*., 1997); a key role for GAGs in controlling the rate of protease inactivation by serine protease inhibitors ("serpins"; Potempa *et al*., 1994); a role for GAGs in tethering molecules to the extracellular matrix (Roberts *et al*., 1988; Moscatelli, 1992; Emerling and Lander, 1997); and an important role for GAGs in some types of cell–cell adhesion (Cole *et al*., 1985, 1986; Reyes *et al*., 1990; Storms *et al*., 1996).

Understanding how GAGs participate in these phenomena has been hindered by the enormous chemical heterogeneity that is possible in GAGs, and the relatively limited number of GAGs that are available in sufficient quantity and purity for biochemical and cell biological studies. Indeed, many GAG-binding proteins have primarily been identified by their ability to bind heparin, a GAG of the heparan sulfate (HS) class that is produced only by mast cells, and that is structurally distinct from other heparan sulfates. In many cases, little is known about the interactions of GAG-binding proteins with the GAGs they actually encounter *in vivo*. Even less is known about how most of these proteins interact with intact PGs, even though GAGs are invariably found as PGs, rather than as free polysaccharides, *in vivo*.

Several years ago, we developed an electrophoretic method for studying protein-GAG interactions that measures binding under equilibrium conditions and requires only trace quantities of GAGs (Lee and Lander, 1991; Lim *et al*., 1991; Herndon and Lander, 1997). The method, known as affinity coelectrophoresis, or ACE, readily detects heterogeneity in binding affinity (San Antonio *et al*., 1993) and can also be used to study the binding of proteins to intact PGs (Sanderson *et al*., 1994). Additionally, previous work using ACE has demonstrated its reliability, yielding K_d values similar to those obtained by other measurements of GAG-protein binding (Lee and Lander, 1991). Here we have exploited ACE to study the interactions of GAG-binding proteins in a single tissue—the developing mammalian brain—with those GAGs and PGs that are expressed in that tissue.

The developing brain was chosen for study because it contains a diverse set of developmentally important secreted, cell-surface and extracellular matrix proteins that bind GAGs and are thought to be regulated by such binding, and because a considerable amount has been learned in recent years about the composition and localization of both GAGs and PGs in the developing brain (for reviews, see Lander, 1993; Lander *et al*., 1996). In particular, the major HSPGs of the developing brain are the integral membrane PGs glypican-1, cerebroglycan (glypican-2), and syndecan-3 (Herndon and Lander, 1990; Carey *et al*., 1992; Lander, 1993; Litwack *et al*., 1994; Stipp *et al*., 1994). All three

are highly expressed by neurons, but with distinct temporal and spatial regulation (Litwack *et al*., 1994; Stipp *et al*., 1994; Carey *et al*., 1997; Ivins *et al*., 1997; Litwack *et al*., 1998). The major chondroitin sulfate (CS) PGs are easily solubilized molecules that appear to be components of the extracellular matrix; these include several members of the aggrecan family (neurocan, brevican, versican) as well as a secreted form of a receptor protein tyrosine phosphatase (phosphacan; LeBaron, 1996; Margolis *et al*., 1996; Schwartz *et al*., 1996; Yamaguchi, 1996).

The GAG-binding proteins chosen for the present study were selected both for their importance in brain development, and for their ability to represent the major classes of GAG-binding proteins: laminin-1, fibronectin, and thrombospondin-1 are major extracellular matrix proteins of the developing brain, and are thought to influence neural cell adhesion, migration, axon growth, and axon guidance (Cohen *et al*., 1986, 1987; Stewart and Pearlman, 1987; O'Shea and Dixit, 1988; O'Shea *et al*., 1990; Sheppard *et al*., 1991; Hynes and Lander, 1992; Powell and Kleinman, 1997). NCAM and L1 are abundant cell–cell adhesion molecules of the brain (reviewed in Linnemann and Bock, 1989; Walsh and Doherty, 1997) and null mutations in either one disrupt brain development (Cremer *et al*., 1994; Bateman *et al*., 1996). The serpin protease nexin-1 (PN-1), and the serine proteases thrombin and uPA are thought to influence both axonal growth and neural cell survival (Farmer *et al*., 1990; Dihanich *et al*., 1991; Sumi *et al*., 1992; Dent *et al*., 1993; Mansuy *et al*., 1993; Vaughan *et al*., 1995). The secreted growth factor FGF-2 is thought to control processes as diverse as neural precursor proliferation (DeHamer *et al*., 1994; Palmer *et al*., 1995) and axon targeting in the visual system (McFarlane *et al*., 1996).

In the present study, the binding of these proteins to HS and CS from the developing brain was compared with their binding to heparin and to HS and CS from non-neural sources. In some cases, binding to the HSPGs glypican-1, cerebroglycan, and syndecan-3 was also tested. In all cases, both affinity and selectivity (ability to fractionate GAGs into subpopulations of different affinity) were evaluated. Among the results obtained, it was observed that the range of protein affinities for brain HS was substantially broader than the range of affinities for heparin. The most striking example of this difference involved NCAM and L1, which showed strong binding to heparin, but undetectable binding to brain HS. It was also found that proteins known to fractionate heparin into subpopulations of different affinities generally failed to fractionate brain GAGs into similar subpopulations, or did so to a much smaller degree. Apparently, the potential exists for variations in HS structure to give rise to substantial specificity in GAG-protein interactions, but little evidence was found that this potential is exploited to a large degree in the developing brain. In contrast, analysis of the binding properties of intact PGs provided strong evidence that PG core proteins can greatly influence affinity for some, but not all, GAG-binding proteins. This suggests that some of the specificity in PG–protein interactions *in vivo* may be achieved through an influence, direct or indirect, of core proteins on binding.

Results

Extracellular matrix and secreted heparin-binding proteins expressed in the brain exhibit a broad range of affinities for brain-derived HS and CS

Metabolically labeled HS was prepared from embryonic day 18 (E18) and postnatal day 0 (P0) rat brain that had been dissected, cut into small pieces and cultured for 16–20 h in the presence of $^{35}SO_4$. A membrane fraction was prepared, detergent solubilized, and subjected to DEAE chromatography to isolate PGs. HS chains were prepared by chondroitinase ABC treatment, followed by alkaline-borohydride cleavage and ethanol precipitation.

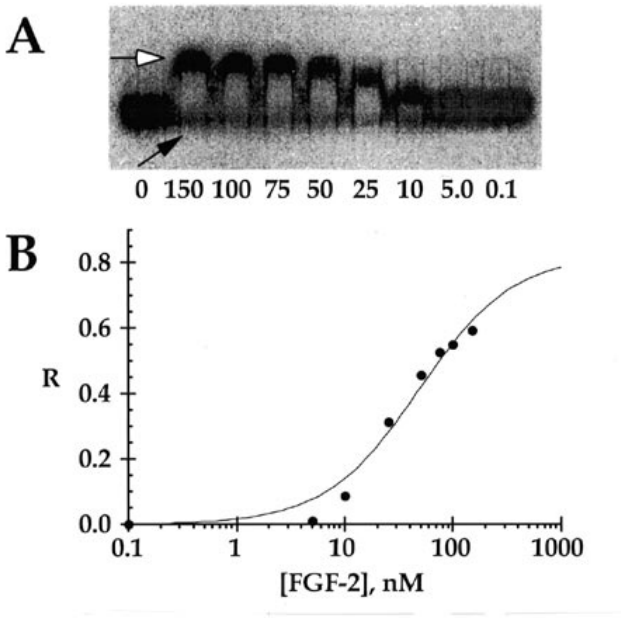

Fig. 1. Affinity coelectrophoresis of P0 brain HS and FGF-2. Metabolically labeled HS purified from a preparation of membrane-associated proteoglycans from P0 rat brain was subjected to electrophoresis through zones containing the indicated concentrations (nM) of FGF-2. (**A**) Electrophoretogram of dried ACE gel. The direction of electrophoresis was from top to bottom in this and all figures. The species that is progressively shifted with increasing FGF-2 concentration is HS (open arrow); the faint band across the bottom of the pattern is a small amount of incompletely digested CS (solid arrow), which shows no binding to FGF-2. (**B**) Calculation of affinity of HS for FGF-2. Retardation coefficients *(R)* were determined at each FGF-2 concentration. The curve was fit to the equation $R = R_\infty/[1 + (K_{d,app}/[\text{FGF-2}_{tot}])]$, and yields an apparent K_d of 47 nM (see *Materials and methods*).

Binding of brain HS to a variety of different proteins—representing secreted and extracellular matrix proteins that are known to bind heparin and are thought to play important roles in brain development—was measured using affinity coelectrophoresis (ACE). Figure 1 shows an electrophoretogram in which the binding of P0 HS to FGF-2 is visualized (panel A). The faint band running at the bottom of the gel is incompletely digested CS, as demonstrated by the loss of this band when the HS preparation was more extensively digested with chondroitinase ABC (data not shown). Measurements of electrophoretic retardation in each of the FGF-2 lanes implied an apparent dissociation constant (K_d) of 47 nM (units of FGF-2 concentration; panel B).

Similar analyses were done for the extracellular matrix proteins laminin-1, fibronectin, and thrombospondin-1, for the secreted proteases thrombin and urokinase plasminogen activator (uPA), and for the secreted serine protease inhibitor protease nexin-1 (PN-1). Table I shows the apparent dissociation constants obtained using P0 HS. Values obtained using E18 HS were not significantly different from those shown (see Table III, below). Also shown are dissociation constants that were measured for the binding of low molecular weight porcine intestinal mucosa heparin to these proteins.

Table I. Binding of extracellular matrix and secreted molecules to neonatal rat brain heparan sulfate and heparin

Ligand	Type of molecule	K_d, brain HS	K_d, heparin	(K_d, brain HS)/(K_d, heparin)
Protease nexin-1	Serpin	35 nM	20 nM[c]	1.8
FGF-2	Growth factor	47 nM	9 nM[a]	5.2
Thrombospondin-1	Extracellular matrix glycoprotein	180 nM	41 nM[a]	4.4
Laminin	Extracellular matrix glycoprotein	891 nM	147 nM	6.1
Thrombin	Serine protease	1025 nM	123 nM[b]	8.3
uPA	Serine protease	2310 nM	312 nM[a]	7.4
Fibronectin	Extracellular matrix glycoprotein	6200 nM	486 nM[b]	12.8

Brain HS was metabolically labeled with $^{35}SO_4$ prior to isolation. HS tested for binding to thrombin and uPA was derived from the soluble fraction; other HS samples were derived from the membrane fraction. Heparin was from porcine intestinal mucosa, end-labeled with tyramine, labeled with ^{125}I, and fractioned to yield a low molecular weight fraction. Binding measurements were made by affinity coelectrophoresis, as described. K_d values are from single determinations except where noted.
[a,b,c]K_d value is mean of two, three, or five determinations, respectively.

Table II. Binding of extracellular matrix and secreted molecules to neonatal rat brain chondroitin sulfate and to cartilage chondroitin sulfate

Ligand	Type of molecule	K_d, brain CS	K_d, cartilage CS	(K_d, brain CS)/(K_d, cartilage CS)
Protease nexin-1	Serpin	158 nM	478 nM	0.33
Thrombospondin-1	Extracellular matrix glycoprotein	235 nM	487 nM	0.48
Thrombin	Serine protease	2.5 μM	10.3 μM	0.24
uPA	Serine protease	21.9 μM	17.1 μM	1.28

Brain CS was metabolically labeled with $^{35}SO_4$ prior to isolation. Brain CS tested for binding to thrombin and uPA was derived from the soluble fraction; brain CS tested for binding to PN-1 and thrombospondin-1 was from the membrane fraction. Cartilage CS was from bovine trachea (predominantly chondroitin-4-sulfate) and was end labeled with hydroxyphenylpropionic acid, prior to radioiodination. Binding measurements were made by affinity coelectrophoresis, as described. K_d values are from single determinations except for PN-1 binding to cartilage CS (mean of four determinations).

Table III. Comparison of dissociation constants determined for multiple GAGs

Ligand	Heparin porcine intestine	HS E18 brain membrane fraction	HS neonatal brain membrane fraction	HS neonatal brain soluble fraction	HS bovine kidney	CS E18 brain membrane fraction	CS neonatal brain membrane fraction	CS neonatal brain soluble fraction	CS bovine cartilage (Ch-4-S)	CS shark cartilage (Ch-6-S)
Protease nexin-1	20[d]	68[a]	35	101	62	223[a]	158	239	478[c]	425
uPA	312[a]	2790	n.d.	2310	n.d.	16,400	n.d.	21900	17,100	n.d.
Thrombin	123[b]	582	n.d.	1025	1710	4410	n.d.	2500	10,300	n.d.
Antithrombin III	16[e]	>10,000	n.d.	n.d.	n.d.	>10,000	n.d.	n.d.	n.d.	n.d.
FGF-2	16[a]	n.d.	47	n.d.	170	n.d.	>1350	n.d.	>1350	n.d.
Fibronectin	486[b]	7400	6200	n.d.	34,400	n.d.	n.d.	n.d.	>17,900	>12,700
Laminin	147	886	891	n.d.	790	>977	>977	n.d.	>1100	>1100
Thrombospondin	41[a]	180	180	n.d.	262	235	235	n.d.	487	648

ACE was carried out and values of K_d measured as described for Tables I and II. Values are given in nM and assume first order binding (n.d., not determined). "Soluble fraction" refers to PGs recovered in soluble form after brain homogenization in isotonic sucrose and clarification by ultracentrifugation (Herndon and Lander, 1990). Where data are reported as greater than a given value, it indicates that no electrophoretic retardation was observed at any protein concentration, and an estimate of the minimum value of K_d was made based on the assumption that the protein concentration $[P]_{tot}$ required for just detectable electrophoretic retardation of HS or CS should occur at a value of $[P]_{tot}/K_d$ that exceeds the highest value of $[P]_{tot}/K_d$ at which no retardation was observed when heparin was tested under that same conditions (typically about 0.2). Thus, minimum values are typically ~5 times the highest ligand concentration tested. K_d values are from single determinations except where noted.
[a,b,c,d]K_d value is mean of two, three, four, or five determinations, respectively.
[e]The value for heparin binding to antithrombin III is that reported by Lee and Lander (1991) for the high affinity fraction of heparin.

Although the order of ligand affinities was nearly identical for both brain HS and heparin, heparin binding was stronger in every case. The factor by which heparin affinity exceeded HS affinity (i.e., K_d(HS)/K_d(heparin)) was not constant, but varied between 1.8 and 13, depending on the ligand. This implies that there must be differences among these proteins in their preferences for particular oligosaccharide structures found within GAGs of the HS/heparin class.

In addition to being tested for binding to labeled brain HS, the protein ligands described above were also tested for binding to the total metabolically labeled GAG population that had been isolated from the brain. Figure 2A shows the results obtained with E18 brain GAGs and PN-1. In this case, two populations of GAGs with different apparent affinities were clearly resolved by the electrophoretogram (arrows). Pretreatment with either chondroitinase ABC (to degrade CS) or heparitinase (to degrade HS) showed that the

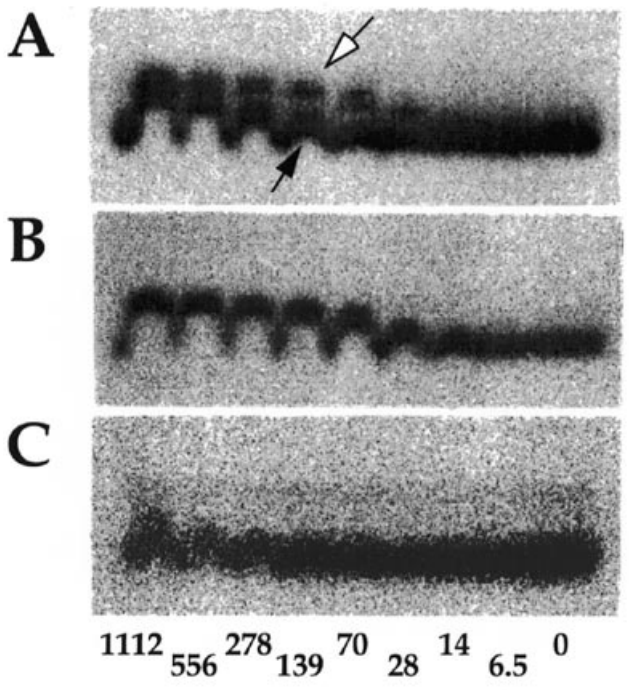

Fig. 2. Binding of brain GAGs to protease nexin-1. Multiple ACE gels were cast with lanes containing PN-1 at the indicated concentrations (nM). Electrophoresis was then carried out with total GAGs from metabolically labeled E18 rat brain membrane-associated PGs (**A**), purified HS (**B**), or purified CS (**C**), the latter two being derived from the GAG preparation used in (**A**). Gels were dried and autoradiographed. HS and CS produce distinct mobility shift patterns, which can be distinguished even in (**A**). An open arrow marks the HS, while a solid arrow marks the CS.

tighter binding species was HS (K_d ~ 72 nM), whereas the weaker was CS (K_d ~ 287 nM; Figure 2B,C). By similar experiments, uPA, thrombin, and thrombospondin-1, but not FGF-2, were also found to bind to brain CS. Table II shows the apparent dissociation constants for CS from P0 brain. Values obtained with CS from E18 brain were not significantly different (Table III). For comparison, measurements were also made using CS from bovine tracheal cartilage (Table II) and shark cartilage (Table III).

As with brain HS, the range of affinities exhibited by heparin-binding proteins for brain CS was broad (139-fold). Thrombospondin-1, PN-1, and thrombin each bound brain CS with a somewhat higher affinity than cartilage CS (2.1-, 3.0-, and 4.2-fold higher, respectively), while uPA bound both GAGs to a similar extent (Table II). These differences most likely reflect structural differences between brain and cartilage CS, and imply that these proteins can be selective, to some degree, for structural variations within GAGs of the chondroitin sulfate class.

The cell adhesion molecules NCAM and L1 bind well to heparin, but poorly, if at all, to brain HS

The developmentally important neural cell adhesion molecule NCAM is known to bind heparin, and several studies have argued that interactions of NCAM with heparin-like molecules on neural cell surfaces (i.e., HS) are important for biological activity (Cole *et al*., 1985, 1986; Cole and Glaser, 1986; Cole and Burg, 1989; Kallapur and Akeson, 1992; Storms *et al*., 1996). When we tested the interactions of E18 and P0 brain membrane HS with postnatal mouse NCAM, however, no evidence for binding was obtained (data not shown). Because many of the studies that have implicated GAGs in NCAM function have focused on retinal, rather than brain, cell adhesion, we also tested HS preparations from E18 whole retina for binding to NCAM. Again, no measurable binding was seen (Figure 3A). In contrast, the same retinal HS preparation bound PN-1 with a K_d close to that of brain HS (K_d = 75 nM; data not shown).

As a control, heparin was tested for binding to identical NCAM preparations (Figure 3B), and a relatively strong affinity (K_d = 126 nM) was measured. This value is reasonably close to a previously reported value obtained using heparin-agarose binding (K_d = 52 nM; Nybroe *et al*., 1989). Interestingly, the ACE pattern indicated that not all heparin molecules bind to NCAM: a significant fraction of the heparin (~50% of total; data not shown) exhibited the same migration as unbound heparin, at all tested NCAM concentrations (arrow in Figure 3B). Thus, approximately half of heparin molecules exhibit little or no binding to NCAM.

At embryonic and early postnatal ages, a significant fraction of NCAM molecules are substituted with large amounts of α2,8-polysialic acid (PSA; Finne *et al*., 1983). This large N-linked glycan is thought to interfere sterically with the binding interactions of NCAM, as well as with the binding interactions of other cell surface receptors localized to the same plasma membrane as NCAM (Rutishauser, 1988, 1992; Zhang *et al*., 1992). Since PSA is polyanionic, PSA moieties on NCAM might be expected to electrostatically hinder the binding of NCAM to GAGs, perhaps explaining our failure to detect NCAM-HS binding. To test this possibility, we also examined the binding of heparin and HS to the adult form of NCAM, which has little PSA; to postnatal NCAM that had been treated with endoneuraminidase N (endo-N) to remove PSA; and to endo-N treated adult NCAM. All three NCAM forms demonstrated apparent K_d values for heparin that were not significantly different from those seen with untreated postnatal NCAM, yet these forms continued to show no detectable binding to brain HS (Table IV). Additional experiments (not shown) also showed that PSA itself (as colominic acid, the form in which it is produced by bacteria), does not bind detectably to NCAM (K_d > 2240 nM), suggesting that PSA on NCAM does not compete for binding of GAGs to NCAM's heparin binding site.

Interestingly, another cell adhesion molecule, L1, behaved quite similarly to NCAM in its interactions both with heparin and brain HS. L1 is structurally related to NCAM, is abundant in the developing nervous system, and appears to mediate both homophilic and heterophilic cell adhesion (reviewed in Linnemann and Bock, 1989). Unlike NCAM, L1 molecules do not bear PSA chains and binding to heparin has not previously been shown for L1. When membrane-associated HS from both E18 and P0 brain were tested for binding to L1, no binding was seen (Table IV). However, L1, like NCAM, bound heparin quite well (K_d = 108 nM; Figure 3C). As with NCAM, not all heparin molecules bound to L1: a nonbinding heparin subpopulation, representing ~50% of total heparin, was readily detected (arrow in Figure 3C).

Subpopulations of brain HSPGs can bear HS chains with moderately different affinities

ACE is well suited for detecting heterogeneity in binding affinity within populations of GAGs, since such heterogeneity manifests itself on electrophoretograms as smearing or splitting of bands, particularly in lanes with ligand concentrations near the (average) value of K_d (San Antonio *et al*., 1993, 1994). In some cases, such as those shown in Figure 3B,C, individual binding and nonbinding populations may be clearly seen; in other cases only a continuum of molecules with differing affinities is observed.

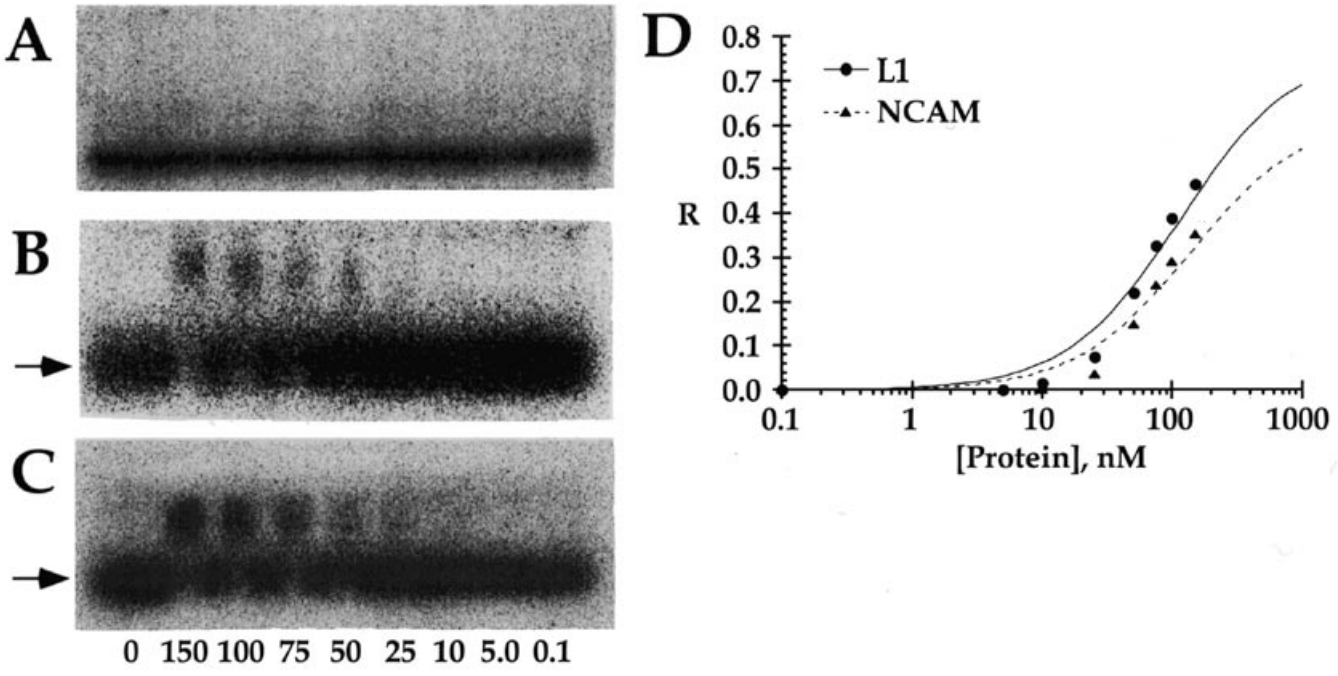

Fig. 3. Affinity coelectrophoresis of the cell adhesion molecules NCAM and L1. ACE gels were prepared with lanes containing NCAM or L1 at the indicated concentrations (in nM). In (**A**), E18 retina-derived $^{35}SO_4$-labeled HS was subjected to electrophoresis through lanes containing early postnatal brain-derived NCAM. In (**B**), ^{125}I-labeled low molecular weight heparin was subjected to electrophoresis through the same NCAM samples. In (**C**), ^{125}I-labeled low molecular weight heparin was subjected to electrophoresis through lanes containing L1. No evidence of retardation of HS mobility by NCAM is detected in (**A**), whereas progressive retardation of mobility is seen with both NCAM and L1, when heparin was used (**B, C**). Arrows in (**B**) and (**C**) point out heparin subpopulations that fail to bind NCAM and L1 at the highest protein concentrations tested. (**D**) Retardation coefficients were determined for the bound heparin populations in (**B**) and (**C**), and were plotted against protein concentration. The curves imply apparent average K_d values of 126 nM and 108 nM for heparin binding to NCAM and L1, respectively (see *Materials and methods*).

Table IV. Binding of NCAM and L1 to heparin and neonatal rat brain membrane-associated heparan sulfate

Ligand	Treatment	K_d heparin	K_d brain HS
Postnatal NCAM (PSA^+)	Untreated	126 nM	>3.15 μM
Postnatal NCAM (PSA^+)	Endo-N treated	88 nM	>4.68 μM
Adult NCAM (PSA^-)	Untreated	104 nM	>5.25 μM
Adult NCAM (PSA^-)	Endo-N treated	119 nM	>5.28 μM
L1	Untreated	108 nM	> 2.75 μM

Heparin and HS were labeled as in Table I, and binding to NCAM and L1 tested by ACE. Where indicated, NCAM samples had been pretreated with endoneuraminidase-N (endo-N) to remove polysialic acid (PSA). Heparin affinities were calculated assuming first order binding (n = 1, in the equation given in *Materials and methods*). In some cases, curves based on second order binding (n = 2) also fit the data well, but values of K_d calculated from those curves differed by less than 30% from those calculated for n=1, and are not shown here. Values of K_d for HS are presented as minimum values, since no electrophoretic retardation was detected over the full range of protein concentrations that were tested. Minimum values were calculated as in Table III. K_d values are from single determinations except for untreated postnatal NCAM binding to heparin (mean of three determinations).

It is interesting that, in the experiments in which we measured the binding of brain HS and CS individually to secreted and extracellular matrix proteins, no such smearing or splitting was seen in any case (Figure 1–2 and data not shown). This contrasts with earlier observations that two of the proteins tested here, laminin and fibronectin, cause marked smearing of heparin, fractionating it into subpopulations that vary more than 10-fold in affinity (San Antonio *et al*., 1993). The data suggest that, if brain GAGs are heterogeneous in their binding properties, either the degree of heterogeneity is relatively small, or the populations that exhibit unusually high or low affinities are relatively rare.

As an alternative approach for looking for subsets of brain GAGs with different protein affinities, we used affinity chromatography to prefractionate brain PGs prior to isolating GAGs from them. PN-1 was chosen for this experiment because it binds well to brain HS, and because there is some evidence that it can fractionate heparin (Rovelli *et al*., 1992), suggesting that it might be able to recognize distinct HS subpopulations. In addition,

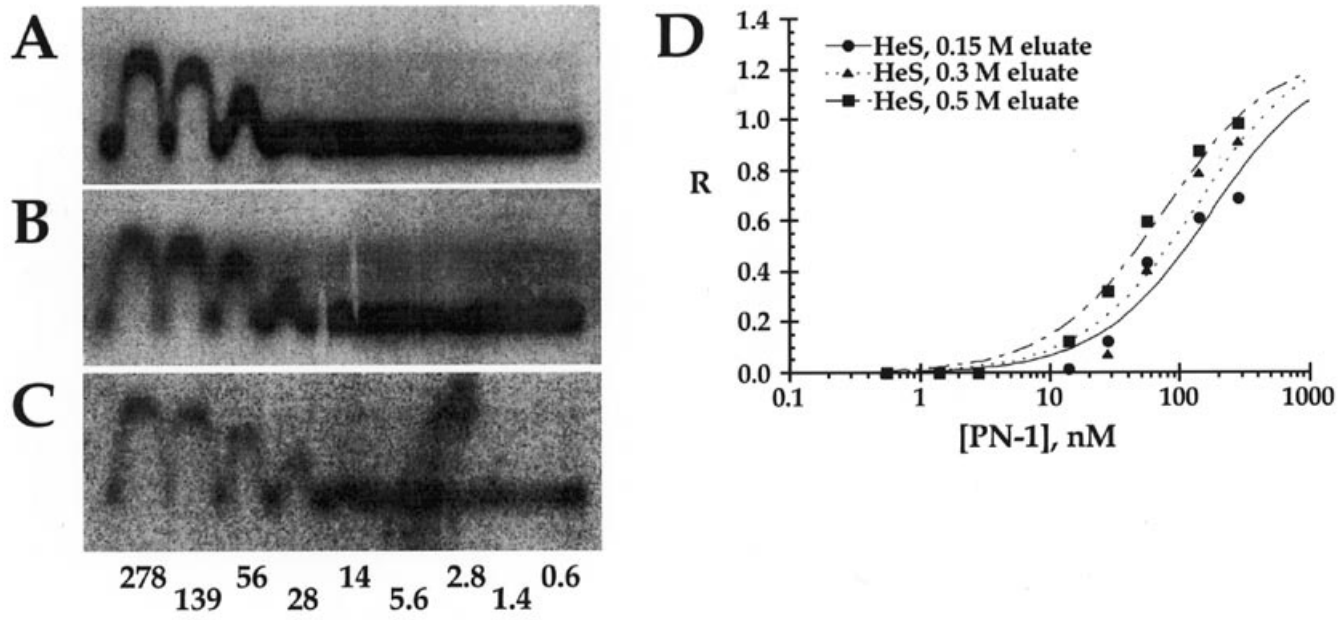

Fig. 4. Binding to protease nexin-1 (PN-1) of HS chains derived from PN-1-binding proteoglycans. Metabolically labeled PGs derived from E18 forebrain were fractionated by affinity chromatography on immobilized PN-1, and eluted with various salt concentration. HS was derived from each of the fractions, and subjected to electrophoresis in ACE gels containing PN-1 at the indicated concentrations (nM). Autoradiograms of the mobility shift patterns obtained with HS isolated from three different salt eluates of the PN-1 column are shown: (**A**) 0.15 M (column flow through), (**B**) 0.3 M, and (**C**) 0.5 M NaCl. (**D**) Retardation coefficients were determined and plotted against PN-1 concentration for each gel. The curves imply apparent K_d values of 162, 114, and 71 nM in the 0.15 M, 0.3 M, and 0.5 M eluates, respectively.

PN-1 is related to antithrombin III, a protein that has so far shown the greatest ability to distinguish among heparin/HS subspecies (Lam *et al*., 1976; Jacobsson *et al*., 1986).

$^{35}SO_4$- and ^{125}I-labeled E18 brain PGs were adsorbed (in parallel experiments) to a PN-1-agarose column in 0.15 M NaCl, and eluted using salt steps of 0.3 M, 0.5 M, 1 M, 1.5 M, and 3 M NaCl. In both cases, ~80% of the radioactivity passed through column, with ~16% eluting at 0.3 M salt, ~3.5% at 0.5 M, and ~1.3% at 1 M (data not shown). When ^{125}I-labeled PGs were used, GAG lyase digestion and SDS–PAGE analysis showed that the PG species in the 0.3 M, 0.5 M, and 1 M fractions were the same, and consisted mainly of glypican-1 (Litwack *et al*., 1994) and cerebroglycan (Stipp *et al*., 1994), the major glycosylphosphatidylinositol-anchored HSPGs of the developing brain (Herndon and Lander, 1990; data not shown). Interestingly, the abundant HSPG syndecan-3 was found mainly in the PN-1 column flow through (0.15 M salt; data not shown).

Using alkaline elimination and chondroitinase digestion, isolated HS chains were obtained from the $^{35}SO_4$-labeled PGs that were recovered in the unbound and eluted fractions, and these chains were tested for binding to PN-1 by ACE. The results, shown in Figure 4, indicate that the HS in the unbound fraction binds PN-1 with a somewhat lower affinity than HS in the 0.3 M eluate, which in turn is not as high as the affinity of HS from the 0.5 M eluate (K_d values were 162, 114, and 71 nM, respectively; Figure 4D). Insufficient material was present in the 1 M eluate to permit testing. Overall, the data indicate that some intrinsic heterogeneity in binding properties does exist among HS chains in the developing brain, but that large differences are not common, at least not when considering binding to the molecules studied here.

Brain HSPGs exhibit an unexpectedly high affinity for laminin-1

In vivo, molecules typically interact with PGs, not isolated GAGs. It is conceivable that, in some cases, the core proteins of those PGs influence the strength or specificity of binding. Indeed, several PG cores are known to bind ligands directly, i.e., independent of the presence of GAG chains (Vogel *et al*., 1984; Andres *et al*., 1989; Heremans *et al*., 1990; Hayashi *et al*., 1992; LeBaron *et al*., 1992; Doege, 1993; Schonherr *et al*., 1995; Aspberg *et al*., 1997; Milev *et al*., 1998). It was therefore decided to measure some of the binding properties of brain HSPGs directly, using ACE. In the developing nervous system, the major HSPGs are glypican-1, cerebroglycan and syndecan-3 (Herndon and Lander, 1990; Stipp *et al*., 1994; Gould *et al*., 1995). Each was immunopurified from PG preparations that had been isolated either from embryonic brain membranes or from embryonic brain growth cone particles (a fraction in which they are enriched; Ivins *et al*., 1997), and radiolabeled with ^{125}I (see *Materials and methods*). Because of the large size and lower overall charge densities of HSPGs, when compared with HS, some measurements of protein affinity could not be made. For example, NCAM and HSPGs exhibited nearly identical electrophoretic mobilities, making it very difficult to use electrophoretic retardation to quantify binding.

One molecule for which ACE analysis worked well was laminin-1. As shown in Figure 5A, binding of ^{125}I-cerebroglycan to laminin-1 was readily visualized as a series of shifts in electrophoretic mobility with increasing laminin concentration. Unlike what had been seen with brain HS, substantial binding heterogeneity was apparent, as evidenced by smearing of the

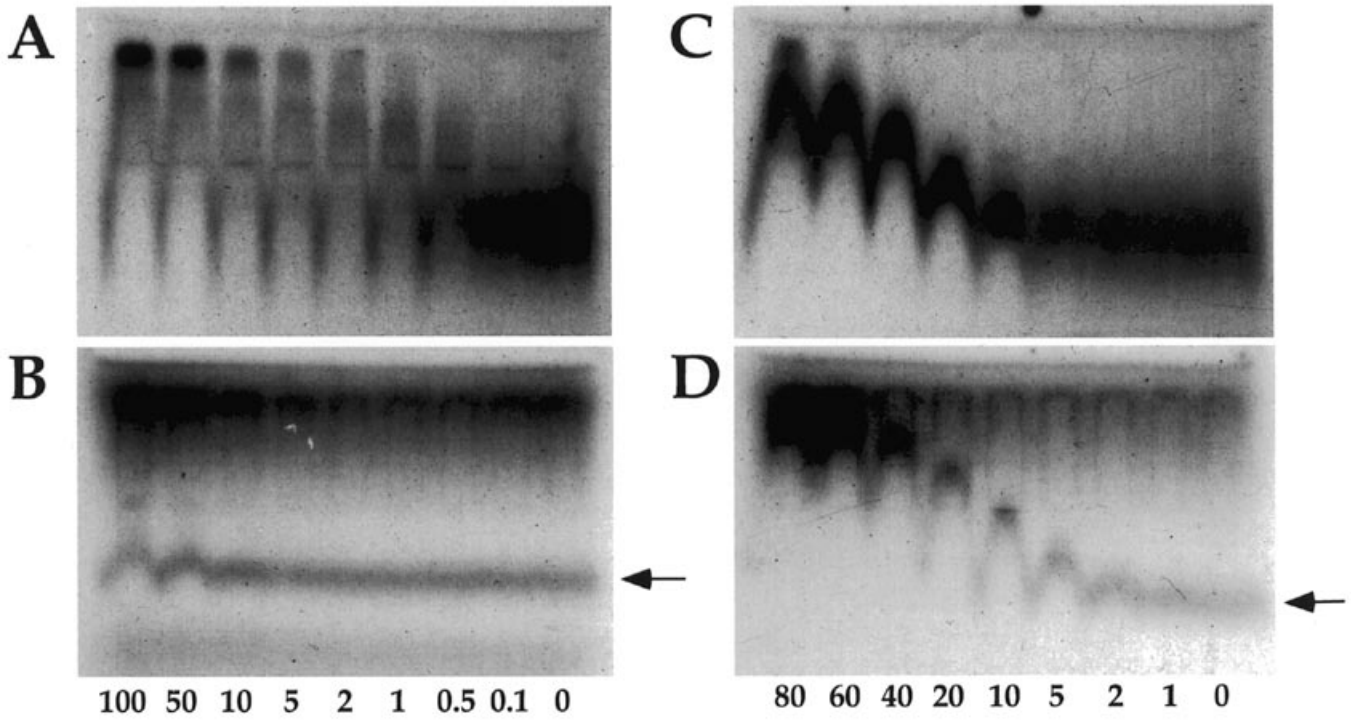

Fig. 5. Binding of cerebroglycan and trypsin-digested cerebroglycan to laminin-1 and FGF-2. Immunopurified ^{125}I-labeled cerebroglycan from embryonic day 16 rat brain was digested exhaustively with trypsin as described in *Materials and methods*. Intact cerebroglycan (**A** and **C**) and trypsin-treated cerebroglycan (**B** and **D**) were tested for binding to laminin-1 (**A** and **B**) and FGF-2 (**C** and **D**) using affinity coelectrophoresis. Concentrations of laminin-1 and FGF-2 are given in nM. Like heparin and HS, intact cerebroglycan migrates with a rapid intrinsic mobility, but is progressively retarded by increasing concentrations of laminin-1 or FGF-2. Trypsin-treated cerebroglycan consists of two species, one that shows little mobility and presumably represents polypeptide fragments that contain no GAG, and another that exhibits a rapid intrinsic mobility, consistent with the presence of GAG (arrows in **B** and **D**). The latter species behaves as a homogenous population whose mobility is only shifted by concentrations of laminin-1 much higher than are needed to shift intact cerebroglycan (**A** vs. **B**). In contrast, similar patterns of retardation are seen when binding to FGF-2 is tested instead (**C** vs. **D**). The data imply that trypsin-digestion greatly reduces the affinity of cerebroglycan for laminin-1, but not for FGF-2. Calculated values of K_d are given in Table V.

migrating front in many of the lanes. Overall, analysis of the average behavior of the labeled cerebroglycan molecules implied an apparent dissociation constant of ~0.5 nM. When analogous experiments were carried out using ^{125}I-labeled glypican-1 and syndecan-3, values of K_d of 2.2 nM and 2.7 nM, respectively, were obtained (Table V). These data imply that all three of these PGs bind to laminin-1 with much higher affinity than brain HS (~890 nM) or even heparin (~150 nM).

Because these PGs had been labeled on their core proteins (with ^{125}I), it was not possible to study the binding of their HS chains after chemical release of those chains. However, because the GAG chains of glypican-1 and cerebroglycan are all attached at a single serine-glycine dipeptide cluster on the core protein (Mertens *et al*., 1996; S. E. Paine-Saunders and A. D. Lander, unpublished observations), it should be possible to obtain proteolytic fragments of these PGs that contain all of the GAG chains attached to a short peptide segment. Since the amino acid sequence of cerebroglycan shows the presence of a tyrosine only five residues C-terminal to the GAG attachment site, it seemed likely that proteolytic digestion of ^{125}I-labeled cerebroglycan could produce a fragment that not only contained all of the HS, but also contained radiolabel. Indeed, autoradiograms of SDS–PAGE gels of trypsin-digested ^{125}I-cerebroglycan showed the presence of numerous, low molecular weight bands and a single broad smear at an apparent molecular size of ~40 kDa. Following heparitinase digestion, the smear resolved to a single labeled band of 7.5 kDa (data not shown). Thus, trypsin digestion apparently produces a single HS-bearing fragment of cerebroglycan which contains $\leq$7.5 kDa of polypeptide.

When the same trypsin digest of ^{125}I-cerebroglycan was tested for binding to laminin-1 using ACE, the pattern shown in Figure 5B was obtained. As expected, much of the radiolabeled material had low electrophoretic mobility (broad smear near the top of the gel in all lanes), consistent with the expected behavior of short peptides no longer attached to GAG. However, one distinct, homogeneous species exhibited a rapid mobility, typical of brain HS (arrow). This species showed mobility retardation in the presence of laminin-1, but only at relatively high laminin concentrations. The data are consistent with an apparent dissociation constant of ~215 nM (Table V), a value in between those previously observed for the binding of heparin and isolated brain HS to laminin-1. Thus, tryptic digestion lowers the apparent affinity of cerebroglycan for laminin-1 by more than two orders of magnitude. In other experiments, reduction and alkylation of the disulfide bonds in cerebroglycan also lowered affinity for laminin, in this case by 15- to 150-fold (data not shown), suggesting that core protein tertiary structure is also required for high affinity binding.

Table V. Binding of neuronal cell-surface HSPGs to laminin-1, FGF-2, and thrombospondin-1

	LN-1		FGF-2		TSP-1	
PG or GAG	K_d (nM)	$(K_d,HS)/(K_d)$	K_d (nM)	$(K_d,HS)/(K_d)$	K_d (nM)	$(K_d,HS)/(K_d)$
Cerebroglycan, E16 rat brain membrane fraction	0.49[b]	1818	50	0.9	33	5.5
Cerebroglycan, neonatal rat brain growth cone particles	0.7[a]	1273	n.d	—	n.d	—
Trypsin-digested cerebroglycan, E16 rat brain membrane fraction	215	4.1	20	2.4	n.d	—
Glypican, neonatal rat brain growth cone particles	2.2	405	n.d	—	n.d	—
Syndecan-3, neonatal rat brain growth cone particles	2.7	330	n.d	—	48	3.8

Cerebroglycan, glypican, and syndecan-3 were isolated and labeled, and cerebroglycan samples subjected to trypsin digestion, as described (see *Materials and methods*). Binding was tested by ACE (see Figure 5), and values of K_d calculated assuming first order binding (n.d., not determined). Also shown for each protein is the ratio of the value of K_d obtained with P0 brain HS to the value obtained with the PGs shown here. Note that intact PGs show binding to laminin-1 that is 300- to 1800-fold stronger than HS. K_d values are from single determinations except where noted.
[a,b]K_d value is mean of two or three determinations, respectively.

The influence of HSPG core proteins on affinity is ligand-specific

The trypsin-sensitive, high affinity interaction between laminin-1 and brain HSPGs suggests that the presence of a core protein can substantially enhance binding between PGs and heparin-binding molecules. Interestingly, this enhancement does not apply to all PG ligands. As shown in Figure 5C,D and summarized in Table V, the binding of ^{125}I-cerebroglycan to FGF-2 was not substantially different from the binding of isolated brain HS to FGF-2, and was not affected by trypsin digestion of the PG. Table V also presents data on the binding of ^{125}I-cerebroglycan and ^{125}I-syndecan-3 to thrombospondin-1. In this case, both PGs did bind with apparent affinities higher than had been seen with isolated HS, but only modestly so (4- to 5-fold higher).

Laminin-1 is a very large, multidomain glycoprotein, and is thought to possess multiple GAG-binding sites. The major sites of GAG binding are thought to reside in the large globule, or "G domain," at the C-terminus of the α1 chain (Ott *et al*., 1982; Skubitz *et al*., 1991; Sung *et al*., 1993). Recently, the G domain has been expressed in a functionally active form as a recombinant protein in insect cells (Yurchenco *et al*., 1993). Interestingly, ACE analysis revealed that the apparent dissociation constant of ^{125}I-cerebroglycan for this recombinant protein was ~29 nM (data not shown). This is 40- to 60-fold weaker than the binding of cerebroglycan to intact laminin, suggesting that structural elements in laminin-1 outside of the G domain contribute to high affinity binding.

Discussion

Protein affinities for neural GAGs

The present study focuses on nine GAG-binding proteins that are thought to play important roles in neural development, and their binding under physiological conditions of pH and ionic strength to the major GAGs of the developing nervous system. At the concentrations tested, seven of these proteins bound brain HS and four of those seven bound brain CS. Apparent dissociation constants for brain HS varied from about 36 nM to 6.2 μM, while those for brain CS varied from about 160 nM to 22 μM (Table I, II). Not surprisingly, protein binding to HS was invariably weaker than to heparin (Table I, III). In other tissues it is commonly observed that HS is 2–3 times less sulfated than heparin (Gallagher and Walker, 1985), with trisulfated disaccharides accounting for ~3% of the structure, as opposed to being the major structural unit in heparin (Turnbull and Gallagher, 1990). The relative difference in affinity between heparin and HS observed in this study was not the same for each protein, however (Table I, IV), implying that affinity is not simply a function of GAG charge.

Although the values of K_d reported here are given in units of protein concentration, it is interesting to compare them with the concentrations of HS and CS that are present in the developing nervous system. In the newborn rat brain, HS and CS are present at ~460 and 1200 μg/ml of tissue volume, respectively (Margolis *et al*., 1975). Assuming an average GAG chain size of 20 kDa, these values correspond to concentrations of 23 μM HS and 60 μM CS. If one assumes that most of these GAGs are restricted to the extracellular space or the cell surface, then local concentrations 5- to 10-fold higher are likely to exist in those locations. If any GAG chains have the potential to bind ligands at more than one site along their length, then the effective concentration of binding sites will be higher still. Clearly, GAG concentrations are sufficiently high in the brain that many of the proteins tested here should be expected to exist in a GAG-bound state a large fraction of the time. Moreover, the data reported here make useful predictions about which type of GAG a protein is more likely to be associated with in the brain. For example, based on the binding of thrombospondin-1 to heparin (K_d = 41 nM) and commercially available CS (K_d = 490–650 nM), one might expect this extracellular matrix protein to associate mainly with HSPGs in the brain. Yet its affinities for brain HS and CS (180 and 235 nM, respectively) are remarkably close. Given the nearly 3-fold higher abundance of CS in the brain, it is reasonable to predict that most thrombospondin-1 in the brain will be found to be associated with CSPGs. These sorts of conclusions have important implications for how experiments ought to be designed to test the biological functions of the GAG-binding proteins discussed here.

A surprising observation in this study was the failure to observe binding of NCAM or L1 to brain HS (Table IV). Both bind heparin, and in some studies of NCAM function evidence has pointed to an important role for interactions with HSPGs

(Cole *et al*., 1985, 1986; Cole and Glaser, 1986; Cole and Burg, 1989; Reyes *et al*., 1990; Kallapur and Akeson, 1992; Storms *et al*., 1996). Given the high concentrations of HS present in the brain, and the high concentrations of NCAM and L1 present there as well (Finne *et al*., 1983), it is possible that interactions weaker than those detectable here could be biologically important. Additionally, it is possible that the affinity of NCAM and L1 for HSPGs is improved by the participation of core proteins in binding (see below).

Selectivity based on GAG fine structure

Previous studies have shown that fibronectin and laminin-1, but not FGF-2 and thrombospondin-1, are capable of fractionating size-selected heparin into subpopulations that differ more than 10-fold in affinity (Lee and Lander, 1991; San Antonio *et al*., 1993). Data presented here (Figure 3B,C) indicate that NCAM and L1 also fractionate heparin into distinct subpopulations that differ considerably in affinity. Such results imply that the binding of these proteins is sensitive to variations in heparin structure, and raise the possibility that these proteins might also discriminate among structural variants of HS. While this may well be true in principle, brain HS was found to contain species that varied only slightly in their affinities for the proteins tested in the present study (Figure 4). It is possible that "high affinity" HS species are present in the brain but occur too infrequently to be detected in these assays, or occur at developmental stages other than the ones studied here (see Nurcombe *et al*., 1993). It is also possible that such species are not found in this tissue, and that the specificity of protein-PG binding is controlled in other ways, or not controlled at all. Identification of the precise oligosaccharide sequences that are preferred by these proteins, such as has been done for antithrombin III (Rosenberg *et al*., 1978; Rosenberg and Lam, 1979), and more recently for FGFs -1 and -2 (Habuchi *et al*., 1992; Turnbull *et al*., 1992; Mach *et al*., 1993), should aid in resolving this issue.

Role of core proteins in PG–protein interactions

An unanticipated finding in the present study was the high affinity observed when cerebroglycan, glypican-1 and syndecan-3 were tested for binding to laminin-1 (Figure 5, Table V). Since these three molecules represent the major HSPGs of the developing brain (Lander, 1993), it is reasonable to assume that their HS chains would be representative of HS derived from the developing brain as a whole. Indeed, when cerebroglycan was subjected to proteolysis, its affinity for laminin fell from nearly 2000-fold over that of brain HS to only 4-fold. The data imply that the cerebroglycan core protein plays a major role in laminin binding, but how it does so is not yet clear. It is possible that the core protein binds directly to laminin-1. Other examples of PG core proteins that bind to ligands independent of their GAG chains have been documented (e.g., binding of betaglycan to TGF-β (Andres *et al*., 1989); decorin and biglycan to collagens (Vogel *et al*., 1984; Schonherr *et al*., 1995); perlecan to integrins and fibronectin (Heremans *et al*., 1990; Hayashi *et al*., 1992;); and members of the aggrecan family to hyaluronan and tenascin-R (LeBaron *et al*., 1992; Doege, 1993; Aspberg *et al*., 1997; Milev *et al*., 1998). In the case of binding to laminin-1, theoretical considerations indicate that core protein-laminin interactions would not need to be of very high intrinsic affinity in order to increase the affinity of an intact HSPG by a few thousand fold over that of HS alone (Creighton, 1984). Alternatively, the role of core proteins in binding may be more indirect, relying solely on the fact that they tether multiple GAG chains together, making possible a greater avidity of binding. One could imagine that, with large ligands such as laminin-1 which have multiple heparin-binding sites (Sung *et al*., 1993; Colognato-Pyke *et al*., 1995), simply tethering multiple HS chains together so they span a very large distance could potentially lead to a stronger ligand interaction. In the case of cerebroglycan, however, this hypothesis is unlikely to explain the data, since the GAG chains of cerebroglycan (and of members of the glypican family in general) appear to be concentrated at a single location on the core protein and would not be separated by trypsin treatment (Lander *et al*., 1996; Mertens *et al*., 1996; S. E. Paine-Saunders and A. D. Lander, unpublished observations). Another possibility is that core proteins do more than merely tether GAG chains, but also position them is such a way as to enhance ligand binding.

Although evidence for core protein participation in binding was directly sought for only a few ligands in this study (laminin-1, thrombospondin-1, FGF-2), there are reasons to suspect that this phenomenon is more widespread. For example, in the experiment described in Figure 4, it was noted that the major HSPGs that bound to a PN-1 affinity column were glypican-1 and cerebroglycan, whereas the abundant HSPG syndecan-3 mostly flowed through the column. Since the data in Figure 4D indicate that the PN-1 affinities of the HS in the flow-through and eluted fractions were only slightly different from each other, the differential fractionation of glypican-1 and cerebroglycan vs. syndecan-3 is likely to depend on differences in the core proteins of these PGs. Another possible example of core proteins playing a role in ligand binding may be the observation that a single HSPG with a core protein size of 120 kDa copurifies through many fractionation steps with NCAM isolated from chick retinal tissue(Cole and Burg, 1989). Given our results that the interaction of NCAM with brain HS was too weak to detect, it is possible that NCAM binds this HSPG because a weak interaction with HS is supplemented by additional interactions with a core protein. If core proteins do play widespread roles in many types of PG–protein interactions, it may help explain the high degree of evolutionary conservation of core protein structure that is observed in some HSPG families, such as the syndecans and the glypicans (Lander *et al*., 1996).

Materials and methods

Materials

Several researchers generously provided molecules from their laboratories: Jack Lawler (Harvard Medical School, Boston, MA) provided human platelet thrombospondin-1; Denis Monard (Freidrich Miescher Institute, Basel, Switzerland) provided recombinant rat protease nexin-1 (produced in yeast); four forms of mouse NCAM (untreated NCAM from adult and from postnatal (up to 7 day old) mice, and endoneuraminidase N-treated forms of the same preparations) were provided by Carl Lagenauer (University of Pittsburgh School of Medicine, Pittsburgh, PA); mouse brain L1 was the generous gift of Vance Lemmon (Case Western Reserve University, Cleveland, OH). Bob Rosenberg (MIT, Cambridge, MA) provided antithrombin III; Jack Henken (Abbott Labs, Abbott Park, IL) provided human recombinant uPA (produced in *E. coli*); and the recombinant G domain (rG) of laminin-1 was the gift of Peter Yurchenco, (UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ). FGF-2 was purified from bovine brain (Lobb and Fett, 1984). Human plasma thrombin was from Enzyme Research Laboratories, Inc. (South Bend, IN). Fibronectin was from New York Blood Supply (New York, NY). Laminin-1 was purified

from Engelbreth-Holm-Swarm (EHS) sarcoma (Kleinman *et al*., 1982; Timpl *et al*., 1982). The concentrations of thrombin, fibronectin, uPA, protease nexin-1, NCAM, L1, antithrombin III, thrombospondin-1, and laminin-1 stocks were verified by amino acid analysis (MIT Biopolymers Laboratory). rG and FGF-2 concentrations were measured by amido-black binding, using crystalline bovine serum albumin (BSA) as a standard (Schaffner and Weissman, 1973).

Porcine intestinal heparin, bovine tracheal chondroitin sulfate (chondroitin 4-sulfate), hyaluronic acid, heparinase II, insulin, transferrin, progesterone, putrescine, selenium, tyramine, and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleiimide (NEM), Pepstatin A, and EDTA were from Sigma. 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS) was from Boehringer Mannheim. Shark chondroitin sulfate (chondroitin 6-sulfate) was from Fluka. Bovine kidney heparan sulfate and crystalline BSA were from ICN. Colominic acid was from Calbiochem. Triton X-114, Triton X-100, Sulfo-SHPP (sulfosuccinimidyl-3(4-hydroxyphenyl)propionate), and Iodogen were from Pierce Chemical Co. Chondroitinase ABC was from Seikagaku, USA. Heparitinase was prepared from *Flavobacterium heparinum* by hydroxyapatite chromatography as described previously (Linker and Hovingh, 1972). This preparation demonstrated specificity for HS digestion under the conditions used (43°C). Tissue culture media, balanced salt solutions, growth supplements, and enzymes for cell dissociation were obtained from GIBCO/BRL. An antibody to rodent syndecan-3 (MSE-3; Kim *et al*., 1994) was kindly provided by Merton Bernfield (Harvard Medical School, Boston, MA).

Metabolic labeling of neural tissue with $^{35}SO_4$ in vitro

Embryonic day 18 (E18) and neonatal (PO) Sprague Dawley rat brains were dissected and meninges removed as described previously (Herndon and Lander, 1990) cut into prisms (roughly 1.0–5.0 mm × 1 mm × 0.35 mm) with a McIlwain Tissue Chopper, then allowed to settle through calcium- and magnesium-free Hank's balanced salt solution (HBSS). Sulfate-free Dulbecco's modification of Eagle's Medium (SF-DMEM) was formulated from concentrated culture supplements, with $MgCl_2$ replacing $MgSO_4$. Labeling medium was SF-DMEM supplemented to 50 μM with unlabeled Na_2SO_4, 10 μg/ml insulin, 5 mg/ml crystalline BSA, 10 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM selenium, and 100–250 μCi/ml $Na_2{}^{35}SO_4$ (Dupont/NEN). The addition of 50 μM unlabeled Na_2SO_4 to the culture medium was found to be required for optimal incorporation of $^{35}SO_4$ into ethanol-precipitable GAGs (data not shown). Labeling medium was added to tissue prisms at 20 ml per ml of tissue (settled volume) and cultured 16–20 h in 5% CO_2, 37°C, with gentle gyratory rocking.

To obtain labeled retinal tissue, eyes from E18 Sprague Dawley rats were removed while submerged under DMEM. Retinas were detached in fresh medium, transferred to SF-DMEM and cut into pieces (~1 mm^3) using a tungsten microknife. Tissue pieces were treated with 1 mg/ml trypsin for 20 min at 37°C, then soybean trypsin inhibitor was added to 1.4 mg/ml. Cells were dissociated by trituration and incubated for 17 h at 37°C in labeling medium in untreated tissue culture dishes, during which time the cells reassociated.

Purification of proteoglycans and glycosaminoglycans

PGs used in this study were isolated from E16 rat brain, from $^{35}SO_4$-labeled brain and retina (see above), and from neonatal rat brain growth cone particles, prepared according to Pfenninger (Pfenninger *et al*., 1983). E16 rat brain and $^{35}SO_4$-labeled brain tissue were subjected to subcellular fractionation and PGs associated with a soluble fraction and a membrane fraction (material solubilized in 0.3 M sucrose/4 mM HEPES, pH 7.4 and 0.15 M NaCl/1.0% CHAPS/50 mM Tris–HCl, pH 8.0, respectively) were isolated by DEAE-Sephacel (Pharmacia) chromatography as described previously (Herndon and Lander, 1990). $^{35}SO_4$-labeled retinal cells (see above) were resuspended in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1.0% CHAPS, 0.25 mg/ml NEM, 400 μM PMSF, 1 mM EDTA, 1 μg/ml Pepstatin A, at 4°C, and placed in ice water in a bath-type sonicator for 5 min. Insoluble material was removed by centrifugation at 13,000 × *g*, 25 min 4°C, and PGs were extracted from the supernatant and chromatographed as with brain tissue.

To isolate PGs from growth cone particles, such particles at ~1 mg/ml total protein in 1 mM TES, 1 mM $MgCl_2$, ~0.65 M sucrose, were made 50 mM in Tris–HCl [pH 7.4], 0.15 M in NaCl, and 1.0% in CHAPS, and vortexed for 1 min. After centrifuging for 3 min at 12,000 × *g*, the supernatant was recovered and pepstatin A and PMSF were added to 1 μg/ml and 0.4 mM respectively. The supernatant was then loaded onto a DEAE-Sephacel column, and PGs were isolated as described previously (Herndon and Lander, 1990).

For CS or HS purification, $^{35}SO_4$-labeled brain and retinal PGs were first subjected to GAG lyase digestions (Herndon and Lander, 1990): For HS purification, PGs were subjected to chondroitinase ABC at 0.05–0.1 U/ml for 4–20 h at 37°C. To obtain CS, PGs were treated sequentially with heparinase II (0.05U/ ml, 4–6 h, 30°C) then heparitinase (4 μg/ml, 43°C, 4–20 h). Intact GAGs were then cleaved from PG cores by incubation for 1 h at 45°C in 90 mM NaOH and 9 mM $NaBH_4$. The reaction was stopped by restoration of the pH to ~7.0, using 0.5 M acetic acid. To precipitate cleaved GAGs, reactions were brought to 0.2 mg/ml in hyaluronic acid, mixed with 3.2 volumes 100% ethanol, incubated at -20°C for 1 h, and centrifuged at 13,000 × *g* for 30 min at 4°C. Because hyaluronic acid is a very large, unsulfated GAG, its migration in ACE is significantly less than sulfated GAGs; as a result, it does not interfere with the ACE pattern demonstrated by radiolabeled HS or CS (data not shown). After removal of the supernatant liquid, the pellets were briefly air dried, and resuspended in ACE electrophoresis buffer (50 mM 3-(N-morpholino)-2-hydroxypropanesulfonic acid (MOPSO), 125 mM sodium acetate, pH 7.0).

Radioiodination of proteoglycans and glycosaminoglycans

Intact PGs were radioiodinated on their core proteins using the chloramine-T method (Herndon and Lander, 1990). Heparin was derivatized with tyramine, radioiodinated, and chromatographed on Sephadex G-100 to produce a low molecular weight fraction ($M_r \leq 6000$) as described previously (San Antonio *et al*., 1993). Bovine kidney HS was alkaline cleaved to remove attached peptides (1 mg HS in 100 μl of 50% DMSO, 10% ethanol, 0.17 M KOH for 1.5 h at 45°C, then pH adjusted to ~7.0 by adding 5 μl 1 M Tris–HCl, pH 7.0 and 12 μl freshly prepared 1 M HCl), exchanged into water using a 0.9 ml G25 Sephadex spin column, and then vacuum dried prior to derivatization with tyramine as described for heparin (San Antonio *et al*., 1993). Bovine tracheal CS and shark CS were derivatized with hydroxyphenylpropionic

acid: 3 ml of 1 mg/ml CS in O.15 M sodium borate pH 8.5 was added to 330 µl sulfosuccinimidyl-3(4-hydroxyphenyl)proprionate (Sulfo-SHPP, 25 mg/ml in dimethylformamide), incubated 4 h at room temperature, dialyzed exhaustively against water, lyophilized, and resuspended in 1 ml of H_2O. Derivatized GAGs were radioiodinated using the Iodogen method (San Antonio *et al*., 1993). Labeled GAGs and PGs were radioprotected by the addition of crystalline BSA to 0.25 mg/ml, and stored at -80°C. The purity of both ^{125}I and $^{35}SO_4$-labeled GAGs was confirmed by susceptibility to and resistance to digestion by appropriate GAG lyases followed by PAGE analysis.

Immunopurification of cerebroglycan, glypican, and syndecan-3

Affinity purified anti-cerebroglycan (521–2) and anti-glypican-1 (343–1) polyclonal antibodies (Ivins *et al*., 1997) were bound to protein A–Sepharose and crosslinked with dimethyl-pimelimidate using an Immunopure Protein A IgG Orientation Kit (Pierce). ^{125}I-labeled PGs from E16 rat brain or from P0 rat brain growth cone particles were diluted 5-fold with 50 mM Tris–HCl, 0.5% CHAPS, and crystalline BSA was added to a final concentration of 0.1 mg/ml; 0.05 to 0.1 volumes (packed beads) of affinity matrix were added, and PGs were allowed to bind overnight at 4°C. Beads were washed for 20–60 min with rotation at room temperature with 1 ml each of (1) 50 mM Tris–HCl, 0.15 M NaCl, 0.5% CHAPS and (2) 0.5M NaCl, 50 mM Tris–HCl, 0.5% CHAPS, and then briefly with 10 mM Tris–HCl, 0.5% CHAPS. Bound PGs were eluted with 100 mM NaH_2PO_4/H_3PO_4 [pH 2.5], 0.5% CHAPS for 10 min and immediately neutralized with 0.2 volumes of 0.5 M Na_2HPO_4.

Syndecan-3 was immunoprecipitated from ^{125}I-labeled PGs derived from postnatal day 0 (P0) rat brain growth cone particles using MSE-3 antibody (Kim *et al*., 1994) and immobilized protein A beads. Bound material was eluted with 0.05 M sodium formate, 6 M urea, 0.2 M NaCl, 0.1% Triton X-100, pH 3.5.

Trypsin digestion of cerebroglycan

To prepare tryptic fragments of labeled cerebroglycan, 6 µl (36 µg) of freshly prepared trypsin (TRTPCK grade, Worthington Biochemical Co.) in phosphate buffered saline was added to 30 µl (~90,000 c.p.m.) of ^{125}I-labeled cerebroglycan, immunopurified as described above, and the sample was incubated for 2 h at 37°C. 12 µl (72 µg) of soy bean trypsin inhibitor in phosphate-buffered saline was added, and the sample was incubated an additional 1 h at 37°C.

Affinity coelectrophoresis (ACE) and analysis

In ACE, a labeled PG or GAG is induced to migrate through lanes containing a test ligand embedded, at various concentrations, in agarose . The shifts in electrophoretic mobility of the PG or GAG as a function of ligand concentration are used to determine a dissociation constant. ACE was carried out exactly as described previously (Lee and Lander, 1991; Lim *et al*., 1991; San Antonio *et al*., 1993; Herndon and Lander, 1997) except that, in experiments involving thrombospondin-1, 2 mM calcium acetate was added to all buffers. ACE gels were dried, exposed to storage phosphor screens (Molecular Dynamics, Sunnyvale, CA), and measurements of GAG or PG mobility were made and converted to retardation coefficients (the mobility shift in a given lane divided by the mobility of the PG or GAG in a protein-free lane), as previously described (San Antonio *et al*., 1993). Data were then fit, using a nonlinear least squares approach (Kaleidagraph, Synergy Software), to the equation $R = R_\infty /[1 + (K_d/[P_{tot}]^n)]$, where R is the retardation coefficient, $[P_{tot}]$ is the protein concentration in a given lane of an ACE gel, and n is the number of protein molecules binding to each PG or GAG molecule, i.e., the order of binding. In most cases, the data were best fit using n = 1. The predicted effects of multiple protein binding sites in ACE are small (see Lim *et al*., 1991), and in no cases did K_d values vary by more than 2-fold from the n = 1 value when n = 2 was used. Because GAGs and PGs are labeled to high specific activity and only trace amounts are used in the gel, their concentrations do not enter into the calculation of K_d (Lee and Lander, 1991; Lim *et al*., 1991; Herndon and Lander, 1997). The variables that were fit simultaneously were K_d, the dissociation constant, and R_∞, the maximum value of R. In cases where the highest value of $[P_{tot}]$ tested was not > K_d, the data were sometimes insufficient to accurately determine both K_d and R_∞. However in many cases an independent estimate of R_∞ could be supplied from other experiments involving the same protein ligand, and the above equation could then be solved for K_d alone (Herndon and Lander, 1997).

Fractionation of PGs by protease nexin-1

Protease nexin-1 (PN-1) was coupled to Affigel-10 (Bio-Rad) at a concentration of 150 µM PN-1 (packed column volume) and used to fractionate whole E18 rat forebrain PGs: $^{35}SO_4$-labeled PGs were prepared from cultured whole E18 rat forebrain tissue as described above, and a small fraction of the purified PGs were ^{125}I-labeled on their core proteins. ^{125}I-labeled PGs, $^{35}SO_4$-labeled PGs, and ^{125}I-labeled heparin were then applied to three identical PN-1 affinity columns run in parallel. PGs were eluted stepwise with buffers containing 50 mM Tris–HCl pH 8.0, 0.5% CHAPS, and NaCl at concentrations of 0.15 M, 0.3 M, 0.5 M, 1 M, 1.5 M, and 3 M. To see which core proteins were eluted at each salt concentration, eluates from the column loaded with ^{125}I-labeled PGs were subjected to GAG lyase digestion and SDS–PAGE (Herndon and Lander, 1990) The corresponding fractions from the column that had been loaded with $^{35}SO_4$-labeled PGs were chondroitinase ABC-treated and subjected to alkaline borohydride cleavage to release HS chains, which were then tested by ACE for binding to PN-1.

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Abbreviations

ACE, affinity coelectrophoresis; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; CS, chondroitin sulfate; DMEM, Dulbecco's modified eagle medium; EDTA, ethylenediamine tetraacetic acid; endo-N, endoneuraminidase N; E18, embryonic day 18; FGF, fibroblast growth factor; GAG, glycosaminoglycan; HBSS, calcium- and magnesium-free Hank's balanced salt solution; HS, heparan sulfate; NCAM, neural cell adhesion molecule; NEM, N-ethylmaleiimide; P0, postnatal day 0 (rat); PG, proteoglycan;

PMSF, phenylmethylsulfonyl fluoride; PN-1, protease nexin-1; PSA, polysialic acid; uPA, urokinase plasminogen activator; R, retardation coefficient; rG, recombinant G domain of laminin-1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SF-DMEM, sulfate-free DMEM; DMSO, dimethylsulfoxide.

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