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A Diverse Set of Developmentally Regulated Proteoglycans Is Expressed in the Rat Central Nervous System

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Summary

Cellular interactions in neural development are influenced by various extracellular proteins, many of which bind glycosaminoglycans or proteoglycans. Precise functions of nervous system proteoglycans remain unknown, in part because neural proteoglycan composition is poorly understood. In this study, 25 putative proteoglycan core proteins were identified in subcellular fractions of rat brain. Levels of many of these varied considerably during development. Membrane-associated proteoglycans included two heparan sulfate proteoglycans (cores of 50 and 59 kd) that are covalently linked to glycosyl-phosphatidylinositol lipid, as well as several that appear to aggregate either with themselves or with copurifying proteins. These data indicate that brain proteoglycans exhibit the abundance, structural diversity, and developmental regulation that would be anticipated for molecules with diverse developmental functions.

Introduction

The behaviors of developing neural cells—e.g., directed cell migration, axon outgrowth and navigation, synaptogenesis, and selective cell death—require the precise orchestration of cell-cell and cell-matrix interactions. Nervous system molecules that are believed to play a role in mediating such interactions include members of diverse categories, such as extracellular matrix glycoproteins, cell surface adhesion molecules, polypeptide growth factors, and extracellular proteases and protease inhibitors (Lander, 1987; Sanes, 1989; Walicke, 1989). Interestingly, a large proportion of nervous system molecules belonging to these categories exhibit binding to glycosaminoglycans (GAGs) and/or proteoglycans (PGs). Examples include laminin, fibronectin, thrombospondin, tenascin, N-CAM, myelin-associated glycoprotein (reviewed by Lander, 1989), retinal purpurin (Schubert et al., 1986), amyloid β-protein precursor (Schubert et al., 1989), acidic and basic fibroblast growth factor (Lobb et al., 1986), a novel glial mitogen (Ratner et al., 1988), and protease nexin-1 (Gloor et al., 1986).

The observation that so many developmentally important extracellular proteins bind GAGs and/or PGs is intriguing, because it suggests that PGs might influence the localization, availability, or biological activities of many of these proteins in vivo. Indeed, the likelihood that PGs are involved in a wide variety of important events in neural development has been suggested by studies of neurulation (Morris-Kay and Crutch, 1982), neural cell-substratum adhesion (Schubert and LaCorbiere, 1982; Akeson and Warren, 1984, J. Cell Biol., abstract; Schubert et al., 1986, 1987), N-CAM-mediated cell adhesion (Cole et al., 1985), migration of neural crest cells (Perris and Johansson, 1987), laminin-stimulated neurite outgrowth (Muir et al., 1989), axon guidance (Carbonetto et al., 1983; Snow et al., 1990a), responsiveness to trophic factors (Schubert et al., 1987; Neufeld et al., 1987; Damon et al., 1987, Soc. Neurosci., abstract; Walicke, 1988), acetylcholine receptor clustering (Kidokoro and Hirano, 1988, Soc. Neurosci., abstract; Gordon and Hall, 1989; Gordon and Hall, 1989, Soc. Neurosci., abstract), and glial growth control (Ratner et al., 1985).

The possibility that PGs have diverse biological functions during development raises an interesting question: Do distinct PGs with unique and highly specific biological activities exist in the nervous system? On the one hand, the fact that protein-PG interactions appear to be mediated by GAGs, of which only a handful of types exist (heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate), suggests that the specificity of protein-PG interactions may be quite limited (Ruoslahti, 1989). On the other hand, examples of PGs exhibiting considerable specificity have emerged recently. For example, syndecan, a cell surface PG that contains heparan sulfate, interacts with fibronectin, but not laminin (Saunders and Bernfield, 1988; Saunders et al., 1989), even though both fibronectin and laminin bind purified preparations of heparan sulfate. Other cell surface PGs apparently distinguish among serine protease inhibitors in a cell type-specific manner (Cunningham et al., 1986; Marcum et al., 1987). Of two heparan sulfate PGs that bind laminin, only one is associated with an ability to block laminin's neurite outgrowth-promoting activity (Muir et al., 1989). Specificity in protein-PG interactions may be achieved through recognition of particular GAG modifications (e.g., Lindahl et al., 1984), or may involve contributions of PG core proteins to binding or function.

If PGs play multiple highly specific roles in nervous system development, it would be logical to expect that a diverse set of PGs would be expressed in the nervous system during development and that the expression of at least some nervous system PGs would be temporally and/or spatially regulated during development. Recent immunohistochemical observations support the latter expectation (Aquino et al., 1984; Levine and Card, 1987; Stallcup and Beasley, 1987; Hoffman et al., 1988; Zaremba et al., 1989), but adequate biochemical studies of the diversity and timing of PG expression are lacking. Instead, biochemical studies of nervous system PGs have tended to focus on characterization of one or a few major species (e.g., Kiang et al., 1981; Klinger et al., 1985; Ripellino and Margolis,

1989). A notable exception is the recent study of Oohira et al. (1988), which suggests that as many as five chondroitin sulfate PGs can be isolated from a single subcellular fraction of 10-day-old rat brain.

The following study represents an initial attempt to assess the diversity and timing of expression of nervous system PGs. The experimental approach is similar to that developed by Bretscher (1985) and Lories et al. (1987) for analyzing the complexity of PGs expressed on the surfaces of cultured cells. The results indicate that a wide variety of heparan sulfate and chondroitin sulfate PGs can be identified in the mammalian brain, that individual PGs associate with particular subcellular fractions, that some integral membrane PGs of the brain are covalently attached to lipid, and that levels of many brain PGs change significantly during development from late fetal stages to adulthood. Using this approach, it may be possible to identify PGs with biochemical properties and patterns of expression suggestive of involvement in particular developmental events. The specificity of interaction of such PGs with appropriate neural PG binding proteins could then be evaluated.

Results

Isolation of Proteoglycan-Enriched Fractions

Anion exchange chromatography was used to isolate PGs from two subcellular fractions of rat brain—a "soluble fraction" and a detergent extract of crude membranes—that had previously been shown to contain PGs (Margolis et al., 1975a; Kiang et al., 1981; Klinger et al., 1985). After adsorption of crude material to the ion exchange matrix, a series of buffers was employed to wash away non-PG proteins (see Bretscher, 1985). Subsequent application of a high salt buffer (buffer G) eluted a putative PG-containing fraction representing ≤0.3% of initial protein. Data from the fractionation of newborn (postnatal day zero; P0) brain are presented in Table 1. A comparison of overall recovery of protein in the buffer G-eluted fractions of embryonic (embryonic day 18; E18), P0, and adult brain is shown in Table 2.

Given the small amounts of protein in buffer G eluates (compared with the amounts eluted by previous washes), it was important to show that the material eluted by buffer G does not consist simply of residual amounts of abundant molecules, most of which had already been eluted by buffers C–F. To address this question, the buffer G eluate from P0 brain membranes was radioiodinated and a small amount was mixed with a fresh, unlabeled membrane extract. The mixture was then subjected to anion exchange chromatography. As shown in Table 3, although approximately 5% of the labeled material failed to rebind the ion exchange matrix (see Discussion), very little of what did bind was eluted by buffers C–F (about 4%). Thus, the bulk of the molecules described below appear to be molecules specifically eluted by high salt (buffer G) and resistant to elution by moderate salt, urea, or low pH (buffers C–F). Table 3 also suggests that molecules eluted by buffer G may be particularly susceptible to loss due to irreversible binding to the ion exchange matrix, since material eluted by buffer G is not quantitatively recovered following rechromatography (see Discussion).

Identification of Proteoglycans

In recent years, SDS–PAGE has been widely used for the characterization of PGs. Typically, the presence of GAG chains causes PGs to migrate as diffuse smears of anomalously high apparent M_r. Enzymatic removal of GAG chains, however, generates PG core proteins (or, alternatively, "core preparations" [Hassell et al., 1986]) that behave similarly to other proteins and glycoproteins when subjected to SDS–PAGE. Thus, electrophoretic bands that appear in response to digestion of a sample with a single GAG lyase can be taken as evidence of individual PGs bearing GAG chains of the class recognized by that enzyme.

This approach was used to characterize PG fractions (i.e., buffer G eluates) derived from brain. To improve sensitivity and to avoid the possibility that protein bands contributed by GAG lyases themselves would be mistakenly identified as core proteins, PG fractions were radioiodinated as described by Lories

Table 1. DEAE Purification of a Proteoglycan-Enriched Fraction from P0 Rat Brain

	Membrane-Associated Proteoglycans[a]	Soluble Fraction-Associated Proteoglycans[b]
Crude homogenate	7.4 ± 2.1[c]	15.6 ± 2.5
DEAE column pools:		
Flowthrough	3.6 ± 1.5	4.2 ± 0.65
Buffer D	0.84 ± 0.10	1.4 ± 0.02
Buffer E	1.00 ± 0.35	10.9 ± 6.1
Buffer F	0.02 ± 0.022	0.049 ± 0.007
PG fraction	0.01 ± 0.004[c]	0.015 ± 0.0045

See the text for a description of the purification procedure. Values are given as mg of protein per g of brain (wet weight).
[a] Mean ± standard deviation for four independent preparations.
[b] Mean ± range based on two independent preparations.
[c] Only three determinations were available for these data points.

Table 2. Recovery of Proteoglycans from Embryonic, Newborn, and Adult Rat Brain

Brain Subcellular Fraction	Crude Homogenate[a]	Proteoglcan Fraction[b]	Recovery[c]
Membrane fraction			
E18[d]	2.62	8	0.30
P0[e]	7.41	10	0.13
Adult[f]	16.9	20	0.12
Soluble fraction			
E18[d]	10.5	12	0.11
P0[e]	15.6	14.5	0.09
Adult[f]	35.0	15	0.04

[a] Values are given as mg of protein per g of brain (wet weight).
[b] Values are given as µg of protein per g of brain (wet weight).
[c] Values are given as % of crude homogenate protein.
[d] Values from one preparation.
[e] Mean values of three independent preparations.
[f] Mean values of two independent preparations.

et al. (1987) and core proteins were identified by autoradiography. GAG lyases that digest chondroitin sulfate (chondroitinase AC), chondroitin sulfate and dermatan sulfate (chrondroitinase ABC), and heparan sulfate (heparitinase) were used. In early experiments, silver staining was also used to identify core proteins. This procedure adequately identified the most abundant PG cores. It was difficult, however, to estimate levels of PGs because many core proteins, when stained as described by Wray et al. (1981), gave rise to "negative bands," i.e., unstained areas over a lightly stained background (data not shown). In all cases in which core protein bands could be identified by silver staining, the same bands could also be found by autoradiographic analysis of radioiodinated material.

Figure 1 shows the results obtained when samples derived from the membrane fraction (A) and soluble fraction (B) of P0 brain were analyzed in this way. Each GAG lyase produces a characteristic pattern of new bands. Bands that do not change in response to digestion with GAG lyases are also seen (Figure 1, asterisks); presumably, these represent proteins that are not PGs, or are refractory to the GAG lyases used. Neuraminidase digestions were also performed to determine whether the presence of sialic acid could account for the tendency of some putative PG core protein bands to appear less sharp than others.

To minimize the possibility that some of the bands identified as core proteins actually result from proteolysis during diagnostic digestions with glycosaminoglycan lyases (see Kato et al., 1985), all enzymatic digestions were carried out in the presence of fresh protease inhibitors and carrier protein. Enzymes were tested at several concentrations and used only at concentrations well below those that resulted in detectable degradation of non-PG proteins. Time course studies (data not shown) indicate that the bands identified as core proteins all "appear" at about the same time after exposure to enzyme, and remain stable in M_r and abundance for >4 hr at 37°C, the longest time tested (e.g., high M_r bands were not seen to "chase" into bands of lower M_r).

Analyses similar to those in Figure 1 were performed for material derived from E18 and adult brain (data not shown). To facilitate unambiguous identification and comparison of putative core protein species at each developmental stage, apparent M_r values were also determined from 5%, 10%, and 5%–15% (exponential gradient) gels run under both reducing and nonreducing conditions. Figure 2 shows examples of 10% nonreducing gels that compare the effects of chondroitinase ABC and heparitinase digestion on neuraminidase-treated membrane-associated and soluble PG fractions from all three developmental stages (equal amounts of radioactivity were loaded in each lane). The same samples were also analyzed on 5% gels to resolve high molecular weight PGs better (data not shown). Results from Figure 1, Figure 2, and analyses not shown are summarized by Tables 4 and 5. Putative core proteins of the membrane fraction are designated M1–M16; those of the soluble fraction,

Table 3. Fractionation of ^{125}I-Labeled Proteoglycans Mixed with Crude Homogenate

Purification Step	Percent Input cpm
Labeled PGs + crude homogenate	100%
DEAE column pools	
Flowthrough	4.7%
Buffer D	0.9%
Buffer E	2.1%
Buffer F	1.0%
PGs recovered (Buffer G)	
Peak (38 ml)	39.2%
Tail (500 ml)	8.5%
Not recovered	43.6%

Radioiodinated PGs purified from P0 membrane preparation (300 ng, 1.24×10^7 cpm) were added to a fresh P0 membrane preparation (52 mg of protein) and subjected to DEAE-Sephacel fractionation (50 ml column volume) as outlined in the text, except that buffers C–F contained 0.5% CHAPS instead of Triton X-100. Values shown are the percentage of initial radioactivity recovered in the protein peaks eluted at the indicated steps. For discussion, see text.

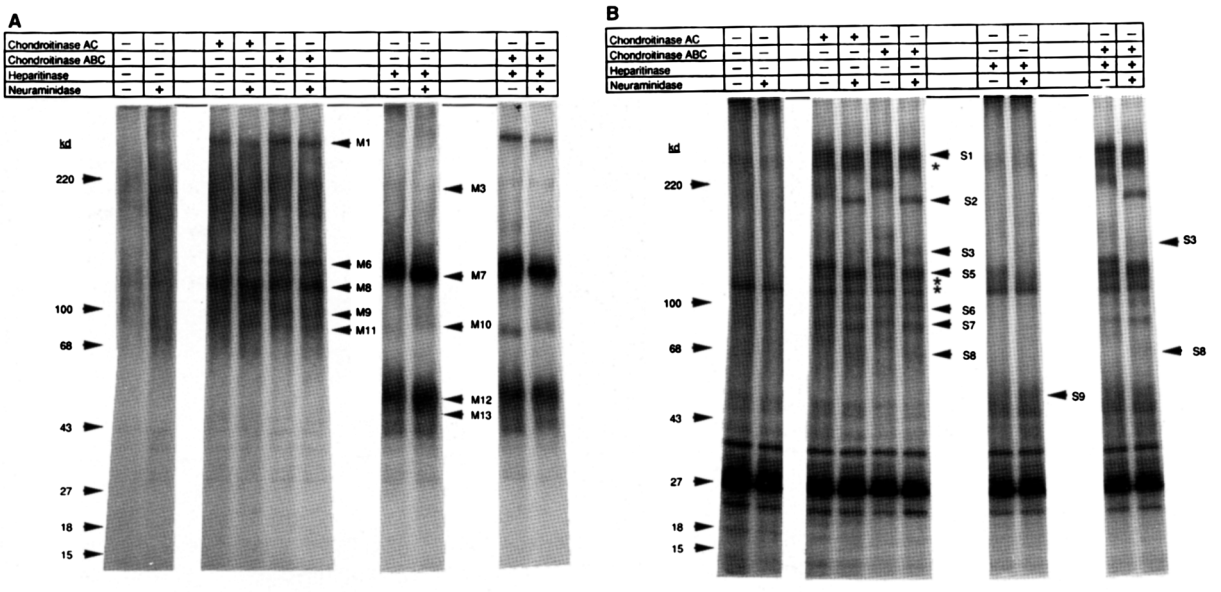

Figure 1. SDS–PAGE Analysis of Proteoglycan Preparations from P0 Rat Brain

Purified and radioiodinated samples were subjected to enzymatic digestion, as described in the text, and analyzed on 5%–15% exponential gradient gels run under nonreducing conditions. Samples derived from a membrane preparation (A) and from the soluble fraction (B) of brain are shown. (+) and (–) symbols indicate whether a sample was treated with an enzyme. Molecular weight markers are shown to the left of each gel; arrowheads at right identify putative PG core proteins mentioned in Tables 4 and 5. Asterisks mark some of the non-PG proteins routinely found in these preparations.

S1–S9 (in descending order of M_r). Arrows in Figures 1 and 2 mark the positions of most of the neuraminidase-treated putative core proteins mentioned in Tables 4 and 5. Not all core proteins are visible on every gel: M16 is seen only after reduction and therefore is not shown by these nonreducing gels. Bands representing M2 and M3 are too faint to be seen in Figure 2A, but are easily resolved by 5% gels of the same samples (data not shown). Also, in Figure 2A, bands representing M10 (a heparan sulfate PG) and M11 (a chondroitin sulfate PG) have been pointed out only in the lanes in which they overlap (the sample treated with both chondroitinase and heparitinase); separate bands representing M10 and M11 are present in samples treated with either heparitinase or chondroitinase, respectively, but are difficult to see in Figure 2A; they are more easily appreciated in Figure 1A. In Figure 2B, chondroitin sulfate PGs S4 and S5 overlap, but are readily distinguished by 5% gels (data not shown).

Tables 4 and 5 also contain information about salt gradient elution profiles of the PGs that give rise to certain putative core protein species, as well as pictorial representations of the relative amounts of putative core proteins at each of the developmental ages studied.

Gel Filtration Analysis of Membrane-Associated Proteoglycans

Although the methods used for isolating PG fractions are apparently highly selective for PGs over other proteins, some non-PGs are clearly not eliminated (Figures 1 and 2, asterisks). These may represent proteins that remain highly anionic even at low pH, perhaps as a result of sulfation or phosphorylation. Alternatively, these may be proteins that strongly associate with PGs and thereby copurify with them. The latter possibility seemed particularly worth investigating in the PG fraction derived from brain membranes, because starting material could easily have contained extracellular matrix material associated with membranes. There is little information available on the behavior of extracellular matrix during subcellular fractionation of brain homogenates. However, at least one form of organized extracellular matrix, the synaptic junctional complex, is known to be recoverable from a membrane preparation similar to the one described here (Wang and Mahler, 1976).

A small amount of radiolabled P0 membrane-associated PGs was therefore fractionated on Sepharose CL4B. To reduce nonspecific aggregation, the column buffer contained 0.5 M NaCl, 0.5% CHAPS, and carrier protein (1 mg/ml crystalline BSA). Fractions were analyzed by nonreducing SDS–PAGE before and after triple digestion with neuraminidase, chondroitinase ABC, and heparitinase.

The results, shown in Figure 3, indicate that some components of the PG fraction behave as expected for typical nonaggregating macromolecules, i.e., they elute as relatively compact peaks in order of their apparent M_r values. Examples of such molecules include M1, M3, M12, M13 (Figure 3B), and non-PG proteins of 43, 37, and 28 (indicated on Figure 3A by

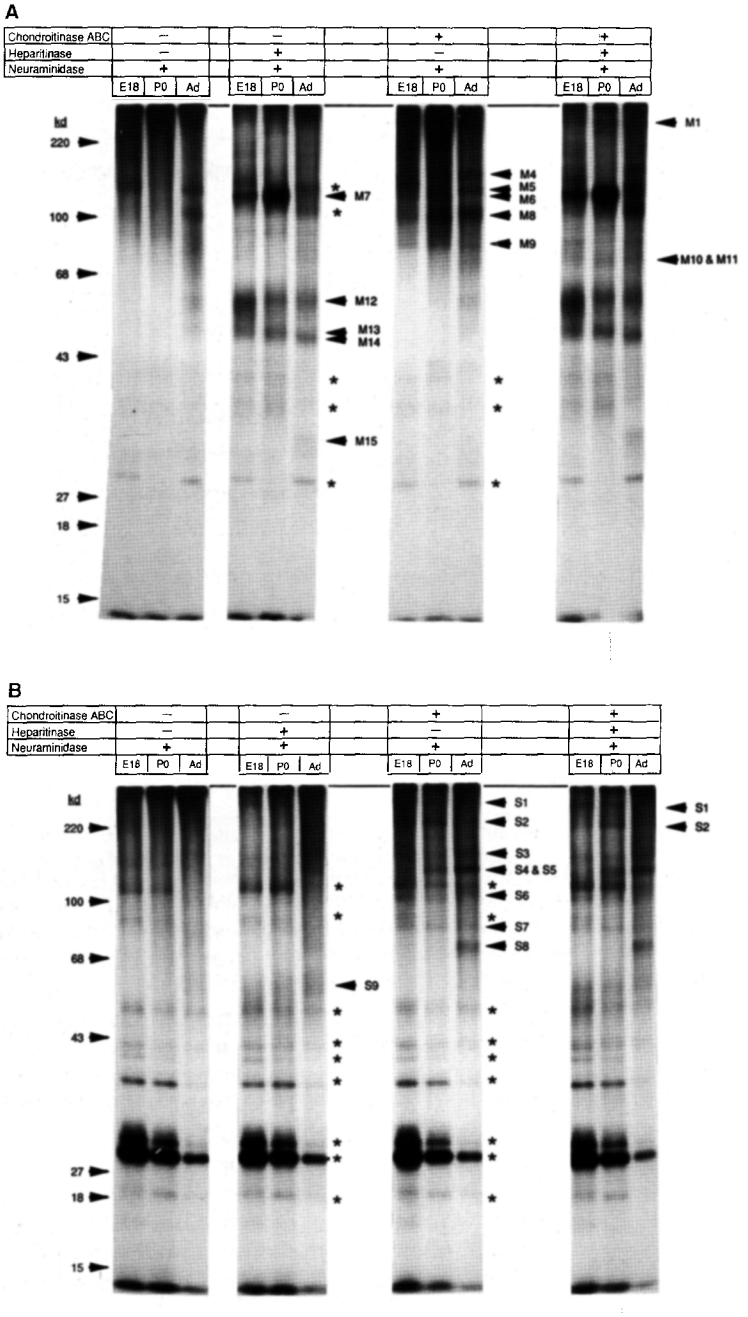

Figure 2. Comparison of Proteoglycan Core Protein Expression in Embryonic, Newborn, and Adult Rat Brain

Equivalent counts per minute of radioiodinated samples from E18, P0, and adult rat brain were subjected to enzymatic digestion and analyzed by SDS-PAGE under nonreducing conditions on 10% gels. (+) and (−) symbols indicate whether a sample was treated with an enzyme. PGs from membrane fractions are shown in (A); PGs from soluble fractions are shown in (B). Molecular weight markers are shown to the left of each gel. Arrowheads to the right of gels identify putative PG core proteins (see Tables 4 and 5).

diamonds). In contrast, several molecules exhibit behaviors suggestive of aggregating species, such as coelution with molecules of much higher apparent M_r values, or a broad elution profile smeared across nearly the entire column profile. Examples of these include M7, M8, M9 (Figure 3B), and non-PG proteins of 135, 115, 98, 46, 41, 35, and 30 kd (indicated on Figure 3A by asterisks). Preliminary results of velocity sedimentation of P0 membrane-associated PGs also suggest aggregation of the same species. Specifically, M1, M12, and M13 sedimented as compact peaks (at 7S, 3S, and 3S, respectively), whereas M7, M8, M9, and non-PG proteins of 46, 41, and 35 kd were found together throughout all sucrose gradient fractions from 7S to the tube bottom (>26S) (unpublished data).

Identification of Putative Integral Membrane Proteoglycans

Studies of cultured cells have identified cell surface PGs that are integral membrane components, as well as PGs that are only peripherally associated with the plasma membrane. To identify brain PGs that are integral membrane components, labeled PG fractions from each developmental age (E18, P0, and adult) were subjected to hydrophobic partitioning using the detergent Triton X-114. This method identifies molecules that

Table 4. Proteoglycans of the Membrane Fraction

Name	GAG Type	Apparent M_r of Core Protein				DEAE Elution	Relative Abundance		
		Non-reduced		Reduced					
		-N'dase	+N'dase	-N'dase	+N'dase	(M NaCl)	E18	P0	Adult
M1	ChS	440	420	440	415	0.5			
M2	ChS	330	270	270	260				
M3	HeS	230	230	NS	NS				
M4	ChS	162	160	170	NS				
M5	ChS	140	140	150	135				
M6	ChS	135	135	140	145				
M7	HeS	145	130	145	135	0.46			
M8	ChS	130	120	120	115				
M9	ChS	85	85	90	85	0.43			
M10	HeS	75	77	75	80				
M11	ChS	75	75	85	80				
M12	HeS	60	59	67	65	0.41			
M13	HeS	48	50	57	55	0.42			
M14	HeS	NS	49	NS	51				
M15	HeS	35	35	39	39				
M16	HeS	NS	NS	25	25				

^{125}I-labeled membrane-associated PGs from E18, P0, and adult rat brain were subjected to SDS gel analysis after enzymatic treatments. Electrophoretic bands that appear in response to either chondroitinase or heparitinase treatment are taken as evidence of chondroitin sulfate (ChS) or heparan sulfate (HeS) PGs, respectively (see text). Putative PGs are listed in descending order of apparent core protein M_r in kd, as determined from 5%, 10%, and 5%–15% reducing and nonreducing SDS polyacrylamide gels. Changes in apparent core protein M_r in response to digestion with neuraminidase (N'dase) from Arthrobacter ureafaciens are also noted. Identical results were seen with neuraminidase from Clostridium perfringens (data not shown). In a few cases, electrophoretic bands were very faint or were obscured by other bands, and an accurate M_r could not be determined; in these instances the core protein is described as "not seen" (NS). Information on the relative abundances of PG species was obtained by comparing SDS gel analyses of equivalent counts per minute of PGs from each developmental stage. Horizontal bars of four different widths have been used to convey a qualitative ranking of PGs into those that are of very high (▬), high (—), moderate (—), and low (—) abundances, as determined from autoradiograms such as those in Figure 2. Data on salt gradient elution profiles are also presented for several abundant PGs. These results, which reflect analysis of P0 material only, are expressed as the NaCl concentration at which the peak of eluted material is found.

contain hydrophobic domains sufficient to permit their incorporation into detergent micelles (Bordier, 1981). The results of analysis of PG fractions derived from brain membranes, shown in Figure 4, indicate that heparan sulfate PGs identified by cores M12 and M13, as well as two adult-specific non-PG proteins of 100 and 120 kd, partition significantly into a Triton X-114-rich phase. The data also suggest that these molecules may normally be integral membrane components. Small amounts of two other PGs, identified by cores M7 and M8, can also be recognized in the detergent-rich phase. However, since these PGs are among those suspected of some form of aggregation (see discussion of Figure 3, above), their presence in the detergent phase should be interpreted with caution: macromolecular aggregates sufficiently large to pellet at 1000 × g would almost certainly have been collected along with the (lower) detergent phase irrespective of actual detergent binding (or lack thereof).

Triton X-114 partitioning was also carried out using labeled PG fractions derived from the soluble fraction of E18, P0, and adult brain. In these experiments, no molecules were found to partition into the detergent-rich phase (data not shown).

Identification of Phosphatidylinositol-Linked Integral Membrane Proteoglycans

Recent studies suggest that some integral membrane PGs are anchored in the membrane by attachment to glycosyl-phosphatidylinositol (GPI) lipids (Ishihara et al., 1987; Carey and Evans, 1989; Yanagishita and McQuillan, 1989). GPI linkages are also characteristic of other developmentally regulated nervous system molecules such as N-CAM-120 (He et al., 1986) and Thy-1 (Low and Kincade, 1985). To determine whether any of the integral membrane PGs identified in rat brain exhibit this type of linkage, labeled membrane-associated PGs were treated with neuraminidase,

Table 5. Proteoglycans of the Soluble Fraction

Name	GAG Type	Apparent M_r of Core Protein				DEAE Elution	Relative Abundance		
		Non-reduced		Reduced					
		-N'dase	+N'dase	-N'dase	+N'dase	(M NaCl)	E18	P0	Adult
S1	ChS	500[a]	400	ND	ND	0.53			
S2	ChS	235	215	230	195				
S3	ChS	165	155	180	165	0.45			
S4	ChS	140	135	160	155				
S5	ChS	140	130	155	150	0.45			
S6	ChS	110	110	115	115				
S7	ChS	93	90	100	100				
S8	ChS	85	80	87	80				
S9	HeS	63	60	60	60	0.41			

[125] I-labeled soluble fraction PGs from E18, P0, and adult rat brain were subjected to SDS gel analysis after enzymatic treatments. For explanation, see Table 4. ND, not determined (standards appropriate for accurate M_r determination in this range were not available). Horizontal bars of four different widths have been used to convey a qualitative ranking of PGs into those that are of very high (▬), high (—), moderate (—), and low (—) abundances, as determined from autoradiograms such as those in Figure 2.

then chondroitinase ABC and heparitinase with or without the addition of phosphatidylinositol-specific phospholipase C (PI-PLC). Samples were then subjected to Triton X-114 phase partitioning, and the aqueous and detergent-rich phases were analyzed by SDS-PAGE.

The results, shown in Figure 5, indicate that the detergent phase partitioning of two PGs, M12 (a major heparan sulfate PG) and M13 (a heparan sulfate PG found only in E18 and P0 samples), is significantly reduced by pretreatment with PI-PLC. In addition, a 100 kd non-PG protein found only in the adult sample (Figure 5, lower asterisk) is also shifted from the detergent to aqueous phase by PI-PLC treatment. Importantly, the detergent partitioning behavior of some molecules (e.g., a 120 kd non-PG in the adult sample [Figure 5, upper asterisk]) was not altered by PI-PLC, indicating that presence of the enzyme does not interfere with the partitioning process itself. Thus, the results imply that the detergent binding properties of M12, M13, and the 100 kd protein depend on covalent linkage to a phosphatidylinositol lipid.

Discussion

Isolation of Brain Proteoglycans

This report describes the isolation and identification of rat brain PGs and the characterization of changes in PG core protein expression during brain development. The method used for isolation of a PG-enriched fraction relies on the ability of sulfated PGs to remain bound to DEAE-cellulose even under chaotropic (6 M urea) and acidic (pH 3.5) conditions. The material obtained is sufficiently depleted of non-PG proteins that individual PGs can be identified by the bands that appear on SDS gels following digestions with GAG lyases. Such methods have been used by others (e.g., Bretscher, 1985; Woods et al., 1985; Coster et al., 1986) for isolating PGs of cultured cells and, when combined with radioiodination (Lories et al., 1987), appear to provide a general and highly sensitive approach for analyzing the PG composition of cells or tissues.

PGs were isolated from subcellular fractions of rat brain in the amounts indicated in Table 2. These figures probably underestimate the abundance of brain PGs for four reasons: First, protein was measured using amido black binding; this anionic dye might be expected to bind less well to PGs than to other proteins. Second, some PGs may fail to bind initially to DEAE-Sephacel because they associate with other proteins in the tissue extract (this phenomenon could explain why, when labeled PGs were added back to initial tissue extracts, 5% of labeled material failed to rebind DEAE-Sephacel [Table 3]). Third, some brain PGs may elute prematurely during fractionation (e.g., PGs with low GAG content, or PGs with unsulfated GAGs); such PGs would not have been detected in this study. Fourth, some PGs may be lost because of failure to elute from DEAE-Sephacel. Indeed, even PGs that have been eluted once from the ion exchange matrix are not completely reelutable from a second round of chromatography (note loss of 44% in Table 3). Apparently, such losses result from the fact that application of a "step" of high salt to DEAE-Sephacel results in a PG peak followed by a "tail" of material that continues to elute for many column volumes (and is usually too dilute for recovery to be practical). Enzymatic analysis has so far detected no differences in PG composition between such peak and tail fractions, nor has treatment of columns with 1.2 M or 2.0 M

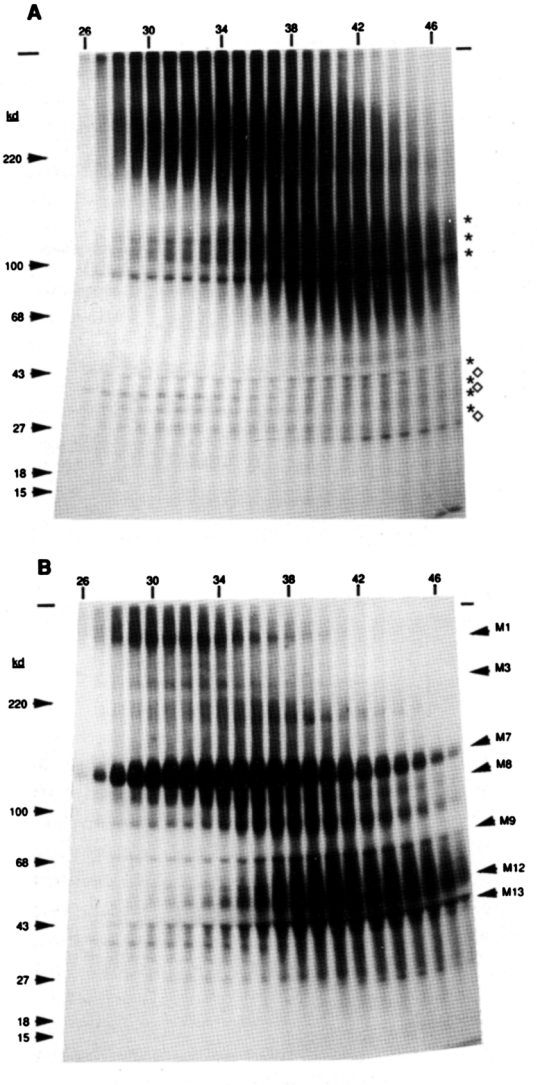

Figure 3. Gel Filtration Analysis of Membrane-Associated Proteoglycans

Radioiodinated P0 membrane-associated PGs were subjected to gel filtration as described in the text. Samples of each column fraction were subjected to 5%–15% SDS–PAGE (nonreducing) either before (A) or after (B) digestion with neuraminidase, heparitinase, and chondroitinase ABC. V_0 and V_t were at fractions 27 and 50, respectively. Column fraction numbers are indicated at top. Molecular weight markers are shown to the left of each gel; arrowheads at right identify putative PG core proteins mentioned in Table 4. Bands identified by asterisks in (A) represent non-PG proteins that exhibit fractionation behavior suggestive of aggregation. Several non-PG proteins that do not exhibit this behavior are marked in (A) with diamonds. Although the same non-PG proteins are present in (B), only the PG core proteins have been marked. For further discussion, see text.

NaCl eluted PGs that are any different in amount or composition from what is eluted by 0.75 M NaCl (unpublished data).

Despite these potential sources of loss or of underestimation of PG content, it is noteworthy that the amounts of PG–protein in Table 2 are nonetheless in the same range as the amounts of total sulfated GAG reported in similar subcellular fractions (see Margolis et al., 1975a; Werz et al., 1985a, 1985b).

Evidence of Proteoglycan Diversity

The results summarized in Tables 4 and 5 suggest that as many as 25 PG core proteins may be expressed in the mammalian brain at various times during development. To minimize the possibility that some of the molecular species identified result from proteolysis during homogenization or fractionation, all steps were carried out quickly, in the cold, and in the presence of protease inhibitors. Significant differences in molecular weights or relative abundances of core proteins among independent preparations were not seen, nor were changes in apparent PG composition detected following long-term storage of either tissue extracts or purified PGs. Steps were also taken to ensure that proteolysis did not occur during exposure of PGs to GAG lyases (see Results). Indeed, the unique patterns of fractionation exhibited by PGs subjected to gel filtration, Triton X-114 partitioning, and salt gradient elution from DEAE-cellulose (Tables 4–5; Figures 3–5) imply that multiple distinct PG species clearly exist *before* treatment of samples with GAG lyases.

Even barring proteolysis, however, it is possible that Tables 4 and 5 might still overestimate the diversity of PG species in the brain. Some species identified in the membrane fraction may be identical or related to species of similar M_r that are found in the soluble fraction (e.g., M1 and S1; M12 and S9). Some putative core proteins that are similar in M_r may actually represent forms of a single protein that have been modified (e.g., by glycosylation) differently (e.g., S4 and S5; M5 and M6; M13 and M14). Ultimately, questions of relatedness among PG species will need to be settled using techniques such as peptide mapping or with antibody probes (e.g., Lories et al., 1989). Recently, Oohira et al. (1988) used tryptic peptide mapping to show that three chondroitin sulfate PGs of the 10-day-old rat brain are unrelated to each other. Significantly, the extraction properties and M_r values of those PGs suggest that they are identical to species S2, S3, and S4, whereas the two less abundant species identified by Oohira et al. (1988) may correspond to S5 and S8.

There are also reasons to suspect that the brain contains PGs not included on Tables 4 and 5. Unambiguous identification of putative core proteins was difficult in some cases because of overlapping of electrophoretic bands, and low-abundance PGs may have been missed. Indeed, faint signals on some autoradiograms suggested the possible existence of additional PG cores of 850, 600, and 140 kd in brain soluble fractions and 850 and 540 kd in brain membrane fractions. In addition, any keratan sulfate PGs that are present in brain would not be included in Tables 4 and 5, since keratanase or endo-β-galactosidase digestions were not carried out. Biochemical evidence indicates that very little keratan sulfate is present in brain (Werz et al., 1985a, 1985b; Eronen et al., 1985). However, recent immunohistochemical studies suggest that

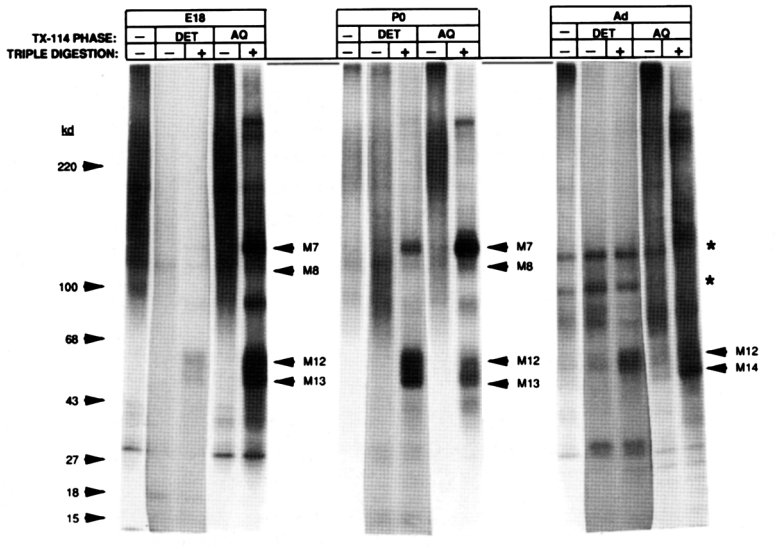

Figure 4. Triton X-114 Phase Partitioning of Membrane-Associated Proteoglycans

Radioiodinated membrane-associated PGs from E18, P0, and adult brain were subjected to Triton X-114 partitioning as described in the text. Detergent-enriched (DET) and aqueous (AQ) phases of partitioning were subjected to nonreducing 5%–15% SDS–PAGE before (–) and after (+) digestion with neuraminidase, heparitinase, and chondroitinase ABC (TRIPLE DIGESTION). Molecular weight markers are shown to the left of each gel. Some proteins exhibit slightly altered mobility in DET lanes, presumably because of the high concentrations of Triton X-114 in these samples. Although the figure shows results from a single gel, lanes marked DET were exposed 3-fold longer than AQ lanes. In general, molecules that partitioned into detergent phases did not do so quantitatively. This was particularly true of intact PGs. In contrast, detergent partitioning of PG cores (following GAG lyase digestion) tended to be more efficient (see Figure 5), consistent with the widely held view that highly hydrophilic moieties (such as GAG chains or other carbohydrates) hinder partitioning into detergent phases (see Clemetson et al., 1984; Volk and Geiger, 1986). Alternatively, biphasic partitioning can reflect the existence of distinct forms of a single protein, as has been suggested for human erythrocyte band 3 protein (Swanson et al., 1988).

the small amount of keratan sulfate present is restricted to a developmentally interesting structure, the roofplate (Snow et al., 1990b). It will undoubtedly be important in the future to extend the data reported here to include keratan sulfate PGs.

These considerations notwithstanding, the data presented suggest that distinct sets of PGs are found in the membrane-associated and soluble fractions of rat brain; that heparan sulfate PGs are more abundant in the membrane-associated fraction, whereas chondroitin sulfate PGs are more abundant in the soluble fraction; that dermatan sulfate PGs, if they are present in brain, are relatively rare (none were detected); and that the expression of PGs changes considerably during nervous system development. These data are in good aggreement with available information on brain GAGs, namely, that heparan sulfate is most abundant in membranes, whereas chondroitin sulfate is found mostly in the soluble fraction (Margolis et al., 1975a); that low levels of dermatan sulfate are found in brain (Werz et al., 1985a, 1985b; Burkart and Wiesmann, 1987); and that levels of GAGs change considerably during brain development (Margolis et al., 1975b; Werz et al., 1985b; Burkart and Wiesmann, 1987).

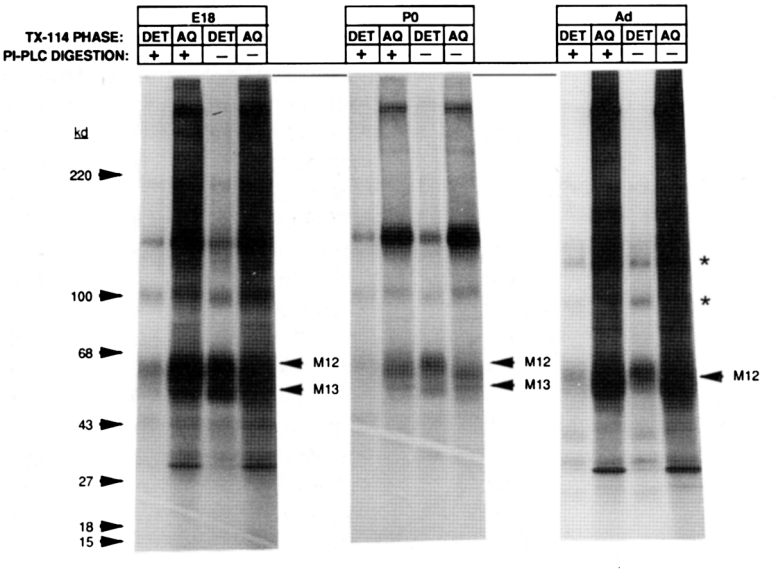

Figure 5. Glycosyl-Phosphatidylinositol-Linked Proteoglcans

Radioiodinated membrane-associated PGs from E18, P0, and adult brain were digested with neuraminidase, then heparitinase and chondroitinase ABC either with (+) or without (–) the addition of PI-PLC. Samples were then subjected to Triton X-114 partitioning, 5%–15% nonreducing SDS–PAGE, and autoradiography as described in the text. Molecular weight markers are shown to the left of each gel. Some proteins exhibit slightly altered mobility in DET lanes, presumably because of the high concentrations of Triton X-114 in these samples. Two major heparan sulfate PGs (M12 and M13) and a non-PG protein found only in adult brain (M_r 100 kd; lower asterisk) demonstrate a significant shift from the detergent to the aqueous phase when pretreated with PI-PLC, indicating that these proteins are GPI-anchored membrane proteins. For further discussion see text.

The data in Figure 1 and Tables 4 and 5 also suggest that many PG cores are sialylated. In particular, the electrophoretic band representing M7, the most abundant species found associated with neonatal membranes, shifts and sharpens considerably with neuraminidase treatment, suggesting that sialic acid might contribute significantly to the net charge of M7. The data in Figures 1 and 2 and Tables 4 and 5 do not provide evidence that PGs contain more than one type of GAG. It is possible, however, that some of the core proteins that were identified as having a single type of GAG may contain small amounts of a second GAG type (see Ripellino and Margolis, 1989). Also, if some core proteins exist predominantly as PGs containing either heparan sulfate or chondroitin sulfate, and at lower levels as hybrid heparan sulfate/chondroitin sulfate PGs, it is unlikely that the contributions of the hybrid species to gels such as those in Figures 1 and 2 would have been noticed.

Most of the putative core protein species in Tables 4 and 5 exhibit M_r values that are relatively similar when reduced and nonreduced. Given the inaccuracies inherent in estimating M_r under nonreducing conditions, the data are consistent with the interpretation that most brain PG cores consist of single polypeptide chains. One possible exception is M16. On nonreducing gels, no putative core protein with an M_r similar to that of M16 is detected, suggesting that M16 may be disulfide-linked to another polypeptide. Given its small apparent size, M16 could have been derived from one of the higher M_r core proteins without significantly altering the mobility of the latter. However, comparison of the gel filtration behavior of M16 with that of larger heparan sulfate PG cores (by analysis of the samples in Figure 3 under reducing conditions) has so far failed to suggest a particular species with which M16 is associated (data not shown). Thus, M16 may derive from a unique species whose presence prior to reduction is masked by other electrophoretic bands.

Biochemical Properties and Developmental Changes: Implications for Potential Functions of Brain Proteoglycans

One striking implication of Tables 4 and 5 is that the expression of several PG core proteins in the nervous system changes dramatically over the course of development. Previous studies had shown that GAG levels, GAG synthesis, and GAG turnover change considerably during brain development (Margolis et al., 1975b; Werz et al., 1985b; Burkart and Wiesmann, 1987). Although such changes could result from alterations in GAG biosynthesis and breakdown per se, the present study raises the alternative possibility that such changes primarily reflect shifts in the types and amounts of core proteins expressed by neural cells.

The developmental changes seen in Tables 4 and 5 may provide clues about the physiological roles of PGs. For example, PGs that appear only postnatally, such as M4, M5, M14, and M15, may be involved in late developmental events such as myelination or in stabilization of synapses, whereas PGs such as M2, M3, M6, and M13, which are restricted to embryonic or neonatal brain, might play a role in processes such as cell migration or axon outgrowth. Conceivably, better correlation of PG expression with developmental events could be achieved by comparing PG expression among regions of the brain that develop at distinctly different times.

The biochemical properties of PGs can also suggest functional roles. For example, integral membrane PGs are thought to mediate cell–cell and cell–substratum adhesion by associating with molecules such as N-CAM (Cole et al., 1985) and fibronectin (Saunders and Bernfield, 1988). It would therefore be interesting to examine the binding of PGs such as M12 and M13 to neural cell adhesion molecules and molecules of the neural extracellular matrix.

PGs that are associated with the membrane fraction but do not exhibit hydrophobic partitioning may represent PGs of the extracellular matrix. This possibility was raised in connection with PGs such as M7, M8, and M9, which exhibit unusual gel filtration behavior suggestive of aggregation (Figure 3). Extracellular matrix PGs of the nervous system are currently thought to play a role in the storage of neurotrophic factors such as the fibroblast growth factors (Walicke, 1989), as well as in the localization of synapse-specific molecules such as acetylcholinesterase (Brandan et al., 1985) and the modulation of neurite outgrowth (Muir et al., 1989; Snow et al., 1990a).

At present it is unclear whether M7, M8, and M9 aggregate with themselves, with each other, or with non-PG proteins. It is noteworthy, however, that such behavior was observed when dilute samples of PGs were analyzed (in Figure 3, PGs were applied at 17 μg of protein per ml, implying that individual PG species were in the range of 1–70 nM), as well as when samples were chromatographed in moderately chaotropic buffers. In an experiment similar to that described in Figure 3, P0 membrane PGs were chromatographed in the presence of 6 M urea and 0.75 M NaCl and broadly smeared elution was still observed for M7 and for 46, 41, and 35 kd non-PGs (data not shown); these observations suggest that at least M7 participates in interactions of relatively high affinity.

Two membrane-associated PGs (M12 and M13) were found to be linked to GPI anchors. So far, GPI-linked PGs have been detected only on the surfaces of hepatocytes, Schwann cells, and ovarian granulosa cells (Ishihara et al., 1987; Carey and Evans, 1989; Yanagishita and McQuillan, 1989). Since M12 and M13 are relatively abundant brain PGs, it is possible that they, along with N-CAM-120 and Thy-1, are among the major GPI-linked constituents of neural cell surfaces. Currently, the physiological role of GPI anchors is not understood, although it has been pointed out that many GPI-linked proteins are thought to be involved

in cell adhesion or cell signaling and that shedding of GPI-linked molecules can be triggered by cell-associated PI-PLC, with the concomitant production of diacylglycerols (Low and Saltiel, 1988). Interestingly, the properties of S9, a PG of the soluble fraction, are sufficiently similar to those of M12 to suggest that the former may represent a shed form of the latter.

In summary, the mammalian brain, a structure that contains a diverse set of developmentally important proteins capable of binding GAGs and PGs, apparently also contains a diverse set of developmentally regulated heparan sulfate and chondroitin sulfate PGs. Uncovering the functions of these PGs will require identifying both the cells that express them and the molecules that bind them. In this regard, it will be important to determine whether any of the PGs described here correspond to neural PGs that have been defined immunochemically (e.g., NG2 [Levine and Card, 1987; Stallcup and Beasley, 1987], Cat-301 [Zaremba et al., 1989], and an HSPG of PC12 cells [Matthew et al., 1985]), neural PGs that have been described in other species (e.g., TAP-1 [Carlson and Wight, 1987] and cytotactin binding PG [Hoffman et al., 1988]), or PGs found in tissues outside the central nervous system.

Experimental Procedures

Materials

Heparitinase was prepared from Flavobacterium heparinum by hydroxyapatite chromatography as described by Linker and Hovingh (1972). Chondroitinase ABC and chondroitinase AC were obtained from Sigma, neuraminidase from Arthrobacter ureafaciens was from Calbiochem, and PI-PLC from Bacillus cereus was from Boehringer Mannheim. Triton X-100 and Triton X-114 were obtained from Sigma; CHAPS was from Boehringer Mannheim. Protease inhibitors, obtained from Sigma, were phenylmethylsulfonyl fluoride, N-ethylmaleimide, pepstatin A, and EDTA. Unless otherwise stated in the text, the term "protease inhibitors" should be taken to mean the combination of 1 mM EDTA, 1 μg/ml pepstatin, 0.25 mg/ml N-ethylmaleimide (added within 1 hr of use), and 0.4 mM phenylmethylsulfonyl fluoride (added within 5 min of use).

Subcellular Fractionation

Whole brains from embryonic, neonatal, and adult Sprague-Dawley rats were obtained as follows. Adult rats were asphyxiated with CO_2, and brains were rapidly removed to dishes of ice-cold saline (0.9% NaCl). Newborn (P0) rats were anesthetized by cooling on ice, and brains removed to ice-cold saline. E18 animals were dissected under ice-cold saline. Brains were stripped free of all visible meninges, washed in saline, resuspended in 9 vol of ice-cold buffer A (0.3 M sucrose, 4 mM HEPES [pH 7.5] containing protease inhibitors), and homogenized using a Teflon-on-glass homogenizer (Thomas Scientific) with pestle rotation provided by a Wheaton overhead stirrer (model 903475, setting 4). Homogenization and all subsequent steps were carried out quickly at 4°C.

Homogenates were centrifuged at low speed (12,000 × g for 30 min), and pellets were rehomogenized in buffer A. Following recentrifugation, the two supernatants were pooled. This material was then centrifuged at high speed (378,000 × g for 30 min). The resulting supernatant, the brain soluble fraction, was clarified by centrifugation at 378,000 × g for 60 min, followed by 0.2 μm filtration. The membrane-containing pellet from the first high speed spin was then homogenized in buffer B (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 1.0% CHAPS, 1 mM EDTA, 1 μg/ml pepstatin A) and centrifuged at 378,000 × g for 1 hr. The resulting pellet was rehomogenized in buffer B and centrifuged at 423,500 × g for 40 min. The two buffer B supernatants, representing a detergent extract of a crude brain membrane fraction, were pooled and clarified by 0.2 μm filtration. Protein concentrations were determined using amido black binding (Schaffner and Weissman, 1973).

Proteoglycan Purification and Radioiodination

PGs were purified by anion exchange chromatography on DEAE-Sephacel (Pharmacia) equilibrated in buffer C (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 0.5% CHAPS). Samples in buffer B were loaded directly onto columns containing the gel; samples in buffer A were first made 0.15 M in NaCl and 0.5% in CHAPS. Column volumes of 0.5 ml of packed gel per of mg protein were used. Columns were eluted stepwise with buffer C, buffer D (50 mM Tris-HCl [pH 8.0], 0.25 M NaCl, 0.1% Triton X-100), buffer E (50 mM Tris-HCl [pH 8.0], 6 M urea, 0.25 M NaCl, 0.1% Triton X-100), and buffer F (50 mM sodium formate [pH 3.5], 6 M urea, 0.2 M NaCl, 0.1% Triton X-100). Column pH was restored with 50 mM Tris-HCl (pH 8.0), 0.5% CHAPS before elution of PGs with buffer G (50 mM Tris-HCl [pH 8.0], 0.75 M NaCl, 0.5% CHAPS).

Material eluted with buffer G was diluted 5-fold with 50 mM Tris-HCl (pH 8.0), 0.5% CHAPS and batch-adsorbed to 100 μl of packed volume DEAE Spectra/Gel M (Spectrum Scientific), equilibrated in buffer C. The gel was washed extensively with 50 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and bound material was radioiodinated using chloramine-T, as described by Lories et al. (1987) with the modification that 5 mCi of ^{125}I were used per reaction and reaction time was reduced to 3 min. After extensive washing with buffer C, radioiodinated material was eluted with buffer G. After measurement of protein concentration and radioactive specific activity, crystalline grade BSA was added to 1 mg/ml and aliquots were frozen and stored at −80°C.

Enzymatic Analysis of Proteoglycans

Neuraminidase digestions were carried out in 25 mM Tris-acetate (pH 5.0) containing 0.16 mg/ml hemoglobin (human, 4× recrystallized; Sigma) and protease inhibitors. The enzyme was used at 0.05 U/ml for 1 hr at 37°C. Heparitinase, chondroitinase AC, and chondroitinase ABC digestions were carried out in 50 mM Tris-phosphate (pH 7.0) containing 0.16 mg/ml hemoglobin and protease inhibitors. Heparitinase was used at 0.4 μg/ml for 1 hr at 37°C, conditions empirically determined to give complete digestion of heparan sulfate PGs without demonstrable chondroitinase activity. Chondroitinase AC and ABC were used at 0.05 U/ml for 1 hr at 37°C. PI-PLC digestion was carried out in 50 mM Tris-phosphate (pH 7.5), 0.16% Triton X-114, 0.16 mg/ml hemoglobin, 1 mM EDTA, 1 μg/ml pepstatin A; the enzyme was used at 0.05 U/ml for 1 hr at 37°C. For all enzyme reactions, volumes were adjusted so that NaCl contributed from the PG-containing sample reached a final concentration of <0.2 M or, in the case of phospholipase C digestion, <0.01 M.

Enzyme-treated samples were analyzed by SDS-PAGE (Laemmli, 1970) under reducing and nonreducing conditions. Controls included both untreated and sham-digested samples. Molecular weights were determined using prestained protein standards (Bethesda Research Laboratories) and, in some experiments, samples of mouse laminin and human plasma fibronectin (detected by Coomassie blue staining). Gels were dried and autoradiographed against preflashed Kodak XAR film at −80°C.

Gel Filtration of Neonatal Membrane Proteoglycans

To 100 μl of PGs in buffer G, solid sucrose was added to 5%, and the mixture was loaded onto a Sepharose CL-4B (Pharmacia) column (0.5 × 18 cm), equilibrated in 50 mM Tris-HCl (pH 8.0), 0.5 NaCl, 0.5% CHAPS, 1 mg/ml crystalline BSA, and protease inhibitors. Flow rate was 0.4 ml/hr, and ~85 μl fractions were collected. V_0 and V_t were determined by elution of β-galactosidase aggregates and $^{35}SO_4$, respectively.

Triton X-114 Partitioning

PGs and enzyme-digested PGs were subjected to Triton X-114 phase partitioning as described by Bordier (1981). Briefly, PGs were diluted into 100 μl of 50 mM Tris–phosphate (pH 7.0), 2% Triton X-114 (sufficient to decrease the concentration of CHAPS to <0.005%) and were layered over a cushion of 6% sucrose, 0.06% Triton X-114 in 50 mM Tris–phosphate (pH 7.0). After incubation for 5 min at 35°C, the tube was centrifuged at 1000 × g for 10 min at 25°C. The upper phase was removed, Triton X-114 was added to 0.5%, and the mixture was layered again over the original sucrose cushion. After incubation and centrifugation as above, the pellet (~10 μl) was retrieved and diluted to 100 μl in 50 mM Tris–phosphate (pH 7.0) (detergent-rich phase). The top phase was also saved and retreated with Triton X-114 at 2%, incubated, and centrifuged. The top phase was saved (aqueous phase).

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References

Aquino, D. A., Margolis, R. U., and Margolis, R. K. (1984). Immunocytochemical localization of a chondroitin sulfate proteoglycan in nervous tissue. II. Studies in developing brain. J. Cell Biol. *99*, 1130–1139.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. *256*, 1604–1607.

Brandan, E., Maldonado, M., Garrido, J., and Inestrosa, N. C. (1985). Anchorage of collagen-tailed acetylcholinesterase to the extracellular matrix is mediated by heparan sulfate proteoglycans. J. Cell Biol. *101*, 985–992.

Bretscher, M. S. (1985). Heparan sulphate proteoglycans and their polypeptide chains from BHK cells. EMBO J. *4*, 1941–1944.

Burkart, T., and Weismann, U. N. (1987). Sulfated glycosaminoglycans (GAG) in the developing mouse brain: quantitative aspects on the metabolism of total and individual sulfated GAG in vivo. Dev. Biol. *120*, 447–456.

Carbonetto, S., Gruver, M. M., and Turner, D. C. (1983). Nerve fiber growth in culture on fibronectin, collagen, and glycosaminoglycan substrates. J. Neurosci. *3*, 2324–2335.

Carey, D., and Evans, D. M. (1989). Membrane anchoring of heparan sulfate proteoglycans by phosphatidylinositol and kinetics of synthesis of peripheral and detergent-solubilized proteoglycans in Schwann cells. J. Cell Biol. *108*, 1891–1897.

Carlson, S. S., and Wight, T. N. (1987). Nerve terminal anchorage protein one (TAP-1) is a chondroitin sulfate proteoglycan: biochemical and electron microscopic characterization. J. Cell Biol. *105*, 3075–3086.

Clemetson, K. J., Beinz, D., Zahno, M. L., and Luscher, F. F. (1984). Distribution of platelet glycoproteins and phosphoproteins in hydrophobic and hydrophilic phases in Triton X-114 phase partition. Biochim. Biophys. Acta *778*, 463–469.

Cole, G. J., Schubert, D., and Glaser, L. (1985). Cell–substratum adhesion in chick neural retinal depends upon protein–heparan sulfate interactions. J. Cell Biol. *100*, 1192–1199.

Coster, L., Carlstedt, I., Kendall, S., Malmstrom, A., Schmidtchem, A., and Fransson, L.-A. (1986). Structure of proteoheparan sulfates from fibroblasts: confluent and proliferating fibroblasts produce at least three types of proteoheparan sulfates with functionally different core proteins. J. Biol. Chem. *261*, 12079–12088.

Cunningham, D. D., VanNostrand, W. E., Farrell, D. H., and Campbell, C. H. (1986). Interactions of serine proteases with cultured fibroblasts. J. Cell. Biochem. *32*, 281–291.

Eronen, I., Mononen, T., and Mononen, I. (1985). Isolation and partial characterization of keratan sulphate from human brain. Biochim. Biophys. Acta *843*, 155–158.

Gloor, S., Odink, K., Guenther, J., Nick, H., and Monard, D. (1986). A glia-derived neurite promoting factor with protease inhibitory activity belongs to the protease nexins. Cell *47*, 687–693.

Gordon, H., and Hall, Z. W. (1989). Glycosaminoglycan variants in the C2 muscle cell line. Dev. Biol. *135*, 1–11.

Hassell, J. R., Kimura, J. H., and Hascall, V. C. (1986). Proteoglycan core protein families. Annu. Rev. Biochem. *55*, 539–567.

He, H.-T., Barbet, J., Chaix, J.-C., and Goridis, C. (1986). Phosphotidylinositol is involved in the membrane attachment of NCAM-120, the smallest component of the neural cell adhesion molecule. EMBO J. *5*, 2489–2494.

Hoffman, S., Crossin, K. L., and Edelman, G. M. (1988). Molecular forms, binding functions, and developmental expression patterns of cytotactin and cytotactin-binding proteoglycan, an interactive pair of extracellular matrix molecules. J. Cell Biol. *106*, 519–532.

Ishihara, M., Fedarko, N. S., and Conrad, H. E. (1987). Involvement of phosphatidylinositol and insulin in the coordinate regulation of proteoheparan sulfate metabolism and hepatocyte growth. J. Biol. Chem. *262*, 4708–4716.

Kato, M., Oike, Y., Suzuki, S., and Kimata, K. (1985). Selective removal of heparan sulfate chains from proteoheparan sulfate with a commercial preparation of heparitinase. Anal. Biochem. *148*, 479–484.

Kiang, W.-L., Margolis, R. U., and Margolis, R. K. (1981). Fractionation and properties of a chondroitin sulfate proteoglycan and the soluble glycoproteins of brain. J. Biol. Chem. *256*, 10529–10537.

Klinger, M. M., Margolis, R. U., and Margolis, R. K. (1985). Isolation and characterization of the heparan sulfate proteoglycans of brain. J. Biol. Chem. *260*, 4082–4090.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Lander, A. D. (1987). Molecules that make axons grow. Mol. Neurobiol. *1*, 213–245.

Lander, A. D. (1989). Understanding the molecules of neural cell contacts: emerging patterns of structure and function. Trends Neurosci. *12*, 189–195.

Levine, J. M., and Card, J. P. (1987). Light and electron microscopic localization of a cell surface antigen (NG2) in the rat cerebellum: association with smooth protoplasmic astrocytes. J. Neurosci. *7*, 2711–2720.

Lindahl, U., Thunberg, L., Backstrom, G., Riesenfeld, J., Nordling, K., and Bjork, I. (1984). Extension and structural variability of the antithrombin-binding sequence in heparin. J. Biol. Chem. *259*, 12368–12376.

Linker, A., and Hovingh, P. (1972). Heparinase and heparitinase from flavobactera. Meth. Enzymol. *28*, 902–910.

Lobb, R. R., Harper, J. W., and Fett, J. W. (1986). Purification of heparin-binding growth factors. Anal. Biochem. *154*, 1–14.

Lories, V., DeBoeck, H., David, G., Cassiman, J.-J., and VanDenBerghe, H. (1987). Heparan sulfate proteoglycans of human lung fibroblasts: structural heterogeneity of the core proteins of the hydrophobic cell-associated forms. J. Biol. Chem. *262*, 854–859.

Lories, V., Cassiman, J.-J., VanDenBerghe, H., and David, G. (1989). Multiple distinct membrane heparan sulfate proteoglycans in human lung fibroblasts. J. Biol. Chem. *264*, 7009–7016.

Low, M. G., and Kincade, P. W. (1985). Phosphatidylinositol is the membrane anchoring domain of the Thy-1 glycoprotein. Nature *318*, 62–64.

Low, M. G., and Saltiel, A. R. (1988). Structural and functional roles of glycosyl-phosphatidylinositol in membranes. Science *239*, 268–275.

Marcum, J. A., Reilly, C. F., and Rosenberg, R. D. (1987). Heparan sulfate species and blood vessel wall function. In Biology of Proteoglycans. T. N. Wight and R. P. Mecham, eds. (Orlando: Academic Press, Inc.), pp. 301–343.

Margolis, R. K., Margolis, R. U., Preti, C., and Lai, D. (1975a). Distribution and metabolism of glycoproteins and glycosaminoglycans in subcellular fractions of brain. Biochemistry *14*, 4797–4804.

Margolis, R. U., Margolis, R. K., Chang, L. B., and Preti, C. (1975b). Glycosaminoglycans of brain during development. Biochemistry *14*, 85–88.

Matthew, W. D., Greenspan, R. J., Lander, A. D., and Reichardt, L. F. (1985). Immunopurification and characterization of a neuronal heparan sulfate proteoglycan. J. Neurosci. *5*, 1842–1850.

Morris-Kay, G. M., and Crutch, B. (1982). Culture of rat embryos with beta-D-xyloside: evidence of a role for proteoglycans in neurulation. J. Anat. *134*, 491–506.

Muir, D., Engvall, E., Varon, S., and Manthorpe, M. (1989). Schwannoma cell-derived inhibitor of the neurite-promoting activity of laminin. J. Cell Biol. *109*, 2353–2362.

Neufeld, G., Gospodarowicz, D., Dodge, L., and Fujii, D. K. (1987). Heparin modulation of the neurotrophic effects of acidic and basic fibroblast growth factors and nerve growth factor on PC12 cells. J. Cell. Physiol. *131*, 131–140.

Oohira, A., Matsui, F., Matsuda, M., Takida, Y., and Koboki, Y. (1988). Occurrence of three distinct molecular species of chondroitin sulfate proteoglycan in the developing rat brain. J. Biol. Chem. *263*, 10240–10246.

Perris, R., and Johansson, S. (1987). Amphibian neural crest cell migration on purified extracellular matrix components: a chondroitin sulfate proteoglycan inhibits locomotion on fibronectin substrates. J. Cell Biol. *105*, 2511–2521.

Ratner, N., Bunge, R. P., and Glaser, L. (1985). A neuronal cell surface heparan sulfate proteoglycan is required for dorsal root ganglion neuron stimulation of Schwann cell proliferation. J. Cell Biol. *101*, 744–754.

Ratner, N., Hong, D., Lieberman, M. A., Bunge, R. P., and Glaser, L. (1988). The neuronal cell-surface molecule mitogenic for Schwann cells is a heparin-binding protein. Proc. Natl. Acad. Sci. USA *85*, 6992–6996.

Ripellino, J. A., and Margolis, R. U. (1989). Structural properties of the heparan sulfate proteoglycans of brain. J. Neurochem. *52*, 807–812.

Ruoslahti, E. (1989). Proteoglycans in cell regulation. J. Biol. Chem. *264*, 13369–13372.

Sanes, J. R. (1989). Extracellular matrix molecules that influence neural development. Annu. Rev. Neurosci. *12*, 491–516.

Saunders, S., and Bernfield, M. (1988). Cell surface proteoglycan binds mouse mammary epithelial cells to fibronectin and behaves as a receptor for interstitial matrix. J. Cell Biol. *106*, 423–430.

Saunders, S., Jalkanen, M., O'Farrell, S., and Bernfield, M. (1989). Molecular cloning of syndecan, an integral membrane proteoglycan. J. Cell Biol. *108*, 1547–1556.

Schaffner, W., and Weissman, C. (1973). A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal. Biochem. *56*, 502–514.

Schubert, D., and LaCorbiere, M. (1982). The specificity of extracellular glycoprotein complexes in mediating cellular adhesion. J. Neurosci. *2*, 82–89.

Schubert, D., LaCorbiere, M., and Esch, F. (1986). A chick neural retina adhesion and survival molecule is a retinol-binding protein. J. Cell Biol. *102*, 2295–2301.

Schubert, D., Ling, N., and Baird, A. (1987). Multiple influences of a heparin-binding growth factor on neuronal development. J. Cell Biol. *104*, 635–644.

Schubert, D., LaCorbiere, M., Saitoh, T., and Cole, G. (1989). Characterization of an amyloid β precursor protein that binds heparin and contains tyrosine sulfate. Proc. Natl. Acad. Sci. USA *86*, 2066–2069.

Snow, D. M., Lemman, V., Carrino, D. A., Caplan, A., and Silver, J. (1990a). Sulfated proteoglycans present in astroglial barriers during development in vivo inhibit neurite outgrowth in vitro. Exp. Neurol., in press.

Snow, D. M., Steindler, D. A., and Silver, J. (1990b). Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier. Dev. Biol. *138*, 359–376.

Stallcup, W. B., and Beasley, L. (1987). Bipotential glial precursor cells of the optic nerve express the NG2 proteoglycan. J. Neurosci. *7*, 2737–2744.

Swanson, M. L., Keast, R. K., Jennings, M. L., and Pessin, J. E. (988). Heterogeneity in the human erythrocyte band 3 anion-transporter revealed by Triton X-114 phase partitioning. Biochem. J. *255*, 229–234.

Volk, T., and Geiger, B. (1986). A-CAM: a 135 kD receptor of intercellular adherens junctions. I. Immunoelectron microscopic localization and biochemical studies. J. Cell Biol. *103*, 1441–1450.

Walicke, P. A. (1988). Interactions between basic fibroblast growth factor (FGF) and glycosaminoglycans in promoting neurite outgrowth. Exp. Neurol. *102*, 144–148.

Walicke, P. A. (1989). Novel neurotrophic factors, receptors, and oncogenes. Annu. Rev. Neurosci. *12*, 103–126.

Wang, Y.-J., and Mahler, H. R. (1976). Topography of the synaptosomal membrane. J. Cell Biol. *71*, 639–658.

Werz, W., Fischer, G., and Schachner, M. (1985a). Glycosaminoglycans of rat cerebellum: I. Quantitative analysis of the main constituents at postnatal day 6. J. Neurochem. *44*, 900–906.

Werz, W., Fischer, G., and Schachner, M. (1985b). Glycosaminoglycans of rat cerebellum: II. A developmental study. J. Neurochem. *44*, 907–910.

Woods, A., Couchman, J. R., and Hood, M. (1985). Heparan sulfate proteoglycans of rat embryo fibroblasts. J. Biol. Chem. *260*, 10872–10879.

Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. (1981). Silver staining of proteins in polyacrylamide gels. Anal. Biochem. *118*, 197–203.

Yanagishita, M., and McQuillan, D. J. (1989). Two forms of plasma membrane-intercalated heparan sulfate proteoglycan in rat ovarian granulosa cells: labeling of proteoglycans with a photoactivatable hydrophobic probe and effect of the membrane anchor-specific phospholipase C. J. Biol. Chem. *264*, 17551–17558.

Zaremba, S., Guimaraes, A., Kalb, R. G., and Hockfield, S. (1989). Characterization of an activity-dependent, neuronal surface proteoglycan identified with monoclonal antibody Cat-301. Neuron *2*, 1207–1219.