Cerebroglycan: An Integral Membrane Heparan Sulfate Proteoglycan That Is Unique to the Developing Nervous System and Expressed Specifically during Neuronal Differentiation

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Abstract. Heparan sulfate proteoglycans (HSPGs) are found on the surface of all adherent cells and participate in the binding of growth factors, extracellular matrix glycoproteins, cell adhesion molecules, and proteases and antiproteases. We report here the cloning and pattern of expression of cerebroglycan, a glycosylphosphatidylinositol (GPI)-anchored HSPG that is found in the developing rat brain (previously referred to as HSPG M13; Herndon, M. E., and A. D. Lander. 1990. *Neuron.* 4:949–961). The cerebroglycan core protein has a predicted molecular mass of 58.6 kD and five potential heparan sulfate attachment sites. Together with glypican (David, G., V. Lories, B. Decock, P. Marynen, J.-J. Cassiman, and H. Van den Berghe. 1990. *J. Cell Biol.* 111:3165–3176), it defines a family of integral membrane HSPGs characterized by GPI linkage and conserved structural motifs, including a pattern of 14 cysteine residues that is absolutely conserved. Unlike other known integral membrane HSPGs, including glypican and members of the syndecan family of transmembrane proteoglycans, cerebroglycan is expressed in only one tissue: the nervous system. In situ hybridization experiments at several developmental stages strongly suggest that cerebroglycan message is widely and transiently expressed by immature neurons, appearing around the time of final mitosis and disappearing after cell migration and axon outgrowth have been completed. These results suggest that cerebroglycan may fulfill a function related to the motile behaviors of developing neurons.

PROTEOGLYCANS (PGs)[1]—proteins that bear glycosaminoglycan (GAG) side chains—are abundant surface components of all adherent cells, and are thought to play important roles in cell growth, morphogenesis and cancer (7, 23, 57). Most commonly, cell-surface PGs bear GAG chains of the heparan sulfate (HS) class and, because of this, have the potential to interact with many extracellular heparin-binding proteins, including extracellular matrix molecules, cell-cell adhesion molecules, cell-surface enzymes, and growth factors (35, 43). It has been proposed that cell-surface HSPGs act as "co-receptors" for such proteins, functioning to bring them together with more conventional molecules (e.g., signal-transducing receptors) on the cell surface (7). Strong evidence for this model comes from studies of the interaction of basic fibroblast growth factor with one of its tyrosine kinase receptors, an interaction that fails to occur in the absence of HS (36, 54, 78). Additional evidence has also come from studies of cell-surface protease inhibition by heparin-binding anti-proteases (17, 49), homophilic cell adhesion mediated by the neural cell adhesion molecule NCAM (16, 55), and integrin-mediated cell–matrix interaction (44, 75).

The co-receptor model provides a useful framework within which to understand the general functions of HSPGs, but leaves open the question of whether specific functions are associated with individual molecular species of HSPG. Currently, five distinct polypeptides are known to be major carriers of HS on mammalian cells. Four of these are transmembrane proteins, and form a gene family, the syndecans, on the basis of strong evolutionary conservation in their cytoplasmic and transmembrane domains (7). The fifth is a glycosylphosphatidylinositol (GPI)-anchored protein known as glypican (19). The expression of these PG core proteins varies dramatically from tissue to tissue, especially during the course of development (7). Tissue-specific and developmental variation also occurs in the structure of the HS that is found on these core proteins, and such variation can give rise to substantial differences in the binding properties of intact PGs (49, 51, 58, 62, 63). Taken together, the data sug-

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1. *Abbreviations used in this paper*: GAG, glycosaminoglycan; GAPDH, glyceraldehyde-phosphate dehydrogenase; GPI, glycosylphosphatidylinositol; HS, heparan sulfate; PG, proteoglycans.

gest that individual HSPGs may indeed be specialized to carry out particular functions.

To explore this possibility, we have chosen to focus on the developing mammalian nervous system, a tissue in which heparin-binding molecules mediate numerous cell–cell, cell–matrix, and cell-growth factor interactions (42), and in which developmental processes occur with precise timing and anatomical localization. Previously, we demonstrated that ~25 PG core proteins can be detected in the developing and adult rat brain (30). Of these, three species exhibited detergent partitioning properties indicative of integral membrane proteins. These three molecules, all of which are HSPGs, are thus likely to represent the major cell surface PGs of the central nervous system. One of these ("M7," ref. 30) is indistinguishable in biochemical properties and timing of expression from a member of the syndecan family, N-syndecan, or syndecan III, which is expressed in perinatal brain, peripheral glia, developing limbs, and other tissues (11, 28). The second ("M12") is the GPI-anchored HSPG glypican (46). The third species ("M13") is also a GPI-anchored HSPG, and could be isolated from the developing brain, but not from adult (30).

We report here on studies that identify the M13 core as a novel membrane protein. We confirm that it is expressed only during development and demonstrate that, unlike other PGs, M13 is restricted in expression to a single tissue—the nervous system. Moreover, although this PG is widely expressed in many regions of the developing nervous system, the data suggest that expression is associated with neurons and is closely linked, in each region, to the time period when postmitotic neurons undergo migration and axon outgrowth, their major morphogenetic movements. The deduced amino acid sequence of this PG, which we call cerebroglycan, indicates that it, together with glypican, defines a new family of cell-surface HSPGs, the GPI-anchored PGs. Interestingly, the core proteins in this family exhibit an unexpectedly high degree of structural homology, raising the possibility that these molecules have evolved for more specialized functions than simply to be the passive bearers of GAG chains.

Materials and Methods

Materials

Heparitinase was purified from Flavobacterium heparinum by hydroxyapatite and phosphocellulose chromatography (45). The GAPDH cDNA clone was the generous gift of Dr. Timothy Hayes (National Institutes of Health [NIH], Bethesda, MD). All restriction enzymes were from New England Biolabs (Beverly, MA). Chondroitinase ABC, Triton X-100, NEM (*N*-ethylmaleimide), pepstatin A, and PMSF, Hoechst 33258 dye (bisbenzimide), and PVP-40 (polyvinyl pyrrolidone) were obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin, RNAse A, CHAPS (3-[(3 Cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate), and yeast RNA were from Boehringer Mannheim Corp. (Indianapolis, IN). RNA and DNA ladders were from GIBCO BRL (Gaithersburg, MD). Nitrocellulose was from Schleicher and Schuell (Keene, NH). DEAE-Sephacel, dextran sulfate, salmon sperm DNA, and Taq DNA polymerase were from Pharmacia Fine Chemicals (Piscataway, NJ). Formamide was from Fluka (Buchs, Switzerland). α^{32}P-labeled dCTP and α^{35}S-labeled UTP were from DuPont NEN (Boston, MA). All other reagents were from Mallinckrodt (Paris, KY), except as noted.

Purification and Microsequencing of HSPG M13

Detergent extracts of a crude membrane fraction of neonatal (P0) Sprague-Dawley rat brains were prepared (30) and stored at −80°C. Extracts from a total of 46.4 g (wet weight) of brain were used to purify PGs by anion exchange chromatography on DEAE-Sephacel (30). At this point, 0.3 μCi of ^{125}I-labeled, membrane-associated PGs, purified and radiolabeled as previously described (30), was added to the PG pool as a tracer.

Centriprep-10 tubes (Amicon Corp., Beverly, MA) were used to concentrate the eluate and exchange the elution buffer for 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% Triton X-100. The sample was then treated with Chondroitinase ABC (0.09 U/ml) in the presence of 1 mM EDTA, 1 μg/ml pepstatin, and 0.4 mM PMSF for 2 h at 37°C, and loaded immediately onto a DEAE-Sephacel column (0.2 ml packed volume), equilibrated in 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% Triton X-100. The column was washed successively with 0.2 M NaCl, 100 mM sodium acetate, pH 3.5, 0.1% Triton X-100; 0.1 M Tris-HCl, pH 8.0, 0.1% Triton X-100; 50 mM Tris-HCl, pH 8.0, 0.1% Triton X-100; and 25 mM ammonium acetate, pH 7.0, 0.1% Triton X-100. An HSPG-enriched fraction was eluted with a 20-ml gradient of NaCl from 0.15 to 0.75 M, in 25 mM ammonium acetate, pH 7.0, and 0.1% Triton X-100. All radioactive fractions eluted by ≥0.3 M NaCl were pooled. In this manner, approximately 80 μg of HSPG were recovered (as determined by amino acid analysis).

A Centricon-10 (Amicon Corp.) tube was used to concentrate the pooled sample and exchange the buffer to 50 mM NaCl, 25 mM ammonium acetate, 0.1% Triton X-100. The sample was made 25 mM in Tris-HCl (pH 7.1 at 37°C), and protease inhibitors added along with heparitinase to a final concentration of 9 μg/ml. The HSPG sample was digested for 3 h at 37°C, concentrated in a Centricon-10 tube and loaded to a 9% SDS-PAGE gel. A control digest of identical composition but with no HSPGs was loaded in an adjacent lane of the gel. After electrophoresis, the gel was electroblotted overnight at 25 V and 4°C to nitrocellulose in 25 mM Tris-HCl, pH 8.3, 20% (vol/vol) ethanol, 0.005% SDS. Bands were visualized by staining with Amido Black (64), and the band corresponding to HSPG M13 was excised, as was a corresponding region from the control lane.

The nitrocellulose-bound HSPG M13 was blocked with PVP-40 (0.5% in 100 mM acetic acid at 37°C for 30 min) and then digested with trypsin for 18 h at 37°C in 100 mM ammonium carbonate/acetonitrile (95:5 by volume) (68). Released digestion products were analyzed by reverse phase HPLC on a Hewlett Packard model 1090L HPLC equipped with a Vydac 218TP52 C18 column (Vydac, Hesperia, CA), and multiple peaks of absorbance at 210 nm were collected and subjected to microsequencing by automated Edman degradation.

PCR Amplification of an HSPG-M13 cDNA

Degenerate PCR primers were designed based on the sequence of peptide M13-22 (see Results and Table I). The sequences of the primers were 22L: 5′-GGCCTCTAGA(T/C)ATGCA(T/C)GA(T/C)GCNGA-3′ and 22R: 5′-GCGCGGGCCCCT(G/A)TCNGC(G/A)TA(C/T)TG(C/T)TG 3′. PCR reactions were performed using 25 pmol of each PCR primer, 2 μg of P0 rat brain cDNA as template, 80 μM of each dNTP, and 2 U of Taq polymerase. The reaction profile was denature at 95°C for 60 s, anneal at 50°C for 90 s, and extend for 90 s at 72°C.

Library Construction and Screening

P0 rat brain polyA-enriched RNA was prepared by fractionation on oligo dT cellulose using a Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). cDNA was synthesized from 5 μg of poly(A)$^+$ RNA using an oligo dT primer with the ZAP cDNA synthesis kit (Stratagene, La Jolla, CA). The resulting cDNA was ligated into EcoRI–XhoI digested and dephosphorylated Uni-ZAP XR vector arms (Stratagene) and packaged for plating (Giga Pack Gold II; Stratagene). The library was amplified once before plating on XL1-Blue host cells. The library was screened with a probe generated by a PCR reaction (as described above), which included α^{32}P-dATP.

A primer-extended library was synthesized from 5 μg of polyA-enriched RNA (prepared as described above) and 120 pmol of primer M13A-165 (based on the sequence of cDNA M13-A, see Results) using a Timesaver cDNA synthesis kit (Pharmacia Fine Chemicals). The resulting cDNA, ligated to EcoRI adaptors, was cloned into EcoRI-digested, dephosphorylated λ-gt10 arms (Stratagene), packaged (Giga Pack Gold II; Stratagene), and plated on NM514 host cells for screening with a probe generated from the 100 bp BglII fragment of M13-A (see Results) by random-primer labeling (70200 Random Primed DNA Labeling Kit; United States Biochemical Corp., Cleveland, OH).

DNA Sequencing

Gel purified fragments were subcloned into pBluescript SK$^-$ (Stratagene)

or pSL301 (Invitrogen). cDNA inserts were sequenced by the dideoxy chain termination method using a modified T7 DNA polymerase (Sequenase kit 2.0; United States Biochemical Corp.). T3, T7, and M13 (−40) primers, as well as custom made primers, were used in reactions with denatured, supercoiled plasmids. All sequences were determined with both dGTP and dITP.

Southern Blot Analysis

Rat genomic DNA was prepared from Sprague-Dawley rat thymocytes according to Sambrook et al. (60), and stored at 4°C under ethanol. 20 μg of DNA, digested to completion with HindIII, KpnI, or XhoI, was electrophoresed and blotted to nitrocellulose according to Sambrook et al. (60). Prehybridization, hybridization, and washing, were done according to Church and Gilbert (14). The filter was autoradiographed with XAR-5 film (Eastman Kodak Co., Rochester, NY).

Northern Blot Analysis

Total RNA was isolated by guanidinium isothiocyanate extraction (13). 20 μg of total RNA from embryonic day 18 (E18), P0, and adult rat brain was used for the Northern analysis, performed according to Sambrook et al. (60), except that yeast RNA (200 μg/ml) and salmon sperm DNA (100 μg/ml) were included in the hybridization buffer. Filters were exposed to Kodak XAR-5 film or imaged with a phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA). The blot was re-probed for glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA to normalize for different loading and transfer efficiences between the lanes.

In Situ Hybridization

Freshly dissected rat embryos were quick-frozen in isopentane on dry ice and stored at −80°C. Before sectioning, samples were equilibrated to −20°C and embedded in OCT medium. 12–20-μm cryostat sections were collected on gelatin-subbed slides or on Probe-On Plus slides (Fisher Biotech; Pittsburgh, PA) and stored at −80°C with desiccant. Sections were equilibrated to room temperature, fixed in 4% paraformaldehyde in PBS at room temperature for 5 min, and washed twice in 2× SSC. Prehybridization treatments, hybridization, and post-hybridization washes were performed according to Simmons et al. (67), except that the proteinase K step was omitted. After hybridization, sections were exposed to hyperfilm βmax (Amersham Corp., Arlington Heights, IL) for 5–7 d at −80°C. Selected sections were dipped in Kodak NTB-2 liquid emulsion that had been diluted 1:1 in ddH_2O, and exposed for 12–21 days at −80°C. Developed sections were stained in Hoechst 33258 (bisbenzimide) at 1 μg/ml in ddH_2O or cresyl violet (0.5% in ddH_2O), and examined by fluorescence, bright field, and dark field illumination on a Zeiss Axiophot microscope, or a Nikon Diaphot microscope.

Results

Molecular Cloning of HSPG M13

To establish the molecular identity of the M13 core protein, a PG-enriched fraction was prepared by DEAE-chromatography of a detergent extract of neonatal rat brain membranes (30). The PG fraction was depleted of chondroitin sulfate PGs by treatment with chondroitinase ABC and refractionation on DEAE-Sephacel. After heparitinase digestion, separation by SDS-PAGE, and electroblotting to nitrocellulose, Amido Black staining revealed two major species. On the basis of the previously characterized molecular weights of neonatal rat brain membrane HSPG cores (30) one species was identified as HSPG M13, and the other as M12. Both bands were excised from the membrane and separately digested in situ with trypsin. The peptides released were separated by HPLC chromatography and selected peaks chosen for amino acid sequencing.

The data obtained from HSPG M12 indicated that this molecule is the rat homologue of glypican (46). In contrast, the four M13-derived peptides that were obtained (Table I) did not closely match any sequences in the NBRF database, but could be aligned with human glypican so as to reveal an overall sequence identity of 58%. These data suggested that M13 might be a glypican-related molecule.

Table I. HSPG M13 Peptide Sequence

Peptide	Sequence
M13-9	R(K)SS(W)C(S)EERPPTTA(R)A(G)G(KY)TN(L)LXR(H)
M13-10	RAL(KSS)VAAR
M13-21	K(R)A(I)S(C)E(S)GL(W)LGMHLQENSVK
M13-22	KA(RI)C(W)AAL(G)GQDLDMHDADEDASGSGGGQQYADD (22L … 22R)

Selected M13 tryptic peptides were subjected to automated Edman degradation after separation by reverse-phase HPLC. Multiple letters indicate ambiguities in the sequence, bold letters indicate the sequence which was later verified from the deduced amino acid sequence of M13 cDNA. 22L and 22R: bars indicate the unambiguous amino acid sequence upon which the PCR primers 22L and 22R were based.

To facilitate further characterization of HSPG M13, a cDNA was obtained from neonatal rat brain by PCR. Using primers 22L and 22R, which were based on unambiguous sequence at either end of peptide M13-22 (Table I), a major PCR product of ~80 bp was obtained. This product was subcloned and two independent clones were isolated and sequenced. Both contained the sequences of primers 22L and 22R, and the intervening sequences of both gave a deduced protein sequence which was an exact match for peptide M13-22 (data not shown), verifying that a genuine M13 cDNA had been amplified.

To obtain a complete cDNA, a neonatal rat brain cDNA library was screened with a probe generated by PCR using α^{32}P-labeled dATP and primers 22L and 22R, with the gel-purified 80-bp M13 cDNA fragment as the template. Three positive plaques were identified out of 900,000 screened in the amplified library. All three were plaque purified, and all contained inserts of ~1,600 bp. Preliminary sequence data supported the conclusion that all three inserts were identical products of a single cloning event. Additional sequencing of this cDNA, referred to hereafter as M13-A, showed that it contained an 862-bp open reading frame followed by 659 bp of 3′ untranslated sequence, and was unlikely to encode full length M13. Therefore, a primer extended library was constructed using neonatal rat brain RNA and a primer based on sequence 165 bp from the 5′ end of M13-A. The library was screened with a random-primer labeled probe generated from the 100 bp BglII fragment of M13-A (see Fig. 1), and 52 positives were identified out of 9×10^4 plaques. Two of these, M13PX-1 and M13PX-2, were plaque purified and found to contain inserts of ~1,200 and ~1,100 bp, respectively. The combined length of M13-A and the overlapping M13PX-1 is an excellent match for the size of the M13 mRNA observed in Northern analyses (see below), indicating that together, M13-A and M13PX-1 constitute an es-

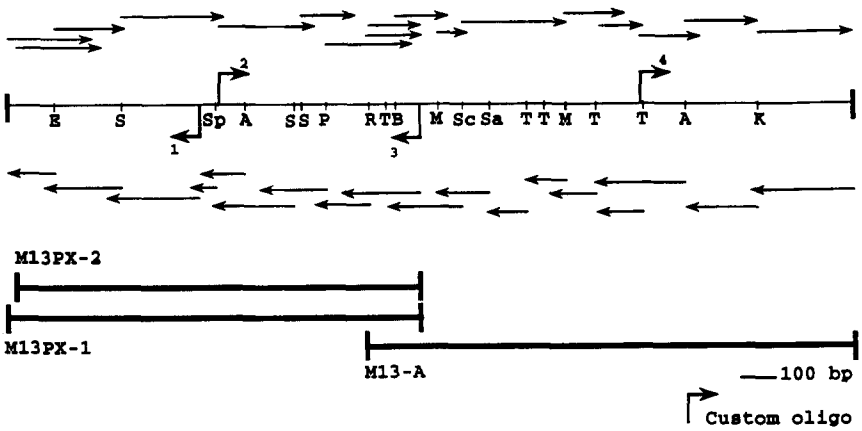

Figure 1. HSPG M13 cDNA sequencing strategy. The restriction sites used in subcloning and sequencing are indicated by letters: *A*, ApaI; *B*, BglII; *E*, EagI; *K*, KpnI; *M*, MspI; *P*, PstI; *R*, EcoRV; *S*, SmaI; *Sa*, Sau3AI; *Sc*, SacI; *Sp*, SphI; and *T*, TaqI. Arrows indicate individual sequencing reactions. The custom oligonucleotides used in sequencing are numbered. Oligonucleotide three was used as the primer in the construction of the primer extended library. The three M13 cDNAs are indicated by the heavy lines below the restriction map.

sentially full length M13 cDNA. Fig. 1 illustrates how the complete M13 cDNA sequence was obtained from M13-A, M13PX-2, and M13PX-1 by a combination of subcloning and the use of custom sequencing primers.

Sequence Analysis

The combined sequences of M13-A, M13PX-2, and M13PX-1 comprise a cDNA of 2,631 bp, shown in Fig. 2. The first ATG codon (at position 70), occurs in a poor context for initiation of translation (40), and the open reading frame that follows terminates after 240 bp. The second ATG codon (at position 236) occurs in a favorable context, with a G in the −3 position. Beginning with this codon, an open reading frame extends 1,740 bases before terminating at position 1975. An AATAAA cleavage and polyadenylation signal is found at position 2582, followed 13 bases later by a poly-A tract. The deduced amino acid sequence of the long open reading frame correctly predicts the sequences of all four M13 tryptic peptides (underlined in Fig. 2), including the three that were not used in screening the cDNA library.

The deduced amino acid sequence features a stretch of hydrophobic amino acids at both the amino and carboxy termini, which could serve as a signal sequence and a GPI-attachment signal respectively. HSPG M13 was previously shown to be GPI-linked on the basis of its ability to be released from Triton X-114 micelles by phosphatidylinositol specific-phospholipase C (30). With the removal of these sig-

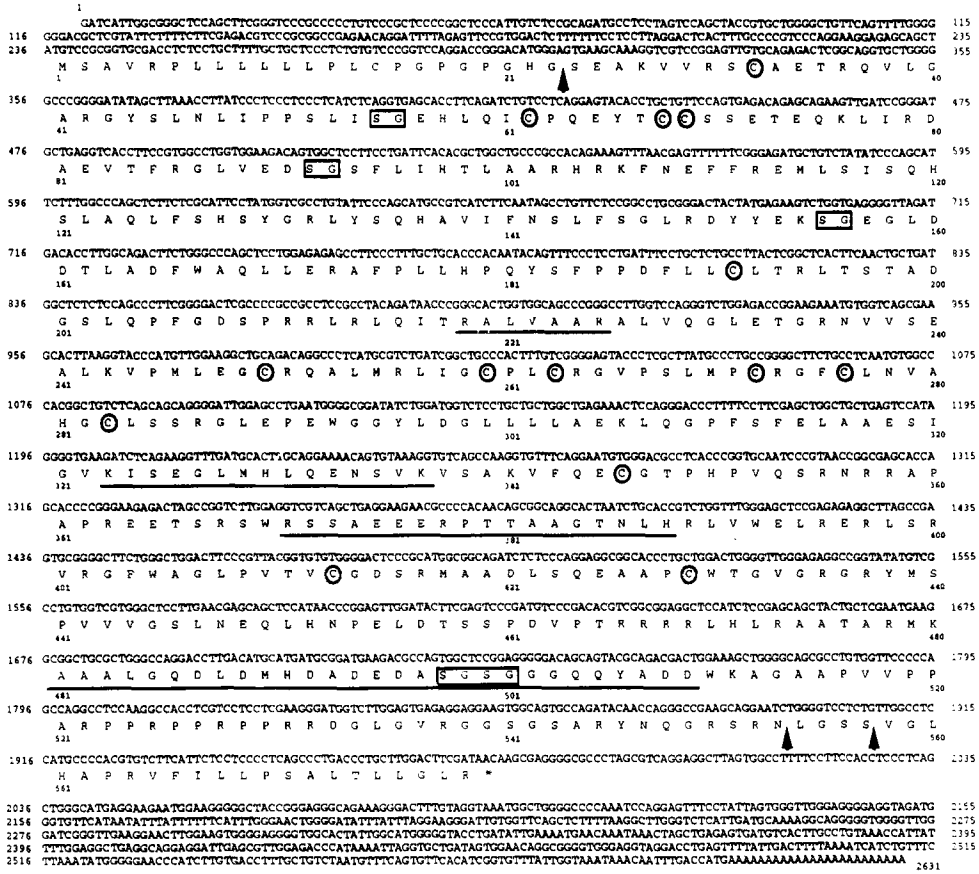

Figure 2. Nucleotide sequence of HSPG M13 cDNA and translation. The merged sequences of cDNAs M13-A, M13PX-1, and M13PX-2 are given along with the deduced amino acid sequence of M13. Potential glycosaminoglycan attachment sites are boxed. Cysteine residues are circled. Arrowheads indicate potential cleavage sites of the signal sequence (after Gly^{21}) and the GPI attachment signal sequence (after Asn^{553} or Ser^{557}). (See text for discussion.) Sequences that correspond to M13 peptides (Table I) are underlined. The cerebroglycan cDNA sequence data are available from GenBank/EMBL under accession number L20468.

nal sequences, the mature protein would have a predicted molecular mass of 58.6-kD, in good agreement with the size of the M13 core protein, as observed on reducing SDS-PAGE gels (57 kD) (30). The protein contains seven "ser-gly" sequences, five of which (boxed in Fig. 2) are potential GAG attachment sites (see Discussion). No "N-X-S" or "N-X-T" N-linked glycosylation consensus sequences are present.

As Fig. 3 illustrates, the M13 protein sequence shows significant homology to two other molecules, rat glypican (37, 46), and the deduced protein sequence of OCI-5, a developmentally regulated transcript cloned from a primitive intestinal epithelial rat cell line (25). The homology is moderate but extensive, with islands of highly conserved sequence surrounded by divergent sequences. Most striking is the conservation of cysteine residues (heavy lines in Fig. 3): Of the 14 cysteines in the predicted sequence of the mature M13 protein, all are conserved in both glypican and OCI-5. In fact, the only cysteines in mature glypican and OCI-5 that are not found in M13 are a single pair near the COOH terminus. In addition, glypican and M13 share three putative sergly GAG attachment sites (Fig. 3, *boxes*), and one of these is present in OCI-5 as well. M13 and glypican are more closely related to each other, with an amino acid identity of 37%, than either M13 to OCI-5 or glypican to OCI-5, with 20% and 21% identities, respectively.

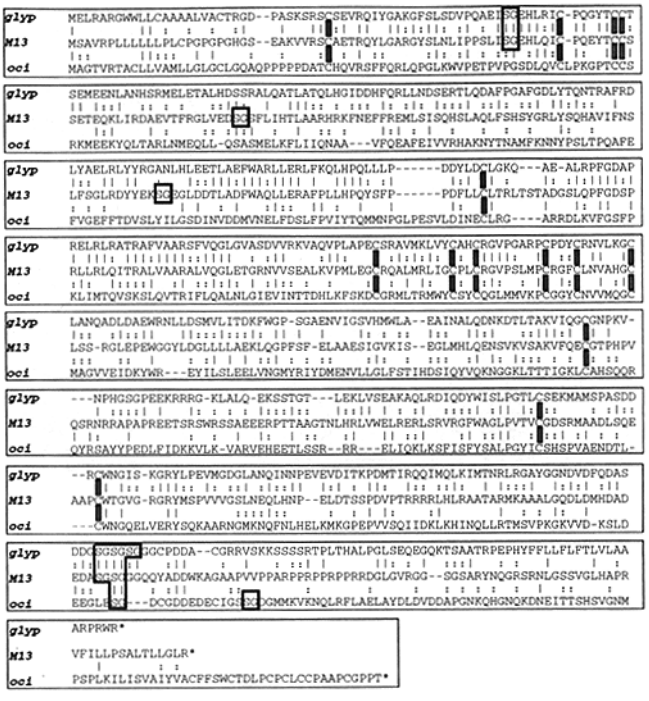

Figure 3. HSPG M13 is related to glypican and OCI-5. The protein sequence of HSPG M13 has been aligned with those of the GPI-linked HSPG, glypican, and the predicted protein product of OCI-5, a developmentally regulated transcript isolated from a rat primitive intestinal epithelial cell line (25). Solid lines indicate sequence identities, and dotted lines indicate conservative substitutions. The cysteine pattern of the three molecules is absolutely conserved as indicated by the heavy lines. Potential glycosaminoglycan attachment sites are boxed.

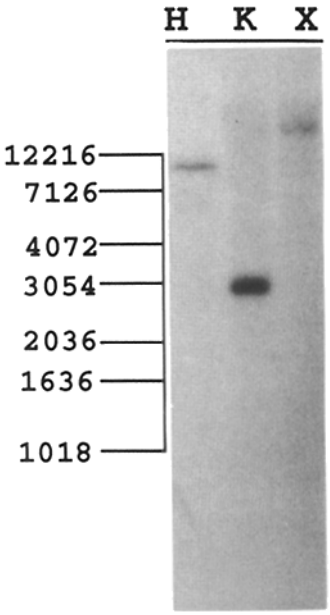

Figure 4. Southern analysis. 20 μg of rat genomic DNA was digested to completion with one of three restriction enzymes, separated by electrophoresis, blotted to nitrocellulose, and probed with an α^{32}P-dCTP–labeled M13 probe. The sizes of DNA standards are indicated. The restriction enzymes used were HindIII (*H*), KpnI (*K*), and XhoI (*X*).

Southern Hybridization

To assess whether M13 is present as a single copy in the genome, 10 μg of rat thymus DNA was digested to completion with HindIII, KpnI, or XhoI, separated by agarose gel electrophoresis, and transferred to nitrocellulose. The filter was probed with a random primer-labeled probe made from the 100-bp BglII fragment of the M13-A cDNA. The resulting autoradiogram is shown in Fig. 4. In each lane a single band appears indicating that the gene encoding M13 is present as a single copy in the rat genome.

Developmental Expression

M13 is observed in the membrane fraction of embryonic day 18 (E18) and newborn (P0) rat brain, but not in the adult (30). To assess the expression of M13 mRNA, Northern blot analysis was performed using 20 μg of total RNA from each of these three developmental stages, and a random-primer–

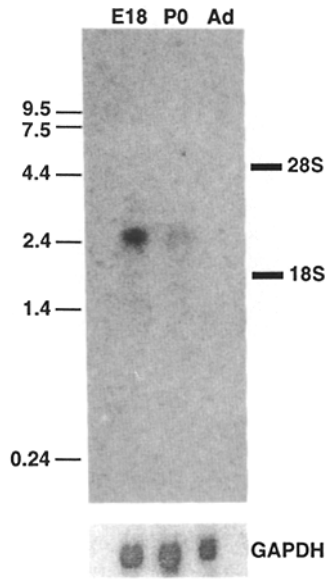

Figure 5. Northern analysis. 20 μg of rat brain total RNA isolated from embryonic day 18 (*E18*), newborn (*P0*), and adult (*A*) rats was subjected to electrophoresis through formaldehyde agarose and transferred to nitrocellulose. A 2.7-kb transcript was detected with an α^{32}P-dCTP–labeled M13 probe. (*Lower panel*) Reprobing of the blot with a GAPDH probe demonstrated that a similar amount of RNA was present in each lane. The positions of molecular weight markers and ribosomal RNA bands are indicated.

labeled probe generated using cDNA M13-A as the template (Fig. 5). A single transcript of ~2.7 kb was seen that is relatively abundant at E18, less so at P0, and undetectable in the adult sample. The filter was re-probed with a glyceraldehyde-phosphate dehydrogenase (GAPDH) probe (Fig. 5, *lower panel*), demonstrating that there was not an appreciable difference in the amount of RNA loaded in each lane. Thus, the developmental expression of M13 mRNA agrees with that previously observed for M13 protein (30).

To localize M13 expression in the developing animal, in situ hybridization was performed. The 306 bp SacI or the 223 bp ApaI–KpnI pBluescript subclones of the M13 cDNA, M13-A (Fig. 1), were used to generate α^{35}S-UTP labeled RNA probes. Fig. 6 shows the results obtained when these probes were hybridized to fresh-frozen sections of embryonic day 14 (E14), 16 (E16), and 19 (E19), and newborn (P0) animals. The results are presented as "reverse contrast" images, printed directly from autoradiograms of the sections.

In E14 and E16 animals (Fig. 6, *B* and *D*) strong M13 expression occurs along the entire neuraxis, including the brain, spinal cord, dorsal root ganglia, and cranial nerve

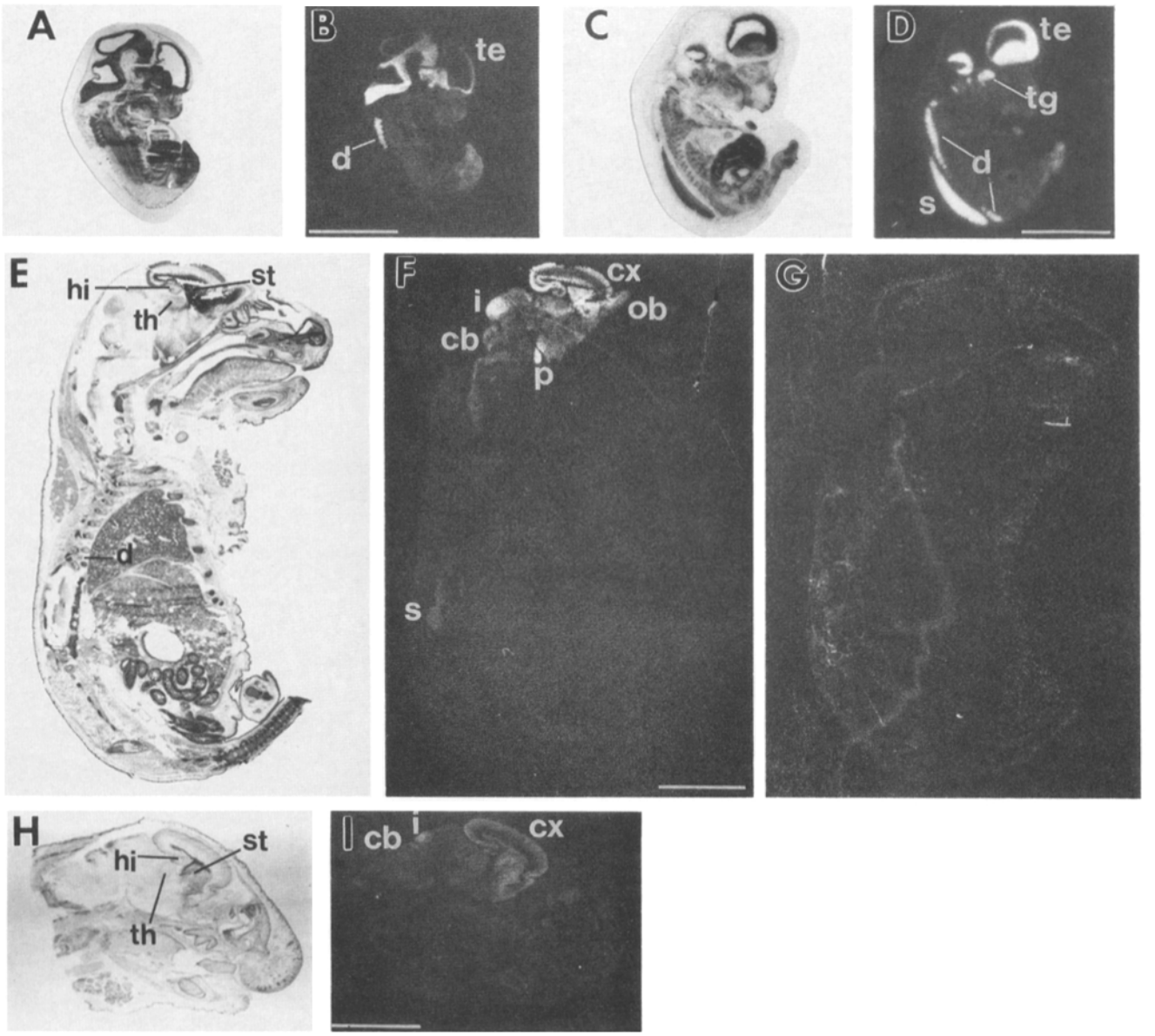

Figure 6. PG M13 expression in the developing nervous system. (*A*, *C*, *E*, and *H*) Cresyl violet stained sections. (*B*, *D*, *F*, *G*, and *I*) reverse contrast images printed directly from autoradiograms of the same sections hybridized to α^{35}S-UTP labeled, in vitro transcribed, M13 RNA probe. (*A* and *B*) embryonic day 14 whole embryo; (*C* and *D*) embryonic day 16 whole embryo; (*E–G*) embryonic day 19 whole embryo; (*H* and *I*) neonatal head. (*s*) spinal cord; (*te*) telencephalon; (*cx*) cerebral cortex; (*cb*) cerebellar cortex; (*d*) dorsal root ganglia; (*p*) pontine nuclei; (*i*) inferior colliculus; (*ob*) olfactory bulb; (*hi*) hippocampus; (*st*) striatum, (*th*) thalamus. *G* shows the result when a sense orientation (negative control) probe was hybridized to an adjacent section of the embryonic day 19 animal; the image was printed at high contrast to allow visualization of the faint background. Bar, 5 mm.

ganglia. No expression in any non-neural tissue could be detected. By E19 (Fig. 6 *F*), expression in the spinal cord and dorsal root ganglia is dramatically lower than in the many regions of the brain that still express high levels of M13 message, including the cerebral cortex, hippocampus, corpus striatum, inferior colliculus, and pontine nuclei. As at earlier stages, hybridization to non-neural tissues was not above background. In the P0 head (Fig. 6 *I*), strong expression persists in the cerebral cortex, hippocampus, striatum, inferior colliculus, and cerebellar cortex, but only weak expression is seen elsewhere in the brain. Again, expression was not observed outside the nervous system. Fig. 6 *G* shows an example of the results obtained when the opposite strand control probe was hybridized to a nearly adjacent section (in this case, in the E19 animal). No hybridization above background was observed in any tissue.

As Fig. 6 illustrates, M13 is globally expressed in the nervous system at early stages. As neural development proceeds expression is selectively lost from certain structures while remaining in others. A closer examination of the pattern of expression suggested that M13 mRNA remains in structures for several days after neurogenesis—the production of postmitotic neurons—has ended (see Discussion). This raised the possibility that M13 expression might be associated with neuronal precursors and/or immature neurons.

To more accurately examine the relationship between M13 expression and neuronal development, in situ hybridization was carried out using sections of postnatal day 7 (P7) brain. At this age, the generation and early development of neurons has finished in most of the brain, but persists in three late-developing areas: the external granule layer of the cerebellum, the dentate gyrus of the hippocampus, and interneurons of the olfactory bulb (34). As shown in Fig. 7, M13 is expressed at this stage in precisely these three areas: the folia of the cerebellum, the dentate gyrus of the hippocampus, and the olfactory bulb. As with the earlier experiments, no hybridization with the opposite-strand control probe was detected (not shown).

A higher magnification view of the cerebellum (Fig. 8 *A*) reveals that M13-expressing cells are found in the external granule layer (EGL), the zone in which granule cells (cerebellar interneurons) are generated and undergo initial stages (~28 h) of development (26). In contrast, the internal granule layer (IGL), which contains mature granule cells, was essentially devoid of hybridization. A higher magnification view of the olfactory bulb (not shown) revealed that M13-expressing cells are localized to the subventricular zone, the site of postnatal production of olfactory bulb interneurons (granule cells and periglomerular cells) (48). In contrast, layers containing mature olfactory bulb neurons were devoid of M13 hybridization (these layers lie anterior to the indicated region of hybridization in Fig. 7, and were visualized by Hoechst 33258 fluorescence) (not shown).

These results suggest that, at least in these tissues, expression of M13 is restricted to an early stage in the development of neurons. An indication of how early M13 appears can be obtained by examining the distribution of cells within the external granule layer of the cerebellum. As shown in Fig. 8 *A*, M13-expressing cells are strongly concentrated within the deeper half of the external granule layer, the so-called

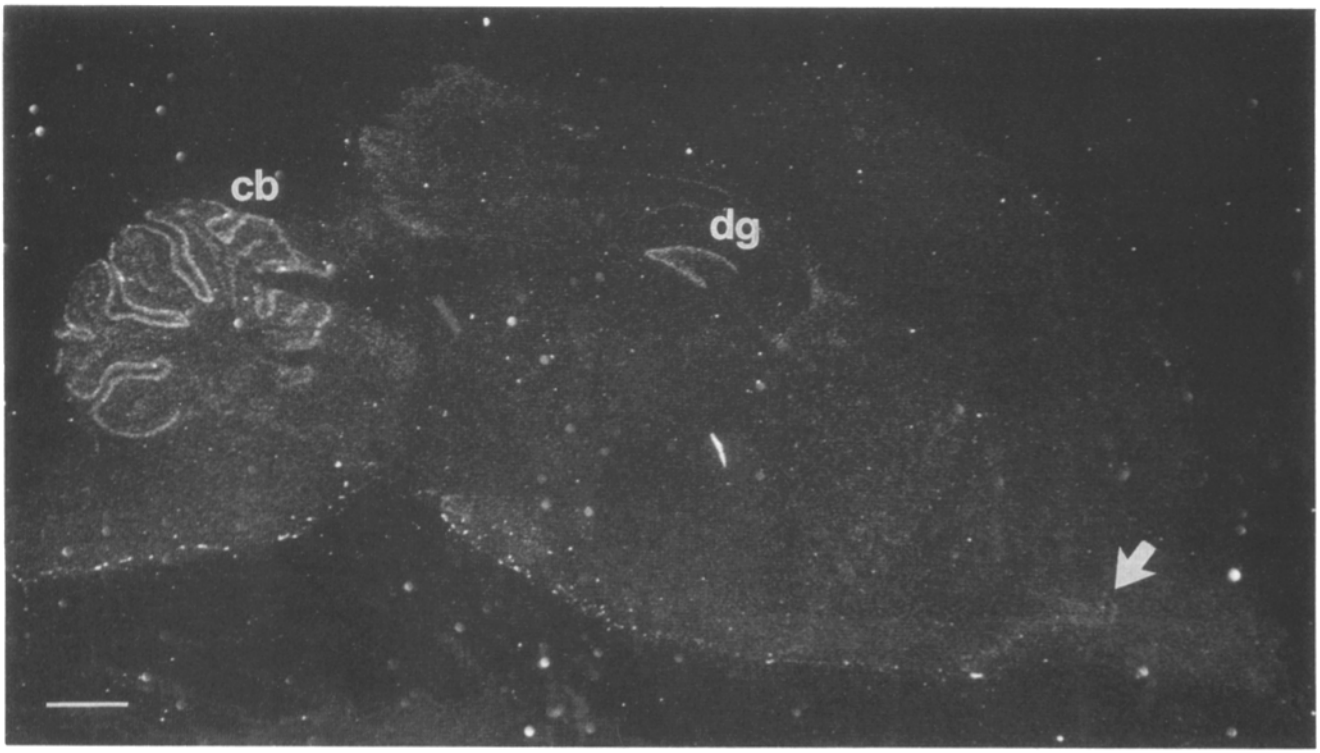

Figure 7. Expression of HSPG M13 in postnatal rat brain. Darkfield photomicrograph of a postnatal day 7 rat brain after in situ hybridization with an α^{35}S-UTP labeled M13 RNA probe. Hybridization is detected in the cerebellum (*cb*), the dentate gyrus of the hippocampus (*dg*), and in migrating granule cell precursors of the olfactory bulb (*arrow*). No hybridization was detected with the sense orientation (negative control) probe (not shown). Bar, 1 mm.

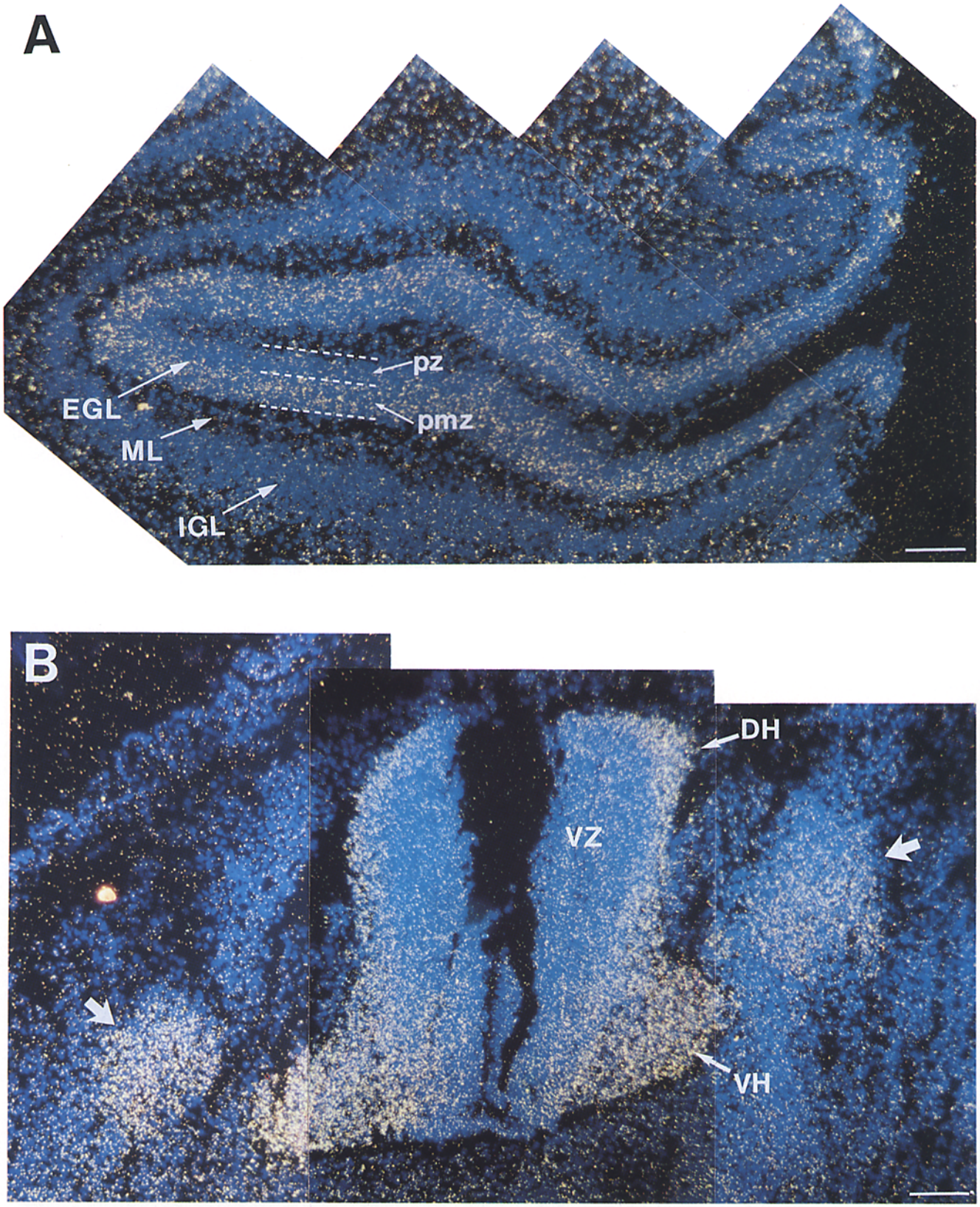

Figure 8. HSPG M13 is expressed by postmitotic neurons. Simultaneous visualization of M13 mRNA (darkfield optics) and cell nuclei (Hoechst blue fluorescence). (*A*) P7 cerebellum. Hybridization is localized to the external granule layer, and is concentrated in the premigratory zone, which contains newly postmitotic granule neurons. *EGL*, external granule layer; *ML*, molecular layer; *IGL*, internal granule layer; *pz*, proliferative zone; *pmz*, premigatory zone. (*B*) E12 spinal cord. Hybridization is detected in the forming dorsal horns (*DH*), and ventral horns (*VH*) of the spinal cord, and the dorsal root ganglia (*arrows*), but not the ventricular zone (*VZ*). The cleft in the ventricular zone of the spinal cord was an artifact of sectioning. No signal was detected in adjacent sections hybridized with sense orientation (negative control) probe (not shown). Bar, 100 μm.

premigratory zone (26). This zone consists of newly generated, postmitotic neurons, whereas the superficial half of the external granule layer contains the proliferating cells that give rise to those neurons. This result indicates that M13 expression begins in neurons at or about the time they become postmitotic.

To further test this hypothesis, in situ hybridization was used to examine M13 expression in the embryonic day 12 (E12) spinal cord. At this stage, the spinal cord consists of a large central proliferative zone (the ventricular zone), from which newly postmitotic neurons migrate laterally to form the nascent ventral and dorsal horns (3). As Fig. 8 *B* illustrates, M13 is strongly expressed by regions of the E12 spinal cord that contain postmitotic neurons, but weakly expressed, if at all, by the still-proliferating neural precursors of the ventricular zone. In addition, expression can be seen in the dorsal root ganglia (DRG) located on either side of the spinal cord. These structures also contain recently post-mitotic neurons (3), the progeny, in this case, of neural crest cells.

These data suggest that M13 is specifically and transiently expressed by neurons during their immediate post-mitotic period. As discussed below, the window of expression of this molecule correlates particularly well, throughout the nervous system, with the time period during which neuronal migration and axon outgrowth take place.

Discussion

Molecular Cloning of Cerebroglycan

Several lines of evidence confirm that the cDNA obtained in this study encodes the GPI-anchored HSPG core protein previously designated as M13 (30): The cDNA encodes all four of the peptides that were obtained from a tryptic digest of M13; it encodes a protein of the correct size, which contains likely GPI anchorage and GAG-attachment sequences (see below); and it hybridizes to an mRNA that is present in embryonic and neonatal rat brain, but not in adult brain (matching the pattern previously observed for M13 protein) (30). In situ hybridization demonstrated that, at every stage so far examined, expression of HSPG M13 is limited to the nervous system. This result is surprising because no other proteoglycan is known to be restricted in expression to a single tissue. To reflect the nervous system-specific expression of this proteoglycan, we have chosen to name PG M13 "cerebroglycan."

Sequence Analysis

The deduced amino acid sequence of cerebroglycan contains seven serine-glycine pairs, representing possible GAG attachment sites. Of these, ser^{155}, ser^{498} and ser^{500} all match the sequence SGXG, and all occur within regions of protein sequence that are rich in acidic residues. Both features have been proposed to define a consensus for GAG attachment (7, 8). In addition, two other serine-glycine pairs (ser^{55} and ser^{92}) occur either just before or just after an acidic residue, and might also represent GAG attachment sites (7). Interestingly, ser^{498} and ser^{500} were sequenceable residues of peptide M13-22. Since substitution of serine with GAG chains should result in a blank cycle in Edman degradation, it is possible that these sites are not actually substituted with GAG chains, or that only a fraction of cerebroglycan core proteins are substituted at both these sites.

The cerebroglycan protein sequence has a stretch of hydrophobic amino acids at both the amino and carboxy termini, as indicated in Fig. 2. These sequences could serve as the signal sequence and a GPI-attachment signal respectively, being cleaved off in the mature protein. The precise cleavage sites are unknown, and those indicated in Fig. 2 are predictions based upon the $-1,-3$ rule (72), for the signal sequence, and the ω, $\omega + 2$ rule (39), for the GPI-attachment signal. Of the two potential GPI-attachment sites indicated, ser^{557} has a slightly higher probability than asn^{553}, based on amino acid preferences at the ω and $\omega+2$ sites of other GPI-anchored proteins. After the candidate GPI-attachment sites are a potential "hinge" region (rich in proline and charged amino acids), and a stretch of hydrophobic amino acids; both the hinge and hydrophobic stretch are features of GPI attachment signals (39).

Cerebroglycan Defines a Family of GPI-Anchored PGs

The deduced amino acid sequence of cerebroglycan shares significant homology with two other molecules, the GPI-anchored HSPG glypican, and the predicted product of a developmentally regulated transcript, OCI-5, that was cloned from a rat primitive intestinal epithelial cell line (Fig. 3). The most striking feature of the homology is the absolute conservation of 14 cysteine residues. It is likely that these residues are involved in stabilizing a highly compact tertiary structure, since both glypican and cerebroglycan exhibit apparent molecular masses on SDS-PAGE gels that are ~10 kD lower under nonreducing conditions than under reducing conditions (30). A search of protein databases revealed no other molecules with a similar pattern of cysteines in their primary structures. Thus, cerebroglycan, glypican, and OCI-5 apparently constitute a new family of proteins characterized by similar core protein size, GPI linkage, the presence of potential GAG attachment sites, and a unique cysteine pattern. Since the protein encoded by the OCI-5 transcript has yet to be purified, it remains to be seen whether this molecule actually bears GAG chains in vivo.

Of the four major transmembrane HSPGs that have been identified to date, all were recently classified into a single gene family, on the basis of homology with syndecan (7). The data presented here suggest that the GPI-anchored HSPGs—which are also major PGs in many cells (10, 21, 33, 77)—may also be classifiable into a single gene family. It is interesting that members of the syndecan family exhibit significant sequence similarity only in their transmembrane and cytoplasmic domains, while their extracellular domains have diverged rapidly, even among orthologues in different mammalian species (7). These results have suggested that the extracellular domains of syndecans may serve no conserved function other than as a protein scaffold onto which GAG chains are attached. In contrast, the GPI-anchored HSPG family exhibits a high degree of sequence similarity among homologues, and appears to be evolving more slowly than the syndecans (e.g., rat and human glypican are 91% identical in their mature protein sequences [37, 46], while the extracellular domains of mouse and human syndecan-1 are only 70% identical [7]).

This analysis raises the possibility that the cerebroglycan, glypican, and OCI-5 polypeptides subserve functions other than just the anchorage of GAG chains to the cell surface.

For example, these core proteins might bind as yet unknown ligands. Other ligand-binding PG core proteins include the core proteins of betaglycan (which binds TGF-β) (12) and decorin (which binds to collagen fibrils) (41, 71).

Alternatively, the conserved protein structure of the GPI-anchored HSPGs may play a regulatory role in post-translational processing. For example, the protein might interact with components of the Golgi apparatus in order to influence the type of GAG that it receives (e.g., HS versus chondroitin sulfate), or even the sequence of GAG modifications (e.g., sulfation) that is subsequently generated. Recent studies underscore the importance of differences in GAG modification on the biological properties of HSPGs (51, 61, 63).

Cerebroglycan Expression Marks a Distinct Stage in Neuronal Development

Examination of patterns of cerebroglycan message expression by in situ hybridization suggests that cerebroglycan mRNA (*a*) is produced by neurons, (*b*) appears at around the time of a neuron's terminal mitosis, and (*c*) disappears by the time that neuronal migration and axonal extension have been completed.

Strong support for this interpretation comes from examination of the developing cerebellum, in which neuronal birth, migration and maturation occur in distinct anatomical layers (66). In that structure, cells that proliferate in the superficial part of the external granule layer give rise to postmitotic neurons that reside in the deep part of the same layer, and these in turn migrate rapidly through the molecular layer to reach their final positions in the internal granule layer. The concentration of cerebroglycan mRNA in the deep half of the external granule layer (Fig. 8 *A*) implies that cerebroglycan begins to be expressed at or after final mitosis, while its absence from the internal granule layer implies that cerebroglycan message disappears by the time cell body migration has finished. From studies of the timing of cerebellar neuron migration (26), it can be concluded that the window of cerebroglycan mRNA expression in granule cells is ~36 h or less.

The view that cerebroglycan is first expressed when neurons become postmitotic is supported by results in the early spinal cord, in which mRNA is found only in regions containing newly generated neurons (Fig. 8 *B*), and by the fact that all sites of cerebroglycan mRNA expression throughout the nervous system contain neurons that have been generated within the preceding 2–3 d (e.g., E12-E16 spinal cord and DRG [Figs. 6 and 8; ref. 3]; E14-P0 cerebral cortex [Fig. 6; ref. 6]; E19-P0 inferior colliculus [Fig. 6; ref. 2]; E19 pontine nuclei [Fig. 6; ref. 1]; E18 retina [not shown; ref. 79]; and P7 hippocampus and olfactory bulb [Fig. 7; refs. 4, 5]). In contrast, little if any cerebroglycan mRNA is found in the proliferating neural precursors that form the ventricular zone, both in the E12 spinal cord (Fig. 8 *B*) and throughout the developing brain (not shown).

The time of disappearance of cerebroglycan message in at least one structure other than the cerebellum also correlates with completion of neuronal migration: cells expressing cerebroglycan are found in the subventricular zone pathway along which olfactory bulb interneuron precursors migrate (48), but not in the layers of the olfactory bulb that contain interneurons that have completed their migration. In other parts of the nervous system, however, a simple correlation between cerebroglycan expression and neuronal cell body migration cannot be drawn. The ventral horn of the spinal cord, the dorsal root ganglia, the E19 thalamus, the superficial layers of the E19 and P0 cerebral cortex, and the dentate gyrus of the hippocampus (Figs. 6–8), all primarily consist of neurons that have reached their final positions, yet express cerebroglycan. Interestingly, although the cell bodies of these neurons are not migrating at these times, their axons are generally still growing. If the same cells are examined after their axons have reached target areas, cerebroglycan message is undetectable (e.g., after E20 for thalamus [22]; E18 for dorsal root ganglia [38]; and P2-P5 for cerebral cortex [52]). Since axon growth and cell migration are both forms of cell motility (9, 18), the data suggest a general correlation between cerebroglycan expression and periods of time during which neurons engage in motile behaviors.

Although the data presented cannot rule out the possibility that some glial cells transiently express cerebroglycan, neither the timing nor pattern of expression of cerebroglycan match the timing or sites of glial production in the brain, which is largely postnatal (34). In addition, several of the structures in the developing brain that highly express cerebroglycan mRNA contain few if any glial cell bodies, e.g., the external granule layer of the cerebellum (Fig. 8) and the E18 retina (data not shown). Interestingly, recent evidence indicates that, in the adult rat brain, glypican is also expressed largely, if not exclusively, by neurons (46).

Possible Roles for Cerebroglycan in the Developing Nervous System

If cerebroglycan is expressed by neurons specifically at times of cell migration and axon pathfinding, it raises the possibility that cerebroglycan plays a role in these events. Many of the molecules that have been implicated in the control of these cell behaviors—including extracellular matrix glycoproteins, polypeptide growth factors, and cell–cell adhesion molecules—share a common feature: they bind GAGs, especially HS and the related GAG heparin (42). The abundance (30) and pattern of expression of cerebroglycan in the developing nervous system suggest that it could play an important role in the recognition of these classes of molecules.

For example, axons that leave the cerebral cortex navigate within a layer that contains both fibronectin (65) and laminin (32), two extracellular matrix proteins that promote axon growth in vitro. Both of these proteins bind HSPGs, and recent studies suggest that cell surface HSPGs are essential for the interactions of central nervous system neurons with fibronectin (29). Importantly, expression of cerebroglycan in the cerebral cortex occurs during the major period of cortical axon growth through this region (20). Laminin is also present along pathways followed by retinal axons (15, 50), as well as those followed by the peripheral axons of spinal motoneurons and dorsal root ganglion neurons (56). In each case, we now know that these pathways are navigated by neurons that, at the time, express cerebroglycan mRNA. Moreover, if the cellular distribution of cerebroglycan is similar to that of other GPI-anchored proteins expressed by neurons, this HSPG is likely to be found on the surface of axons (e.g., 24, 27, 76).

Other potential ligands for cerebroglycan in the developing nervous system include the cell adhesion molecule NCAM—which appears to require an interaction with

HSPGs for at least some of its functions (16, 55)—and heparin binding growth factors such as FGF-1, FGF-2, and FGF-5, which are known to influence neuronal survival and neurite outgrowth in vitro (31, 70, 73, 74), and which apparently require HSPGs to interact with cells (54, 78).

The fact that cerebroglycan is a GPI-anchored molecule also raises the possibility that the binding of ligands to cerebroglycan could lead to the production of intracellular signals. Crosslinking of any of a wide variety of GPI-anchored proteins on the surface of leukocytes is known to trigger changes in intracellular calcium levels, protein secretion and cell proliferation (47, 53). The mechanism of GPI-mediated signal transduction has not been determined, but may involve the interaction of GPI-anchored proteins with transmembrane proteins such as tyrosine kinases (69), or the phospholipase-induced cleavage of the GPI-anchor, followed by rebinding of the anchor to a specific signal-transducing receptor (59). Future studies aimed at determining precisely which molecules cerebroglycan interacts with, and what the intracellular consequences of those interactions are, will be critical in defining the in vivo functions of this molecule.

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