F is the Faraday constant, R is the gas constant, and T is absolute temperature; k_i^0 and k_{-i}^0 define values at zero membrane potential; z_i is known to be positive for Na translocation (*4, 5*).

9. As shown in Table 1, Na_i-Na_o exchange flux is predicted to saturate at extreme positive potentials if $\delta_1 = 0$ (only k_1 affected by potential) and extreme negative potentials if $\delta_2 = 1$ (only k_{-2} affected by potential) but to decline toward zero at extreme positive and negative potentials for all other values of δ_i. It can also be shown that (i) ^{22}Na efflux–voltage plots must be bell-shaped (flux vanishes at the voltage extremes) in all cases in which both steps are voltage sensitive ($z_1 > 0$ and $z_2 > 0$), regardless of the values of δ_i, and (ii) insertion of electroneutral transitions between steps one and two does not alter the results in Table 1, but (iii) insertion of a voltage-sensitive step, irrespective of the value of its δ, again causes the exchange to cease at both voltage extremes. In other words, only when k_{first} (Na_i binding) or k_{-last} (Na_o rebinding) is the sole voltage-sensitive step in the reaction chain will saturating exchange rates be observed at extreme potentials; all other models predict bell-shaped flux-voltage plots.
10. Voltage independence of k_1 and k_{-1} implies that no charge movement accompanies translocation of nNa ions through the remaining $(1 - \lambda)V$, presumably because equal but opposite charges are supplied by the ion binding sites (*4, 5*).
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12. The model in Fig. 2B yields a Hill equation for unidirectional Na efflux, $\Phi = \Phi_{max}/(1 + K$ [illegible] where Φ_{max} is unidirectional efflux at [illegible] or hyperpolarization and $K_{0.5}$ is the voltage-dependent, half-activating $[Na]_o$ given by $K_{0.5} = K_{0.5}^0 \exp(\lambda U)$, where $K_{0.5}^0$ is the value at 0 mV.
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16. In scheme 1, if hyperpolarization were to increase the ratio k_{-1}/k_1, the steady-state concentration of the intermediate E-PnNa would decline, and so the apparent affinity for Na_o would be enhanced.
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20. Although the voltage-induced transient pump currents (*18, 19*) relax relatively slowly (~40 s^{-1} at 0 mV and 20°C), they are still readily explained by our model (Fig. 2B) provided that the rate constants for occlusion and deocclusion (k_1 and k_{-1}) and for Na_o rebinding and release (k_{-2} and k_2) have appropriate magnitudes. According to our model, a voltage jump is expected to cause a virtually instantaneous charge movement (obscured by the membrane-capacitance current) as Na ions migrate along the access channel in response to the field change. The ensuing redistribution of enzyme intermediates subsequent to Na_o rebinding or release would induce a much slower, hence detectable, charge transient reflecting further Na-ion movement along the access channel, now rate-limited by the conformational changes [P. Läuger and H.-J. Apell, *Biochim. Biophys. Acta* **944**, 451 (1988)].
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26. The small pump current at 0 mV is unlikely to reflect Na,K exchange because K contamination of the nominally K-free seawater was ~25 μM whereas, under these conditions, the half-activating $[K]_o$ is 4 mM (*2*), but it is presumably mediated by electrogenic $3Na_i$-$2Na_o$ exchange [K. H. Lee and R. Blostein, *Nature* **285**, 338 (1980); H.-J. Apell, V. Häring, M. Roudna, *Biochim. Biophys. Acta* **1023**, 81 (1990)]. In all such experiments, H_2DTG-sensitive ^{22}Na efflux and ΔI averaged 14.0 ± 1.1 pmol cm^{-2} s^{-1} and −0.010 ± 0.003 μA cm^{-2} (±SEM, $n = 7$), respectively, at −60 mV, and 9.5 ± 0.6 pmol cm^{-2} s^{-1} and −0.034 ± 0.003 μA cm^{-2} ($n = 8$) at 0 mV, indicating that even at 0 mV, electrogenic $3Na_i$-$2Na_o$ exchange could have contributed, on average, ≤10% to the H_2DTG-sensitive ^{22}Na efflux.
27. The model is reduced to scheme 1 by setting $k_{-2}^0 = k'_{-2}[Na]_o^n$ and $z_2 = n\lambda$.
28. Supported by NIH grant HL-36783 and the Irma T. Hirschl Trust (D.C.G.), NIH grant NS-22979 (R.F.R.), and NIH grant NS-11223 (P.D.W.). We thank C. M. Armstrong for comments on the manuscript and R. W. Ratzlaff for programming assistance.

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Developmental Regulation of Neural Response to FGF-1 and FGF-2 by Heparan Sulfate Proteoglycan

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Murine neural precursor cells and cell lines derived from them are stimulated by members of the heparin-binding fibroblast growth factor (FGF) family. The activity of FGF is regulated by heparan sulfate proteoglycans (HSPGs), and this interaction is an essential prerequisite for the binding of growth factor to the signal transducing receptors. Messenger RNA for FGF-2 was detectable in the neuroepithelium at embryonic day 9, and the HSPGs produced by these cells at this time preferentially bound FGF-2. However, at embryonic day 11, when messenger RNA for FGF-1 was first detectable, there was a switch in the binding specificity of the HSPG to FGF-1. Thus, a single species of HSPG undergoes a rapid, tightly controlled change in growth factor–binding specificity concomitant with the temporal expression of the FGFs.

The mammalian central nervous system is developmentally derived from the cells of the neural tube. The heparin-binding growth factors FGF-1 and FGF-2 are potent developmental regulators of the proliferation, migration, and differentiation of murine neural precursor cells (*1*). FGF activity is regulated in part by heparin analogs such as the glycosaminoglycan (GAG) heparan sulfate (*2*). HSPGs are a highly diverse group of macromolecules, each of which consists of a core protein to which highly sulfated GAG side chains of heparan sulfate are covalently attached (*3*). They are ubiquitous constituents of mammalian cell surfaces and of most extracellular matrices, including the specialized basal laminae that surround neural tissue (*2, 4*). FGF binding to HSPGs appears to offer protection against proteolytic degradation, thus creating a reservoir of growth factor in tissues (*5*). More recent studies have demonstrated that HSPG-FGF interaction is an essential prerequisite for the presentation and subsequent binding of growth factor to signal transducing receptors (*6*). However, the timing of expression of the FGFs and HSPGs and the interactions between them during the early proliferative phases of brain development remain largely unknown.

To determine the precise timing of the expression of FGF-1 and FGF-2 during neural development, we performed specific amplification of the mRNA sequences for these factors on mesencephalic and telencephalic neuroepithelial tissue at embryonic days 9, 10, 11, and 13 (E9, E10, E11, and E13) by polymerase chain reaction (PCR). The two factors were expressed in different temporal patterns: FGF-2 mRNA was readily detectable at E9 and throughout subsequent ages, but FGF-1 mRNA was detectable only from E11 on (Fig. 1). Immunohistochemical studies with antibodies to FGF support this differential pattern of expression (*7*). A similar differential expression of the FGFs was observed in a cell line originally derived from E10 neuroepithelium, 2.3D (*1*). At low cell density, this cell line expressed only FGF-2 mRNA. In contrast, when these cells were maintained

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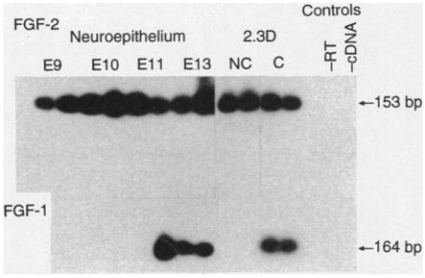

Fig. 1. Expression of FGF-1 and FGF-2 mRNA in the murine embryonic central nervous system and the murine neuroepithelium-derived 2.3D cell line. The PCR products amplified from cDNA templates prepared from E9, E10, E11, and E13 neuroepithelial tissue mRNA and 2.3D nonconfluent (NC) and confluent (C) cell line mRNA were analyzed by Southern (DNA) blotting with internal end-labeled oligonucleotides as probes (*19*). Results are representative of four independent replications. RT, reverse transcriptase.

as confluent monolayers for periods greater than 4 days, expression of FGF-1, in addition to expression of FGF-2, became readily detectable (Fig. 1). These results show that individual neuroepithelial cells can differentially regulate the expression of FGFs during development.

As HSPGs are essential for the presentation of FGFs to their signal transducing receptors, it would seem that appropriate HSPGs must be present for differential FGF expression to have functional significance. To determine the presence of endogenously produced HSPGs during development, we established neuroepithelial cell cultures from both E9 and E11 (*1*) and allowed them to condition serum-free culture media for 24 hours. We have previously shown that neuroepithelial cells secrete essentially one species of proteoglycan at these early embryonic stages and that this species is an HSPG (*8*). The respective conditioned media were chromatographed on DEAE columns under conditions that result in the elution of HSPGs (*9*). The elution profiles of the conditioned media derived from the E9 and E11 neuroepithelial cells were remarkably similar: a major peak of material that absorbs at 280 nm was eluted with 0.40 M NaCl for both preparations. These peaks were identified as HSPGs (Fig. 2A) by enzymatic digestion with heparitinase, which abolishes the high molecular weight species by digesting the heparan sulfate side chains (*10*). The high molecular weight HSPG complexes run as a broad smear with an approximate molecular weight of 460,000 for both E9 and E11. Further confirmation that these molecules were HSPGs was obtained by nitrous acid deamination and by their failure to be digested with either chondroitinase ABC, dermatan lyase, or hyaluronidase (*9*). The HSPG profiles detectable in 2.3D-conditioned media from nonconfluent or confluent cells showed identical properties; they were also identical to the properties of media conditioned with E9 and E11 neuroepithelium (*7*).

To examine the binding specificity of the HSPGs from both E9 and E11 neuroepithelium, we coupled both species to Affi-Gel 10 affinity support and decanted them into small chromatographic columns. Both ^{125}I-labeled FGF-1 and ^{125}I-labeled FGF-2 were passed over column beds to which DEAE-purified HSPGs from either E9- or E11-conditioned medium or from confluent and nonconfluent 2.3D cells had been coupled. The amount of radioactivity applied to the columns was monitored, as were the number of counts that flowed through the column (the unbound fraction) and the number of counts recoverable after a 2 M NaCl wash (the bound fraction). The results (Fig. 2B) revealed a switch in the binding affinity of the HSPGs isolated from the two ages and from the two states of confluency. HSPGs derived from E9 preparations were over four times more effective in binding FGF-2 than binding FGF-1, whereas HSPGs derived from E11 neuroepithelium were six times more effective at binding FGF-1 than binding FGF-2. This change in binding specificity also occurred in 2.3D cells: nonconfluent 2.3D HSPGs preferentially bound FGF-2, whereas the HSPGs isolated from 2.3D cells confluent for 6 days displayed a much greater capacity to bind FGF-1 than to bind FGF-2.

To determine if this change in binding specificity was a result of the differential expression of HSPG core proteins or differential glycosylation of the same core protein, we collected conditioned media from E9 and E11 cells maintained in either [^{35}S]methionine to label the core protein or in $^{35}SO_4$ to label the GAG side chains. When labeled E9 and E11 HSPGs were stripped of GAGs with heparitinase, the core proteins appeared to have similar molecular weights of 45,000 (Fig. 3A). This similarity was further substantiated by immunoprecipitation with an affinity-purified rabbit antibody raised against the core protein of the nonconfluent 2.3D HSPG. This antibody was able to precipitate core proteins with a molecular weight identical to that of proteins obtained from the original DEAE isolates from both E9 and E11 (Fig. 3A) and 2.3D cells. These molecular weights, which differ from other neural HSPGs from older rat embryos (*11*) and from those present in more mature basal laminae (*12*), together with preliminary amino acid sequencing of core protein fragments (*7*), indicate that neuroepithelial cells secrete a single, unique species of HSPG. When these core proteins were digested with trypsin, the resultant peptides yielded profiles on SDS-polyacrylamide gels and reversed-phase high-pressure liquid chromatography that were essentially identical (*7*).

Preliminary stoichiometric analysis of $^{35}SO_4$-labeled GAG side chains after gel filtration showed that the average side chain from E9-conditioned medium was

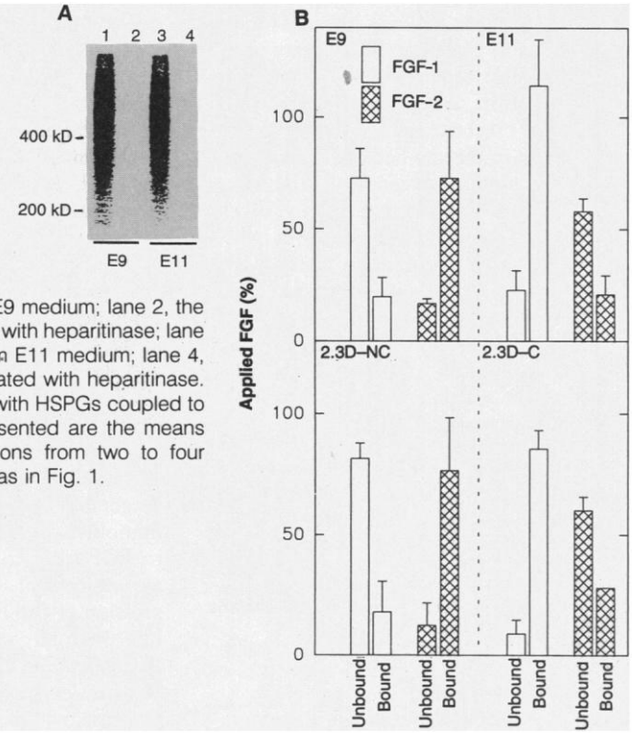

Fig. 2. (**A**) Analysis of HSPG molecular complexes. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of the eluate fractions was obtained after DEAE chromatography. Radioactive fractions from DEAE chromatography were run on 3 to 10% or 15% SDS-PAGE gels and subjected to autoradiography (*9, 10*). Lane 1, $^{35}SO_4$-labeled HSPGs from E9 medium; lane 2, the same preparation pretreated with heparitinase; lane 3, $^{35}SO_4$-labeled HSPGs from E11 medium; lane 4, the same preparation pretreated with heparitinase. (**B**) Affinity chromatography with HSPGs coupled to Affi-Gel 10 (*20*). Values presented are the means and SDs of six determinations from two to four experiments. NC and C are as in Fig. 1.

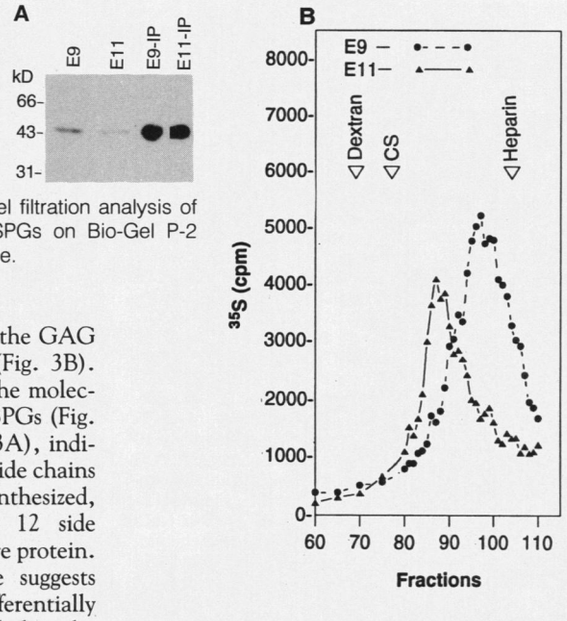

Fig. 3. Analysis of HSPG core proteins. (**A**) [^{35}S]methionine-labeled, heparitinase-stripped HSPG core proteins from E9 and E11 neuroepithelium were separated by 15% SDS-PAGE. IP, core protein immunoprecipitated with rabbit polyclonal antibodies made against 2.3D cell HSPG core protein (*21*). (**B**) Gel filtration analysis of GAG chains from E9 and E11 HSPGs on Bio-Gel P-2 columns (*21*). CS, chondroitin sulfate.

approximately 20 kD; the size of the GAG side chains at E11 was 35 kD (Fig. 3B). These data, in association with the molecular weight data on the intact HSPGs (Fig. 2A) and the core proteins (Fig. 3A), indicate that at E9 approximately 20 side chains are attached per core protein synthesized, whereas at E11 approximately 12 side chains are attached per HSPG core protein. Overall, the available evidence suggests that the same protein core is differentially glycosylated at the two ages and that the FGF binding specificity resides in the GAG side chains of the HSPGs.

To test whether HSPG binding specificity coincides with the ability of each factor to stimulate cell proliferation, we isolated E11 neuroepithelial cells, pretreated them with trypsin to remove endogenous HSPGs and attached growth factors, and then exposed them to either FGF-1 or FGF-2 in the presence of HSPGs obtained from E9 or E11 neuroepithelium (Fig. 4). E11 HSPG was more effective (approximately four times) at stimulating cell division with FGF-1 than with FGF-2, whereas the E9 HSPG was more effective (approximately seven times) with FGF-2. This response to the HSPGs was dose-dependent within the range 0.01 to 10 μg/ml. At concentrations greater than 10 μg/ml, the HSPGs interfere with the mitogenic response, as has been found with heparin (*1*, *3*). The HSPGs added to the cultures in the absence of growth factors had no detectable mitogenic effect (Fig. 4). To verify that the HSPG recognized by the core protein antibody is actually mediating the biological activity, we immunodepleted purified preparations of E9 or E11 HSPGs with the antibody and protein A before adding them to the E10 neuroepithelial cultures. The immunodepleted HSPG preparations of both ages had greatly reduced mitogenic potential, which demonstrates that the HSPGs are the biologically active molecules in the conditioned medium. It also indicates that the core proteins that mediate the E9 and E11 response are immunologically identical. Control incubations with other antibodies did not greatly reduce the bioactivity. Thus, the functional activities of the HSPGs reflect their binding specificities. Although these results argue in favor of the core proteins being identical, it nevertheless remains possible that the response to FGF-1 is due to a minor, contaminating proteoglycan. This seems unlikely as it would require a coordinated loss of the FGF-2 binding response. It is notable that the HSPGs from more mature tissue (Fig. 4, postnatal day 1 brain preparation, P1) are less effective at stimulating proliferation, which suggests that binding specificities for other growth factors apply at these later times.

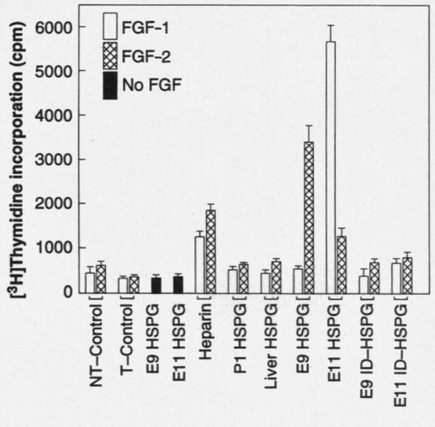

Fig. 4. [^{3}H]Thymidine incorporation into neuroepithelial cells maintained in either FGF-1 or FGF-2 with supplemental glycosaminoglycan. E10 neuroepithelial cells were trypsinized (0.1% w/v trypsin) to remove surface and adherent proteoglycans, allowed 2 hours to recover, and then plated onto HL-A plates (Lux, Sydney, Australia) in the presence of FGF-1 or FGF-2 (5 ng/ml) at a cell density of 1500 cells per well (*1*). After 36 hours, the cells were pulsed for 16 hours with [^{3}H]thymidine, harvested, washed, and counted. Cultures remained unsupplemented or were supplemented with heparin, postnatal day 1 brain HSPG preparation (P1), commercially obtained HSPGs from liver (Sigma), or E9 HSPG or E11 HSPG, all at 10 μg/ml. In some experiments, the cells were either not pretreated with trypsin (NT-Control), treated with trypsin (T-Control), or not supplemented with growth factors (E9 HSPG, E11 HSPG, and no FGF). In preparations where the purified HSPG preparations were immunodepleted (ID), procedures were similar to those of Edgar and co-workers (*18*). E9 and E11 HSPG preparations were mixed with antibody to the 2.3D core protein (10:1 v/v for 2 hours), mixed with Pansorbin (Calbiochem; 10:1 v/v for 2 hours), clarified, and the procedure repeated. Experiments with $^{35}SO_4$-labeled HSPGs demonstrated that greater than 88% of HSPGs are removed by this procedure. Equivalent volumes of the immunodepleted HSPGs were then added back to the cultures. Values are the means and SDs of six determinations.

The results of this study show that at very early embryonic stages, before neurons have begun to differentiate from the actively proliferating murine neural precursor cell population, an HSPG selective for FGF-2 is secreted into the neuroepithelial environment. This finding further supports previously published evidence that FGF-2 regulates neural precursor cell division (*1*). Neuroepithelial cells then appear to use identical but differentially glycosylated core proteins to preferentially bind FGF-1 at stages when neuronal differentiation is being initiated. Because large amounts of FGF-1 are specifically associated with neuronal populations, whereas FGF-2 is more ubiquitously expressed throughout the embryo, it is likely that FGF-1 is more important for neural development (*13*). The results from the 2.3D cells, an immortalized cell line clonally derived from E10 neuroepithelial cells, reinforce the concept that a switch in binding properties can occur in a homogeneous population of cells and not merely in discrete subpopulations. Thus, the synthesis of FGF-1 by precursor cells is tightly coordinated with the synthesis of differentially glycosylated HSPG species. This type of regulatory mechanism does not rely on changes in cell surface receptor expression or cessation of growth factor production and allows for rapid changes in cell signaling during development.

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19. Neuroepithelial tissue from ten E9, E10, E11, and E13 mouse embryos was dissected free of mesodermal tissue (*1*) and processed for polyadenylated RNA as described (*14*). Approximately 0.5 μg of mRNA was preincubated in a volume of 10 μl of H_2O with either 100 ng of p7 (FGF-1) or p2 (FGF-2) primers at 75°C for 5 min then chilled on ice. First-strand cDNA was prepared according to the manufacturer's instructions with the reverse transcription system (Promega). PCR was performed with 5 μl of the cDNA template in a reaction volume of 50 μl that contained 100 ng of 5′ and 3′ primers (p8 and p9 define a 164-bp fragment for FGF-1 and np2 and p1 define a 153-bp fragment for FGF-2), 200 μM deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate, 10 mM tris HCl (pH 8.3 at 25°C), 50 mM KCl, 0.001% gelatin, and 4.0 mM $MgCl_2$. The primers were designed across intron 1 and intron 2, both approximately 16 kb in the FGF-1 and FGF-2 genes, respectively (*15*), so that the 164-bp and the 153-bp fragments could have arisen only from mRNA. Recombinant Taq1 polymerase (Cetus, Emoryville, CA) was added at 2.0 units per reaction, and PCR was carried out for 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min in a GM10 Amplifier (Innovonics, Australia). Oligonucleotides were designed from the published cDNA sequences for murine FGF-1 and FGF-2 (*16*) and are 5′ to 3′ p7: 5′-CGCACTTTCCGCACTGAGCTG; p8: 5′-AATGTGCTGGTCGCTCCTGTCCCTTCT; p9: 5′-GAAGGGGAGATCACAACCTTCGCA; p10: 5′-AGGTTCAACCTGCCTCTAGGAAAACTAC; p1: 5′-GTGTCTATCAAGGGAGTGTGTGCC; ip2: 5′-CTGGCTTCTAAGTGTGTTACAGAAGAGTGT; np2: 5′-AACTGGAGTATTTCCGTGACCGGTAA; and p2: 5′-ACTGCCCAGTTCGTTTCAGTGCCA. Size analysis of PCR products was carried out by electrophoresis of 10 μl of the PCR reaction on a 1.5% agarose gel. PCR products were transferred to Zeta-probe membrane (Bio-Rad, Sydney, Australia) by alkaline blotting. Blots were rinsed in 10× SSC (1.5 M NaCl and 0.15 M sodium citrate at pH 7) and prehybridized at 42°C in 5× SSC, 25 mM NaH_2PO_4 (pH 6.5), 5× Denhardt's, denatured salmon sperm DNA (100 μg/ml), and 0.5% SDS for 2 to 4 hours. The 164-bp and 153-bp PCR products for FGF-1 and FGF-2 were detected by hybridization to their respective internal oligonucleotides, p10 for FGF-1 and ip2 for FGF-2. The oligonucleotides were end-labeled with [γ-^{32}P]adenosine triphosphate (Amersham) with T4 polynucleotide kinase (Promega). Blots were hybridized at 42°C overnight in the above prehybridization buffer adjusted to 15% formamide and with the addition of 100 ng of end-labeled oligonucleotide. Blots were washed twice in 2× SSC and 0.5% SDS followed by 0.5× SSC and 0.5% SDS for 15 min at 42°C and exposed to autoradiographic film (Kodak XAR5).
20. Serum-free media conditioned over 2.3D cells or E9 or E11 neuroepithelial cell cultures (10^5 cells per 16-mm well for 24 hours) were filtered through 0.45-μm mesh and chromatographed through a low-pressure Econo-Pac Q Sepharose cartridge (Bio-Rad) at a final concentration of 2 ml/min. The column was washed with 0.15 M tris-buffered saline (TBS) (pH 7.4) until the absorbance at 280 nm reached base line. The bound material was then released with a NaCl gradient from 0.15 to 1.0 M and collected in 3-ml fractions. In some experiments, we maintained the cells in Dulbecco's modified Eagle's medium (DMEM) containing $^{35}SO_4$ in order to detect GAG side chains; in other experiments we maintained the cells in DMEM containing [^{35}S]methionine in order to detect HSPG core proteins. Purified HSPG preparations from either E9- or E11-conditioned media or 2.3D cell media were ligated to the affinity agarose support Affi-Gel 10 (Bio-Rad) in carbodiimide coupling buffer according to the manufacturer's instructions. Approximately 100 μg of each HSPG preparation was bound to each 1-ml column volume of gel bed. The bound support was then decanted into small chromatographic columns and washed in 0.15 M TBS (pH 7.4). For each experiment, 100 ng of either ^{125}I-labeled FGF-1 or ^{125}I-labeled FGF-2 in 0.15 M TBS was passed over the gel bed at a flow rate of 0.5 ml/min. The flow-through material was collected as the unbound fraction, and the column was then washed with 20 column volumes of 0.15 M TBS. The bound fraction (2 ml) was then eluted from the column with 2.0 M NaCl-TBS at a rate of 0.5 ml/min. The radioactivity in the applied (1.65×10^6 cpm for FGF-1 and 1.83×10^6 cpm for FGF-2), unbound, and bound fractions was then quantitated. Preliminary control experiments demonstrated that other factors such as transforming growth factor–β1, insulin-like growth factor–1, nerve growth factor, ciliary neurotrophic factor, leukemia inhibitory factor, and the heparin-binding factor midkine were not retained by the HSPG columns. When the HSPG columns were pretreated with 10 μg of heparitinase (Sigma) for 1 hour at 37°C, they lost their ability to bind either FGF-1 or FGF-2.
21. To reveal the molecular weight of the core proteins, we pretreated HSPG complexes collected after chromatography with heparitinase III (heparinase I, EC 4.2.2.8, Sigma; 10 μg/ml at 37°C for 1 hour) before being applied to a 3 to 10% SDS-polyacrylamide gel. To prove the antigenic similarity of the E9 and E11 core proteins, we raised a rabbit polyclonal antibody against the 2.3D core protein (*17*). Affinity-purified antibodies were then used to immunoprecipitate the core proteins from both E9 and E11 HSPG preparations according to the method of Edgar and co-workers (*18*). Carbohydrate chains of the E9 and E11 HSPGs were prepared from a 100-μl sample of immunopurified proteoglycan layered onto a Bio-Gel P-2 column equilibrated in TBS, collected in the void volume, and digested with Pronase (1 mg/ml) for 4 hours at 25°C. The samples were concentrated to 50 μl by dialysis against solid polyethylene glycol at 25°C for 2 hours and adjusted to 4 M guanidinine hydrochloride–50 mM tris (pH 7.0). The eluted fractions were counted in Aquasol (NEN, Du Pont, Sydney, Australia). The column was calibrated with samples of ^{14}C-labeled dextran (70 kD), [^{3}H]chondroitin sulfate (50 kD), and [^{3}H]heparin (12 kD).
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