Development 129, 2223-2232 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV4583

Cerebellar proteoglycans regulate sonic hedgehog responses during development

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Accepted 31 January 2002

SUMMARY

Sonic hedgehog promotes proliferation of developing cerebellar granule cells. As sonic hedgehog is expressed in the cerebellum throughout life it is not clear why proliferation occurs only in the early postnatal period and only in the external granule cell layer. We asked whether heparan sulfate proteoglycans might regulate sonic hedgehog-induced proliferation and thereby contribute to the specialized proliferative environment of the external granule cell layer. We identified a conserved sequence within sonic hedgehog that is essential for binding to heparan sulfate proteoglycans, but not for binding to the receptor patched. Sonic hedgehog interactions with heparan sulfate proteoglycans promote maximal proliferation of postnatal day 6 granule cells. By contrast, proliferation of less mature granule cells is not affected by sonic hedgehog-proteoglycan interactions. The importance of proteoglycans for proliferation increases during development in parallel with increasing expression of the glycosyltransferase genes, exostosin 1 and exostosin 2. These data suggest that heparan sulfate proteoglycans, synthesized by exostosins, may be critical determinants of granule cell proliferation.

Key words: Sonic hedgehog, Proteoglycans, Ext, Cerebellum, Granule cells, Mouse

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INTRODUCTION

During development, sonic hedgehog (SHH) is crucial for patterning tissue, promoting differentiation and stimulating proliferation (Goodrich and Scott, 1998; Ming et al., 1998; Weed et al., 1997). The pleiotrophic effects of SHH result in part from the ability of SHH to elicit distinct responses as a function of concentration. This has been best characterized in the developing neural tube where progenitors differentiate into several different cell types in response to an apparent gradient of SHH (Briscoe and Ericson, 1999; Ericson et al., 1997a; Hynes et al., 2000). However the spectrum of SHH activities also derives from interaction with other factors that modulate responses to SHH. Some modulators, such as cAMP, function as rheostats, making cells more or less sensitive to SHH (Concordet et al., 1996; Hammerschmidt et al., 1996; Klein et al., 2001). Other modulators alter the nature of the cellular response. Soluble factors such as bone morphogenetic proteins, fibroblast growth factors, Wnts and insulin/insulin-like growth factors, can act synergistically with SHH to promote chondrogenesis (Murtaugh et al., 1999), myogenesis (Munsterberg et al., 1995; Pirskanen et al., 2000) or patterning (Liem et al., 2000; Dale et al., 1997; Stull and Iacovitti, 2001; Laufer et al., 1994). Transcription factors such as PAX1 and PAX6 cooperate with SHH in the control of proliferation in developing somites (Furumoto et al., 1999) or patterning of the neural tube (Ericson et al., 1997b), respectively. In addition, extracellular matrix glycoproteins, such as laminin and vitronectin, may modulate proliferative and differentiation responses to SHH (Pons et al., 2001).

How modulators and co-acting factors influence SHH responses is not yet known. We became interested in evaluating the effect of heparan sulfate proteoglycans (HSPGs) on SHH signaling when work in *Drosophila* suggested that long-range hedgehog (HH) signals depend upon the normal synthesis of HSPGs. Mutation of the HSPG synthetic enzyme Tout-velu (TTV) results in a phenotype similar to the HH mutation. The TTV phenotype has been ascribed to a disruption of the ability of HH to diffuse and establish a concentration gradient (Bellaiche et al., 1998; The et al., 1999). Additionally, HH-HSPG interactions may modulate cellular responses to HH.

We asked whether HSPGs were important for vertebrate SHH signaling and if so, how. We evaluated the CNS expression of the *Ttv* orthologs, the exostosins (Exts) and found the highest level of *Ext1* and *Ext2* in the cerebellum. As cerebellar granule cells require SHH for proliferation (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999), this seemed an ideal system for studying the interaction of SHH and HSPGs. We report here that SHH interacts with HSPGs through a highly conserved heparin-binding domain. This interaction is not required for binding to patched (PTCH) but is necessary for maximal proliferative response to SHH. The influence of HSPGs on SHH induced proliferation increases with age during the neonatal period and is temporally correlated with an increase in expression of *Ext1* and *Ext2*, as well as increased binding of SHH to in situ HSPGs and a dramatic change in the SHH dose-response curve. This mature curve is bell-shaped, with peak proliferation elicited only by a sharply narrowed range of SHH concentrations. Together these data provide a molecular basis for SHH-heparin/HSPG interactions and identify HSPGs as important modulators of SHH-induced proliferation.

MATERIALS AND METHODS

Mutagenesis

The biologically active N-terminal fragment of murine SHH (amino acids 25-198) cloned into APtag4 (Flanagan and Leder, 1990) was from Andrew McMahon. Mutations were introduced by Quikchange (Stratagene, La Jolla, CA) according to manufacturer's instructions. Primer sequences were designed to introduce the desired amino acid changes as well as a new *Nhe*I (AlaSHH:AP and GlnSHH:AP) or *Dra*I (Arg+SHH:AP) restriction site to allow for clone selection. Sense mutagenesis primer sequences are as follows with base changes in bold: AlaSHH:A, 5′ GGAAAG**GC**GCG**C**CACCCC**GC**AAAGCTGACCCC**GC**TAGCC 3′; GlnSHH:AP, 5′ GGAAAG**CA**GCGGCACCCC**C**AAAAGCTGACCCC**GC**TAGCC 3′; and Arg+SHH:AP, 5′ CCCGGCAGGGGGTTT**AA**AAAGAGGCGG. Mutagenized DNA was used to transform Ultracomp MC1061/P3 amp^r *E. coli* (Invitrogen, Carlsbad, CA). *Nhe*I or *Dra*I digest of plasmid DNA was used to screen for mutants. Desired mutations were identified by direct sequencing of purified plasmids.

Transient transfection

Plasmids containing sequences for SHH:AP, AlaSHH:AP, GlnSHH:AP, Arg+SHH:AP and AP alone were transiently transfected into COS 7 cells by the Lipofectamine method (Gibco BRL, Rockville, MD). Plasmid DNA (4-8 µg) was mixed with 60 µl of lipofectamine per 100 mm tissue culture dish. Transfection proceeded for 9 hours. Transfected cells were maintained in serum free DMEM/F12 without supplements or antibiotics. Culture supernatants were collected every 24 hours and assayed for alkaline phosphatase activity. Protein was analyzed by western blotting with an antibody to alkaline phosphatase (Biomeda, Foster, CA).

Column chromatography

Wild-type or mutant SHH transfection supernatants (1 ml) were applied to a 3 ml heparin-agarose column (Sigma, St Louis, MO) in equilibration buffer (20 mM Tris pH 7.4, 150 mM NaCl and 0.1% Triton X-100). The column was washed with two volumes of equilibration buffer. Then, 20 ml of a salt gradient from 0 to 2 M NaCl in 20 mM Tris pH 7.4 and 0.1% Triton X-100 was applied. Fractions (0.5 ml) were obtained from the time of protein application until the end of the gradient. Elution of wild-type and mutant SHH was detected by alkaline phosphatase activity of each fraction. The peak of elution was determined by curve fitting the gradient profile to y=mx + b and deriving a value for y (molarity of NaCl) at the peak (x=fraction number) of elution.

Alkaline phosphatase assay

Determination of alkaline phosphatase activity was accomplished by incubation with 2 M diethanolamine (Sigma), 0.5 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin (BSA) and 12 mM p-nitrophenylphosphate (Sigma 104$^{®}$ phosphatase substrate). Reactions proceeded at 37°C for 20 minutes and the reaction product was quantitated by measuring sample absorbance at 405 nm.

Section binding assay

In situ HSPG binding was evaluated by methods based on those of Friedl (Friedl et al., 1997). Briefly, brains from postnatal day 3 and 6 BALB/c mice were removed and fixed in 4% paraformaldehyde for 24 hours and cryoprotected in 30% sucrose. Sections were treated, or not, with a combination of 1 mU/ml of heparinase I (Sigma) and 1 mU/ml heparinase III, overnight at 4°C (Sigma). Autofluorescence was diminished by treatment with 0.05% sodium borohydride for 10 minutes at room temperature, followed by treatment with 0.1 M glycine at 4°C overnight. Non-specific ligand binding was blocked with 1% BSA in phosphate-buffered saline (PBS) for 1 hour at room temperature. Equimolar amounts of SHH:AP or AlaSHH:AP were added for 1 hour at room temperature. Sections were washed with PBS containing 0.5 M NaCl to dissociate any low affinity interaction between ligands and HSPGs. Washed sections were incubated with rabbit anti-human alkaline phosphatase for 1 hour at room temperature (Biomeda). Ligand-antibody complexes were visualized with a Cy3-conjugated goat anti-rabbit IgG, for 1 hour at room temperature (Jackson Immunoresearch). High magnification views of binding were examined by DeltaVision® restoration fluorescence microscopy (Applied Precision, Issaquah, WA), viewed with a 60× objective. z-series comprising 20 0.2 µm serial optical sections were acquired and deconvolved with softWoRx imaging software (Applied Precision). Final images are single optical sections rendered in softWoRx volume viewer.

In situ hybridization

In situ hybridization was performed as described (Klein et al., 2001). Briefly, brains from postnatal day 8 BALB/c mice were removed and fixed in 4% paraformaldehyde for 24 hours and cryoprotected in 30% sucrose. Sagittal sections (15 µm) were obtained and treated with 20 µg/ml proteinase K for 10 minutes at room temperature. Sections were fixed in paraformaldehyde and washed in PBS. Hybridization was performed with digoxigenin (DIG)-labeled sense and antisense RNA probes for 20 hours at 65°C in hybridization buffer (50% formamide, 5× SSC, 100 µg/ml yeast tRNA, 100 µg/ml heparin, 1× Denhardt's, 0.1% Tween 20, 0.1% CHAPS, 5 mM EDTA). Sections were washed with 0.2× SSC, 0.1% Tween 20 at 65°C and treated with 20% sheep serum to block non-specific binding. Hybridized DIG-labeled probes were visualized with an antibody to DIG according to manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany).

Probe preparation

Plasmid containing full-length mouse *Ext1* was from Dominique Stickens and Glenn Evans. Plasmid containing rat *Ext 2* probe was from IMAGE consortium (IMAGE clone ID: UI-R-EO-dd-h-11-0-UI.s1). Sequence and orientation of each probe was confirmed by direct sequencing. Sense and antisense digoxigenin-labeled riboprobes were synthesized using DIG/Genius labeling kit according to the manufacturer's instructions (Boehringer Mannheim*)*.

Northern blot analysis

RNA was obtained from pooled cerebella using Trizol according to manufacturer's instructions (Gibco BRL). Total RNA (25 µg) was electrophoresed on a 1.2% agarose formaldehyde gel and transferred to GeneScreen membrane (NEN Life Science Products, Boston, MA). Prehybridization and hybridization was performed as previously described (Klein et al., 2001). ^{32}P-labeled full-length *Ext1* and 389 bp *Ext2* antisense probes were generated by random primed DNA synthesis (Promega Life Sciences, Madison, WI).

Primary culture

Primary cultures of neonatal mouse cerebellum were established as previously described (Klein et al., 2001). Briefly, cerebella were dissected and meninges were removed. After incubation with 0.1% trypsin (Sigma) in HBSS with 125 units/ml DNase (Sigma), 0.5 mM

EDTA for 20 minutes at 37°C, cells were pelleted in a clinical centrifuge. Cell pellets were washed three times with HBSS. The final cell suspension was passed through a 100 μM nylon mesh cell strainer (Falcon, Franklin Lakes, MI). Cells were diluted to 2×10^6 cells/ml in DMEM/F12 (Gibco BRL) supplemented with N2 (Gibco BRL), 20 mM KCl, 36 mM glucose and penicillin/streptomycin, SHH, SHH:AP or AlaSHH:AP as indicated, and plated at 2×10^5 cells/well onto a 96-well tissue culture dish coated with 15 μg/ml poly-ornithine (Sigma). Control cultures were treated with an equivalent volume of media conditioned by non-transfected COS cells. For heparinase treatment, a mixture of heparinase I 1 mU/ml (Sigma) and heparinase III 1 mU/ml (Sigma) was added at 24 hours post-plating.

Proliferation assay

At 36-40 hours post-plating cultures were treated with 5 μCi/well of [^{3}H]thymidine (New England Nuclear, Boston, MA). After 4 hours at 37°C, Triton X-100 was added to a final concentration of 1% and cells were lysed for 10 minutes at room temperature. Ice-cold trichloroacetic acid was added to a final concentration of 10% and DNA was precipitated for 1 hour on ice. Precipitated DNA was collected by vacuum filtration through phosphocellulose membranes (Pierce, Rockford, IL). Filters were washed with ice-cold 10% TCA, dried with 100% ethanol and then solubilized in Scintisafe (New England Nuclear) and counted. Each experiment was performed in quadruplicate. Representative experiments are presented as mean DPM±s.e.m. Statistical significance was determined by two-tailed Student's *t*-test.

Binding assay

Primary cerebellar cultures prepared as above were washed with PBS. Measurements of specific binding were conducted on unfixed cultures or cultures that had been fixed in 4% paraformaldehyde for 10 minutes at room temperature. In all cases, non-specific binding was reduced by treatment of cultures with 1% BSA in PBS for 1 hour on ice.

Total specific binding

Cultures treated or not with heparinase were incubated with 1 nM SHH:AP for 2 hours on ice in the absence or presence of 100 nM unconjugated SHH. Cultures were washed three times with buffer containing 20 mM Tris pH 7.4 and 0.75 M NaCl. Bound ligand was then measured by assessing alkaline phosphatase activity as described above. Binding experiments were carried out in quadruplicate and data are presented as mean±s.e.m.

Competition binding

Fixed cultures were incubated with increasing concentration of SHH:AP or AlaSHH:AP (0.7-35 nM) in the absence or presence of 100 nM unconjugated SHH. Cultures were washed, and cell-associated alkaline phosphatase activity was determined as above. Specific binding was derived by subtracting the cell-associated alkaline phosphatase activity measured in the presence of excess unconjugated SHH from the cell-associated alkaline phosphatase activity measured in the absence of unconjugated SHH. Each determination was done in triplicate and data are presented as the mean cell-associated AP activity±s.e.m.

Scatchard analysis

SHH:AP (1 nM) was added and incubated on ice for 2 hours in absence or presence of 5-500 nM unconjugated SHH. Concentration of bound SHH was calculated as the product of (cell-associated alkaline phosphatase activity/total applied alkaline phosphatase activity) and (concentration of total SHH). Free SHH was calculated as the difference between total applied SHH and bound SHH. Each determination was made in triplicate and data are presented as mean bound/free±s.e.m. versus the mean bound (nM). Values for K_d and B_max were derived from a linear curve fit to the steepest region of the relationship.

RESULTS

SHH has a heparin-binding domain

The ability of hedgehog proteins to interact with heparin is well established (Bumcrot et al., 1995; Lee et al., 1994). However, the molecular basis for heparin binding is as yet unknown. We examined the sonic hedgehog (SHH) sequence for identifiable motifs that might bind to heparin/HSPGs. We discovered a highly conserved Cardin-Weintraub consensus sequence for heparin binding at the N terminus of the biologically active fragment of SHH (Fig. 1A) (Cardin and Weintraub, 1989). This motif, XBBBXXBX, is characterized by a cluster of basic amino acids (B) that allows for electrostatic interaction between the positive charges on the protein and the negatively charged sulfates of HSPGs. All hedgehog proteins contain the sequence with slight variations in amino acid composition (Fig. 1C). Two basic amino acid positions within the potential heparin-binding motif are absolutely conserved between *Drosophila* and the family of vertebrate hedgehogs. In order to assess the role of this sequence in heparin/HSPG binding, we mutated these two basic amino acids, Arg33 and Lys37, to alanine or glutamine (Fig. 1B). The mutation to both alanine and glutamine allowed us to assess the contribution of hydrogen bonding to interactions between heparin and this domain. In addition, we created an alternative mutation in which we added an extra arginine in position 31 as a tool for evaluating whether total positive charge influenced SHH-HSPG interactions. Sequences of mutants discussed in this paper are given in Fig. 1B. All mutations were introduced into the biologically active conjugate protein comprised of the N-terminal fragment of mouse SHH and human placental alkaline phosphatase developed by Yang et al. (Yang et al., 1997).

To determine whether mutation of this domain altered the interaction between SHH and HSPGs, we evaluated the binding of wild-type SHH (SHH:AP) and the mutant SHHs (AlaSHH:AP, GlnSHH:AP and Arg+SHH:AP) to a heparin-agarose column. Gradient salt elution from heparin-agarose columns allowed us to characterize the relative affinities of wild-type and mutant SHHs for heparin. Wild-type SHH:AP eluted from the column with two peaks of activity at 0.48 and 0.76 M NaCl (Fig. 2A,B). Mutation of position 33 and 37 to either Ala or Gln resulted in the loss of the higher affinity peak (0.76 M), but maintenance of the lower affinity peak (0.5 M and 0.45 M respectively). As the behavior of these two mutants was indistinguishable in this assay (Fig. 2B), hydrogen-bonding alone does not appear to be sufficient for the higher affinity interaction. Addition of a basic amino acid (Arg+SHH:AP) had no significant effect on elution (Fig. 2B) and therefore affinity does not appear to be a simple function of the total number of positive charges. Alkaline phosphatase alone did not bind to the column (data not shown). Elution from heparin-agarose at 0.5 M NaCl has been described for positively charged proteins that do not possess specific heparin-binding domains (Klagsbrun, 1990). This lower affinity peak may therefore represent a non-specific electrostatic interaction. Alternatively, it could indicate that other, non-mutated, moieties contribute to specific, lower affinity, interactions between SHH and heparin. Together these data indicate that the Cardin-Weintraub sequence is the domain that mediates high affinity interactions between SHH and heparin. Given that substitution with either Ala or Gln produced similar changes

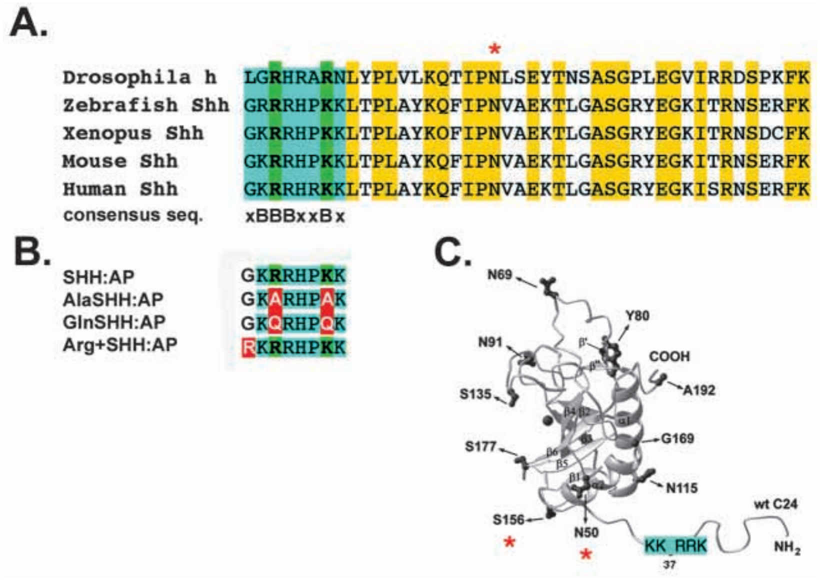


Fig. 1. Sonic hedgehog contains a Cardin-Weintraub consensus sequence for heparin binding. (A) Alignment of vertebrate sonic hedgehog and *Drosophila* Hedgehog protein sequences. Identical amino acids are in yellow and the putative heparin-binding domain is in blue. Green highlights indicate two absolutely conserved basic amino acid positions. Pictured below the sequence alignment is the Cardin-Weintraub consensus sequence for heparin binding; B represents basic amino acids. Red asterisk identifies N50, an amino acid involved in binding to PTCH. (B) Sequences of mutant SHH:AP generated in these studies. (C) Molecular model of SHH demonstrating the Cardin-Weintraub sequence (blue), and N50 and S156 (red asterisks), two amino acids involved in binding to patched [adapted from Pepinsky (Pepinsky et al., 2000)].

in heparin binding, further experiments to characterize the function of SHH-HSPG interactions were limited to the comparison of SHH:AP and AlaSHH:AP.

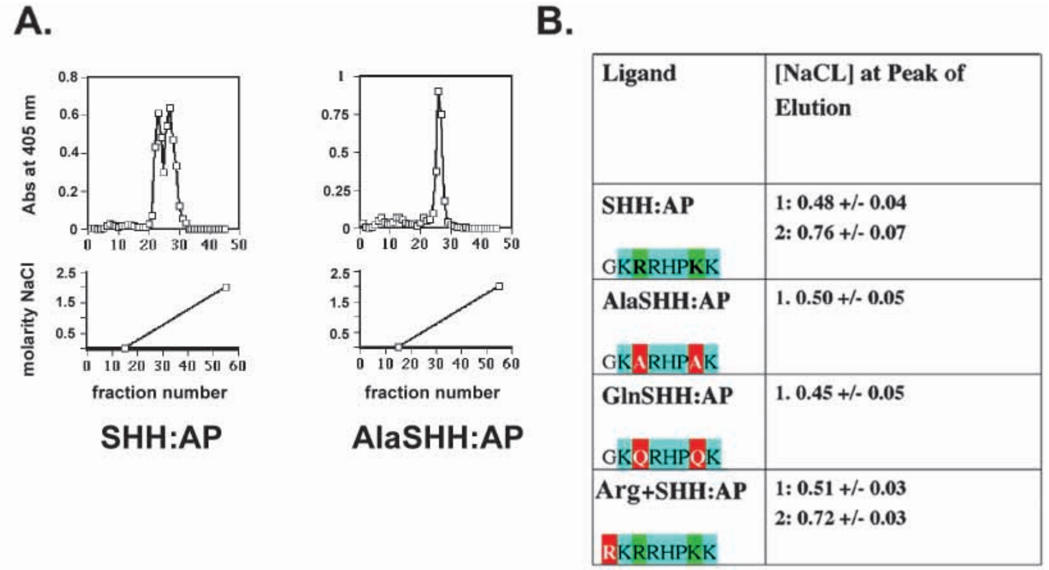
Ttv homologs *Ext1* and *Ext2* are expressed in the postnatal cerebellum

The evolutionary conservation of the heparin-binding domain among the family of hedgehog proteins suggests that it plays an important role in hedgehog biology. Genetic analyses in *Drosophila* support this notion. Mutation of *Ttv*, a glycosyltransferase involved in HSPG synthesis, has been demonstrated to phenocopy the *Hh* mutation (Bellaiche et al., 1998; The et al., 1999; Toyoda et al., 2000). We examined the expression of the closest vertebrate homologs of *Ttv*, *Ext1* and *Ext2* (Ahn et al., 1995; Stickens et al., 1996) in developing mouse brain in order to identify developmental systems in which to evaluate interactions of SHH and HSPGs. In the neonatal mouse brain *Ext1* (Fig. 3A,B) and *Ext2* (Fig. 3B) exhibited overlapping patterns of expression, with the highest levels of mRNA evident in the cerebellum. Expression was also detectable in the hippocampus as well as the olfactory and neo-cortices. Within the cerebellum, *Ext1* and *Ext2* are expressed by granule cells of both the internal (IGL) and external (EGL) granule cell layers as well as by Purkinje cells (Fig. 3C). Northern blot analysis indicated that expression of *Ext 2* is developmentally regulated in the cerebellum (Fig. 4A). No *Ext2* mRNA was evident in total RNA samples from P0 mice. However at P2 and P4, equivalent, low levels of mRNA were detected and expression of *Ext2* increased 4.5-fold from P4 to P9. *Ext1* expression was similarly regulated during cerebellar development, with a comparable fourfold increase in expression between P4 and P9 (Fig. 4B). The increase in *Ext* expression parallels the increase in granule cell proliferation observed in vivo during the first postnatal week (Mares et al., 1970). This early proliferation requires SHH (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Together, these findings suggest that HSPGs, synthesized by the gycosyltransferases *Ext1* and *Ext2*, might be present at an appropriate time and place to regulate SHH-induced proliferation during cerebellar development.

SHH interacts with in situ HSPGs through the Cardin-Weintraub sequence

We next sought to determine whether Ext expression in the cerebellum was associated with synthesis of HSPGs that interact with SHH. Growth factor interactions with low-affinity proteoglycan binding sites can be evaluated using the method of Friedl (Friedl et al., 1997). In this assay, excess amounts of ligand are bound to both low-volume, high-affinity specific receptor-binding sites as well as to high-volume, lower-affinity proteoglycan receptor-binding sites in fixed tissue sections. Bound ligand is then visualized immunohistochemically. This approach has been used to demonstrate decreased association of Indian hedgehog with HSPGs in *Ext1*-deficient embryos (Lin et al., 2000). We found that in sections from P3 mice, when *Ext* expression is low, SHH:AP did not bind significantly to any layer of the cerebellum (Fig. 5A). However, SHH:AP bound extensively to the EGL at P6, when *Ext* expression has increased twofold and granule cell proliferation in vivo is at a maximal level (Mares et al., 1970). In addition, lower levels of SHH:AP binding were observed at the pial surface of the cerebellum and in the IGL. Similar patterns of SHH:AP binding were seen at P9 (data not shown). At P3 the binding of the AlaSHH:AP mutant was similar to that seen with wild-type SHH:AP. By contrast, at P6 (Fig. 5A) and P9 (data not shown), binding of AlaSHH:AP was significantly less than what was observed for SHH:AP. Thus, increasing *Ext* expression appears to be associated with synthesis of a proteoglycan species to which SHH can bind with relatively high affinity. This binding depends upon an intact Cardin-Weintraub sequence, suggesting that the relevant proteoglycan is likely to be an HSPG.

To determine directly whether SHH was binding to HSPGs in the EGL and IGL of cerebellar sections, we treated tissue sections with heparinase I and III to remove heparan sulfate side chains. After heparinase treatment, SHH:AP binding to the EGL and IGL was greatly diminished (Fig. 5B), indicating that SHH:AP binds to endogenous HSPGs within the granule cell layers. Low levels of SHH:AP binding to heparinase treated sections was most evident at the pial surface of the cerebellum. Similarly, residual binding of AlaSHH:AP was predominantly localized to the pia (Fig. 5A). Thus SHH:AP binds to HSPGs in the EGL and to a lesser extent the IGL. In addition, SHH:AP



Ligand	[NaCL] at Peak of Elution
SHH:AP GKRRHPKK	1: 0.48 +/- 0.04 2: 0.76 +/- 0.07
AlaSHH:AP GKARHPAK	1. 0.50 +/- 0.05
GlnSHH:AP GKQRHPQK	1. 0.45 +/- 0.05
Arg+SHH:AP RKRRHPKK	1: 0.51 +/- 0.03 2: 0.72 +/- 0.03

Fig. 2. Sonic hedgehog interacts with heparin through the Cardin-Weintraub sequence. (A) Elution profile of SHH:AP and AlaSHH:AP from a heparin-agarose column in a continuous salt gradient. SHH:AP and AlaSHH:AP content of each fraction was quantified by its alkaline phosphatase activity. Two peaks are evident for SHH:AP but only one for AlaSHH:AP. (B) Elution behavior of SHH:AP and three mutants. The molarity of peak ligand elution was calculated from curve fits to the plot of NaCl concentration as a function of fraction number. Data are the mean±s.e.m. of three separate experiments. SHH:AP and Arg+SHH:AP behave identically with peaks of elution at ~0.5 and ~0.75 M NaCl. By contrast, AlaSHH:AP and GlnSHH:AP each display only a single peak of elution at ~0.5 M.

apparently binds to a non-heparan sulfate containing constituent of the pia.

At higher magnification, SHH:AP binding localizes in a lattice-like pattern around granule cell bodies in the EGL (Fig. 5C). The pattern suggests that the bulk of the SHH:AP-HSPG interactions occur at the surface of granule cells and/or in the extracellular matrix. The ability of SHH:AP to bind to these sites is reduced by mutation of the Cardin-Weintraub sequence.

Comparison between the binding of SHH:AP to sections of P3 and P6 mouse cerebellum demonstrates that the ability of HSPGs in the granule cell layers to bind to SHH is developmentally regulated. The increased binding of SHH:AP to P6 relative to P3 sections parallels the increases in *Ext1* and *Ext2* expression and is temporally correlated with increased granule cell proliferation in vivo (Mares et al., 1970).

Loss of SHH-HSPG interactions decreases SHH-induced proliferation

We next asked whether interaction of SHH with HSPGs is important for biological effects of SHH. Granule cells proliferate postnatally (Altman, 1972a; Altman, 1972b) and SHH is a potent mitogen for this proliferation (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). We evaluated whether mutation of the Cardin-Weintraub sequence (AlaSHH:AP) affects the ability of SHH to promote proliferation of cerebellar cells. Granule cell cultures derived from P6 mice, the stage of maximal granule cell proliferation, exhibited a bell-shaped proliferation dose-response curve in response to SHH:AP (Fig. 6A, white squares) or SHH (Fig. 6B, white squares). Peak proliferative responses were observed for 1.5 μg/ml (35 nM) conjugate protein or 0.28 μg/ml (14 nM), SHH. When we compared the effects of equimolar amounts of SHH:AP and AlaSHH:AP, we found that loss of SHH-HSPG interactions was associated with a reduction in the proliferative responses (Fig. 6A, black diamonds compared to white squares). The peak of proliferation in response to AlaSHH:AP was decreased to 60% of the response to wild-type SHH:AP (n=8, *P*<0.02), but occurred at the same dose.

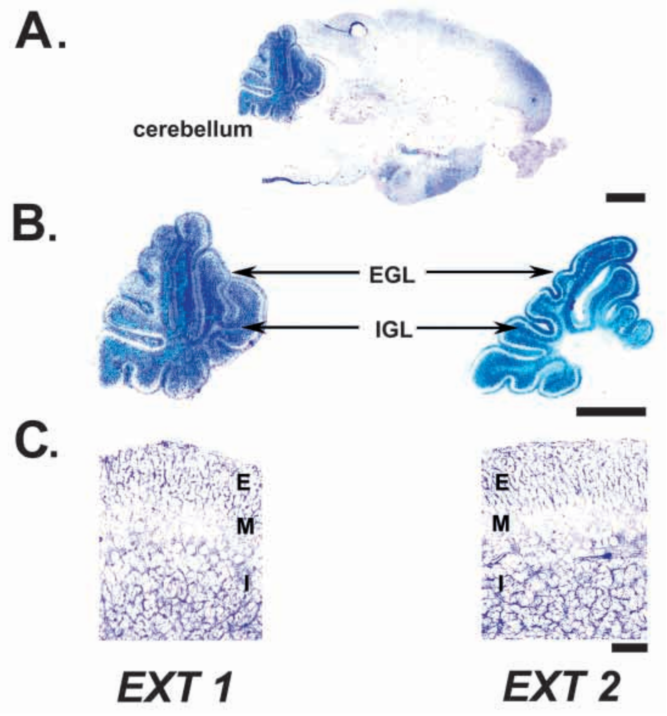


Fig. 3. *Ext1* and *Ext2* expression during early postnatal development in mouse brain. (A) In situ hybridization for *Ext1* in a postnatal day 8 mouse brain. *Ext1* mRNA is most abundant in the cerebellum. Scale bar: 1 mm. (B) *Ext1* and *Ext2* have similar patterns of expression in postnatal day 8 mouse cerebellum. Scale bar: 1 mm. (C) Higher magnification views of the cerebellum reveal that both *Ext1* and *Ext2* are expressed by granule cells of the EGL and IGL as well as by Purkinje cells. E, EGL; M, molecular layer; P, Purkinje cell layer; I,IGL. Scale bar: 25 μm.

We questioned whether disrupting the SHH-HSPG

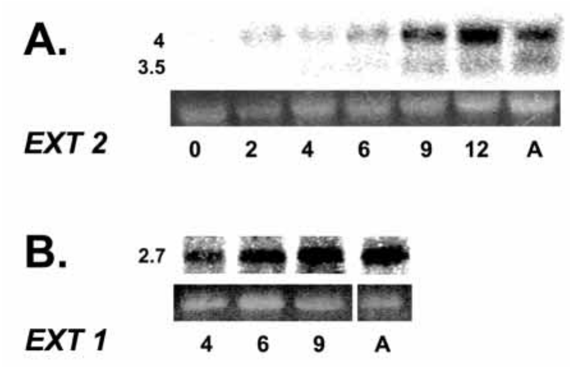


Fig. 4. *Ext1* and *Ext2* expression increases during postnatal cerebellar development. (A) Northern blot analysis of *Ext2* expression in developing and adult mouse cerebellum reveals that expression increases with increasing postnatal age, particularly from P4 to P9. *Ext2* mRNA is present as two transcripts of 3.5 and 4 kb. (B) *Ext1* expression demonstrates a similar increase between P4 and P9. *Ext1* mRNA is present as a single species of 2.7 kb. Ethidium Bromide images corresponding to each northern blot are shown as loading controls (numbers refer to postnatal age; A, adult).

interactions in other ways would yield the same results. We therefore treated cultures with a mixture of heparinase I and III to digest the heparan sulfates (HS). P6 cultures, treated with heparinase, exhibited decreased proliferative responses to SHH (Fig. 6B, compare black diamonds with white squares). Similar to the results seen with AlaSHH:AP, peak responses were decreased to 65% of control (*n*=20, *P*<0.001). A reduction of the peak proliferative response to 51% of control was also obtained by treating cultures with sodium perchlorate to prevent sulfation of HSPGs (data not shown, *n*=12, *P*<0.001). Together these data demonstrate that direct interaction between SHH and HSPGs is necessary for maximal proliferative response to SHH in P6 cerebellar cultures.

The dependence of granule cell proliferation on SHH-HSPG interactions is developmentally regulated

Given the age-dependent changes in cerebellar *Ext* expression and SHH:AP-HSPG interactions, we asked whether granule cell proliferation also displays age-dependent changes in the requirement for SHH-HSPG interactions. When we examined primary cultures from P3 mice (when *Ext* expression and SHH:AP-HSPG binding are low), we observed a markedly different dose response to SHH when compared with that observed for cultures from P6 mice (when *Ext* expression and SHH-HSPG binding are high). Whereas P6 cultures exhibited a bell-shaped SHH dose-response curve, cultures derived from P3 mice displayed increasing proliferation in response to increasing doses of SHH:AP (Fig. 6C, white squares) or SHH (Fig. 6D, white squares). The magnitude of the peak response was less at P3 when compared with P6 but the range of effective SHH concentrations was broader.

In addition to age-dependent changes in the SHH dose-response curve, there was also an age-dependent change in the impact of SHH-HSPG interactions on these responses. In cultures derived from P3 mice, when *Ext* expression is low, mutation of the Cardin-Weintraub sequence had no effect on proliferative responses (Fig. 6C, compare black diamonds with white squares). Similarly, in P3 cultures, degradation of

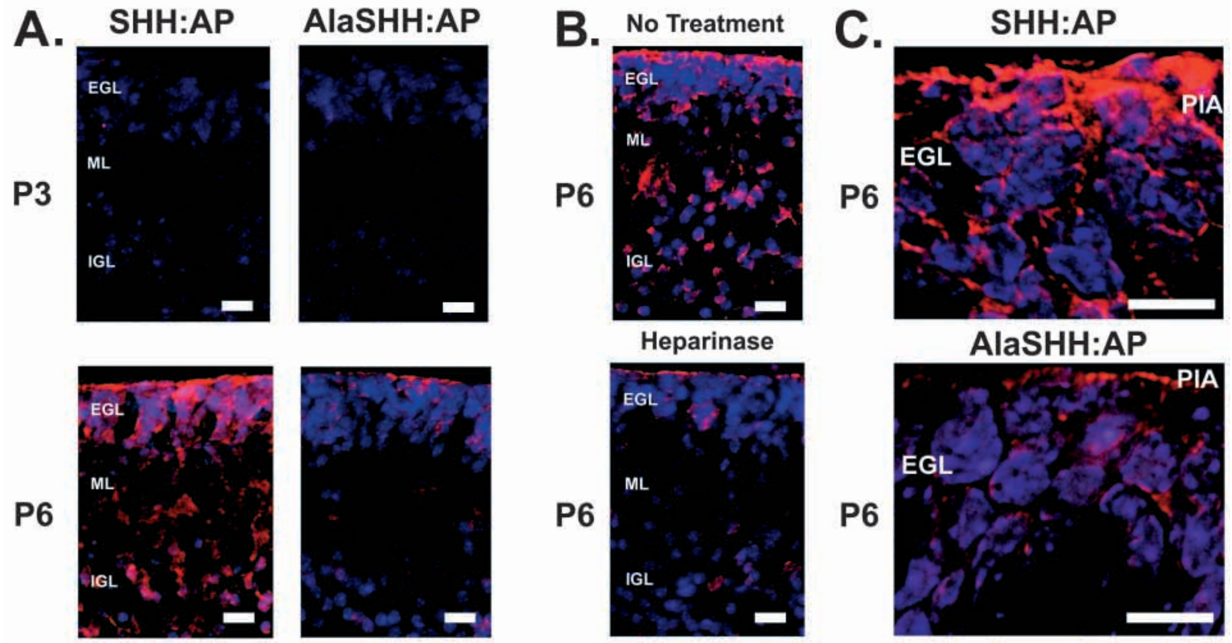


Fig. 5. SHH binds to endogenous HSPGs through the Cardin-Weintraub sequence. The binding of SHH:AP and AlaSHH:AP to endogenous HSPGs in postnatal day 3 and 6 cerebellum was evaluated by modification of the method of Friedl (Friedl et al., 1997). (A) SHH:AP and AlaSHH:AP exhibit very low levels of binding (red) to sections from P3 mice. DAPI-stained nuclei appear blue. Significant amounts of SHH:AP but not AlaSHH:AP are bound by P6 HSPGs, suggesting that SHH:AP requires a wild-type Cardin-Weintraub sequence for this binding. Scale bar: 10 µm. (B) Heparinase I and III treatment results in significantly decreased binding of SHH:AP to the EGL and IGL in sections of P6 cerebellum. Scale bar: 10 µm. (C) Higher magnification view of the SHH:AP localization. Mutation of the Cardin-Weintraub sequence (AlaSHH:AP) reduces the total level of binding. Scale bar: 10 µm.

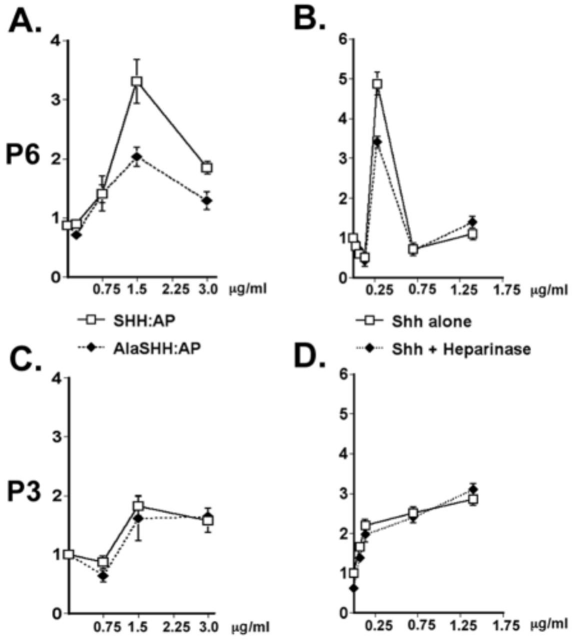


Fig. 6. Interactions between SHH and HSPGs modulate proliferation of more mature granule cells. (A) Proliferation dose response of primary cerebellar cultures from P6 mice to SHH:AP (white squares) or AlaSHH:AP (black diamonds). In this and the following panels, a representative experiment is shown in which data from quadruplicate cultures was averaged and normalized to the mean incorporation observed in the absence of SHH. Values are represented as fold proliferation relative to this control±s.e.m. (B) P6 cultures were treated with SHH in the absence (white squares) or presence (black diamonds) of a mixture of heparinase I and III. In both A and B, P6 cultures display a bell-shaped dose-response to SHH, and disruption of the interaction of SHH and HSPGs reduces the peak proliferative response. (C) Proliferation dose response of primary cerebellar cultures derived from postnatal day 3 (P3) mice to SHH:AP (open squares) or AlaSHH:AP (filled diamonds). (D) P3 cultures were treated with unconjugated SHH in the absence (white squares) or presence (black diamonds) of a mixture of heparinase I and III. In both C,D, P3 cultures display increasing proliferation in response to increasing doses of SHH and disruption of the interaction between SHH and HSPGs has no effect on the proliferative response.

heparan sulfates with heparinase I and III had no effect on the dose response to SHH (Fig. 6D, compare black diamonds with white squares). Taken together, these data suggest that minimal SHH-HSPG interactions are taking place at P3. Of note, the height of peak proliferation in P3 cultures (twofold over control) was similar in magnitude to the peak proliferation observed in P6 cultures treated with AlaSHH:AP or SHH in the presence of heparinase. Thus, the magnitude of the peak of proliferation in response to SHH in the absence of interactions with HSPGs was the same regardless of age. We conclude that age-dependent changes in *Ext* expression are temporally correlated with: (1) increased synthesis of proteoglycan binding sites for SHH; (2) changes in the proliferation dose response of cerebellar granule cells to SHH; and (3) increasing influence of SHH-HSPG interactions on proliferation.

Mutation of the Cardin-Weintraub sequence does not alter binding to patched

HSPGs frequently act as co-receptors that facilitate high affinity binding of ligands to their specific receptors (Bernfield et al., 1999). As mutation of the SHH Cardin-Weintraub sequence abrogated SHH-HSPG interactions and reduced the proliferative response, we asked whether or not AlaSHH:AP retained its high-affinity binding to PTCH. Several observations suggested that disruption of SHH-HSPG interactions did not lead to decreased proliferative responses as a result of a loss in high-affinity binding. A decrease in receptor binding would have shifted the dose-response curve in P6 cultures to the right rather than diminish the peak. Additionally cultures derived from P3 mice were not affected by treatments that abrogated SHH interactions with HSPGs. This argues against an absolute requirement for SHH-HSPG interactions for receptor binding and activation.

We first characterized the high-affinity binding of SHH to primary cultures of cerebellar cells. Scatchard analysis of unconjugated SHH binding revealed a K_d of binding of 23 nM (Fig. 7A), and indicated that there were ~ 50×10^3 high-affinity SHH-binding sites per granule cell. Previously measured values for the K_d of SHH binding have ranged between 0.46 and 7 nM in transfected cells expressing high levels of PTCH (Fuse et al., 1999; Marigo et al., 1996; Pathi et al., 2001; Stone et al., 1996) and are comparable with the values measured here in primary cultures. In addition the Scatchard analysis suggests that proliferative granule cells possess a relatively high number of receptors.

Having characterized the high-affinity binding of SHH to primary cerebellar cultures we asked whether HSPGs function as co-receptors for specific SHH binding. To do so, we examined the high-affinity binding of SHH:AP and AlaSHH:AP. Primary cultures derived from postnatal day 6 mouse cerebella were fixed and incubated with increasing amounts of either SHH:AP or AlaSHH:AP (0.7 to 35 nM) in the absence or presence of 100 nM unconjugated SHH. Cultures were then washed with high salt to remove SHH bound to HSPG sites and the remaining specific, cell-associated AP activity was quantified. Competition studies revealed that half saturating doses of SHH:AP and AlaSHH:AP were identical at 3.5 nM (Fig. 7B). Saturation occurred by 7 nM for both wild-type and mutant SHH:AP. These data indicate that there is no detectable difference in the specific binding of SHH:AP and AlaSHH:AP.

To assess further whether HSPGs function as co-receptors for SHH, we examined the effect of heparinase on SHH binding. Disruption of the interaction of SHH with HSPGs by heparinase treatment did not diminish specific SHH:AP binding (Fig. 7C). Taken together, these data indicate that HSPGs function as modulators of SHH-induced proliferation but do not function as facilitators of SHH-PTCH binding.

DISCUSSION

Sonic hedgehog possesses a Cardin-Weintraub sequence for heparin and endogenous HSPG binding

It is well established that SHH can interact with heparin (Bumcrot et al., 1995; Lee et al., 1994) and work in *Drosophila* has indicated that HSPGs are essential for hedgehog function (Bellaiche et al., 1998). The molecular basis for these interactions between hedgehog proteins and heparin/HSPGs has not previously been determined. We have identified a highly

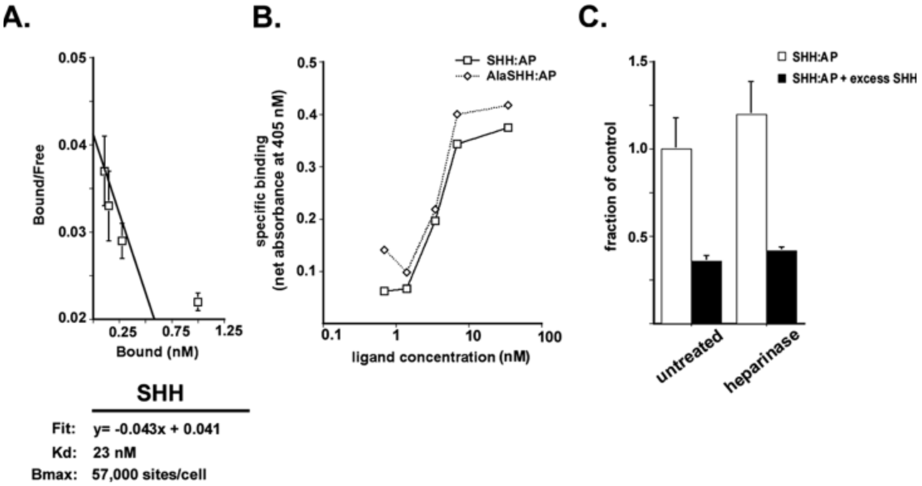


Fig. 7. Mutation of the Cardin-Weintraub sequence and disruption of SHH-HSPG interactions does not alter receptor binding. (A) Fixed cultures were incubated with 1 nM of SHH:AP and increasing amount of unconjugated SHH (5-500 nM). Data are the means of triplicate determinations±s.e.m. Linear fits to the steepest region of the bound/free versus bound curve are given beneath each plot along with calculated values for K_d and B_max. (B) Cells were incubated for 2 hours on ice with increasing doses of SHH:AP or AlaSHH:AP (0.7 to 35 nM) in the absence or presence of 100 nM unconjugated SHH. Specific binding was derived from the difference between triplicate determinations of binding in the absence and presence of excess unconjugated SHH. Specific binding is plotted as net absorbance in OD units at 405 nm. (C) P6 cultures were treated with a mixture of 1 mU/ml heparinase I and III or not as indicated. Specific SHH:AP binding was measured by incubation of 1 nM ligand in the absence (white bars) and presence (black bars) of 100 nM unconjugated SHH. Data are the means of quadruplicate determinations±s.e.m. and are plotted as the fraction of binding measured for SHH:AP alone.

conserved Cardin-Weintraub sequence of the type, XBBBXXBX in the N terminus of all vertebrate hedgehog proteins (Cardin and Weintraub, 1989) and shown that it mediates binding to heparin/HSPGs. The distance between the Cardin-Weintraub sequence and two amino acids necessary for SHH binding to its receptor PTCH has been conserved (Pepinsky et al., 2000), raising the possibility that simultaneous binding to both HSPGs and PTCH is important for SHH function.

The Cardin-Weintraub sequence consists of a cluster of basic amino acids that allow for protein interaction with sulfates contained within the glycosaminoglycan side chains of proteoglycans. The SHH Cardin-Weintraub sequence occurs in the N terminus (amino acids 31-38 of murine SHH) of the biologically active fragment of SHH, a region of the molecule that is lacking in significant tertiary structure (Pepinsky et al., 2000). Freedom of movement in this domain could be important to its function (Cardin and Weintraub, 1989). Mutation of the two conserved basic amino acid positions (33 and 37) within the Cardin-Weintraub sequence to either alanine (AlaSHH:AP) or glutamine (GlnSHH:AP) results in loss of high-affinity interaction with heparin. The finding that two out of five basic amino acids in a Cardin-Weintraub sequence are predominantly responsible for high-affinity interaction with heparin is similar to what has been found for the chemokine SDF-1α (Sadir et al., 2001). Addition of a basic amino acid adjacent to the Cardin-Weintraub sequence (Arg+SHH:AP) had no effect on heparin binding. Together, these data suggest that interaction between SHH and heparin is mediated by the Cardin-Weintraub sequence, requires interactions other than hydrogen bonding and is not a simple function of total positive charge.

The Cardin-Weintraub sequence mediates interaction of SHH with endogenous HSPGs in the cerebellum. When we tested the ability of endogenous HSPGs in P6 cerebellar tissue to bind wild-type SHH:AP, binding was greatly diminished by treatment of tissue sections with heparinase, identifying the relevant proteoglycan as an HSPG. Binding was similarly diminished by mutation of the Cardin-Weintraub sequence (Fig. 5). These data identify the Cardin-Weintraub sequence as an essential domain for the binding of SHH to endogenous HSPGs as well as to heparin.

The identity of the relevant HSPG remains unclear. There are two families of pure heparan sulfate proteoglycans that can be distinguished by their core proteins. The syndecans possess membrane spanning core proteins, while the glypicans are characterized by core proteins that are GPI linked to the cell surface. There is some speculation that glypicans are the HSPGs that are most likely to bind hedgehog proteins (De Cat and David, 2001). However, so far, the proteoglycans that interact with hedgehog proteins have not been identified. Data presented here suggest that SHH-HSPG interactions are determinants of more than just appropriate SHH localization. Thus, more than one type of HSPG may interact specifically with SHH and perform multiple functions.

Interaction between SHH and HSPGs is critical for developmental regulation of proliferation

We have found that there is an age-dependent change in the effect of HSPGs on cerebellar granule cell proliferation in response to SHH. Primary cultures from P3 mice display a sigmoidal dose response curve to SHH, that is not affected by mutation of the Cardin-Weintraub sequence, nor by treatment of cultures with heparinase or sodium perchlorate. At this stage, expression of *Ext1* and *Ext2* are low, and SHH:AP binds at low levels to HSPGs in cerebellar slices. These correlations suggest that HSPGs that participate in SHH responses may not be synthesized during the early neonatal period.

By contrast, proliferation in cultures derived from P6 mice was modulated by SHH-HSPG interactions. Primary cultures derived from P6 mice display a bell-shaped dose-response curve to SHH. Mutation of the Cardin-Weintraub sequence, or treatment of cultures with heparinase or sodium perchlorate, reduces the peak proliferative response to SHH. This developmentally regulated dependence on HSPG interactions is accompanied by increased expression of *Ext1* and *Ext2*, and the synthesis of HSPGs capable of binding to SHH (Fig. 5). Thus, at P6, the developmental stage when granule cell proliferation is maximal, HSPGs contribute to SHH-induced proliferation. Furthermore, SHH binds at highest levels to HSPGs in the EGL, the location of proliferating granule cell precursors. Thus, the regulated synthesis of HSPGs may allow optimal proliferation to occur at both the right time and place.

SHH is one of many growth factors that interact with low-affinity HSPG binding sites as well as with high-affinity primary receptors (Bernfield et al., 1999). Proteoglycans can modulate growth factor signaling by several possible mechanisms. They can increase the likelihood of ligand high-affinity receptor binding by limiting ligand diffusion to the two-dimensional space of the membrane surface rather than the three-dimensional extracellular space (Schlessinger et al., 1995). They can promote the formation of ligand dimers (Moy et al., 1997) and thereby enhance receptor activation. They can regulate internalization (Tyagi et al., 2001) and modulate intracellular signaling (Delehedde et al., 2000). Finally, they can possess independent signaling functions that are initiated by ligand interactions (Kinnunen et al., 1998).

HSPGs do not appear to modulate SHH responses by altering binding of SHH to PTCH. The peak proliferative response in P6 cultures occurred at the same SHH concentration regardless of the presence or absence of intact SHH-HSPG interactions. This indicates that HSPGs do not alter the affinity of receptor binding, in which case treatment with heparinase or mutant SHH would have produced a shift in the dose-response curve to the right. Consistent with this, when directly tested, wild-type and mutant SHH bound with equal affinity to receptor sites on the cell surface. However, the present studies do suggest that SHH biological activity is not a simple function of receptor binding. The coordinated interaction between SHH and HSPGs allows for modulation of receptor signaling in a developmentally regulated fashion. The identification of the molecular basis for the interaction between SHH and HSPGs will facilitate the elucidation of the mechanism by which HSPGs modulate SHH responses.

Two previous reports have evaluated the receptor binding and biological activity of SHH mutants that delete the N-terminal half of what is identified here as a consensus sequence for heparin binding (Fuse et al., 1999; Katsuura et al., 1999). Consistent with data presented here, both Katsuura et al. (Δ25-35) and Fuse et al. (Δ25-34) observed that this sequence was not essential for high-affinity binding to PTCH. Although the heparin binding of Δ25-35 was not evaluated, Fuse found that the Δ25-34 mutant was capable of binding heparin. However, no measurements of the affinity of this interaction were presented. It is therefore not possible to determine whether the deletion mutant had lost the higher affinity (0.75 M NaCl) heparin binding and retained only the lower affinity (0.5 M NaCl) binding in a manner similar to AlaSHH:AP and GlnSHH:AP mutants presented here. Alternatively, it is possible that the high affinity interaction between SHH and heparin is predominantly dependent on Lys37, which is preserved in the deletion mutant but not the AlaSHH:AP mutant described here, and not Arg33, which is altered in both mutants.

A comparison of the biological responses to the mutants is particularly revealing. Katsuura et al. (Katsuura et al., 1999), who evaluated induction of alkaline phosphatase activity, found that Δ25-35 SHH lost all biological activity. By contrast, Fuse et al. (Fuse et al., 1999), who used a neural plate HNF3β induction assay, found that Δ25-34 retained its biological activity. We observed that loss of SHH-HSPG interactions had no effect on SHH-induced proliferation at P3, but resulted in a dramatic decrement in the proliferative potency of SHH at P6. Thus, the modulation of SHH biological responses by HSPGs appears to be strongly context dependent.

The dose-response to SHH induced proliferation is developmentally regulated

Developmental regulation of SHH-induced proliferation is evident in the modulatory actions of HSPGs and also in changes in the shape of the dose-response curve. In cultures from P3 mice, proliferative responses of granule cells to SHH are characterized by a sigmoidal relationship between dose and proliferation. By contrast, cultures from P6 mice display a bell-shaped dose-response curve. While HSPGs modulate the magnitude of the peak response at P6, they do not appear to be responsible for the change in the shape of the dose-response curve. The morphogenetic effects of hedgehog proteins often display bell-shaped responses and depend upon the establishment of a SHH concentration gradient. Within these gradients, individual cell types are induced at limited locations, where the correct dose of SHH occurs (Ingham and McMahon, 2001).

Similarly, the bell-shaped proliferative response could constitute a mechanism for promoting granule cell proliferation exclusively in the EGL. SHH concentrations within the EGL may fall within the narrow proliferative range while those of the molecular layers and internal granule cell layers may be either too high or too low to induce proliferation. Thus the emergence of the bell shaped curve and the anatomic localization of modulatory factors such as HSPGs, SDF (Klein et al., 2001), laminin (Pons et al., 2001) and Notch2 (Solecki et al., 2001) may all work together to promote granule cell proliferation in the correct place and time.

We thank Curis Inc. for the generous gift of sonic hedgehog; Dr Andrew McMahon for the SHH:AP conjugate clone; Drs Glen Evans and Dominique Stickens for *Ext*1 clone; Erin Berry and Maria Pazyra for excellent technical assistance; and Drs Bernard Mathey-Prevot and Charles Stiles for helpful discussions. In accordance with the Harvard conflict of interest guidelines, we note that R. A. S. has a financial interest in Curis, Inc. This work was supported by grants from the NIH (NS37757 to R. A. S. and HD 01393 to J. B. R.), the McDonnell Foundation and the Levy Fund (R. A. S.).

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