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Heparan Sulfate Proteoglycans Exert Positive and Negative Effects in Shh Activity

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Abstract Hedgehog (Hh) proteins are morphogens involved in short- and long-range effects during early embryonic development. Genetic analysis in fly and vertebrate embryos showed that heparan sulfate proteoglycans (HSPGs) are required for Hh transport and signaling. To further understand how HSPGs regulate Sonic hedgehog (Shh), we performed experiments using cell culture and biochemical assays. When the synthesis of HSPGs was reduced, a decrease in Shh activity was observed. Contrary to that, addition of a peptide that competes the binding of Shh to HSPGs resulted in augmentation of Shh activity. From these results, we concluded that HSPGs exert positive and negative effects in Shh activity. This dual effect correlates with the finding that Shh interacts preferentially with two HSPGs. The current model for the role of HSPGs in Shh diffusion is discussed in view of our findings. J. Cell. Biochem. 96: 831–838, 2005.
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Hedgehog (Hh) proteins are a family of secreted proteins, including the mammalian homolog Sonic hedgehog (Shh) that regulates embryonic development and tissue homeostasis. Members of this family function as morphogens; they signal over short- and long-range in embryonic fields and induce different cell fates in a concentration-dependent manner [Ingham and McMahon, 2001]. Hh proteins are synthesized as a 45-kDa precursor, which is first autocatalytically processed and then covalently modified with cholesterol and a palmitate group [Lee et al., 1994; Porter et al., 1996; Pepinsky et al., 1998] producing a fully processed and active Hh molecule (Hh-Np). The Hh-binding receptor Patched (Ptc) and the cell-surface transducer Smoothened (Smo) mediate cellular responses to Hh. In the absence of Hh, Smo is inactive and after Hh binding to Ptc, Smo is relocated to the cell surface and becomes active [Ingham and McMahon, 2001]. Smo triggers a cascade of intracellular events that culminate in the transcriptional activation of Hh-specific targets such as Ptc [Ingham and McMahon, 2001].

The fact that Hh is a lipid-modified protein and exerts short- and long-range effects suggests the existence of a mechanism to regulate its movement across embryonic fields. Genetic analysis in *Drosophila* embryos has demonstrated that heparan sulfate proteoglycans (HSPG) are important players in the regulation of Hh diffusion and signaling [Perrimon and Bernfield, 2000]. HSPGs are cell surface and extracellular matrix (ECM) macromolecules formed by a core protein to which heparan sulfate (HS) glycosaminoglycans (GAGs) are covalently attached [Bernfield et al., 1999]. Based on the core protein structure HSPG can be subdivided in three major families: the single membrane-spanning syndecans, the membrane GPI-anchored glypicans, and basement membrane PGs like perlecan. The first evidence that HSPG are involved in Hh distribution came from the genetic analysis in *Drosophila* of *tout*

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velou (*ttv*) a gene that encodes for a glycosyltransferase involved in the synthesis of HS chains. In wing imaginal discs, the Hh protein is not able to diffuse over a field of *ptc-ttv* double mutant cells indicating that Hh requires HSPGs to move across the embryo [Bellaiche et al., 1998]. In the absence of HS chains the Hh gradient is no longer formed, and the Hh protein accumulates in the cells immediately close to the producing cells [Han et al., 2004a; Takei et al., 2004]. Exostoses (Ext), the mammalian homolog of *ttv*, regulate the range of activity of Indian hedgehog (Ihh), another Hh family member, suggesting that this function for HSPGs is evolutionary conserved [Koziel et al., 2004]. Analysis of loss-of-function mutations in *dally* and *dally-like protein* (*dlp*), the *Drosophila* members of the glypican family, indicate that these molecules are involved in Hh diffusion [Han et al., 2004b].

Classical biochemical and cell culture assays have implicated HSPGs as modulators of growth factor activity; they bind through its HS chains to a variety of growth factors, such as Wnt and FGF's, and regulate their ability to signal [Carey, 1997; Larraín et al., 1998; Bernfield et al., 1999]. Recent results clearly indicate that Dlp is also required for proper Hh signaling [Desbordes and Sanson, 2003; Lum et al., 2003]. The interplay between the effects of HSPGs on diffusion and signaling is still a matter of active research and debate [Lin, 2004]. To further understand the mechanism by which HSPGs regulate Shh activity, we have evaluated the importance of this interaction using cell culture and biochemical assays. When the synthesis of HSPGs was altered by chlorate or heparitinase treatment a decrease in Shh activity was observed. Contrary to this, addition of a peptide that competes the binding of Shh to HSPGs resulted in augmentation of Shh activity. Therefore, we concluded that HSPGs exert positive and negative effects in Shh activity. Using affinity chromatography we have determined that Shh interacts preferentially with two HSPGs with molecular masses of >250 kDa and 60 kDa and based on apparent migration in SDS–PAGE they could correspond to perlecan and glypican, respectively.

RESULTS

HSPGs are Required for Shh Activity

In order to study the role of HSPG in Shh activity, we measured binding of Shh to the cell surface and induction of alkaline phosphatase (AP) in C3H10T1/2 fibroblasts [Pepinsky et al., 1998; Taipale et al., 2000]. Shh-AP fusion protein was used for binding studies. The Shh-AP was specifically bound to the cell surface (Fig. 1A). First we evaluated the effect of chlorate treatment in our cell-binding assay. Sodium chlorate is a specific inhibitor of PG sulfation extensively used to study the role of PGs in growth factor signaling [Carey, 1997; Larraín et al., 1998]. We found that chlorate treatment inhibits binding of Shh-AP to the cell surface (Fig. 1B). The effect of chlorate was rescued by addition of 30 mM sulphate indicating that this effect is specific (Fig. 1C). The type of GAG chains involved in this effect was evaluated by heparitinase (Hase) treatment of the cells. When HS chains were specifically removed by the enzyme, binding of Shh-AP was clearly reduced (Fig. 1D). We evaluated the effect of different GAG chains on Shh-AP binding to the cell surface. We have found that increasing the levels of HS using heparin resulted in a concentration-dependent inhibition of the binding of Shh-AP to cells with an IC_{50} of 1 μg/ml (Fig. 1E), this effect was specific since chondroitin sulfate (CS) chains had almost no effect ($IC_{50} > 100$ μg/ml) and required specific sulfate modifications ($IC_{50} > 100$ μg/ml for de-N-sulfated heparin). HS chains were also able to specifically compete the binding of Shh-AP to the cell surface (IC_{50} 1–2 μg/ml, Fig. 1F), indicating that the binding of Shh was competed by specific sugar sequences on the HS chains and not because of the high levels of sulfation present on Heparin chains (Fig. 1F). These results indicate that proper sulfation of PGs, in particular of HSPG, is required for Shh binding to cell surface.

To determine if sulfation of PGs is important for Shh activity in fibroblasts, we performed assays to measure Shh-induced AP in the presence of chlorate. Shh-Np was used for induction assays. First, we compared a Shh-N (R&D Systems) with a Shh-Np (purified as described in methods), which possess two endogenous lipidic modifications that are absent in Shh-N and are considered to improve Shh activity [Pepinsky et al., 1998]. Shh-Np was 100 times more potent than Shh-N (data not shown). In our cell-signaling assay, chlorate was able to efficiently inhibit the induction of AP by Shh-Np in C3H10T1/2 fibroblasts (Fig. 2). These results indicate that proper sulfation of PGs is required for Shh activity.

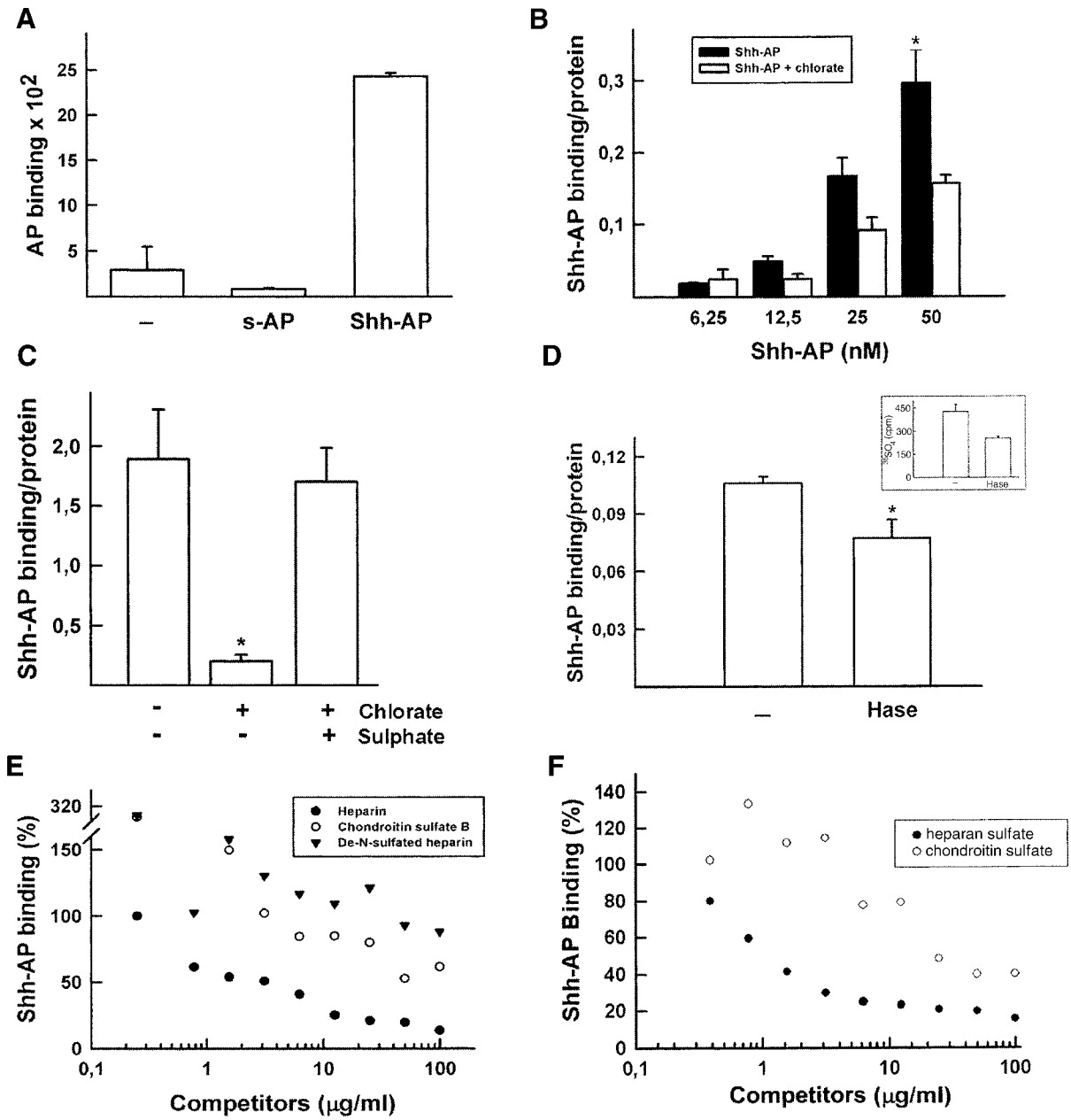

Fig. 1. HSPGs regulate binding of Shh to fibroblasts. **A**: Shh-AP was specifically bound to cell surface. C3H10T1/2 cells were incubated with 20 nM Shh-AP or secreted-AP (s-AP) for 2 h and the amount of bound Shh-AP or s-AP was quantified using a colorimetric AP assay. **B**: Chlorate reduced Shh-AP binding to the cell surface. C3H10T1/2 cells were grown with or without chlorate (30 mM) and were incubated with different concentrations of Shh-AP and the amount of bound Shh-AP was quantified using a colorimetric AP assay. $^*P < 0.001$, when binding of Shh-AP (50 nM) was compared to Shh-AP (50 nM) in the presence of chlorate. **C**: Sulphate rescued the effect of chlorate on the binding Shh-AP to the cell surface. C3H10T1/2 cells were grown with 30-mM chlorate and sulphate and the amount of bound Shh-AP was quantified as above. $^*P < 0.001$, when binding of Shh-AP was compared to control and sulphate. **D**: Heparitinase (Hase) treatment decreased Shh-AP binding to cell surface. The effect of Hase treatment in binding of Shh-AP (20 nM) to cells was determined as above. The inset showed the effectiveness of Hase treatment estimated measuring incorporation of $^{35}S\text{-}SO_4$ to GAGs chains $^*P < 0.05$. **E**: Heparin competes binding of Shh to fibroblasts. C3H10T1/2 cells were incubated with Shh-AP and increasing amounts of different GAGs chains (heparin, chondroitin sulfate B, and de-N-sulfated heparin) and the amount of bound Shh-AP was measured as above. **F**: Heparan sulphate chains competes binding of Shh to fibroblasts. C3H10T1/2 cells were incubated with Shh-AP and increasing amounts of different GAGs chains (heparan sulphate and chondroitin sulfate B and de-N-sulfated heparin) and the amount of bound Shh-AP was measured as above. We have consistently observed that very low concentrations of the different chains resulted in an increase of the binding of Shh-AP, we have no explanation for such an effect but it could be related to the net negative charge present in Shh (estimated pI of 9.4).

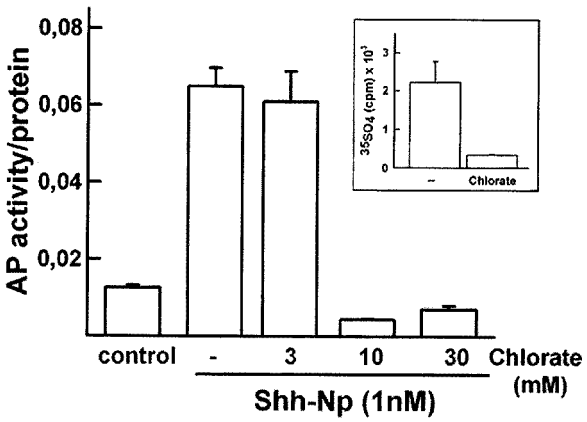

Fig. 2. Proper sulfation of PGs is necessary for Shh activity. Shh-Np activity was reduced by chlorate. Cells were seeded and grown in the presence of 0, 3, 10, and 30 mM chlorate up to 100% confluence (2 days). After that cells were incubated with fresh growth medium containing 1 nM Shh-Np in the presence or absence of chlorate. After 3 days the endogenous levels of AP activity were measured. The effectiveness of the chlorate treatment was determined by measuring ^{35}S-sulfate incorporation to GAGs chains (Inset).

Shh-HBD Peptide Affects Shh Activity

Aiming for a more specific analysis of the role of HSPG-Shh interaction, we designed a peptide that mimics the Cardin–Weintraub consensus sequence for the heparin binding domain (Shh-HBD) present in Shh [Rubin et al., 2002] and a mutant peptide (Shh-HBDm; Fig. 3A). The Shh-HBD peptide, but not the Shh-HBDm, was able to compete the binding of Shh-AP to heparin-agarose indicating its specificity (Fig. 3B). The effect of this peptide in Shh activity was tested in our cell-culture assays. We found that the Shh-HBD peptide, but not the mutant peptide, increased the binding of Shh-AP to the cell surface in a dose-dependent manner (Fig. 3C). The maximal effect was obtained when the peptide was added in a 1,000-molar excess, when higher levels of the peptide were used the positive effect in the binding starts to decrease. Similarly, the Shh-HBD peptide increased the induction of AP by Shh-Np (Fig. 3D). The maximal effect was also observed when a 1,000-fold excess of the peptide over Shh-Np was used.

Shh Interacts Preferentially With Two HSPGs

To determine the HSPGs that specifically interact with Shh, affinity chromatography was used. Trunk explants from ^{35}S-sulfate labeled rat embryos were homogenized and pools enriched in PGs were prepared by anionic exchange chromatography. HSPG-enriched pools were loaded on Shh-Fc or s-Fc affinity columns and after extensive washing with 0.2 M NaCl the PGs bound to the column were eluted with 1 M NaCl (Fig. 4A). 21% of the radioactivity present in the input was eluted from the Shh-Fc column, compared to 4% from the s-Fc column indicating that some population of the PGs obtained from the trunk were specifically bound to Shh. To determine which HSPGs were bound to Shh the eluate was treated with Hase and analyzed by Western blot using the anti-Δ-heparan sulfate antibody. This antibody recognizes the neo-epitope generated in any PG bearing HS chains after Hase treatment [Steinfeld et al., 1996]. We found that Shh binds to all the HSPGs present in the trunk explants without showing preference for any of them (Fig. 4B). The same results were obtained when head and limbs explants were analyzed (data not shown). It is known that Shh has two different interactions with heparin, one that is disrupted at 0.48 M NaCl probably representing non-specific electrostatic interaction and a second one disrupted at 0.76 M NaCl that depends on the Cardin–Weintraub HBD [Rubin et al., 2002]. To further evaluate the selectivity of Shh for HSPGs we performed a similar experiment but now the Shh-Fc columns were extensively washed with 0.5 M NaCl and the PGs eluted with 1 M NaCl. Under these conditions Shh showed clear preference for two HSPG core proteins, one with a molecular weight around 250–350 kDa and the other of approximately 60 kDa (Fig. 4C). The possibility that the HSPGs will just correspond to those more abundant is unlikely because the beads were washed until the radioactivity in the washout was zero. These results indicate that at low ionic strength Shh interacts with all HSPGs and at higher salt concentrations has preference for two HSPGs. The molecular weight of these two HSPGs core proteins is similar to the molecular weight described for glypican and perlecan, two HSPGs involved in Hh activity in the fly embryo [Lum et al., 2003; Park et al., 2003].

DISCUSSION

Our analysis of the role of HSPGs in Shh activity showed that HSPGs are necessary for Shh activity. More important, we have found that HSPGs could also exert a negative effect in Shh activity, a function not described previously.

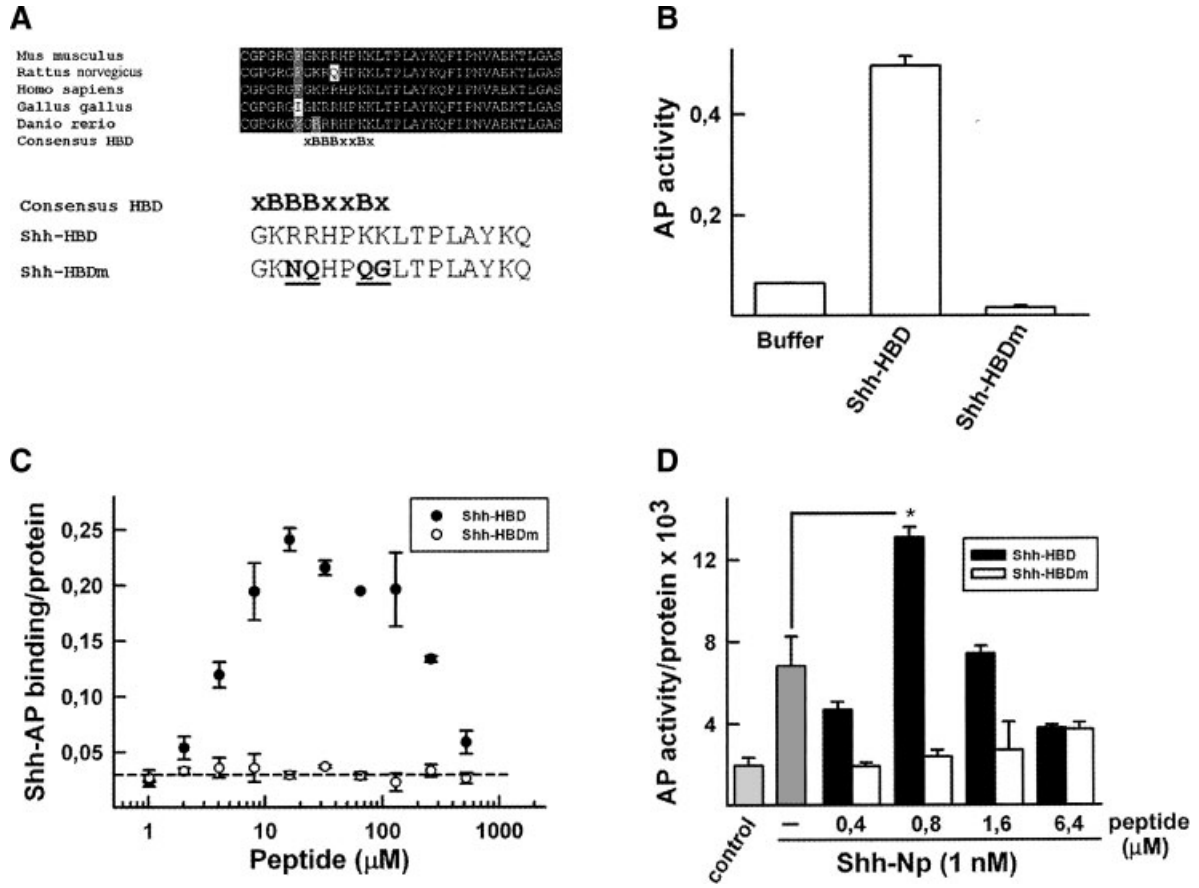

Fig. 3. A peptide mimicking the Shh heparin binding domain increased Shh activity. **A**: Alignment of vertebrate Shh protein sequences. Identical, similar, and different amino acids between sequences are indicated in black, gray, and white highlights, respectively. The Cardin–Weintraub consensus heparin-binding domain (HBD) sequence is shown below the alignment. **B** represents basic amino acids and **X** any aminoacids. The sequence of the Shh-HBD and the Shh-HBD mutant (Shh-HBDm) peptides used in these studies are indicated below. **B**: Shh-HBD peptide displaces Shh-AP from heparin-agarose beads. Shh-AP was bound to heparin-agarose beads and then eluted with the binding buffer alone or containing the Shh-HBD peptide or the Shh-HBDm peptide. AP activity from each eluate was measured. **C**: Shh-AP binding was modulated by Shh-HBD peptide in a concentration-dependent manner. C3H10T1/2 cells were incubated with Shh-AP (20 nM) in the presence of increasing amounts of Shh-HBD or Shh-HBDm peptide, after 2 h at 4°C the amount of Shh-AP bound to the cells was quantified. The punctuated line indicates the amount of Shh-AP bound to the cells in the absence of peptide. **D**: Shh-Np activity was modulated by Shh-HBD peptide in a concentration-dependent manner. Confluent C3H10T1/2 cells were incubated with Shh-Np (1 nM) in the presence of increasing amounts of Shh-HBD or Shh-HBDm peptides, after 3 days the amount of endogenous AP was determined $^*P < 0.001$.

Using affinity chromatography we have determined that Shh interacts preferentially with two HSPGs. One candidate for being the positive regulator or co-receptor could be glypican based on the finding that siRNA for dlp in insect cells results in abolishment of Hh signaling [Lum et al., 2003] and in our findings that chlorate inhibits Shh activity and that Shh binds to a HSPG core protein with a molecular weight similar to glypican.

Our hypothesis to explain the bimodal effect of the Shh-HBD peptide (Fig. 3C,D) is that Shh interacts with two different groups of HSPGs, one that exerts a negative effect on Shh and another one that has a positive effect on this growth factor. In such a case, the increase in Shh activity will result from the ability of the peptide to compete the binding of Shh to the inhibitory HSPG (observed when up to 1,000-fold excess of the peptide was used). At higher levels the peptide would also compete the binding of Shh to the HSPG with a positive effect, overcoming the gain on activity observed when lower amounts of the peptide were used. The fact that chlorate treatment only resulted in a decrease of Shh activity (Figs. 1B and 2B)

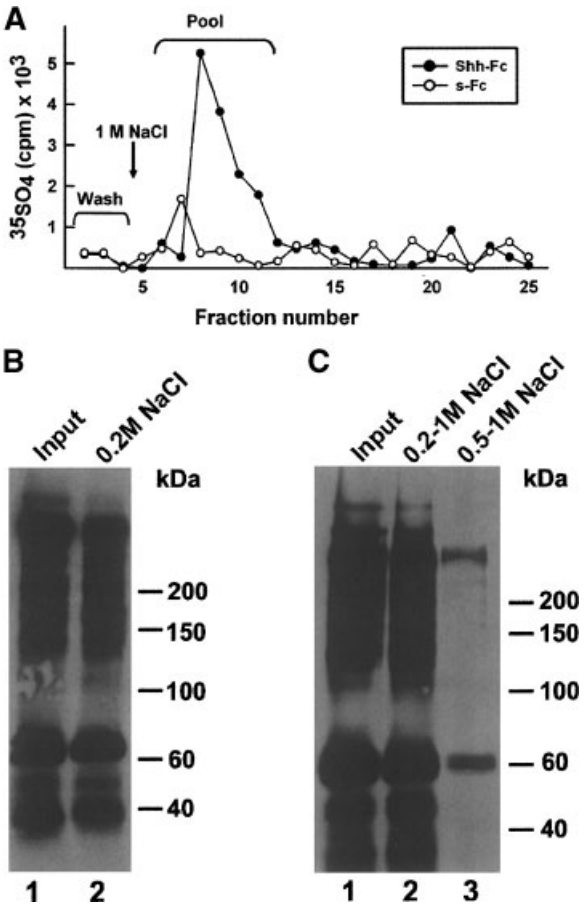

Fig. 4. Binding of HSPGs to Shh-Fc affinity columns. **A**: Shh-Fc affinity chromatography profile of ^{35}S-labeled PGs from rat E13 trunk explants. Extracts enriched in PGs by anionic exchange chromatography were loaded into Shh-Fc and s-Fc affinity columns, washed until no ^{35}S-sulfate counts were detected in the washout and eluted with 1 M NaCl (arrow). **B**: Binding of Shh to HSPG at low ionic strength. Fractions eluted from the Shh-Fc affinity column that contain ^{35}S-labeled PGs were pooled, dialyzed, treated with Hase and analyzed by Western blot using an anti-Δ-heparan sulfate antibody. Lane 1 showed loading of 2% of the total PG input. **C**: Binding of Shh to HSPGs at high ionic strength. Shh-Fc columns were loaded with PG-enriched extracts and then split in two equal parts, each was washed extensively with 0.2 M (lane 2) or 0.5 M (lane 3) NaCl. Lane 1 showed loading of 2% of the total PG input. The HSPGs core proteins bound to Shh in the different conditions was analyzed as above. Note that after washing at 0.5 M NaCl (lane 3) Shh binds selectively to two bands, one with a molecular weight around 250–350 kDa and one of approximately 60 kDa.

indicates that the HSPG with the positive effect is downstream of the inhibitory HSPG and because chlorate treatment abrogates all HSPG function only the role of the downstream component was visualized.

On the other hand we propose perlecan as a candidate to be the negative regulator. The function of trol, the fly perlecan homolog has not been completely defined as it has been shown that mutations in *hh* dominantly enhance the *trol* proliferation phenotype and also that *trol; ttv* / + mutants result in increased Hh signaling [Park et al., 2003]. The excencephaly phenotype observed in mice embryos where perlecan has been gene-targeted [Costell et al., 1999] could be explained by an increase in Shh signaling as shown for other negative regulators of the Shh pathway such as rab23 [Eggenschwiler et al., 2001]. The finding that Ihh activity is increased in mice carrying a hypomorphic mutation in Ext1 gives further support to the in vivo relevance of HSPGs negative effect [Koziel et al., 2004].

Our findings that HSPGs could exert a negative effect in Shh activity could be a novel component to incorporate into the understanding on how a gradient of Hh is established. The current model indicates that Hh diffusion occurs in the extracellular space through a cell-to-cell mechanism rather than free diffusion or an endocytosis mediated process. Dally and Dlp proteins play a central role in such a process, by passing Hh from one cell to the other [Han et al., 2004b; Lin, 2004]. We propose that HSPGs located in the extracellular matrix (ECM), perhaps perlecan, could sequester Hh competing with the Ptc receptor for the binding of this morphogen. This would allow modulation of the Hh gradient considering that its diffusion is restricted by endocytosis through Ptc (Chen and Struhl, 1996) or in a Ptc-independent manner [Torroja et al., 2004]. The observation that the row of cells closer to the source of Hh, in clones mutant for *ttv* and *brother of tout velou* (*botv*), accumulate Hh protein at even higher levels than the normal [Takei et al., 2004] could be explained by the increased availability of Hh for the Ptc receptor, as showed in our cell culture assay in the presence of the Shh-HBD peptide. On the other hand the absence of Hh protein and signaling after the first row of cell and behind the mutant clones could be explained by the internalization of Hh, that is even more efficient in the absence of an ECM HSPG that competes with Ptc for the binding of the morphogen.

METHODS

Preparation of PG Enriched Extracts

Rat E13 embryos were metabolically labeled with ^{35}S-sulfate (100 μCi/ml) for 6 h at 37°C, 5% CO_2, and gentle rocking. After that head,

trunks, and limb buds were explanted and homogenized in buffer A (10 mM Tris pH 7.5, 150 mM NaCl, 0.5% TX-100, and protease inhibitors). Homogenates were enriched in PGs by anionic-exchange chromatography as described [Minniti et al., 2004].

Protein Purification and Chromatography

Shh-Np was purified by immunoaffinity chromatography as described [Pepinsky et al., 1998; Taipale et al., 2000]. The anti-Shh immunoaffinity column was prepared by coupling the 5E1 monoclonal antibody to AffiGel 10. Shh-Fc affinity columns were prepared as described [Larraín et al., 2003]. PGs enriched pools were incubated with Shh-Fc or s-Fc columns at 4°C for 16 h with rocking, then the beads were washed with buffer A containing 0.2 M or 0.5 M NaCl until the radioactivity in the washout was zero and eluted with three column volumes of buffer A containing 1 M NaCl. The eluates were dialyzed against buffer A and treated with 0.5 mU of heparitinase (Seikagaku, Japan). The samples were analyzed by Western blot as described [Minniti et al., 2004].

Cell Surface Binding and AP Induction

C3H10T1/2 fibroblasts were grown to 80% confluence in 96-well plates and Shh-AP binding was performed as described [Larraín et al., 2003]. For chlorate experiments, cells were plated with 30 mM sodium chlorate. For heparitinase treatment, 0.25 mU of heparitinase were added to the cells 1 day and 12 h before the binding assay was performed. Incorporation of ^{35}S-sulfate to GAG chains was measured as described [Minniti et al., 2004]. Shh activity was tested measuring alkaline phosphatase induction in C3H10T1/2 fibroblasts [Pepinsky et al., 1998]. Data is expressed as mean ± standard error for one representative experiment performed in triplicate. Statistical analysis was performed with GraphPad Prism program. One or two ways-ANOVA was performed.

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