Sulfation of chondroitin sulfate proteoglycans is necessary for proper Indian hedgehog signaling in the developing growth plate

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In contrast to the functional role of heparan sulfate proteoglycans (HSPGs), the importance of chondroitin sulfate proteoglycans (CSPGs) in modulating signaling pathways involving hedgehog proteins, wingless-related proteins and fibroblast growth factors remains unclear. To elucidate the importance of sulfated CSPGs in signaling paradigms required for endochondral bone formation, the brachymorphic (bm) mouse was used as a model for undersulfated CSPGs. The bm mouse exhibits a postnatal chondrodysplasia caused by a mutation in the phosphoadenosine phosphosulfate (PAPS) synthetase (Papss2) gene, leading to reduced levels of PAPS and undersulfated proteoglycans. Biochemical analysis of the glycosaminoglycan (GAG) content in bm cartilage via sulfate labeling and fluorophore-assisted carbohydrate electrophoresis revealed preferential undersulfation of chondroitin chains (CS) and normal sulfation of heparan sulfate chains. In situ hybridization and immunohistochemical analysis of bm limb growth plates showed diminished Indian hedgehog (Ihh) signaling and abnormal Ihh protein distribution in the extracellular matrix. Consistent with the decrease in hedgehog signaling, BrdU incorporation exhibited a significant reduction in chondrocyte proliferation. Furthermore, co-immunoprecipitation experiments showed that Ihh binds to the major cartilage CSPG aggrecan via its CS chains. Overall, this study demonstrates an important function for CSPGs in modulating Ihh signaling in the developing growth plate, and highlights the importance of carbohydrate sulfation in regulating growth factor signaling.

KEY WORDS: PAPS, GAG, Proteoglycan, CSPG, Ihh, Chondrocyte, Proliferation, Sulfation, Brachymorphic mouse

INTRODUCTION

Endochondral bone formation is a complex developmental process that begins with differentiation of mesenchymal cells to chondroblasts, which then undergo a period of proliferation followed by exit from the cell cycle and terminal differentiation, first to pre-hypertrophic and then to hypertrophic chondrocytes. The latter undergo programmed cell death and are replaced by osteoblasts, which commence the transition to bone (Karsenty and Wagner, 2002). Several signaling molecules including Indian hedgehog (Ihh), parathyroid hormone-related protein (PTHrP, Pthlp – Mouse Genome Informatics), fibroblast growth factors (FGFs), Wnt proteins and bone morphogenetic proteins (BMPs), function in concert to tightly regulate this multistep process leading to endochondral bone formation (Kronenberg, 2003). Disruption of any of these signaling pathways results in defects in growth plate development (Karsenty and Wagner, 2002).

Ihh signaling is essential for normal chondrocyte maturation, regulating both proliferation and differentiation (St-Jacques et al., 1999). Ihh delays the onset of hypertrophy by inducing expression of PTHrP, which in turn signals to proliferative chondrocytes, preventing them from entering hypertrophy (Lanske et al., 1996). Ihh also regulates proliferation of chondrocytes independently of PTHrP by directly controlling the rate of cell division of columnar/proliferative chondrocytes (Long et al., 2001), and by regulating transition of periarticular (resting) to proliferative chondrocytes (Kobayashi et al., 2005).

The mechanism of Ihh signaling is not clear, but there is evidence to suggest that Ihh can act both as a short and long-range morphogen (Chen et al., 2004; Gritli-Linde et al., 2001), despite being palmitoylated and cholesterol-modified (Pepinsky et al., 1998; Porter et al., 1996). It is postulated that Ihh moves through the extracellular matrix (ECM) to reach its target cells by forming multimeric aggregates (Chen et al., 2004; Vyas et al., 2008) or by association with lipoprotein particles known as argosomes (Eaton, 2006; Panakova et al., 2005).

The ECM is a complex micro-environment that is integral for proper cell-cell and cell-growth factor interactions, but the contribution of the ECM to regulating cell function is poorly understood. Proteoglycans are a major class of ECM molecules, comprised of protein-bound carbohydrate chains termed glycosaminoglycans (Schwartz, 2000) that play a pivotal role in proper cell-cell and cell-growth factor interactions, but the contribution of the ECM to regulating cell function is poorly understood. Proteoglycans are a major class of ECM molecules, comprised of protein-bound carbohydrate chains termed glycosaminoglycans (Schwartz, 2000) that play a pivotal role in regulating cell signaling (Hacker et al., 2005). Heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs) are two major classes of proteoglycans, differentiated by their GAG compositions and sulfation patterns (Habuchi et al., 2004). The importance of HSPGs in development and their role in regulating various signaling molecules, including hedgehog (Hh), have been described in several systems in fly and mouse (Bellaiche et al., 1998; Hacker et al., 1997; Lin et al., 1999; Lin and Perrimon, 1999; Paine-Saunders et al., 2000; Toyoda et al., 2000). In Drosophila, deletion of the gene tout-velu, which encodes a HS polymerizing enzyme, leads to abnormal signaling and restricted distribution of Hh (Bellaiche et al., 1998). In mouse, a hypomorph mutation of Ext1 (mouse homolog of tout-velu) results in increased Ihh distribution in the growth plate (Koziel et al., 2004). Furthermore, biochemical studies have shown that Hh proteins bind HS (Zhang et al., 2007) via a conserved stretch of basic amino acids in the N-terminal region of all Hh proteins (Cardin and Weintraub, 1989; Rubin et al., 2002).
In contrast to HSPGs, the function of CSPGs (which are often the more abundant proteoglycans in tissues) in development is not well understood. Absence of the CSPG aggrecan, in both the nanomelic (nm) chicken and the cartilage-matrix-deficient mouse (cmd), results in lethal phenotypes that are characterized by altered growth plate architecture and significant reduction in the sizes of cartilaginous elements (Kimata et al., 1981; Krueger et al., 1999; Li et al., 1993; Schwartz and Domowicz, 2002; Watanabe et al., 1994). Despite the severe chondrodystrophies displayed by these aggrecan-deficient models, the underlying mechanisms responsible for the observed phenotypes have not been elucidated. An ES cell gene-trap screen for target genes of BMP signaling showed that chondroitin-4-sulfotransferase (C4st1)-deficient mice have a severe chondrodysplasia that is characterized by global reduction in chondroitin sulfate (CS) content in the growth plate and by increased TGFβ signaling (Kluppel et al., 2005). Interestingly, a recent gene trap mutant (JAWS) encoding a putative nucleotidase had a severe chondrodysplasia characterized by undersulfation of CS chains and abnormal synovial joint positioning (Sohaskey et al., 2008). These findings suggest that CSPGs are involved in regulating endochondral bone development, and, more importantly, provide evidence that sulfation of GAG chains is crucial for normal CSPG function.

HSPGs and CSPGs are highly sulfated molecules, and undersulfation of HSPGs results in Wnt and Hh signaling defects in Drosophila, as seen in the sulfatase (Sfl) mutant (Lin and Perrimon, 1999). To elucidate the importance of CS in endochondral bone formation, we are taking advantage of the brachyomorphic (bm) mouse (Sugahara and Schwartz, 1979; Sugahara and Schwartz, 1982a; Sugahara and Schwartz, 1982b). The bm mouse has a mutation in the gene Paps2, which encodes PAPS synthetase 2 (PAPSS2), one of two isoforms in mammals that catalyze the synthesis of the universal sulfate donor PAPS (Kurima et al., 1998), thus resulting in severe undersulfation of CSPGs (Orkin et al., 1976). The bm mouse is characterized by a dome-shaped skull, short thick tail and shortened limbs (Lane and Dickie, 1968; Schwartz and Domowicz, 2002; Schwartz et al., 1978). At birth, bm mice are the same size as wild-type (wt) littermates, but as development proceeds, a limb defect becomes apparent at postnatal day 3. By maturity, bm mice exhibit 50% reduction of limb length and 25% reduction in axial skeleton length (Kurima et al., 1998). Histological studies of bm limbs revealed normally organized growth plates with reduction of both the columnar/proliferative and hypertrophic zones concomitant with undersulfation of CSPGs (Orkin et al., 1976; Schwartz et al., 1978).

In the present study, detailed analysis of the bm growth plate revealed normal HS sulfation and preferential undersulfation of CSPGs, as well as reduced Ihh signaling and abnormal Hh protein distribution. Direct evidence that Ihh binds sulfated CSPGs, specifically aggrecan, suggests a mechanism in which CSPGs together with HSPGs modulate Ihh signaling by controlling the distribution of secreted Ihh across the ECM. This is the first study to demonstrate a role for CSPGs in modulating Ihh signaling and provides an explanation for how Ihh can act as a long-range morphogen by its interaction with ECM proteoglycans.

MATERIALS AND METHODS

Immunohistochemistry

Postnatal day 6 limbs were fixed in 4% paraformaldehyde phosphate-buffered saline (PBS) overnight at 4°C. Paraffin sections (6 μm) were treated with 0.5 U/ml chondroitinase ABC (Seikagaku), and stained with anti-HS (10E4), anti-CS-4 (2-B-6), anti-CS-6 (3-B-3) and anti-CS-0 (1-B-5) (Seikagaku) diluted 1:100. Paraffin-embedded sections were treated with hyaluronidase (Sigma-Aldrich), incubated with anti-Ihh antibody (R&D systems, 1:50), and signal amplification and detection performed using fluorescent tyramide signal amplification (Perkin Elmer) as previously described (Gritli-Linde et al., 2001).

Sulfate labeling and GAG analysis

Day 6 wild-type or bm cartilage (100 mg) was incubated for 24 hours in 200 μCi/ml [35S]H2SO4 then homogenized in 0.5 M guanidine. Proteoglycans were purified by cesium chloride density gradient centrifugation, extensively dialyzed against 100 mM ammonium acetate (pH 7.0) and digested with either chondroitinase ABC (1 U/ml) or heparitinase (0.5 U/ml). Digested proteoglycan samples were TCA/PTA precipitated to quantitate label released and retained after each digestion. Counts were normalized for total protein, and data from three independent experiments was analyzed using GraphPad Prism 4 software.

Fluorophore-assisted carbohydrate electrophoresis (FACE)

FACE was performed as previously described (Calabro et al., 2000a; Calabro et al., 2000b; Calabro et al., 2001) with minor modifications. Briefly, 100 mg of day 6 wild-type or bm cartilage was digested with proteinase K, then digested with either 100 μM of heparitinase (Glyko) or chondroitinase ABC. Disaccharide products were fluorescently labeled with 2-AMAC (Invitrogen). Disaccharide standards for HS/CS (Seikagaku) were labeled as described. Samples were electrophoresed in monosaccharide composition gels (Glyko) at 4°C at a constant current of 60 mA for 40 minutes, and quantified using the Bio-Rad ChemiDoc XRS imaging system. Three independent triplicate-sample experiments were performed and the data analyzed using GraphPad Prism 4.

RNA in situ hybridization

Hind limbs of wild-type and bm day 6 mice were perfused with and fixed in 4% paraformaldehyde in PBS. Gelatin sections (40 μm) were mounted on silane-treated slides and processed as previously described (Domowicz et al., 2008). Probes were generated from the following mouse cDNA fragments: Col11a1 (3’UTR 1-280bp), Ihh (bp1-606), Pch1 (bp3581-4276), Fgr3 (bp1114-1740), Pthr1 (bp1100-1776) and Acan (4083-4652bp).

RNA preparation and Northern blot hybridization

Total RNA was extracted from wild-type and bm day 6 articular cartilage using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. To reduce proteoglycan contamination and prevent RNA degradation, RNA was precipitated with isopropanol/sodium citrate, resuspended in formamide and quantified using the RiboGreen RNA kit (Invitrogen). Northern blot hybridization was performed as previously described (Domowicz et al., 2008).

Semi-quantitative RT-PCR

OneStep RT-PCR mix (Qiagen) was used to amplify target RT-PCR fragments according to the manufacturer’s protocol, using 0.5 μg of total wild-type or bm day 6 cartilage RNA. Cycling parameters for each PCR fragment were optimized by varying the annealing temperature, extension time and number of cycles (30-40) to ensure the amplification was in the exponential range. Primer sequences for each PCR target are available upon request. Amplified DNA was electrophoresed in 1% agarose gels, the bands imaged and quantified using the BioRad ChemiDoc XRS imaging system and results plotted using GraphPad Prism 4 software.

Limb lacZ staining

Lims were fixed for 2 hours at 4°C in 2% paraformaldehyde, 0.2% glutaraldehyde, 0.02% sodium deoxycholate, 0.01% NP-40, 5 mM EGTA, 2 mM MgCl2 in PBS permeabilized for 3 hours in 0.02% sodium deoxycholate, 0.01% NP-40, 2 mM MgCl2 in PBS, incubated in 5 mM K3[Fe(CN)6], 5 mM K4[Fe(CN)6]·3H2O, 2 mM MgCl2, 1 mg/ml X-gal in the dark for 1 hour at 37°C, then overnight at room temperature. Following staining, limbs were washed in PBS, post-fixed in 10% formalin and sectioned. Sections were counterstained with Eosin.
Bromodeoxyuridine (BrdU) (100 μg/g of mouse) was injected intraperitoneally into day 6 wild-type and bm mice (Stickens et al., 2004). After 1 hour of BrdU incorporation, mice were perfused with 4% paraformaldehyde in PBS. Limbs were gelatin embedded and 10 μm sections were permeabilized, blocked and immunostained with an anti-BrdU antibody (Beckton-Dickinson, 1:500). Counting the number of BrdU-positive cells divided by the total number of cells (DAPI-positive) within the proliferative zone yielded the percentage of BrdU-positive cells.

Cloning and expression of N-IhhAP fusion protein
DNA encoding the N-terminal domain of Ihh (amino acids 1-202) was PCR amplified from mouse cartilage using Proofstart DNA polymerase (Qiagen) and cloned into the pAPTagNeo vector using the following primers: Ihh-F, 5’-GCAAGCTTCAACATGTCCTCCAAGCTGGGCTCCGCC3’ and IhhmutR1 (phospho5’-GGGTTCGGTGGCGAGCCGCGGCGCCGCGGGCGGGCCG-3’); Ihh-R, 5’-GAAAGATCGCCAGCTTGTCTTGACGCGCGGCG-3’. Stable cell lines expressing IhhAP were grown in serum-free medium for 4 days, and spent medium collected and concentrated 50-fold using Centricon YM-10 filters (Millipore). IhhAP concentration was determined using a standard protein curve for purified human alkaline phosphatase (Calbiochem).

Generation of N-IhhAP mutant
Multiple point mutations were generated in one step using a modified fusion PCR method. Two DNA fragments encoding the desired mutations were generated by PCR using the following primers: fragment A came from PCR with Ihh-F (5’-GCAAGCTTCAACATGTCCTCCAAGCTGGGCTCCGCC3’) and IhhmutR1 (phospho5’-GGGTTCGGTGGCGAGCCGCGGCGCCGCGGGCGGGCCG-3’); fragment B came from PCR with Ihh-R (5’-GAAAGATCGCCAGCTTGTCTTGACGCGCGGCGGCG-3’); fragment C from PCR with Ihh-F (phospho5’-GGGTTCGGTGGCGAGCCGCGGCGCCGCGGGCGGGCCG-3’), and IhhmutF1 (phospho5’-GGGTTCGGTGGCGAGCCGCGGCGCCGCGGGCGGGCCG-3’); fragment D from PCR with Ihh-R (5’-GAAAGATCGCCAGCTTGTCTTGACGCGCGGCGGCGGCG-3’). Fragments were purified and blunt-end ligated, followed by a second round of PCR using the primers Ihh-F and Ihh-R.

N-IhhAP glycosaminoglycan binding assay
HS, CS-4 and CS-6 (Sigma), and CS-0 (Seikagaku) GAGs were bound to polylysine-treated 96-well plates at a concentration of 5 mg/ml. Plates were blocked with 1% BSA in TBS for 2 hours at 25°C. Serial dilutions of wild-type and mutant IhhAP were bound for 2 hours at 25°C, followed by three 0.5 M NaCl washes. Bound IhhAP was measured for 10 minutes by adding 1 mM 4-methylumbelliferone substrate to measure IhhAP activity. After a 30-minute incubation, fluorescence was measured with a Victor 3 plate reader (Perkin Elmer) at abs355/em460 nm.

RESULTS
Undersulfation of CS chains in the bm growth plate
To characterize the sulfate content of the GAG chains in the bm growth plate, day 6 limb sections were stained with a set of monoclonal antibodies that specifically recognize CS-4, CS-6 and CS-0. Immunohistochemistry revealed a reduction in the amounts of CS-4 and CS-6 epitopes, and increased staining for the CS-0 epitope in the ECM of the bm growth plate compared with cartilage from wt littermates (Fig. 1). The 10E4 antibody, which recognizes N-sulfated HS, showed comparable HS staining in both wild-type and bm growth plate, but significantly less staining compared with CS epitopes (Fig. 1). Note HS staining was localized around the cells rather than distributed in the ECM like the CS epitopes.

To complement the immunohistochemical results and to quantify the observed differences in sulfated CSPGs between wild type and bm, fluorophore-assisted carbohydrate electrophoresis (FACE) of growth plate cartilage treated with chondroitinase ABC showed a 32% (P<0.05) decrease in CS-4 (the predominant isofrom) and a twofold increase of non-sulfated CS-0 (P=0.05) (Fig. 2A). By contrast, treatment of cartilage samples with heparitinase revealed no significant differences in HS-GAG composition (Fig. 2B). The FACE data represent both pre-existing and newly synthesized CSPGs, and are consistent with 35SO4-incorporation experiments that measured only newly synthesized CSPGs, and showed a 41%
(P<0.05) reduction in sulfate incorporation in CSPGs and no significant change in HS sulfate content from bm cartilage compared with wild type (Table 1). These results demonstrate that mutation of PAPSS2 in the bm mouse leads to preferential undersulfation of CS chains, resulting in reduction of the predominant CS-4 species, and establishes the bm mouse as a model for studying the role of chondroitin sulfation in cartilage development.

Analysis of the brachymorphic mouse growth plate

To determine whether the bm growth plate exhibited defects in chondrocyte proliferation or in differentiation or whether signaling pathways were affected by undersulfated CS, in situ hybridization was performed on postnatal day 6 limb growth plate sections with riboprobes against various chondrocyte markers and signaling molecules. In the wild-type growth plate, Papss2 mRNA was predominantly expressed by the pre-hypertrophic chondrocytes with some expression in the proliferative and resting chondrocytes (Fig. 3A). A marked reduction in Papss2 mRNA was observed in bm sections; similar to the reduction demonstrated by northern analysis (Kurima et al., 1998), and RT-PCR (Fig. 3H). Papss1, by contrast, was detected at very low levels in both wild-type and bm cartilage by in situ (data not shown), as previously demonstrated on mouse cartilage at late developmental stages (Stelzer et al., 2007). However, northern blot analysis (see Fig. S1 in the supplementary material) and RT-PCR (Fig. 3H) showed consistent levels of Papss1 mRNA in both wild-type and bm cartilage, probably accounting for the reduced but not complete loss of proteoglycan sulfation in the bm growth plate. Aggrecan (Acan) mRNA levels were comparable by in situ in wild-type and bm growth plates (Fig. 3B), with strong expression in the pre-hypertrophic region and significant expression throughout the proliferative and resting zones, despite the presumed undersulfation of aggrecan GAG chains in the bm growth plate. The dense packing and reduced size of the bm growth plate is well outlined by the aggrecan expression pattern. Collagen type X (Col10a1), a marker of hypertrophic chondrocytes, exhibited mRNA expression levels that were also comparable between normal and bm, despite an overall reduction of the hypertrophic zone in the bm growth plate (Fig. 3C). The PTHrP receptor (Pthr1), which is expressed at high levels in the pre-hypertrophic zone, also had similar levels of expression in the wild-type and bm growth plates (Fig. 3D). By contrast, probes to Fgfr3 (Fig. 3E), and to Ihh (which is predominantly expressed in the pre-hypertrophic zone (Fig. 3F))
Table 1. Sulfate incorporation of postnatal (P6) cartilage in wild-type and bm mice

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<th>Mean SO₄²⁻ incorporation (cpm/mg ss d.)</th>
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<td>wt/wt</td>
<td>3</td>
<td>1.7x10⁴±0.31</td>
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<td>bm/bm</td>
<td>3</td>
<td>2.4x10⁴±0.60</td>
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<td>Chondroitin sulfate</td>
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<tr>
<td>wt/wt</td>
<td>3</td>
<td>9.7x10⁴±0.21</td>
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<tr>
<td>bm/bm</td>
<td>3</td>
<td>5.7x10⁴±0.27</td>
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n, number of independent experiments; s.d., standard deviation; NS, not significant.

and its receptor patched (Ptch1) [which is normally expressed in the proliferative chondrocytes (target cells of Ihh) (Fig. 3G)] revealed decreased mRNA levels in the bm growth plate compared with wild type. To complement the in situ hybridization experiments and to verify the observed mRNA differences between wild-type and bm growth plates, semi-quantitative RT-PCR was performed, and decreases in Papss2, Ptch1 and Fgfr3 mRNA expression in the bm growth plate were confirmed (Fig. 3H). By contrast, no significant changes were observed in the expression of Ihh (Fig. 3H). As Ptch1 has been shown to be a direct transcriptional downstream target of Ihh signaling (Goodrich et al., 1996), the reduction in Ptch1 mRNA in the bm growth plate suggests a Hh signaling defect.

**Altered Ihh signaling in the bm growth plate**

Ihh is a secreted protein known to act as a long-range signaling molecule in the developing growth plate (Gritli-Linde et al., 2001). Staining of wild-type growth plates with a polyclonal antibody that recognizes the mature secreted Ihh showed Ihh protein to be distributed in the extracellular space from the pre-hypertrophic source to the resting zone (Fig. 3F) with greater abundance in the proliferative zone (Fig. 4A). By contrast, bm growth plates had reduced staining overall, and an abnormal Ihh protein distribution pattern that did not appear to be uniformly dispersed between chondrocytes, as seen in wild-type growth plates (Fig. 4B); rather, it was marked by restricted Ihh diffusion (Fig. 4A, arrowhead). The lack of extracellular Ihh protein deposition between and among the chondrocytes is most striking at higher magnifications (Fig. 4A). By contrast, bm growth plates display abnormal Ihh distribution marked by aggregates in the proliferative zone (B). Ihh throughout the ECM from the proliferative zone to the resting zone (A). By contrast, the bm growth plate displays abnormal Ihh distribution marked by aggregates in the proliferative zone (B). Higher magnification views (A', A''/B', B'') show the restricted diffusion of Ihh in the bm growth plate marked by the reduction in Ihh surrounding cells in the resting zone (A', arrowhead), and aggregation of Ihh in the proliferative zone (A'', arrowheads). To investigate whether the abnormal distribution of Ihh protein resulted in downstream defects in Ihh signaling, bm mice were crossed with Ptch1⁺⁻ mice, in which the Ptch1 allele is replaced with the LacZ gene; LacZ staining in Ptch1⁺⁻ accurately represents Ptch1 transcription (Goodrich et al., 1997). Papss2 and Ptch1⁺⁻ crosses generated homozygous bm mice carrying one copy of the Ptch1 mutant allele. β-Galactosidase (β-gal) staining of Papss2 and Ptch1⁺⁻ growth plates showed a gradient distribution of β-gal staining from the proliferative to the resting zone (Fig. 4C, double black arrows). By contrast, Papss2 and Ptch1⁺⁻ mice had reduced distribution of β-gal-positive cells that was restricted to the proliferative zone with only a few β-gal-positive cells in the resting zone (Fig. 4C, yellow double arrows). Although Ptch1 mRNA expression is used as a direct readout for Ihh signal induction, the ratio of Gli1/Gli2 to Gli1 repressor (Gli3) is used to measure Ihh pathway activation (Hilton et al., 2005). Semi-quantitative RT-PCR for Gli1 and Gli3 revealed that homozygous bm mice had a 25% decrease in Gli1 mRNA, resulting in a reduced ratio of Gli1 activator to repressor, providing additional evidence of a decrease in Ihh signaling in the bm mouse growth plate (Fig. 4D).

Although Ihh signaling is clearly affected in the bm growth plate, CSPGs undersulfation may also be modulating other signaling pathways, including BMPs and FGFs, which in turn may affect Ihh signaling (Minina et al., 2002; Minina et al., 2001; Yoon et al., 2006). To determine whether BMP signaling was affected, we examined phosphorylation of the Smad family of proteins, which have been shown to be direct downstream targets of BMP signaling.

![Fig. 4. Abnormal Ihh signaling in the bm mouse growth plate.](image-url)

(A'-B') Representative immunostaining of wild-type (A-A') and bm (B-B') day 6 distal tibias for secreted Ihh (green), counterstained with DAPI (blue). The resting (R), proliferative (P) and hypertrophic (H) zones are indicated, respectively. Wild-type tissue shows graded distribution of Ihh throughout the ECM from the proliferative zone to the resting zone (A). By contrast, the bm growth plate displays abnormal Ihh distribution marked by aggregates in the proliferative zone (B). Higher magnification views (A', A''/B', B'') show the restricted diffusion of Ihh in the bm growth plate marked by the reduction in Ihh surrounding cells in the resting zone (A', arrowhead), and aggregation of Ihh in the proliferative zone (A'', arrowheads). (C) β-Gal staining of proximal tibia growth plates from wild-type and bm mice heterozygous for the Ptch1⁺⁻ mouse allele show that, in the bm mouse, there is a reduction in the range of β-gal-positive cells (black double-headed arrow), highlighted by an increase in the proportion of resting chondrocytes that are not β-gal positive (yellow double-headed arrow). (D) Semi-quantitative RT-PCR for the Ihh signaling activator (Gli1) and Ihh signaling repressor (Gli3), showing a reduction in Gli1 mRNA expression. Quantification of the shown RT-PCR, illustrating the reduction in both Gli1 mRNA and the ratio of Gli1/Gli3 in the bm growth plate.

![Diagram](image-url)
(Kretzschmar and Massague, 1998). Immunohistochemistry with an antibody that recognizes phosphorylated Smad 1,5,8 revealed no detectable differences in BMP signaling in the bm growth plate (data not shown). Furthermore, antibody staining against phospho-STAT1, a direct target of FGFR3-mediated signaling (Su et al., 1997) showed only a slight decrease in phospho-STAT-1 staining (data not shown), in agreement with the decrease in \(Ffgr3\) mRNA observed (Fig. 3H).

**Chondrocyte proliferation is decreased in the bm growth plate**

As Ihh has been shown to regulate chondrocyte proliferation (Long et al., 2001; St-Jacques et al., 1999), we determined whether chondrocyte proliferation was affected in the bm mouse. Decreased BrdU incorporation was observed when day 6 bm growth plate was compared with wild type (Fig. 5A). Quantification of the percentage of BrdU-labeled cells relative to the total number of cells in the proliferative region indicated a 38% reduction (wild type, 20.12±1.4%; bm, 12.47±0.48%; \(P<0.001\)) in BrdU incorporation in bm growth plates compared with wild type (Fig. 5B). The reduction in chondrocyte proliferation in the bm mouse growth plate was more apparent in the distal proliferative zone, near the resting/proliferative junction, which directly correlates with the region marked by restricted Ihh diffusion and decreased \(Pch1\) activation, suggesting that undersulfation of CSPGs in the bm growth plate affects the rate of chondrocyte cell division, which is likely to be attributable to a disruption in Ihh signaling.

**Indian hedgehog interacts with CSPGs**

Sonic hedgehog has been shown to interact with HSPGs via its highly conserved Cardin-Weintraub domain, xBBxxBBBx (Rubin et al., 2002) (Fig. 6A); however, there have been no biochemical studies to determine whether Ihh likewise interacts with other proteoglycans, particularly CSPGs. To test this possibility, HS and CS GAG chains were immobilized on poly-d-lysine treated plates. To measure Ihh binding to the GAG chains, the N-terminal signaling domain (amino acids 1-202) was fused to alkaline phosphatase (IhhAP) and AP activity was used to detect binding. Binding curves were generated for HS, CS–4, CS–6 and CS–0, respectively (Fig. 6B), which showed that IhhAP binds HS \((K_d=2.73\pm0.1\mu M)\) [as previously demonstrated for sonic hedgehog (Rubin et al., 2002)], as well as CS–4 \((K_d=3.8\pm0.1\mu M)\) and CS–6 \((K_d=4.7\pm0.5\mu M)\) chains, albeit with lower binding affinity (Fig. 6C). However, in agreement with the observed defects in Ihh protein distribution in the bm mouse, we observed even lower Ihh binding affinity for unsulfated CS \((K_d=5.0\pm0.4\mu M)\) compared to CS–4 (Fig. 6C), the predominant
sulfated species in the murine growth plate (Fig. 2). To demonstrate that the Ihh interaction with CS chains is specific (via its N-terminal Cardin–Weintraub motif), the charged xXRRPPRRXX domain was mutated to xXAAAPPXX. This mutation resulted in complete loss of IhhAP binding to both HS and CS chains (Fig. 6B), suggesting that the basic domain of the Hh family of proteins is required for the interaction of Hh proteins with both HS and CS.

To determine whether Ihh directly interacts with the CSPG aggrecan, E14 chicken cartilage lysates were incubated with IhhAP fusion protein and then immunoprecipitated using the S103L aggrecan core protein-specific monoclonal antibody (Krueger et al., 1990b). A threefold increase \( (P<0.05, n=3) \) in the amount of IhhAP co-immunoprecipitated with the S103L aggrecan antibody was observed compared with a control without antibody (Fig. 7). The interaction between aggrecan and Ihh was specific, as co-immunoprecipitation of IhhAP mutant protein lacking the Ihh proteoglycan binding domain showed no binding to aggrecan (supplementary material Fig. S2). Furthermore, treatment of the cartilage lysates with chondroitinase ABC, which specifically degrades CS chains, resulted in a 40% \( (P<0.05, n=3) \) decrease in IhhAP binding to aggrecan compared with non-treated samples (Fig. 7), demonstrating that this interaction was mediated by the CS component. The in vitro binding data using defined CS structures and the co-immunoprecipitation of IhhAP with aggrecan support a direct interaction between Ihh and CSPGs.

**DISCUSSION**

**Undersulfation of CSPGs in PAPSS2 deficient mice**

Biochemical analysis of the bm growth plate revealed preferential reduction of CS sulfation, whereas HS sulfation remained normal, making the non-lethal bm mouse an excellent model with which to study the effect of the loss of PAPSS2 activity, and the contribution of sulfated CSPGs in postnatal cartilage development. Preferential undersulfation of CSPGs can be explained by the large amount of PAPS required to properly sulfate the CSPG-rich ECM, as aggrecan is the major CSPG produced by chondrocytes and requires high levels of PAPS in order to sulfate the numerous (>100) GAG chains per proteoglycan molecule (Krueger et al., 1990a). By contrast, cartilage HSPGs, like the hybrid CS/HS-bearing perlecan are sparse in quantity and contain far fewer GAG chains (3–4) that need to be sulfated (Knox and Whitelock, 2006); therefore, the requirement for PAPS is significantly less for HSPG and presumably satisfied by residual PAPSS1. Furthermore, kinetic parameters (e.g. higher affinity for PAPS) favoring HS sulfotransferases may result in preferential sulfation of HS chains when the cellular levels of PAPS are decreased. In agreement with our results, a recently reported mouse model harboring a mutation in a novel Golgi PAP phosphatase \( (gPAPP) \) resulted in a severe chondrodysplasia marked by undersulfation of CS and normal HS sulfation. It was postulated that the accumulation of PAP in the Golgi may alter PAPS use, by preferentially affecting CS sulfotransferases (Frederick et al., 2008). The preponderance of undersulfated CS chains in the bm mouse growth plate, the nucleotidase mutant JAWS (Sohaskey et al., 2008) and the PAP phosphatase mutant \( (gPAPP) \) (Frederick et al., 2008) highlight the importance of regulating sulfation to promote cartilage development and bone growth.

Overall, the severe-to-moderate spectrum of chondrodysplasias observed in models deficient in CSPG synthesis and/or modifications directly correlates to the location of the underlying mutations in the biosynthetic pathway. Absence of CSPG core protein \( (nm \) and \( cmd) \) or reduction in CS chain content \( (C4sst1) \) all lead to lethal phenotypes (Kluppel et al., 2005; Krueger et al., 1999; Li et al., 1993; Schwartz and Domowicz, 2002; Watanabe et al., 1994), whereas insufficient sulfation of CS chains \( (bm) \) is non-lethal, but still results in a severe growth retardation disorder.

**Reduced chondroitin sulfation and reduction in long-range Ihh signaling in the postnatal growth plate**

Several key signaling pathways have been implicated in the control of limb elongation, including Ihh and PTHrP (Lanske et al., 1996), which function together in a feedback loop to regulate the rate of proliferation and differentiation of growth plate chondrocytes (Vortkamp et al., 1996). Furthermore, Ihh signaling has recently been shown to be essential for postnatal growth plate maintenance (Maeda et al., 2007). In the present study, analysis of the postnatal bm growth plate revealed defects associated with Ihh signaling, including: (1) abnormal Ihh distribution in the ECM of the bm growth plate marked by reduced Ihh diffusion and abnormal aggregation; (2) reduction in Pith1 mRNA expression which is a direct target of Ihh signaling and therefore indicative of reduced Hh signaling (Goodrich et al., 1996); (3) reduction in the ratio of the Gli1 activator to Gli3 repressor mRNA expression; (4) decreased range of \( \beta \)-gal-positive cells in the growth plate of Pith1\( ^{LacZ} \) reporter mice; and (5) decreased chondrocyte proliferation, presumably as a consequence of reduced Ihh signaling. Altogether, the experimental evidence suggests that undersulfation of CSPGs results in restricted Ihh diffusion, which leads to reduced proliferation that significantly impacts cartilage development and postnatal skeletal growth. Interestingly, the phenotype associated with reduced chondroitin sulfation in the bm mouse is the opposite of the phenotype seen in HS synthesis mutants, particularly the Ext1 gene trap mutant, in which reduction of HS results in an increased range of Hh signaling marked by increases in Pith1 and Pthrp mRNA, as well as increased chondrocyte proliferation and expansion of the proliferative zone (Koziel et al., 2004). The significant differences between the bm phenotype and that of the HS-deficient mutants, combined with our new data, provide evidence that HS likely does not contribute to the bm growth disorder. Thus, it would appear that sulfated CSPGs can function as modulators of Hh signaling, and in concert with HSPGs actively control long-range Ihh movement in the ECM. Disruption of the synthesis or sulfation of either one of these GAG types may result in increased or decreased Hh signaling. The HS/CS hybrid...
A stretch of basic amino acids (xBBBxxBx) conserved in all mammalian Hh family members (Fig. 6A). In vitro binding assays using an IhhAP fusion protein revealed that, similar to Shh, Ihh can also bind HS with high affinity (Fig. 6C). Furthermore, Ihh also binds CS-4, CS-6 and CS-0, albeit with decreasing affinities and binding capacities (Fig. 6C). As mutations in the Cardin-Weintraub motif completely abolish Ihh binding, the interaction between Ihh and CS is solely mediated via this motif. Interestingly, structural data revealed a second HS binding site necessary for Ihh signaling of the core protein of perlecan, HS- and CS-chains, from that of aggrecan and its CS chains.

**Indian hedgehog can directly interact with CSPGs**

The Hh family of proteins have been shown both genetically and biochemically (Bellaiche et al., 1998; Rubin et al., 2002; Vyasa et al., 2008) to interact with HSPGs through the Cardin-Weintraub motif, a stretch of basic amino acids (xBBBxxBx) conserved in all mammalian Hh family members (Fig. 6A). In vitro binding assays using an IhhAP fusion protein revealed that, similar to Shh, Ihh can also bind HS with high affinity (Fig. 6C). Furthermore, Ihh also binds CS-4, CS-6 and CS-0, albeit with decreasing affinities and binding capacities (Fig. 6C). As mutations in the Cardin-Weintraub motif completely abolish Ihh binding, the interaction between Ihh and CS is solely mediated via this motif. Interestingly, structural data revealed a second HS binding site necessary for Ihh signaling of the core protein of perlecan, HS- and CS-chains, from that of aggrecan and its CS chains.

**Undersulfation of CSPGs and other signaling pathways**

BMP and FGF signaling also control growth plate proliferation and differentiation through opposing actions (Minina et al., 2002). BMP signaling is needed to maintain normal chondrocyte proliferation and prevent premature differentiation (Minina et al., 2001), whereas FGF signaling negatively regulates chondrocyte proliferation through FGFR3 and accelerates hypertrophic differentiation (Deng et al., 1996; Liu et al., 2002). The lack of detectable changes in phospho-Smad1, downstream targets of BMP, suggest minimal contribution of BMP signaling to the bm phenotype. By contrast, the reduction in Fgfr3 expression (Fig. 3E) and the reduction in phospho-STAT-1 (data not shown) suggest that undersulfated CSPGs may negatively modulate FGF signaling to some extent. Reductions in Fgfr3 should result in increased cell proliferation and overgrowth, which could be altered in the bm growth plate as a mechanism to compensate for the decrease in Ihh signaling. However, studies on the role of FGFR3 suggest that FGF signaling may play a less significant role in postnatal, compared with embryonic, growth plate development (Naski et al., 1998). Furthermore, loss of postnatal Ihh signaling in cartilage results in a severe defect which can not be compensated by other signaling pathways such as FGF (Maeda et al., 2007), suggesting that in the postnatal growth plate Ihh is the primary pathway regulating proliferation. Alternatively, Ihh signaling may affect FGF signaling...
by regulating Fgf3 expression in the proliferative chondrocytes or by inducing FGF expression from the perichondrium, as previously hypothesized (Omtzit and Marie, 2002). Recent studies in the nanometric chick model suggest that loss of aggrecan results in defects in both Ihh and FGF signaling in early growth plate development (Domowicz et al., 2009), expanding the role of CSPGs in signaling and suggesting that CSPGs may be playing different roles in modulating growth factor signaling as cartilage development progresses.

**Sulfated HSPGs and CSPGs are necessary for normal Ihh signaling in the growth plate**

Based on previous data from HS synthesis mutants (Ext1) and the present study, we propose a mechanism in which cell-surface-associated HSPGs and matrix-associated CSPGs such as aggrecan function in concert to establish a morphogen gradient, thereby modulating Ihh signaling in the epiphyseal growth plate. HSPGs, which have higher affinity for Ihh, can act at the surface of the cells that are the source of Ihh, causing them to retain a high local concentration of Ihh and thus establish a sharp signaling gradient. Matrix-associated CSPGs are then needed for formation of the Ihh gradient, either through affecting the diffusion of Ihh by aiding in its trafficking, or by protecting Ihh from degradation. Finally, cell-surface HSPGs act at the target cells to bring Ihh close to the membrane for interaction with its receptor (Fig. 8). Therefore CSPGs and HSPGs probably work together as modulators to fine-tune signaling pathways during development.

The ability of Ihh proteins to bind with different affinities to HSPG and differently sulfated CSPGs adds another level of complexity to understanding how the Ihh proteins act as long-range morphogens and how gradients of these signaling molecules are established. Furthermore, the strength of the interactions between the Ihh proteins and sulfated proteoglycans may also be responsible for differential potencies observed among the three Ihh isoforms (Pathi et al., 2001). Importantly, despite the lower binding affinities and capacities observed for CS compared with HS chains in the in vitro assays, CSPGs are significantly more abundant than HSPGs in cartilage, therefore their contribution to Ihh distribution and signaling may be more important than previously recognized.

In summary, this is the first study to demonstrate that CSPGs can modulate Ihh signaling, and highlights the importance of the ECM in development. Owing to this new role of CSPGs in fine-tuning signaling pathways, it will be important to determine whether sulfated CSPGs are also required in modulating signaling pathways that regulate development in other tissues where proteoglycans are prevalent.

We thank Drs Miriam S. Domowicz and Leslie A. King for helpful discussions and technical advice, Judy Henry for tissue section preparation, and James Mensch for critical reading of this manuscript. The Ptc1 reporter mouse was a generous gift from Dr Wei Du. This work was supported by grants from the National Institute of Health, HD-017332 (to N.B.S.). Deposited in PMC for release after 12 months.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/10/1697/DC1

**References**


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Fig. S2

![Graph showing RFU levels for IHHAP and IHHAPmut]