Regulation of Chondrocyte Differentiation by Indian Hedgehog: Modulation by Parathyroid Hormone-related Peptide, Engrailed-1, and Heparan Sulfate Glycosaminoglycans

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ABSTRACT

During the process of endochondral bone formation, Indian Hedgehog (Ihh), a member of the hedgehog (Hh) family of secreted morphogens, and parathyroid hormone-related peptide (PTHrP) play key roles in the regulation of cartilage cell (chondrocyte) differentiation. Previous studies have established that Ihh coordinates chondrocyte differentiation indirectly by activating PTHrP expression, which in turn delays their transition to the terminal hypertrophic state. In the present study, using an *in vitro* system of cultured CFK-2 chondrocytic cells, we have explored the possibility that Ihh may also influence chondrocytic differentiation directly and investigated how this process is modulated by PTHrP, Engrailed-1 (En-1), and heparan sulfate glycosaminoglycans (HS-GAG). We show that Ihh signaling enhances, rather than inhibits, expression of markers of chondrocytic differentiation and that an inactivating missense mutation mapping to its NH_2-terminal domain (N-Ihh^{W160G}) abolishes this capacity. Moreover, activation of protein kinase A (PKA) by PTHrP-signaling also perturbs Ihh-mediated differentiation. Indicative of a novel regulatory mechanism, Ihh in turn downregulates PKA activity downstream of the PTH/PTHrP-receptor (PTH1R). En-1, detected in prehypertrophic growth plate chondrocytes *in situ*, influences chondrocytic differentiation and impedes Hh-signaling *in vitro*, suggesting a novel role for this protein in cartilage development. Finally, we show that HS-GAGs are required for proper mediation of Hh-signaling and thus also participate in modulating chondrocytic differentiation. Taken together, this study provides experimental evidence that Ihh harbors the capacity to directly induce rather than impede chondrogenic differentiation, and that this function is modulated by the cellular actions of PTHrP, En-1, and HS-GAG.

RÉSUMÉ

Au cours du processus de la formation d'os endochondrale, la protéine Indian Hedgehog (Ihh), un membre de la famille hedgehog (Hh) des morphogènes sécrétés, et la protéine liée à l'hormone parathyroïde (PTHrP), jouent des rôles clefs dans la différenciation des cellules du cartilage (chondrocytes). Des études antérieures ont établi que Ihh coordonne la différenciation des chondrocytes par l'activation de l'expression de PTHrP, qui à son tour retarde la transition des chondrocytes à l'état terminal hypertrophique. Dans cette étude, en utilisant une système *in vitro* des cellules cultivées CFK-2 chondrocytique, nous avons examiné la possibilité que Ihh peut aussi directement influencer la différenciation des chondrocytes, et nous avons étudié la manière dont ce processus est modulé par PTHrP, Engrailed-1 (En-1), et héparane-sulfate glycosaminoglycane (HS-GAG). Nous avons montré que la signalisation de Ihh améliore l'expression des marqueurs de la différenciation des chondrocytes, et qu'une mutation faux-sens qui induit l'inactivation et qui se trace au domaine N-terminal (N-Ihh^{W160G}), abolit cette capacité. De plus, la différenciation mediée par Ihh, est perturbé par l'activation de protéine kinase A (PKA) par la signalisation de PTHrP. Ihh, à son tour, diminue l'activité de PKA en aval du récepteur PTH/PTHrP, ce qui suggère un nouveau mécanisme de régulation. En-1, détecté *in situ* dans les chondrocytes du cartilage de conjugaison pré-hypertrophiques, influence la différenciation des chondrocytes, et gêne la signalisation de Hh in vitro, ce qui suggère un nouveau rôle de cette protéine dans le développement du cartilage. Finalement, nous avons montré que HS-GAGs sont nécessaire à la médiation de la signalisation de Hh, et par conséquent, participent à la modulation de la différenciation des chondrocytes. Donc, cette étude fournit des preuves expérimentales que Ihh maintient

la capacité d'induire directement, plutôt que gêner, la différenciation chondrogénique, et que cette fonction est modulée dans les cellules par PTHrP, En-1, et HS-GAG.

Preface

This Ph.D. thesis was written in accordance to the Guidelines for Submitting a Doctoral or Master's thesis, by the Faculty of Graduate studies and Research, McGill University.

The thesis guidelines state:

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:
1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication...2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceeding and following each manuscript are mandatory...3. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent..."

Manuscripts presented in chapters 2-4 inclusive, as they are published or will be submitted for publication.
The contributions of authors of chapters 2-4 are as follows:

Chapter 2: Ron A. Deckelbaum, George Chan, Dengshun Miao, David Goltzman, Andrew C. Karaplis

Chapter 3: Ron A. Deckelbaum, Dengshun Miao, David Goltzman, Andrew C. Karaplis

Cahpter 4: Ron A. Deckelbaum, Dengshun Miao, David Goltzman, Andrew C. Karaplis

The candidate was responsible for all of the work presented in these chapters. In chapter 2, George Chan contributed valuable discussion and helped in establishing the assay for protein kinase A. Dengshun Miao provided valuable assistance in cytochemical analysis of cells. In chapter 3 and 4, Dengshun Miao contributed to *in situ* hybridization and immunohistochemistry for detection of EXT1/2 mRNA and En-1 protein, respectively.

All work presented here was conducted under the supervision of Andrew C. Karaplis and David Goltzman.

Chapter 2 was previously published as the following research article and appears here in manuscript form with no additional changes:

Ron A. Deckelbaum, George Chan, Dengshun Miao, David Goltzman, Andrew C. Karaplis (2002). Ihh enhances differentiation of CFK-2 chondrocytic cells and antagonizesPTHrP-mediated activation of PKA. *J Cell Sci* 115, 3015-3025

Other manuscripts to which the candidate contributed:

1. Chan GK, **Deckelbaum RA**, Bolivar I, Goltzman D, Karaplis AC. PTHrP Inhibits Adipocyte Differentiation by Down-Regulating PPAR-gamma Activity via a MAPK-Dependent Pathway. *Endocrinology*. 2001 Nov; 142(11): 4900-9.

2. He B, **Deckelbaum RA**, Miao D, Lipman ML, Pollak M, Goltzman D, Karaplis AC. Tissue-specific targeting of the *pthrp* gene: the generation of mice with floxed alleles. *Endocrinology*. 2001 May; 142(5): 2070-7.

3. Karaplis AC, **Deckelbaum RA**. Role of PTHrP and PTH-1 receptor in endochondral bone development. *Front Biosci.* 1998 Aug 1; 3: D795-803.

4. Sidler B, Alpert L, Henderson JE, **Deckelbaum R**, Amizuka N, Silva JE, Goltzman D, Karaplis AC. Amplification of the parathyroid hormone-related peptide gene in a colonic carcinoma. *J Clin Endocrinol Metab*. 1996 Aug; 81(8):2841-7.

Special Dedication

The work presented here is dedicated to the memory of my beloved uncle, Itzhak "Itzik" Tal, who passed away during the last days of writing this document. He will truly be missed and my memories of him will remain close to my heart.

עבודה זו מוקדשת לזיכרו של דודי היקר יצחק איציק טל אשר הלך לעולמו ימים ספורים לפני תום כתיבת מאמר זה. יהי זכרו ברוך.

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With deepest gratitude and admiration I extend my thank you to my one love and best pal, Mayla Hsu.

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List of abbreviations

ALP	Alkaline Phosphatase
Col2a1	Type II collagen gene/mRNA
Col10a1	Type X collagen gene/mRNA
EXT1	Exostosin 1
EXT2	Exostosin 2
Dhh	Desert hedgehog
En-1	Engrailed 1
Hh	Hedgehog
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
Ihh	Indian hedgehog
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PTH	Parathyroid hormone
PTHrP	Parathyroid Hormone-related Peptide
PTH1R	Type 1 PTH/PTHrP-receptor
Shh	Sonic Hedgehog
GAG	Glycosaminoglycan

Note: Other standard abbreviations are defined within the text.

Chapter 1

Literature review

Ihh Signaling and Chondrocyte differentiation

1.1 Endochondral ossification- An overview

The vertebrate skeleton is formed by two major processes. In the first, termed endochondral ossification, bone develops through a cartilage intermediate and this process governs the formation of most skeletal elements. The second, intramembranous ossification, involves the direct differentiation of bone forming osteoblasts from mesenchymal precursors, a process that is restricted mainly to the craniofacial bones and clavicles. The initial step in endochondral ossification is the formation of mesenchymal condensates at locations of primordial skeletal elements that subsequently differentiate into cartilage (Horton, 1993). Early chondrocytic cells are distinguished by their unique expression of aggrecan and type II collagen (*Col2a1*), which differentiates them from their surrounding cells forming a structure, termed the perichondrium. The earliest known determinant of mesenchymal condensation is Sox-9, a member of the high-mobility group (HMG) box-containing transcription factors whose sequence shares 50% identity with that of the sex determining factor SRY (Wagner et al., 1994). The generation of chimeric mice harboring *Sox-9*-null cells that failed to be included in wild type mesenchymal condensations and subsequent cartilaginous elements, provided the definitive experimental basis for this conclusion (Bi et al., 2001). These cells also failed to express *Col2a1*, *Col11a2*, and aggrecan. In addition, Sox-9 was shown to bind to and activate transcription of the *Col2a1* promoter, suggesting that in addition to mesenchymal condensation, it may be required for early chondrogenic gene expression (Lefebvre et al., 1997). The conversion of early chondrocytes, typified by secretion of chondrogenic extracellular matrix proteins (ECM), into proliferating or non-hypertrophic chondrocytes marks the

initiation of the formation of the growth plate, a structure comprised of chondrocytes organized in a columnar fashion which can be spatially divided according to their differentiated state. This critical step involves yet other members of the Sox family, L-Sox-5 and Sox-6. These factors, devoid of transactivation domains, were nonetheless shown to collaborate with Sox-9 in activating *Col2a1* expression (Lefebvre et al., 1998). Furthermore, L-Sox-5 and Sox-6 compound mutant mice display a severe form of chondrodysplasia characterized by loss of growth plate columnar structure, failure in expression of non-hypertrophic chondrogenic markers, and ectopic expression of late differentiation (hypertrophic) markers (Smits et al., 2001). The fact that Sox-9 expression and early mesenchymal condensations were not affected in L-Sox-5/Sox-6-ablated animals suggests that these proteins act downstream of Sox-9 and are required for the specification of chondrocytes per se.

One of the major factors controlling the proliferative capacity, and thereby the differentiation, of non-hypertrophic chondrocytes is fibroblast growth factor (FGF). Activating mutations in the FGF-receptor 3 (FGFR3) are associated with achondroplasia and thanatophoric dysplasia, characterized by the virtual absence of non-hypertrophic chondrocytes (Naski et al., 1996; Rousseau et al., 1994). In contrast, mice lacking *Fgfr3* display a phenotype of elongated bones accompanied by expansion of the proliferative zone within the growth plate (Deng et al., 1996). Although the identity of the FGF protein mediating these effect is not clearly identified, *Fgf-18* deficient mice display an expanded zone of hypertrophic chondrocytes suggesting that this may be the preferred ligand for FGFR3 (Liu et al., 2002). The mechanism by which FGFR3 activation, normally associated with proliferative enhancement in other cell types, elicits anti-proliferative

effects in chondrocytes is not fully understood but appears to involve STAT1 activation and a subsequent increase in the expression of the cell cycle inhibitor p21 (Sahni et al., 1999).

In contrast to FGF, Indian hedgehog (Ihh), a member of the hedgehog (Hh) family of secreted morphogens, was shown to positively govern non-hypertrophic chondrocyte proliferation. Thus, *Ihh* ablation results in severe reduction in proliferation of non-hypertrophic chondrocytes (St-Jacques et al., 1999), while the conditional inactivation of its receptor, Smoothened (Smo), in chondrocytes is accompanied by diminished cyclin D1 expression (Long et al., 2001). In addition, Ihh also coordinates a negative feedback loop to enhance expression of parathyroid hormone-related peptide (PTHrP) expression in the articular perichondrium thereby reducing the rate of chondrocyte differentiation to their final hypertrophic state (Lanske et al., 1996; Vortkamp et al., 1996). The nature of this critical regulatory mechanism is central to this study and will be discussed in further detail below.

Hypertrophic chondrocytes are distinguished from non-hypertrophic chondrocytes by the absence of proliferative capacity, large cell size, absence of *Col2a1*, and the unique expression of type X collagen (*Col10a1*). Hypertrophic chondrocytes are also characterized by expression of vascular endothelium growth factor (VEGF) and low levels of the osteoblast determinant, Cbfa1. The requirement of Cbfa1 for hypertrophic differentiation became apparent upon the observation that animals lacking this transcription factor displayed a lack of hypertrophic chondrocytes, in addition to absence of bone (Inada et al., 1999; Kim et al., 1999). Furthermore, misexpression of Cbfa1 in

non-hypertrophic chondrocytes induced these cells to inappropriately hypertrophy and express Ihh (Takeda et al., 2001).

Growth plate chondrocytes undergoing the temporal and spatial progression of proliferation and differentiation eventually culminate in programmed cell death, apoptosis, allowing for the concomitant invasion of vasculature and osteoblasts into the cartilage-osseous junction. The timely advent of chondrocytic death is therefore of critical importance to proper bone formation, and the roles of PTHrP and its receptor, the type 1 PTH/PTHrP–receptor (PTH1R), in regulating this important process are therefore discussed in further detail in a subsequent section.

Finally, required for *bona fide* bone formation is the step in which vascular invasion proceeds into the ECM of the mature hypertrophic zone, concomitantly introducing mesenchymal progenitors of the osteoblast (bone forming) and osteoclast (bone resorbing) lineages. Mediating these processes are metalloprotease 9 (MMP-9) and VEGF which degrade the cartilage ECM and promote angiogenesis in the chondro-osseous junction (Engsig et al., 2000; Haigh JJ, 2000; Vu et al., 1998). In addition, it appears that Cbfa1 is also required for vascular invasion and VEGF expression, probably by directly binding to and enhancing its promoter activity (Zelzer et al., 2001).

2. PTHrP and Chondrocyte Biology

2.1 PTHrP: Introduction

Already defined, PTHrP was first discovered as the causative agent of humoral hypercalcemia of malignancy. This paraneoplastic complication, most commonly observed in squamous carcinomas of the lung, renal carcinomas, and breast carcinomas, is

characterized by elevated serum calcium levels and hypophosphatemia, biochemical alterations consistent with hyperparathyroidism (high PTH secretion) (Kemp et al., 1987; Moseley et al., 1987; Suva et al., 1987). That PTHrP elicits similar humoral physiological effects to those of PTH, the endocrine regulator of calcium and phosphate homeostasis, is reflected by the high homology that these two peptides share at their first 13 NH_2-terminal amino acid residues. Moreover, NH_2-terminal (1-34) fragments of PTH and PTHrP were shown to bind a common receptor, PTH/PTHrP-receptor or PTH1R, and lead to its full activation (Juppner et al., 1988). This similarity is not reflected by their respective gene structures as *PTH* is comprised of three exons while the gene for *Pthrp* encompasses five exons in mice and eight in humans. Transcription of *Pthrp* is complex and initiates through at least three promoters in the 5'-flanking region that yield an array of mRNA species and three main translation products of 139, 141, and 173 amino acids (Philbrick et al., 1996). All three of these products are identical in their 1-139 region, encoded by exon 4, whereas amino acids 140-141 and 140-173 within the 1-141 and 1-173 species are derived from exon 6 and 5, respectively; these are absent from non-human forms of PTHrP. As appropriate for a secretory peptide, PTHrP contains a "prepro" sequence (initiating at aa -36) encoded by exons 3 and 4 that includes a signal sequence, thereby directing the peptide to the constitutive or regulatory secretory pathway. A "pro" sequence, consisting of 5-10 amino acids preceding the alanine of the mature peptide at position +1, is believed to be cleaved by prohormone convertase, furin (Liu et al., 1995).

In contrast to PTH, which is expressed only by the parathyroid glands, hypothalamus, and thymus and functions in regulating systemic calcium and phosphate levels through its skeletal and renal actions, PTHrP is expressed by virtually every tissue in the body and has

a wide range of physiological and developmental functions. These varied functions are also exhibited by the post translational proteolytic processing that PTHrP undergoes to yield multiple derivatives with diverse actions. The NH_2-terminal species (aa 1-36) is generated upon cleavage of the PTHrP prohormone at the monobasic Arg^{37} and is similarly active to PTH (1-34) in activating their common receptor (Everhart-Caye et al., 1996). In addition, three mid-region secretory forms of PTHrP, all initiating at Ala^{38} and terminating at amino acids 94-101, were shown to functionally act through a distinct receptor that activates the phosphatidylinositol/protein kinase C pathway (Orloff et al., 1996; Soifer et al., 1992; Wu et al., 1993). One of the important physiological functions for the mid-region species is in the maintenance of transplacental calcium transport to the fetus (Kovacs et al., 1996). A third functional PTHrP peptide is probably generated by proteolytic processing, yielding a carboxy terminal fragment comprised of residues 107-139. This fragment was shown to inhibit osteoclastic bone resorbing activity, increase osteoblast activation, and was therefore termed "osteostatin" (Fenton et al., 1991; Fenton et al., 1994).

Reiterating yet another structural-functional divergence, PTHrP harbors a nuclear/nucleolar localization sequence between amino acid 88-106, which appears to mediate intracrine functions of PTHrP that culminate in enhanced cell proliferation and survival (Henderson et al., 1995). This region, comprised of an Arg-Arg hinge flanked by Lys-rich sequences, resembles the nucleolar localization sequences (NoS) described in a number of eukaryotic nucleolar proteins (HSP70) as well as retroviral regulatory proteins (HIV-1 rev, HIV-1 tat). It was shown that this domain is necessary and sufficient to mediate nucleolar targeting of PTHrP in CFK-2 chondrocytic cells and enhance their survival under pro-apoptotic conditions (Henderson et al., 1995). Further studies have

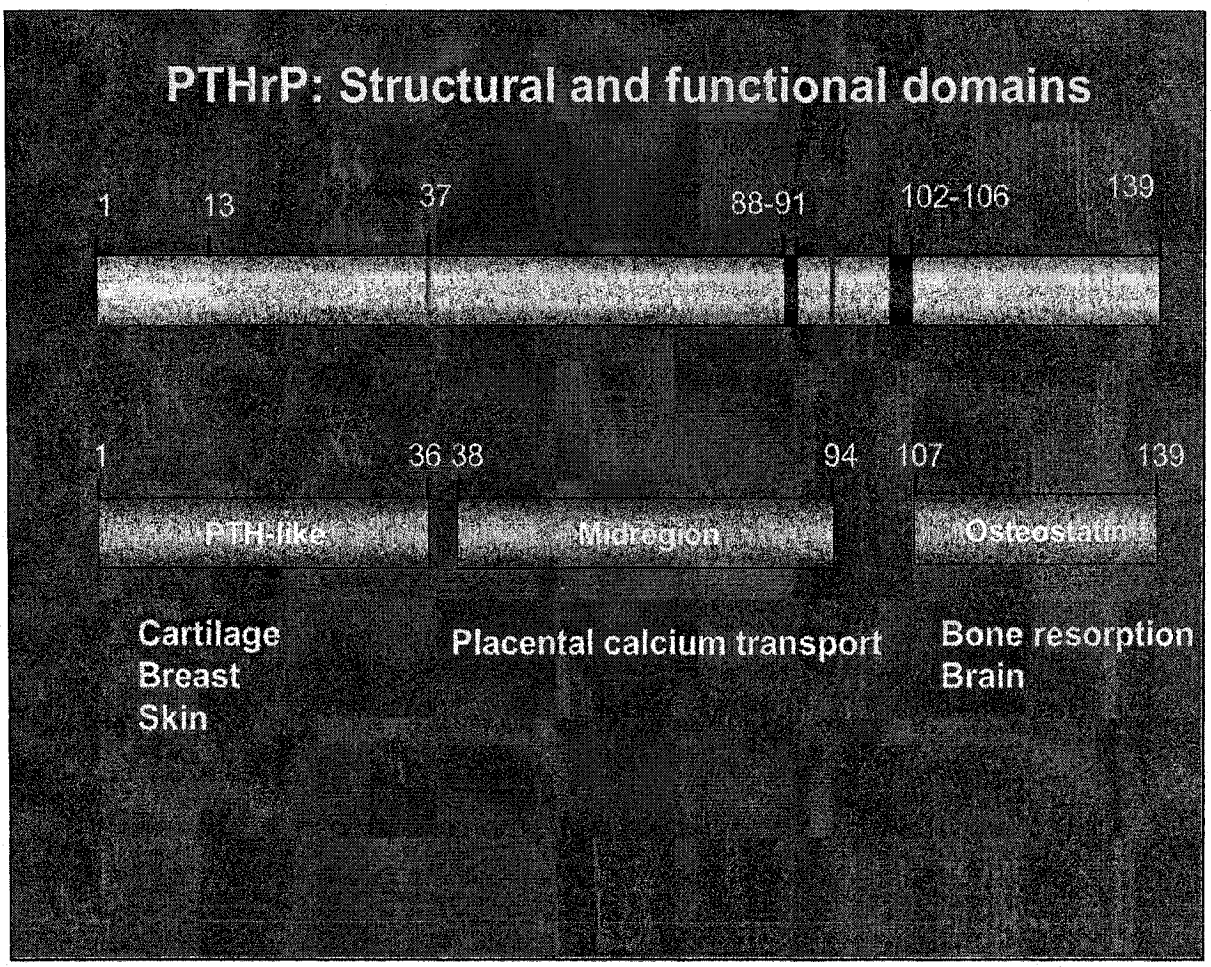
also identified a region adjacent to, and partly overlapping with, the NoS (amino acids 61-94) as comprising a nuclear targeting sequence (NTS; aa 89-94) and two potential phosphorylation sites for CDC2-CDK2 kinase (residue Thr^{85}) and casein kinase II (residue Ser^{61}), respectively. Largely based on homology to the SV40 large T antigen NTS-like sequence, this domain was determined to be important for cell-cycle regulated targeting of PTHrP to the nucleus/nucleolus. It was shown that PTHrP associates most strongly with the nucleolus in cells during the G_1 phase and that its cytoplasmic concentration increases during the G_2 and M phases (Lam et al., 1999b; Lam et al., 1997). Moreover, CDC2-CDK2 phosphorylation of Thr^{85} inhibits nuclear translocation of PTHrP during the G2/M phases and, by inhibiting NTS/NoS function, sequesters it to the cytoplasm. Subsequent analysis of this domain showed that cytoplasmic-nuclear shuttling occurs via binding of PTHrP (through amino acids 66-94) to importin ß, a process dependant on the GTP-binding protein Ran but independent of importin a (Lam et al., 1999a).

The physiological actions of PTHrP reflect its diverse tissue distribution. Thus, PTHrP functions in several developmental processes where epithelial-mesenchymal interactions are important for tissue induction and differentiation. During hair follicle development these interactions occur between PTHrP-expressing keratinocytes in the epidermis and PTH1R-expressing mesenchymal cells in the underlying dermis; signaling by PTHrP inhibits the development of dermal hair follicle cells (Wysolmerski et al., 1994). Similar interactions were demonstrated during mammary gland development where PTHrP is expressed in the mammary epithelium while its receptor localizes to the mammary stromal cells. Both overexpression and underexpression experiments have demonstrated

that signaling by PTHrP is essential for proper mammary bud formation and branching (Wysolmerski et al., 1995; Wysolmerski et al., 1998).

In other systems, experimental data indicate paracrine-autocrine actions for PTHrP in mediating proliferation or cell survival of pancreatic β cells (Gaich et al., 1993; Vasavada et al., 1996). In addition, PTHrP was shown to be a relaxant of vascular smooth muscle; acting through its cognate receptor it increases cAMP levels and leads to vasodilation of many types of blood vessels (Maeda et al., 1996; Massfelder et al., 1996). Interestingly, PTHrP was shown to have both mitotic (Pirola et al., 1993) and anti-mitotic (Massfelder et al., 1997) effects on vascular smooth muscle cells when these were treated with exogenous peptide or transfected by the cDNA, respectively. Despite the complex diversity in the physiological actions of PTHrP, its most striking effects appear to be on the developing skeleton, a system in which PTHrP appears to mediate most of its actions via its NH_2-terminal receptor (see below).

Figure 1. PTHrP structure


PTHrP: Structural and functional domains
1
13
37
88-91
102-106
139
1
36
38
94
107
139
PTH-like
Midregion
Osteostatin
Cartilage
Breast
Skin
Placental calcium transport
Bone resorption
Brain

2.2 Signal transduction by the type I PTH/PTHrP-receptor

The shared receptor for PTH and PTHrP (PTH1R), characterized as the first and primary receptor to mediate calcium and phosphate homeostasis by PTH, is a member of the seven transmembrane-domain (7-TM, serpentine) G-protein coupled receptor (GPCR) superfamily. This receptor, similarly structured and homologous to the calcitonin and secretin receptors, is characterized by seven transmembrane α–helical domains, a large NH_2-terminal extracellular domain, and a large COOH-terminal intracellular tail (reviewed in Segre, 1996). Biochemical and pharmacological studies on ligand interactions with PTH1R have focused primarily on the NH_2-terminal peptides of PTH (aa 1-34) and PTHrP (aa 1-34/36) as no experimental evidence exists suggesting that other regions of the intact ligands interact with the receptor (Pines et al., 1994). In addition to PTH1R, two other receptors termed PTH2R and PTH3R have been recently cloned. Whereas PTH1R can bind and be activated by both PTHrP and PTH, PTH2R is exclusively activated by PTH but its tissue distribution suggests that it does not function in bone (Usdin et al., 1995). Interestingly, PTH2R has been useful for mapping ligand-receptor interaction and facilitating the identification of residues His5 and Phe23 in PTHrP (Ile and Trp in PTH) as the key determinants for signaling and receptor binding, respectively (Gardella et al., 1996). Conversely, PTH3R, originally cloned in zebrafish is preferentially activated by PTHrP; however, its biological function and mammalian expression profile have not yet been determined (Rubin and Juppner, 1999). Nevertheless, in bone, PTH1R is responsible for mediating most known biological effects of PTH and PTHrP and is also the best characterized.

Two major signaling cascades are known to be activated by the PTH1R: the G_s-coupled adenylate cyclase (AC)/ protein kinase A (PKA) pathway and the G_q-coupled phopholipase C (PLC)/protein kinase C (PKC) pathway (Abu-Samra, 1992). Interestingly, structure-functional analysis demonstrated that the AC and PLC activated pathways can be uncoupled by mutations to the second intracellular loop which results in abolishment of PTH-stimulated PLC activity (Iida-Klein et al., 1997). G_s and G_q are members of the large family of heterotrimeric guanine nucleotide binding (G)-proteins (comprised of α, β, and γ subunits) that transmit signals from cell-surface receptors to downstream effectors such as AC or PLC (Weinstein et al., 2001). Individual members of this family are distinguished by their class of α-subunits ($G_s\alpha$, $G_q\alpha$, $G_i\alpha$) which possess the capacity to recognize specific receptors, covalently bind GDP/GTP, and catalyze GTP hydrolysis through their intrinsic GTPase activity. In their inactive state $G_s\alpha$ and $G_q\alpha$ covalently bind GDP, are in a trimeric state with the β and γ subunits, and associate with the cell membrane through lipid modifications on the γ subunit and palmitoylation of the α subunit. Upon agonist-mediated receptor activation the Gα subunit dissociates from the $\beta\gamma$ dimer, GDP is released and replaced by GTP and Gα is depalmitoylated. GTP-bound Gα is therefore conformationally altered and is able to directly interact with and activate its downstream effectors, AC or PLC. Return to its inactive state depends on the intrinsic GTPase activity of Gα and in the case of $G_q\alpha$, the activity of an RGS (regulator of G-protein signaling).

Coupling of $G_s\alpha$ to AC leads to its activation and the conversion of adenosine monophosphate (AMP) to its cyclic form, cAMP, which serves as the second messenger that binds the regulatory subunits of PKA causing them to release the now-activated

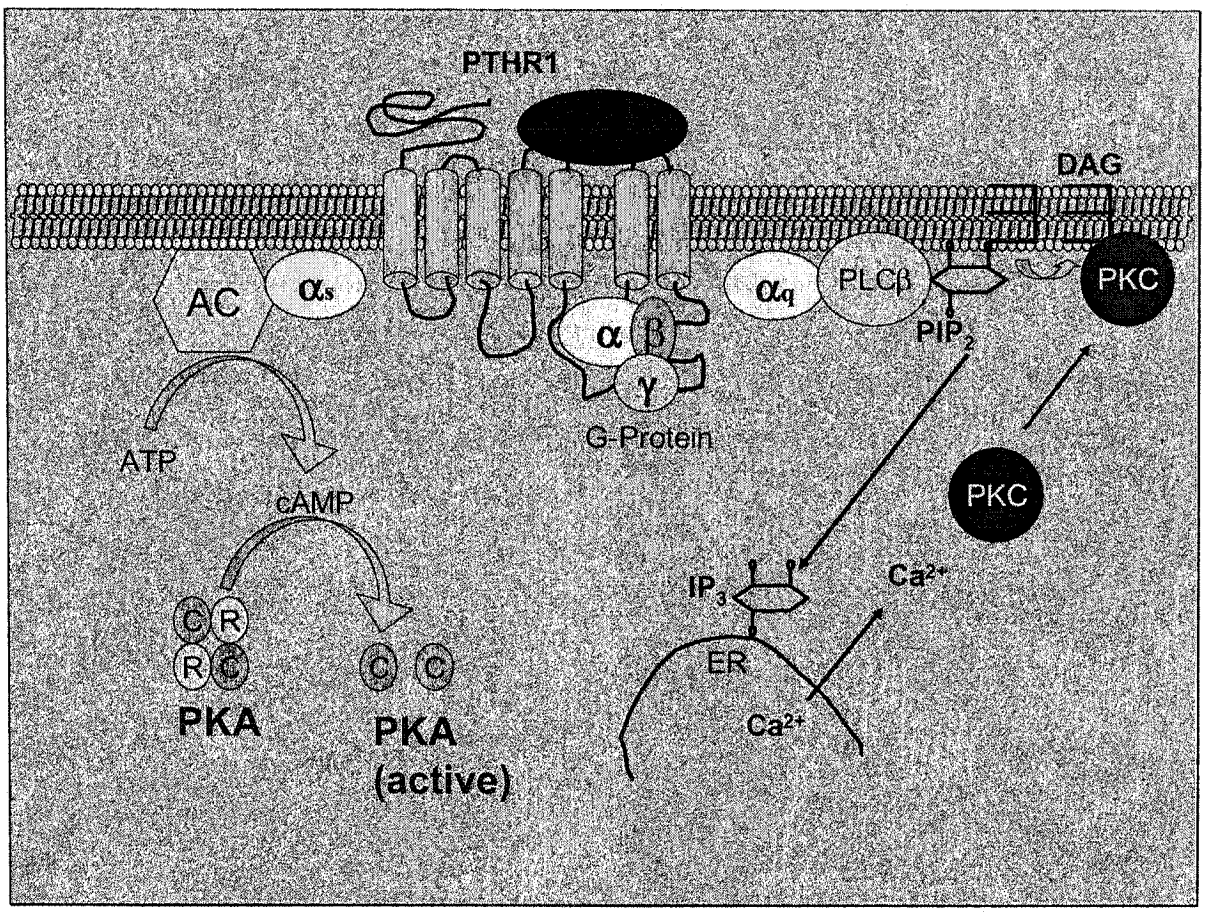
catalytic subunits (Hanks et al., 1988). Activated PKA can then phosphorylate cAMP response element binding protein (CREB) which in its phosphorylated form enters the nucleus and activates transcription from promoters harboring CRE elements, a process involving the synergistic actions of CREB binding protein (CBP). In chondrocytes, both PTH and PTHrP were shown to bind PTH1R and elicit a robust elevation in cAMP levels, an effect associated with increases in glycosaminoglycan synthesis and activation of ornithine decarboxylase (Takano et al., 1985; Takigawa et al., 1980; Takigawa et al., 1981). In addition, the cAMP/PKA activated pathway is believed to be responsible for mediating the inhibitory effects of PTHrP on hypertrophic differentiation (Guo et al., 2001). These actions of PTH1R are to be discussed further in a subsequent section.

PTHR-activated $G_q\alpha$ couples to and enhances the enzymatic activity of PLC which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP_3) (Dunlay and Hruska, 1990). IP_3 enhances intracellular Ca^{2+} levels upon binding to specific receptors on the rough endoplasmic reticulum, which leads to activation of Ca-calmodulin-dependent kinases. Concomitantly, accumulation of DAG in the plasma membrane recruits and activates PKC. Interestingly, PKC activity appears to be involved in desensitization and downregulation of PTH1R cell-surface expression (Ferrari et al., 1999).

By comparison to PKA activation, it has been shown that PTH1R activates the PLC/PKC pathway less effectively, requiring higher ligand concentrations and receptor numbers (Guo et al., 1995). The biological significance of PTHrP-mediated activation of PLC/PKC is not fully understood but some studies suggest that this may contribute to the ligand's inhibitory effects on hypertrophy, possibly through inhibition of p38 MAPK and

activation of bcl-2 (Zhen et al., 2001). Other studies suggest that activation of PKC by PTH in chondrocytes is responsible for increases in vitamin D receptor expression (Klaus et al., 1994).

Figure 2. Signal transduction by the type 1 PTH/PTHrP-receptor


PTHR1
DAG
AC
α_s
α_q
PLCβ
PKC
PIP$_2$
α
β
γ
G-Protein
ATP
cAMP
PKC
IP$_3$
Ca^{2+}
ER
Ca^{2+}
C
R
R
C
C
C
PKA
PKA
(active)

2.3 PTHrP and chondrocyte proliferation

The first clear evidence to implicate PTHrP as a central regulator of cartilage and bone development arose from studies on mice harboring an ablation of the PTHrP gene (Karaplis et al., 1994). These animals exhibited wide-spread defects to the endochondral skeleton of which one consequence is their postnatal lethality resulting from possible asphyxia due to the malformation of the rib cage. The phenotypic features of these animals, which were most strikingly manifested in bone and cartilage, were typical of a form of skeletal dysplasia characterized by premature ossification of endochondral bone elements and accelerated maturation of chondrocytes. By comparison, *Pthr1*-ablated mice presented with similar skeletal features, albeit to a greater severity, but unlike *Pthrp*-null animals underwent premature lethality at stage E11.5-E12.5 (Lanske et al., 1996). These similarities between *Pthrp*-null and *Pthr1*-ablated animals supported the view that PTHrP mediates most of its skeletal actions through PTH1R. A closer analysis, however, suggested that overexpression of a constitutively active form of PTH1R in cartilage could not correct for the early lethality of *Pth1r*-null embryos and that this may be attributed to a function of PTH1R in the heart (Soegiarto et al., 2001).

A closer analysis of bones from PTHrP-null mice revealed that the number of proliferative epiphyseal chondrocytes was greatly reduced and that these exhibited inappropriate expression of advanced differentiation markers (Amizuka et al., 1996; Karaplis et al., 1994). Furthermore, a diminished ^{3}H-thymidine incorporation index and PCNA immunoreactivity exhibited by *Pthrp*-null epiphyseal chondrocytes suggested that PTHrP may harbor mitogenic properties that are necessary for normal cartilage growth.

This hypothesis is supported by a number of studies that demonstrate the ability of PTHrP to elicit stimulatory effects on proliferation. Thus, constitutive expression of PTHrP in CFK-2 chondrocytic cells was reported to enhance their proliferative capacity (Henderson et al., 1996). Furthermore, this mitogenic effect was suggested to be dependent on a secreted fragment of PTHrP residing outside of its NH_2-terminal, as exogenous PTHrP (1-34) or constitutive expression of a non-secreted form of PTHrP had no significant effects on proliferation. However, other studies have since shown that PTHrP (1-34) does elicit a mitogenic effect on primary rat and mouse chondrocytes as well as on rat chondrosarcoma cells (Beier et al., 2001). A detailed investigation revealed that PTHrP, signaling through PTH1R, promotes chondrocyte proliferation by enhancing cyclin D1 expression consequent to CREB phosphorylation and activation by PKA; phospho-CREB binding to a CRE element on the proximal promoter of cyclin D1 promotes transcriptional activation of this gene (Beier et al., 2001). The cyclin D1 gene, its expression shown to be necessary for normal chondrocyte proliferation *in vivo* (Beier et al., 2001), also harbors an AP-1 site in its promoter that renders further susceptibility to positive regulation by PTHrP. Thus, expression of one of the AP-1 transcription factor members, c-fos, in chondrocytes is activated by PKA-mediated events downstream of PTH1R signaling, and c-fos partakes in mediating a full PTHrP-elicited mitogenic response (Ionescu et al., 2001; Lee et al., 1994a). These studies concluded that PTHrP mediates its mitogenic effects primarily through activation of the PKA-CREB pathway downstream of PTH1R to control cyclin D1 expression in a biphasic manner; immediate phosphorylation and activation of CREB by PKA and a delayed c-fos induction synergize to activate the cyclin D1 promoter.

Inconsistent with the role of CREB as a necessary factor for cyclin D1 expression, is the fact that overexpression of a dominant-negative form of this protein (A-CREB) in growth plate chondrocytes of transgenic mice did not lead to a reduction of cyclin D1 mRNA, despite an observed severe reduction in chondrocyte proliferation (Long et al., 2001). In contrast, mice harboring an ablation of another CRE-activating transcription factor, ATF2, displayed a severe attenuation of cyclin D1 expression (Reimold et al., 1996). Moreover, ATF2 has been shown to synergize with PTHrP in regulating cyclin D1 expression *in vitro* and appears to be directly activated by TGFß signaling (Beier et al., 2001). It is possible that cyclin D1 expression *in vivo* is influenced more directly by TGFß signaling rather than by PTHrP. Furthermore, *in vivo* data suggested that PTHrP signaling is not the primary mediator of CREB phosphorylation (Long et al., 2001) and that other factors such as insulin-like growth factor I (IGF-1), itself important for chondrocyte proliferation (Liu et al., 1993), can activate CREB (Pugazhenthi et al., 1999).

Most notably, CREB inactivation *in vivo* appeared to have a more dramatic effect on chondrocyte proliferation than that observed in PTHrP–null animals. In fact, closer examination revealed that despite the shortening of the proliferative zone in PTHrP-null mice, there was not significantly different in the percentage of cells in the S-phase of the cell cycle when compared to wild-type litter mates, suggesting that perhaps PTHrP does not elicit mitogenic effects on chondrocytes *per se* (Lee et al., 1996). In addition, CREB inactivation in the growth plate independently led to a downregulation of the Ihh signaling pathway, itself a key mediator of chondrocyte proliferation (see below), further suggesting that multiple effectors in addition to PTHrP arbitrate its influences on chondrocyte proliferation. The fact that constitutive activation of PTH1R was unable to rescue the

proliferative deficiency in *Ihh* (-/-) mice further indicates that PTHrP is more likely to have an effect on chondrocytic cell survival, rather than proliferation, thus increasing the pool size of mitotic cells in the growth plate (Karp et al., 2000).

An additional proposed mediator for PTHrP-induction of proliferation in chondrocytes was suggested from studies showing that activation of PKA downstream of PTH1R led to transcriptional repression of *Fgfr3* (McEwen et al., 1999). As FGFR3 activity has a negative influence on cartilage growth, inhibiting its expression concomitantly with CREB activation would enhance the proliferative capacity of chondrocytes.

2.4 PTHrP and chondrocyte apoptosis

In contrast to conflicting reports on the mitogenic effects of PTHrP, its role in prevention of programmed cell death in chondrocytes has been more compelling and has provided an alternative explanation for the *Pthrp*-null chondrodyplastic phenotype. As the analysis of growth plate chondrocytes from *Pthrp* (-/-) animals revealed inappropriate numbers of apoptotic cells, this indicated that PTHrP is likely involved in promoting chondrocytic survival and thus ensuring that the proper number of cells remain in the mitotic state (Amizuka et al., 1996).

Several studies have now indicated that at least two mechanisms exist by which PTHrP mediates inhibition of apoptosis: PTH1R-mediated upregulation of the apoptotic inhibitor bcl-2, and a non-receptor mediated function in the nucleolus that is cell-cycle regulated and promotes quiescence. Non-receptor mediated actions of PTHrP promoting cell survival were first observed upon studying a domain of the molecule located between

amino acid 87-107 (Henderson et al., 1995). As described in section II (A), the NoS/NLS is important for the regulation of nuclear/nucleolar localization of PTHrP in a cell-cycle-dependent manner. Amounting evidence indicates that nucleolar accumulation of PTHrP is a contributing mechanism by which this peptide can drive cellular senescence under conditions promoting apoptosis. In addition to PTHrP localization to a region of the nucleolus associated with ribosome biogenesis (Henderson et al., 1995), it was further demonstrated to maintain a capacity to bind rRNA (Aarts et al., 1999) and by doing so also inhibit its synthesis (Aarts et al., 2001). Thus, PTHrP appears to inhibit cellular protein translation indirectly through interaction with and inhibition of rRNA synthesis, a process that is induced under pro-apoptotic conditions, and thereby enhances cell survival by inducing entry into quiescence.

In addition to the anti-apoptotic actions of PTHrP in the nucleolus there is accumulating evidence implicating receptor-mediated actions that promote chondrocyte cell survival. Thus, treatment of murine growth plate chondrocytes with PTHrP (1-37) or PTH (1-34) results in enhancement of bcl-2 protein expression (Amling et al., 1997). Bcl-2 is the prototypical member of a larger family of apoptotic inhibitors that acts by sequestering apoptotic promoting proteins of the Bax family, consequently preventing the activation of downstream caspases, and thus ensuring cell survival (Adams and Cory, 2001). Examination of growth plates in bcl-2-defficient mice revealed a reduction in proliferative chondrocytes concomitant with accelerated differentiation and premature vascular invasion, suggesting that bcl-2 plays a key role in endochondral bone formation that parallels that of PTHrP (Amling et al., 1997). Furthermore, collagen type II-promoter driven PTHrP overexpression in growth plate chondrocytes demonstrated that bcl-2 can

be activated by, and thus lies downstream of, PTHrP *in vivo* and is probably involved in mediating part of its effects on chondrocytic survival. In agreement with this hypothesis, growth plate chondrocytes from *Pthrp* (-/-) mice displayed a reduction in bcl-2 expression (Lee et al., 1996). Interestingly, further studies have shown that PTHrP or PTH induce bcl-2 upregulation by acting on their cell surface receptor, PTH1R, an effect achieved via activation of the PKC pathway (Zhen et al., 2001).

Several studies have also provided evidence for the involvement of members of the stress-induced mitogen-activated protein kinase (MAPK) superfamily, such as p38 MAPK and ERK 1/2 kinases, in regulation of chondrocyte survival and differentiation (Kim et al., 2002; Shakibaei et al., 2001; Zhen et al., 2001). Thus, under conditions of cellular stress such as cytokine-induced (interleukine-1ß, tumor necrosis factor-a) nitric oxide (NO) production, the consequent activation of ERK1/2 inhibits, while that of p38 kinase elicits apoptosis in a differentiation state-dependant manner (Kim et al., 2002). Enhancement of apoptosis through pharmacological inhibition of ERK 1/2 was associated with increases in expression of caspase-3, an executioner of cell death, and p53, an upstream regulator of caspase-3; opposite effects were obtained from inhibition of p38 kinase. Interestingly, p38 kinase mediated apoptosis is specifically elicited in differentiated chondrocytes and this is abrogated by PTH1R signaling through PKC, resulting in concomitant upregulation of bcl-2 and inhibition of *Col10a1* expression (Kim et al., 2002; Shakibaei et al., 2001).

2.5 PTHrP and chondrocyte differentiation

An additional phenotypic aspect characterizing the PTHrP-null mice was the apparent acceleration in chondrocyte differentiation, as evidenced from increased *Col10a1*

expression and ALP activity, markers of terminal differentiation and increased matrix mineralization (Amizuka et al., 1996; Lee et al., 1996), respectively. These changes were accompanied by an inappropriate spatial organization of *Col10a1*-positive chondrocytes, which were distributed throughout the growth plate instead of being restricted to the hypertrophic zone (Amizuka et al., 1996; Lanske et al., 1996). That similar alterations in chondrocyte differentiation were also observed in mice lacking PTH1R and that chondrocytes from these animals were unresponsive to PTHrP, suggested that the actions of PTHrP pertaining to the delay of terminal differentiation are PTH1R-mediated (Lanske et al., 1996). Furthermore, support for the role of PTHrP in delaying chondrocyte maturation was demonstrated in mice overexpressing PTHrP in growth plate chondrocytes under the control of the *Col2a1* promoter (Weir et al., 1996). These mice presented with a chondrodysplastic phenotype opposite to that of the PTHrP-ablated animals, characterized by a delay in ossification of appendicular and axial skeletal elements coupled to retardation in chondrocyte hypertrophy. An exacerbation of these features was observed in transgenic mice with targeted overexpression of a constitutively active form of PTH1R (harboring the H223R mutation) (Schipani et al., 1997). This mutation was originally identified in patients afflicted with Jansen's type metaphysical chondrodysplasia and was shown to ascribe selective and constitutive activation of the cAMP/PKA pathway downstream of PTH1R (Schipani et al., 1995). In addition to a severe delay in chondrocytic hypertrophy, illustrated by near absence of *Col10a1* expression, transgenic animals harboring the PTH1R (H223R) variant also displayed an impairment of vascular invasion into the ossifying cartilage matrix, a feature not observed in PTHrP overexpressing mice. Moreover, the fact that PTH1R (H223R) expression in the endochondral cartilage

corrected the majority of skeletal defects observed in the PTHrP-null animals suggested that PTHrP mediates most of its actions in chondrocytes through the shared PTH1R. In support of this conclusion, *Pth1r* (-/-) chondrocytes dispersed amongst normal cells within the growth plates of chimeric mice underwent premature hypertrophic differentiation in a cell autonomous manner regardless of their location in the growth plate, demonstrating that PTHrP requires PTH1R to mediate its effects on chondrocytes (Chung et al., 2001).

Despite the detection of low levels of PTH1R expression in proliferating growth plate chondrocytes, the localization of PTH1R and the colocalization of PTHrP protein at high levels within a narrow zone of prehypertrophic cells, was in further agreement with the role of receptor-mediated PTHrP signaling in coordinating the timely transition of these cells to their terminal differentiated state (Lanske et al., 1996; Lee K et al., 1995; Lee et al., 1996). In addition, these prehypertrophic PTH1R-expressing chondrocytes overlap, but are distinct, from the zone of Ihh-expressing cells indicating a possible interplay between these two pathways (see below). Corroborating these *in vivo* observations, several studies have demonstrated a dependence of PTH1R expression on the differentiation state of chondrocytes *in vitro*. Primary chondrocytes from the presumptive prehypertrophic zone, shown to be actively synthesizing proteoglycans, exhibited strong binding affinity for PTH (1-34) that was considerably diminished in chondrocytes from the resting, proliferative, or hypertrophic zones (Iwamoto et al., 1994). Similarly, PTH1R expression was observed to correlate with the onset of chondrogenic differentiation in the ATDC5 embryonic carcinoma cell line, increasing concomitantly with *Col2a1* and aggrecan expression (Shukunami et al., 1996). These findings indicate that chondrocytes

within the prehypertrophic zone, the most susceptible to terminal hypertrophic differentiation, are the primary targets for the receptor-mediated actions of PTHrP.

An accumulating body of evidence suggests that the PTH1R-mediated effects of PTHrP on the delay of chondrocytes differentiation occur primarily via the cAMP/PKA pathway. Early experiments demonstrated that primary chondrocytic cells respond to NH_2-terminal analogues of PTH by increasing intracellular levels of cyclic adenosine 3'-5'-monophosphate (cAMP) levels (Takano, 1985). Furthermore, these elevations in cAMP, shown to be mediated by PTH1R receptor-activation by PTH (1-34) or PTHrP (1-34/36), were strongly associated with decreases in expression levels of the hypertrophic differentiation markers ALP and *Col10a1* (Farquharson et al., 2001; Iwamoto et al., 1994; Zhen et al., 2001). Emphasizing the significance of PTHrP/PTH1R signaling to skeletal development in humans are several chondrodysplasias that arise from missense mutations in *PTH1R*. Jansen's metaphyseal chondrodysplasia is characterized by short-limbed dwarfism and severe agonist-independent hypercalcemia resulting from an activating mutation in PTH1R that causes it to constitutively activate the cAMP/PKA pathway. In contrast, Blomstrand chondrodysplasia is caused by an inactivating mutation in *PTH1R*, leading to skeletal defects resembling those observed in *Pthr1*-knockout mice (Jobert et al., 1998; Karaplis et al., 1998; Karperien et al., 1999). Although the exact mechanism by which the cAMP/PKA pathway mediates inhibition of chondrocytic differentiation is unclear, several studies have indicated potential downstream effectors as being key regulators of these effects.

One of the potential mediators of PTHrP activity in chondrocyte differentiation is c-fos, a member of the AP-1 transcription factor family. c-fos, member of the cellular

immediate-early gene family, is rapidly and transiently upregulated in response to mitogenic (growth factors) and non-mitogenic stimuli such as PTH (Pearman et al., 1996). Its transcriptional activity is dependent upon heterodimerization with members of the Jun protein family (c-Jun, JunB, JunC) through their common basic/leucine-zipper motif (bZIP), thereby enabling DNA binding and target gene activation in a sequence-specific manner. c-fos is expressed by chondrocytes and osteoblasts at the postnatal stage and its overexpression in transgenic mice leads to tumor formation in bone (Grigoriadis et al., 1993; Kameda et al., 1997; Lee et al., 1994b). The biological effects of c-fos on chondrocytes appear to be in suppressing their differentiation since its overexpression leads to strong inhibition of ALP, *Col2a1* and *Col10a1* (Thomas et al., 2000). Furthermore, regulation of c-fos expression by PTH or PTHrP signaling was shown to be mediated by CREB phosphorylation at Ser133 by the cAMP-dependant protein kinase A, an event leading to transcriptional activation from the CRE element present in the c-fos promoter (Ionescu et al., 2001; Pearman et al., 1996; Tyson et al., 1999). In addition, the inhibitory effects of PTHrP on chondrocytic differentiation were abrogated by dominant negative forms of both CREB and c-fos, further suggesting that c-fos is a key mediator of PTHrP action in chondrocytes (Ionescu et al., 2001).

In addition to CREB, Sox-9, was shown to be a direct target for activation by PKA mediated phosphorylation downstream of PTHrP signaling (Huang et al., 2001; Huang et al., 2000). Although Sox-9 plays a major role in early mesenchymal condensation (Bi et al., 2001), its persistent expression in proliferating growth plate chondrocytes and its exclusion from hypertrophic cells suggested a role also in the later stages of endochondral bone formation (Ng et al., 1997; Wright et al., 1995). The clinical manifestation of *SOX-9*

haploinsufficiency in humans (campomelic dysplasia) and its phenotypic recapitulation in heterozygous *Sox-9*-null mice, typified by expansion of the hypertrophic zone with associated premature mineralization and a decline in *Col2a1* expression, suggested that Sox-9 may also play a role in regulating the rate of hypertrophic differentiation (Bi et al., 2001; Mansour et al., 1995). Consistent with a role for Sox-9 in regulating the prehypertrophic-hypertrophic transition, the PKA-phosphorylated form of the protein normally observed in prehypertrophic chondrocytes was absent in cells from *Pth1r*-null animals. In addition, PTH1R was shown to enhance Sox-9 phosphorylation *in vitro* (Huang et al., 2001). Finally, PTHrP-mediated activation of PTH1R resulted in the enhancement of Sox-9 upregulation of a *Col2a1* chondrocytic-specific enhancer, an effect abolished by mutations to two PKA phosphorylation sites on Sox-9 (Huang et al., 2001). Thus, PTHrP may elicit at least two of its biological actions through activation of Sox-9; downregulating hypertrophic differentiation in maturing chondrocytes while activating *Col2a1* expression in proliferating cells.

Section 3: Indian Hedgehog and Chondrocyte Biology

3.1 The hedgehog family of morphogenetic proteins: biochemistry

Hedgehogs are a family of secreted proteins that act as morphogens in a multitude of processes pertaining to invertebrate and vertebrate development. Whereas there is one known invertebrate hedgehog member (hh), three mammalian homologues have been isolated; Sonic hedgehog (Shh), Indian hedgehog, and Desert hedgehog (Dhh). Like most secreted peptides, all Hh proteins harbor a signal sequence at the NH_2-terminal end that is cleaved upon entry into the secretory pathway. Interestingly, *Drosophila* hh and mouse

Ihh differ from other Hh family members by possessing internal signal sequences that are preceded by 50 and 39 amino acids, respectively, and can potentially initiate translation from an alternative AUG codon (Lee et al., 1992; Valentini et al., 1997). This led to speculations that hh and Ihh may be present, to some degree, as type II transmembrane proteins; however, subsequent experiments have shown that *hh* is indeed a secreted protein (Porter et al., 1995). Following entry into the secretory pathway Hh proteins, which are synthesized as ~45-kDa precursors, undergo further posttranslational processing yielding two discrete products; a ~19-kDa peptide corresponding to the NH_2-terminal (N-hh) and a ~25-kDa protein derived from the COOH-terminal of the precursor (C-hh) (Bumcrot et al., 1995; Lee et al., 1992). This unique property shared by all Hh family members is mediated by an autocatalytic activity intrinsic to the COOH-terminal domain (Porter et al., 1995; Porter et al., 1996b). This domain acts intramolecularly to cleave the precursor between universally conserved Gly-Cys residues (amino acids 240-241 in Ihh) through a mechanism initiated by formation of a thioester intermediate between the thiol side chain of Cys^{241} and the carbonyl carbon of Gly^{240}. Cholesterol, acting as a lipophilic nucleophile, covalently attaches to the carbonyl carbon of Gly^{240} resulting in release of a modified NH_2-terminal bioactive peptide and a free COOH-terminal protein (Porter et al., 1996a; Porter et al., 1996b). Structural and functional analysis revealed that C-hh is derived from the intein family of self splicing proteases (Hall et al., 1997). Conserved residues between C-hh and inteins include His^{313} and Thr^{310} which were shown by site-specific mutagenesis to be required for thioester formation (Hall et al., 1997; Porter et al., 1996a). An aspartate residue at position 290 is not found in inteins but only in C-hh and is required for cholesterol activation. Following intramolecular processing, C-hh is secreted

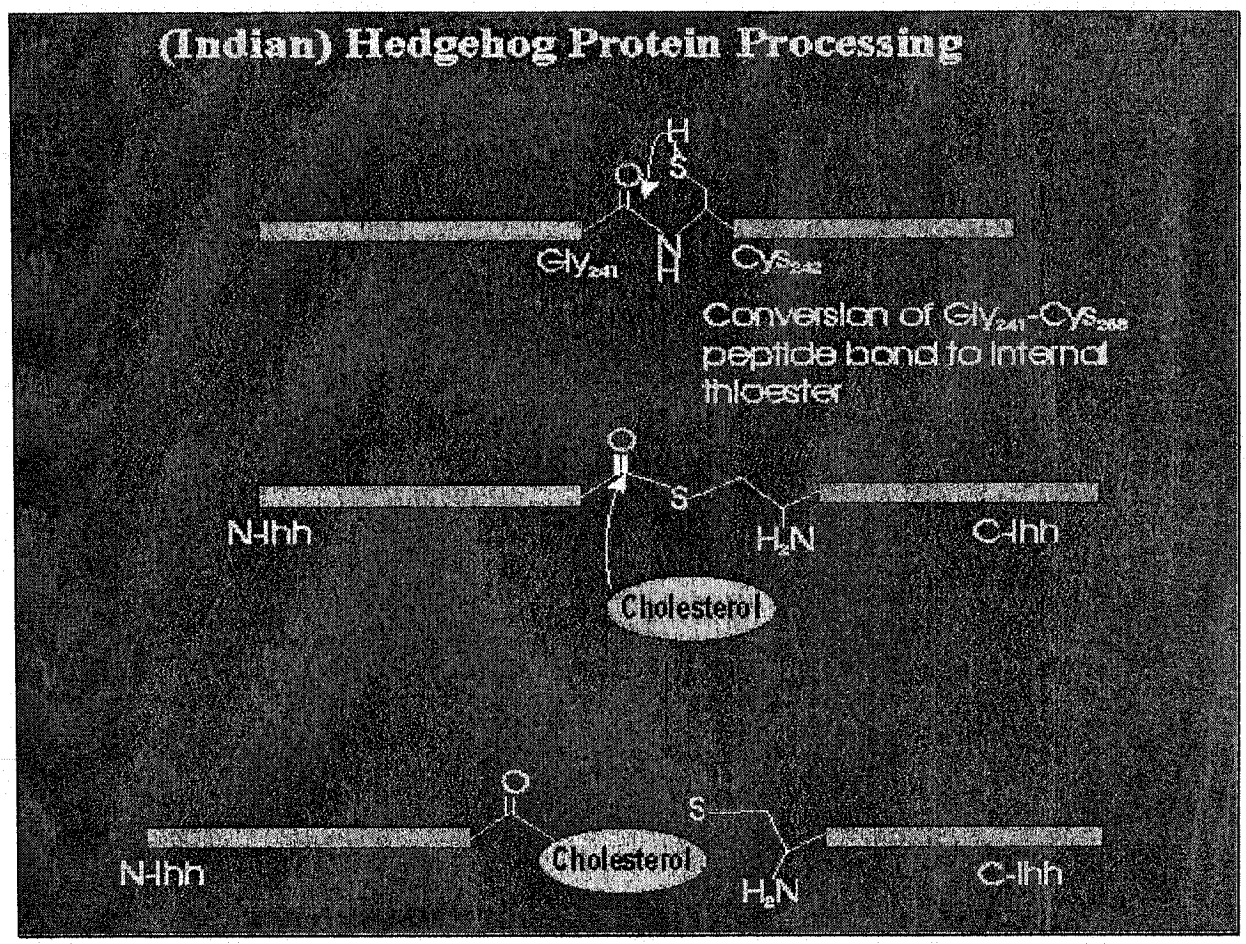
by Hh-expressing cells in culture; however, no evidence exists to suggest independent biological roles for this peptide (Bumcrot and McMahon, 1995; Roelink et al., 1995).

The cholesterol-modified N-hh, harboring all known biological properties of HH proteins, was shown to undergo further lipid modification by being palmitoylated at its most NH_2-terminal Cys^2 residue (Pepinsky et al., 1998). This residue is highly conserved amongst HH family members and when mutated, it results in the generation of a dominant negative form of the protein (Lee et al., 2001). Mutually these modifications contribute significantly to the hydrophobicity of Hh proteins and account for their apparent membrane retention observed in cell culture systems, a phenomenon that was originally difficult to reconcile with their proposed long-range signaling capacities (Bumcrot et al., 1995; Lee et al., 1994a; Porter et al., 1995). The importance of the palmitoyl moiety became apparent following the discovery that the *skinny/sightless* (*ski/sit*) gene, encoding for an acyl transferase, mediates plamitoylation of Hh proteins and its loss of function results in perturbed Hh signaling activity (Chamoun et al., 2001 Sep 14; Lee and Treisman, 2001). Although not required for receptor binding, palmitoylation was shown to potentiate the biological activity of Hh in some *in vitro* assay systems (Pepinsky et al., 1998). Furthermore, replacement of Cys^2 with another hydrophobic residue was sufficient for retention of biological potency suggesting that it is the hydrophobic nature at this position, rather than the palmitoyl moiety *per se*, that is important for Hh function (Taylor et al., 2001).

The importance of the sterol modification to Hh bioactivity was unapparent at first since unmodified forms of N-hh were able to mimic the biological effects, both long- and short-range, of the wild type protein in both invertebrate and mammaliansystems (Fan et

al., 1995; Porter et al., 1995; Roelink et al., 1995). However, studies in *Drosophila* have suggested a role for the cholesterol moiety in the sequestration of Hh by its receptor *Ptc*, such that when a non-cholesterified form of Hh was expressed in the imaginal disc, significant expansion in the expression domain of Hh-target genes occurred (Chen and Struhl, 1996). In addition, cholesterol is imperative for controlled Hh secretion from cells, as implicated from studies on *Drosophila dispatched* (*disp*), a 12-pass transmembrane protein harboring a sterol sensing domain (SSD) similar to those found in *Ptc* and several enzymes of the cholesterol biosynthetic pathway (Burke et al., 1999). These studies strongly suggested that *disp* acts opposite to *Ptc* and serves to actively release the ligand from HH-expressing cells. Cholesterol's role in restricting HH distribution was further corroborated by observations that Hh accumulates in membrane microdomains corresponding to lipid rafts, which function in sorting and concentrating lipid-modified signaling molecules (Rietveld et al., 1999; Tabata and Kornberg, 1994). In contrast, other evidence suggests that in certain circumstances cholesterol may be necessary for long-range distribution of HH. Thus, during patterning of the limb a non-cholesterified form of Shh cannot substitute for its native counterpart in specifying digit formation and this is due to impaired distribution across multiple cell diameters (Lewis et al., 2001). This disparity may reflect differences between mammalian and *Drosophila* forms of Hh and indicates existing divergent mechanisms for regulating its distribution.

Figure 3. Hedgehog protein processing by autocatalytic cleavage


(Indian) Hedgehog Protein Processing
Gly241
Cys
Conversion of Gly241-Cys
peptide bond to internal
thioester
N-Ihh
S
H2N
C-Ihh
Cholesterol
N-Ihh
Cholesterol
S
H2N
C-Ihh

3.2 The role of heparan sulfate proteoglycans in Hh signal transduction and Chondrocyte Biology

Heparan sulfate proteoglycans (HSPGs) are cell-surface molecules that consist of a protein core that is covalently modified by negatively charged heparan sulfate glycosaminoglycan (GAG) chains. These moieties participate in a multitude of biological processes ranging from cell adhesion to intracellular signaling and are particularly abundant in cartilage. The heparin sulfate chains are composed of polymeric repeats of the disaccharide D-glucuronic acid (GlcA) / N-acetyl-D-glucosamine (GlcNAc) that attach to specific serine residues on the glycoprotein via a tetrasaccharide "linkage region" (GlcA-galactose-galactose-xylose) (Perrimon and Bernfield, 2000; Salmivirta et al., 1996). Subsequent to chain assembly GAGs undergo further modifications in the Gogi by N-deacetylase, N-sulfotransferase, GlcA C5-epimerase, and 3-O-sulfotransferase, which results in generation of structurally modified and sulfated sugar chains. It is important to stress that the degree of GAG chain length and modification is highly variable, thus further increasing the complexity of their biological functions.

The first demonstration for involvement of HSPGs in HH signaling came from the identification of the *Drosophila* gene *tout velu* (*ttv*), where its absence resulted in impaired HH diffusion and activation of target genes across the imaginal disc A/P boundary (Bellaiche et al., 1998). Furthermore, *ttv* showed high homology to members of the human exostosin (EXT) gene family (56% identity to EXT1, 25% to EXT2), originally cloned by their linkage to the autosomal dominant disorder hereditary multiple exostoses (HME) (Ahn et al., 1995; Stickens et al., 1996). The involvement of EXT genes in HSPG synthesis became apparent from studies on the ability of herpes simplex virus type-1

(HSV-1) to infect sog9 cells (McCormick et al., 1998). Normally refractile to HSP-1 infection due to a deficiency in GAG synthesis, expression of EXT1 in these cells restored their HSPG synthetic capacity and rendered them susceptible to the virus.

EXT1 and EXT2 encode for peptides of 746 and 718 amino acids in length, respectively, that localize to the endoplasmic reticulum (ER) and Golgi and are classified as type II transmembrane proteins harboring a short cytoplasmic NH_2-terminal and a large lumenal COOH-terminal domain (McCormick et al., 2000; McCormick et al., 1998). Biochemical analysis has shown that EXT1 and EXT2 both possess GlcNAc- and GlcA-glycosyltransferase activities, thus identifying them as polymerases in GAG chain elongation (Lind et al., 1998; McCormick et al., 2000; Senay C, 2000). Differing from EXT2, EXT1 appears to possess significant transferase activity when expressed independently; however, this is further enhanced upon coexpression of EXT2 (McCormick et al., 2000). This finding and the fact that EXT1 and EXT2 form a physical oligomeric complex *in vivo* that accumulates in the Golgi, suggested that EXT1/EXT2 are copolymerases for HSPGs that act synergistically to exert full enzymatic activity. In parallel, studies in *Drosophila* indicated that *ttv* alone probably mediates all HSPG biosynthetic activities (Toyoda, 2000; The, 1999).

The relevance of EXT function to chondrocyte biology is intriguing in light of the fact that these are ubiquitously expressed genes that are central to a fundamental biosynthetic pathway. Yet, the clinical manifestations of HME appear to be limited to cartilage. Thus, patients with HME display benign epiphysial tumors (exostoses) that are histologically similar to the parental bone and are comprised of growth plate-like cartilage surrounded by trabecular bone and periostium that are continuous with the underlying bone (Solomon,

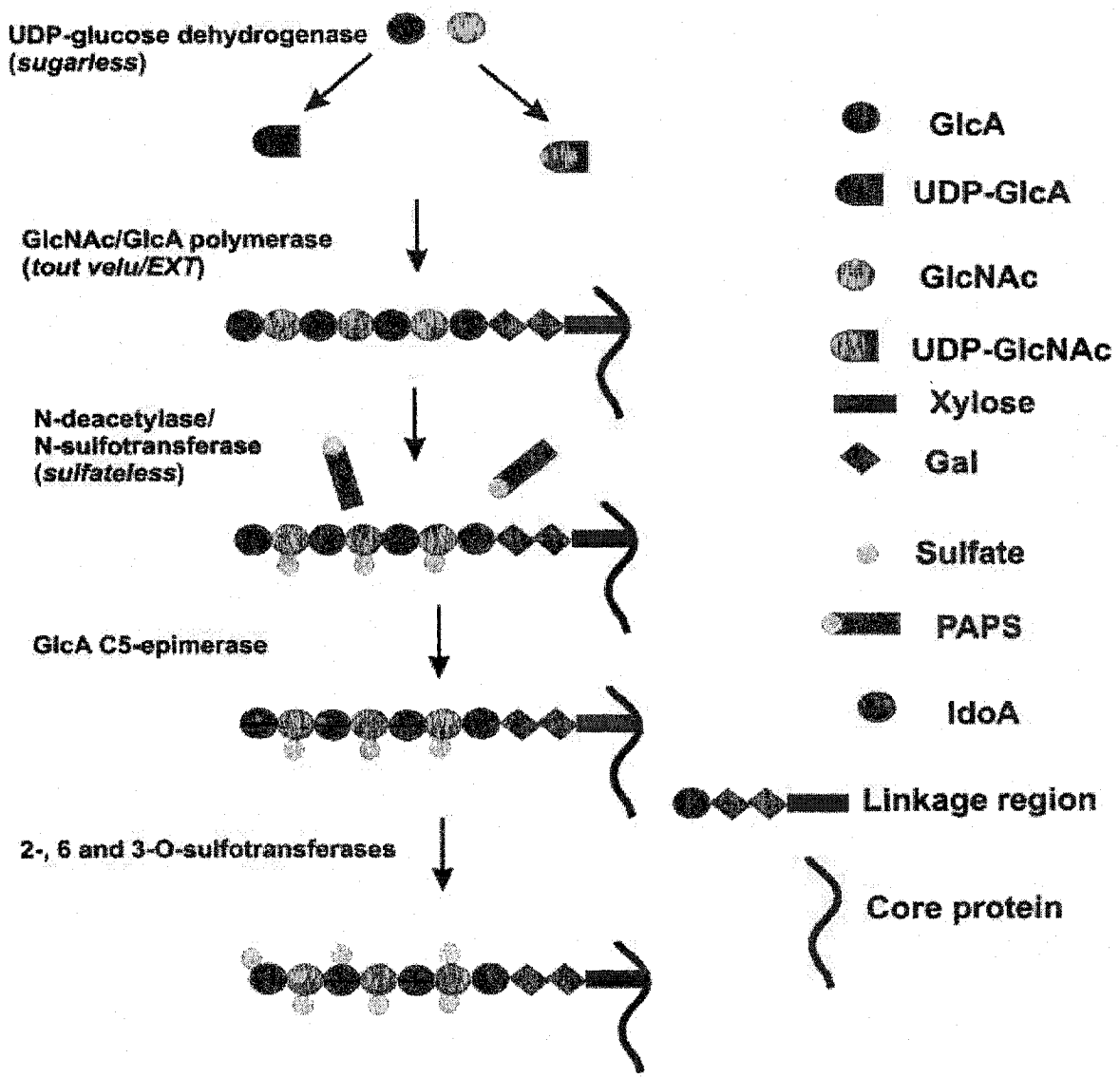
1967). In the most severe form of the disorder and at a low occurrence (0.5-2%), exostoses can undergo malignant transformation into chondrosarcomas or osteosarcomas (Schmale et al., 1994). Loss of heterozygosity of EXT1 or EXT2 has suggested their classification as tumor suppressors, and analysis of genomic DNA from HME patients has demonstrated a wide plethora of EXT1 and EXT2 mutations resulting in either truncated or missense product variants (Wuyts et al., 1995). Yet, in some, but not all cases, these missense variants were shown to be deficient in HS synthesis and cell surface expression, suggesting that the etiological mutations do not always correlate with the presumptive functions of EXT proteins (Cheung et al., 2001; McCormick et al., 2000). These findings are further confounded by the observation that mice lacking EXT1 exhibit arrested growth at an early embryonic stage due to failure in gastrulation and that in the heterozygous state, these animals do not develop exostoses despite compromised HS synthesis (Lin et al., 2000).

The fact that complete or partial loss of EXT function in humans leads to cartilaginous tumors is indicative of their possible role as regulators of chondrocyte proliferation. At the cellular level, however, chondrocytes from patients with HME display alteration in cytoskeletal protein composition and distribution, indicating that EXT1/EXT2 may have important functions in differentiation (Bernard et al., 2000). Accordingly, EXT1 and EXT2 expression were shown to co-localize to non-hypertrophic growth plate chondrocytes in developing mice embryos (Stickens et al., 2000). Taken together, this data led to the speculation that HSGP synthesis by EXT1/EXT2 may be important for facilitating Ihh-signal reception by growth plate chondrocytes (Duncan G, 2001). It is not

clear, however, whether particular HSPGs are required for actual Hh ligand binding or, as suggested from *Drosophila* studies, for Hh diffusion only.

The direct role for EXT1/EXT2 in mammalian Hh-signaling has also not been determined. Interestingly, studies on EXT1-/- animals suggest that HSPG is required for Hh binding to cells, however, no functional studies for determining Hh-responsiveness in EXT1-deficient cells has been reported (Lin et al., 2000). In addition, studies in *Drosophila* have suggested that ttv affects Hh signaling exclusively (The et al., 1999). A parallel and specific role for EXT1 or EXT2 is not clearly evident, however, as HSPGs can affect Wnt and FGF signaling, both of which are required for normal chondrogenesis.

Figure4. Pathway for heparan sulfate proteoglycan synthesis


UDP-glucose dehydrogenase
(sugarless)
GlcNAc/GlcA polymerase
(tout velu/EXT)
N-deacetylase/
N-sulfotransferase
(sulfateless)
GlcA C5-epimerase
2-, 6 and 3-O-sulfotransferases
GlcA
UDP-GlcA
GlcNAc
UDP-GlcNAc
Xylose
Gal
Sulfate
PAPS
IdoA
Linkage region
Core protein

3.3 Hedgehog Signal Transduction: The Patched/Smoothened receptor complex

It is generally accepted that all Hh proteins elicit their biological actions through the activation of a unique signal transduction cascade, initiated upon binding to their cognate receptor, *Patched* (*Ptc*). This large 12-pass transmembrane protein was first identified in *Drosophila* as a segment polarity gene that was later shown in cell culture systems to physically bind Hh through its large extracellular domain (Hooper and Scott, 1989; Marigo et al., 1996; Nakano et al., 1989; Stone et al., 1996). Mutating or deleting this domain of *Ptc* resulted in reduction or abolishment of Hh signaling *in vivo* (Briscoe et al., 2001; Mullor et al., 2000). The affinity between *Ptc* and Hh was further corroborated by observations that the two proteins colocalize to intracellular vesicles in Hh-responsive cells (Martin et al., 2001; Strutt et al., 2001), suggesting that *Ptc* may be involved in sequestering the Hh ligand via endocytosis, a finding that agrees with its action in restricting Hh distribution *in vivo* (Chen and Struhl, 1996). An additional structural feature identified in *Ptc* through homology to proteins involved in the intracellular trafficking of cholesterol was a sequence designated as a sterol sensing domain (SSD), encompassing TM helices 2-6 (Strutt et al., 2001).

Interestingly, in most invertebrate and vertebrate contexts, *Ptc* displays expression patterns indicative of being an actual Hh-target gene. Thus, its tissue distribution correlates with Hh-responsive cells but not with cells expressing Hh itself, and can be ectopically upregulated in response to Hh overexpression (Basler and Struhl, 1994; Goodrich et al., 1996; Tabata and Kornberg, 1994). In mammals, an additional *Ptc* (henceforth *Ptc1*) member, *Ptc2*, was cloned and shown to bind Hh and elicit signaling with equal capacity to that of *Ptc1*; however, its tissue distribution, which in some cases

coincides with *Hh* itself, suggested a possible autocrine function (Carpenter et al., 1998; Motoyama et al., 1998). Nonetheless, genetic analysis in *Drosophila* and mammals suggested that *Ptc* or *Ptc1* activity suppresses Hh target gene expression and that Hh antagonizes this activity (Capdevila and Guerrero, 1994; Dahmane et al., 1997; Goodrich et al., 1996; Ingham et al., 1991). Thus, *Ptc/Ptc1* expression serves as an excellent indicator for Hh-signaling activity.

Mediating the actual Hh signal is a second receptor, Smoothened (Smo), belonging to the 7-TM G-protein coupled receptor family which is required for Hh activity in all invertebrate and vertebrate systems (Chen W, 2001; Chen and Struhl, 1996; Zhang et al., 2001). This categorization of Smo is supported by its demonstrated ability to activate $G_{\alpha i}$ in a *Xenopus* system, and the fact that one of several activating Smo mutations found in human basal cell carcinomas is predicted to disrupt G-protein coupling (DeCamp et al., 2000; Xie et al., 1998). Despite these suggestive findings, there is no sufficient evidence to irrefutably prove that Smo acts in this fashion Moreover,in *Drosophila,* G-proteins mutations do not appear to affect hh-signaling (Wolfgang et al., 2001). It is generally accepted that Smo activity is functionally repressed by Ptc1 and is only reconstituted in the presence of Hh ligand or in the absence of *Ptc1* (Chen and Struhl, 1996; Quirk et al., 1997). The mechanism mediating this novel interaction is still a contentious subject and includes several models. The most accepted of these proposes the occurrence of a physical complex between Ptc1 and Smo, observed to occur in cell culture systems (Murone et al., 1999; Stone et al., 1996), leading to an inactivating conformational alteration in Smo.

Contesting this model, however, are observations that *Drosophila Smo* and *Ptc* do not overlap in their protein expression domains and that their physical interaction does not

occur *in vivo* (Denef et al., 2000; Johnson et al., 2000). Furthermore, overexpression of *Smo* does not lead to increased hh-signaling, suggesting a non-stoichiometric relationship with *Ptc,* but rather that hh mediates increased cell membrane accumulation of *Smo* (Alcedo et al., 2000; Ingham et al., 2000). Intriguingly, the absence of *Ptc* or the presence of hh is accompanied by phosphorylation of Smo which correlates with its active state, and it was proposed that this may occur via inactivation of a Ptc-dependent phophatase (Denef et al., 2000). The model emerging from these data suggests that hh and Ptc act antagonistically to recruit or remove Smo to and from the plasma membrane and that this may occur via specific intracellular transport vesicles emanating from the Golgi. In agreement with this view, Rab23, a member of the small GTP-activated proteins involved in many aspects of membrane trafficking, was shown to act downstream of Shh and to negatively regulate its actions (Eggenschwiler et al., 2001). In addition, there is some evidence that Ptc may control Smo membrane accumulation through its SSD, as mutations in this domain result in loss of Smo inhibiting activity (Martin et al., 2001; Strutt et al., 2001).

3.4 Signal transduction by hedgehog proteins: Post-receptor events

Although the mechanisms by which activated Smo propagates signaling toward the nucleus are poorly understood, there is substantial knowledge on how its primary transcriptional effector is regulated by Hh and other signaling inputs. Whereas in *Drosophila,* all hh-mediated transcriptional output is governed by a single gene product, *Cubitus Interuptus* (*Ci*), in mammals these functions are controlled by three Ci homologues, designated Gli1, Gli2, and Gli3 (after glioblastoma) (Forbes et al., 1993; Methot and Basler, 2001; Ruppert et al., 1990). Characteristic of transcription factors, Ci

and Gli proteins all harbor a zinc finger DNA-binding domain (ZFD; aa 451-603 in Ci) and a COOH-terminal transactivation domain (TA) (Alexandre et al., 1996). In addition, Ci, Gli3, and in some contexts Gli2, also possess an NH_2-terminal transcriptional repressor domain (Alexandre et al., 1996; Aza-Blanc et al., 1997; Ruiz i Altaba, 1998; Sasaki et al., 1999). Interestingly, Ci and Gli3 also encompass a CREB-binding protein (CBP) interacting region and a cluster of consensus protein kinase A (PKA) phosphorylation sites (RRXS/T) located COOH-terminal to the ZFD (Akimaru, 1997; Dai, 1999; Chen, 1998; Chen, 2000), indicative of further regulatory inputs on these factors.

In *Drosophila*, Ci undergoes proteolytic processing to generate a nuclear 75-kDa (Ci75) NH_2-terminal transcriptional repressor form, a process that is inhibited by HH-signaling which promotes the stability and cytoplasmic localization of its full-length 155 -kDa (Ci155) form (Aza-Blanc et al., 1997). Thus, studies conducted on *Ci*-null in combination with *hh*-null mutants revealed that HH acts by promoting the activator form and inhibiting the repressor form of Ci where the balance between the two depends on the HH-signal strength (Methot and Basler, 2001). In mammals, a parallel HH-regulated cleavage has been definitively demonstrated for Gli3 but less convincingly so for Gli2, its cleavage being insensitive to HH activity and this form representing a minor fraction of the protein (Ruiz i Altaba, 1999; Wang, 2000).

The mechanisms that control Ci/Gli transport into the nucleus for transcriptional activation or repression were elucidated primarily in *Drosophila* and pertain to Ci function; however, heterologous expression experiments substituting Ci with Gli proteins in imaginal discs has provided a system for understanding their mammalian functions (Aza-Blanc et al., 2000; von Mering and Basler, 1999). In the absence of HH signal, Ci is found

primarily tethered to microtubules in the cytoplasm where it complexes with the kinesin-related protein Costal-2 (Cos-2), and a serine-threonine kinase, Fused (Fu) (Alves et al., 1998; Ohlmeyer and Kalderon, 1998; Wang et al., 2000b). In this state, Ci is highly susceptible to phosphorylation by PKA which is required for its subsequent cleavage (Johnson et al., 1995; Ohlmeyer and Kalderon, 1998). An additional negative regulator of HH signaling, suppressor of fused [Su(Fu)], was found to complex with Ci and is presumably involved in retaining it to the cytoplasm (Methot and Basler, 2000; Stegman et al., 2000). Mediating the actual cleavage process is the gene *Slmb*, encoding an F-box protein involved in priming protein substrates for proteosome-dependent cleavage.In its absence, Ci accumulates in its full-length form (Wang et al., 2000b). These events lead to the outcome where Ci75 accumulates in the nucleus and represses HH-target genes. In response to HH-signaling, however, ill-understood negative inputs emanating from *Smo* impede Cos-2 activity and reduce the phosphorylation status of Ci, possibly by activating a phosphatase, leading to stabilization of Ci155 and its transactivating action in the nucleus.

In mammals, each of the three Gli proteins appears to encompass a distinct aspect of Ci function that is also context dependent. Thus, Gli1 possess only activator functions and, when overexpressed, it activates HH-target genes in the absence of HH ligand (Ruiz i Altaba, 1998, 1999). It does not appear to be susceptible to proteolytic cleavage or negative regulation by PKA (Dai et al., 1999). In fact Gli1 is not likely to be the primary mediator of HH activity and its ablation has no phenotypic consequences (Park et al., 2000). In contrast to Ci, it appears rather that Gli1 is itself a HH-responsive gene that is transcriptionally upregulated similarly to *Ptc* which indicates that it cannot be the initial mediator of HH-signaling. Nonetheless, other functional aspects such as its ability to

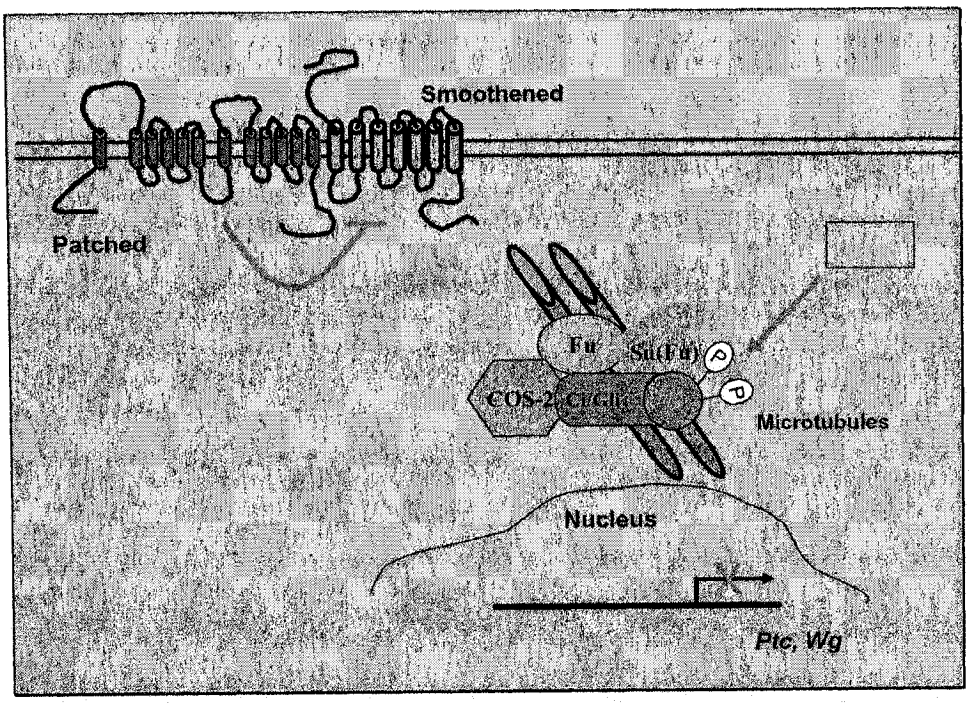
interact with vertebrate Fu and Su(Fu), suggest that its activity can be potentiated by HH-signaling (Murone et al., 2000). That Gli1 may potentiate HH-signaling *in vivo* is suggested from analysis of compound $Gli1^{-/-}/Gli2^{-/+}$ mutants, where a loss of some floor plate cells indicated that these are specified by Gli1.

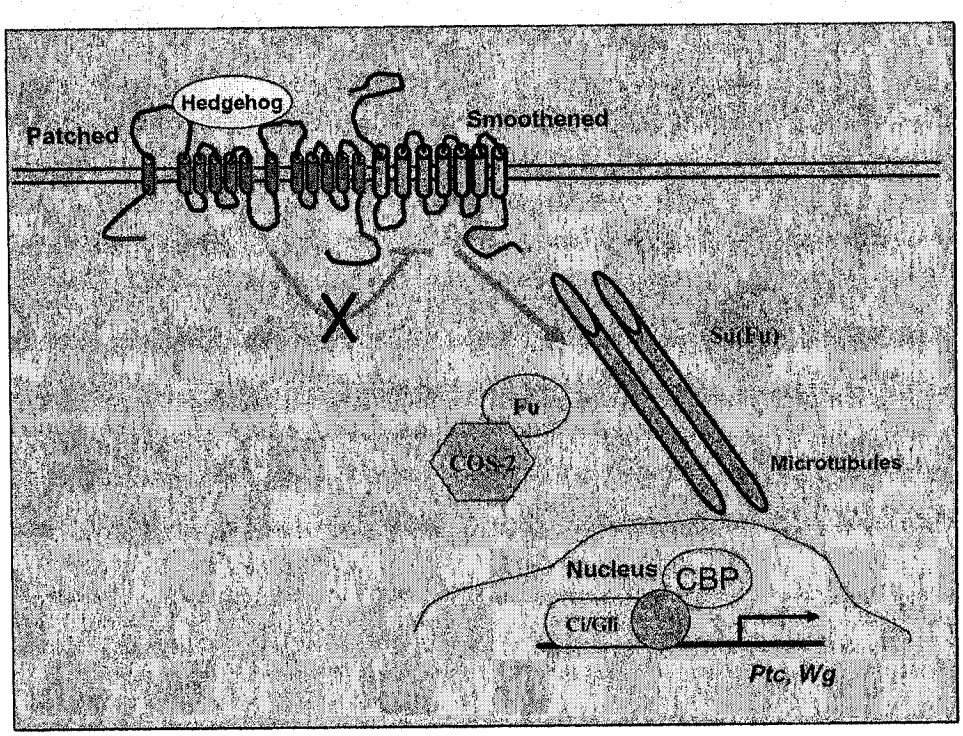
Although Gli1 may mediate minor aspects of HH-signaling, most evidence indicates that *in vivo* the majority of these activating functions are controlled by Gli2. Thus, animals ablated for the ZFD of Gli2 develop severe central nervous system defects manifested by loss of most floor plate and reduction of V3 interneurons, cells normally specified by high HH activity (Ding et al., 1998; Matise et al., 1998). In contrast, experiments based on ectopic expression of Gli2 in *Xenopus* indicated its possible role as a transcriptional repressor of HH-target genes (Ruiz i Altaba, 1998). These experiments, however, do not emulate the status of HH-signaling in mammals and may represent a species-specific phenomenon. In other contexts, Gli2 proteins harboring NH_2-terminal or COOH-terminal truncations acquire constitutive activating or repressing properties (Karlstrom et al., 1999; Sasaki et al., 1999). There is no evidence, however, that these forms of Gli2 exist *in vivo*.

On the other hand, there is ample evidence to suggest that in most contexts, Gli3 mediates repressor functions. These functions observed in cell culture and in the embryo proper require proteolytic cleavage of Gli3 to generate a repressor form of about 83-kDa that accumulates in the nucleus (Ruiz i Altaba, 1998; Wang et al., 2000a). These findings suggest that Gli3 undergoes similar processing to that of Ci although no vertebrate ortholog of Cos-2 has been discovered. In other contexts, Gli3 may also activate HH-target genes. Thus, it is shown that Gli3 can activate the Gli1 promoter *in vitro* and that in *Gli3/Shh* compound mutants there is a loss of Gli1 expression (Dai et al., 1999; Litingtung

and Chiang, 2000), indicating that prior to induction of Gli1 by Shh, Gli3 may have activating functions to promote Gli1 expression.

Figure 5. The Hedgehog signal transduction pathway


Smoothened
Patched
Fu
Su(Fu)
P
P
COS-2
Ci/Gli
Microtubules
Nucleus
Ptc, Wg


Hedgehog
Patched
Smoothened
X
Su(Fu)
Fu
COS-2
Microtubules
Nucleus
CBP
Ci/Gli
Ptc, Wg

4. Mechanisms regulating hedgehog activity and expression

As hedgehog proteins are potent regulators of a plethora of biological processes critical to animal development, it is not surprising that negative mechanisms have emerged to counteract its functions. Negative regulation of Hh-action could be exerted either by transcriptional regulation of *Hh/hh* gene expression or through negative signaling inputs that directly or indirectly interplay with components within the Hh-signaling pathway. Of these, the latter is the better understood, as illustrated by the role of the cAMP-dependent protein kinase A (PKA), whereas knowledge of mechanisms governing *Hh/hh* expression is relatively rudimentary.

Early studies in *Drosophila* indicated that PKA activity mimicks the phenotype caused by loss of *Ptc*, known to function antagonistically to *hh*. Thus, during limb development, PKA suppressed inappropriate *dpp* and *wg* expression but did not interfere with their induction by hh in cells proximal to its secretion (Jiang and Struhl, 1995). Conversely, loss of PKA led to a cell-autonomous, hh-independent, activation of *dpp* and *wg* in the anterior (A) compartment cells of the developing led and wing imaginal disc, respectively (Lepage et al., 1995; Li et al., 1995). Similarly in mammals, ectopic expression of a dominant-negative regulatory subunit of PKA, PKI, led to alterations in patterning of somites, brain, and eye that resembled the phenotype arising from ectopic Hh expression (Hammerschmidt et al., 1996; Ungar and Moon, 1996). These findings, together with the observation that expression of a constitutively active form of PKA resulted in suppression of Hh-induced cell types, suggested that in both invertebrates and mammals PKA acts in Hh-target cells as a common negative regulator (Hammerschmidt et al., 1996).

The mechanistic basis for these phenomena became apparent from observations in *Drosophila*, where cells lacking PKA activity accumulated full length Ci independently of hh-activity (Johnson et al., 1995; Ohlmeyer and Kalderon, 1998). Primary sequence analysis revealed that Ci harbors 7 potential consensus sites for PKA phosphorylation (RR(D/S)QXSXXS) that are also conserved in Gli1, Gli2, and Gli3. Two of these sites are located within the Zn-finger domain while five are downstream of the Ci cleavage site. It was reported that mutating the five PKA sites outside the Zn-finger was sufficient to abolish phosphorylation by PKA *in vitro* and induce ectopic *hh*-target gene expression concomitantly with accumulation of the PKA-insensitive Ci155 (Chen et al., 1999; Chen et al., 1998). These findings suggested that phosphorylation by PKA may be necessary for priming Ci for protease recognition and further promoting its association with Cos-2. By comparison, mammalian Gli proteins appear to be susceptible to modulation by PKA in a selective manner that varies between Gli1, Gli2, and Gli3. Thus, although PKA inhibits Gli1 function, albeit without altering its protein structure or subcellular distribution, it is only Gli3 that is clearly susceptible to PKA-mediated cleavage that generates an NH_2-terminal active repressor form in the nucleus (Kaesler et al., 2000; Ruiz i Altaba, 1998; Wang et al., 2000a). This demonstrates that whereas Gli3 and Ci are similarly regulated by PKA, phosphorylation of Gli1 only prevents its transcriptional activity and promotes its sequestration in the cytoplasm and possible association with Su(Fu) (Ding et al., 1998; Kogerman et al., 1999). In contrast, Gli2 can act as a bipotential transcription factor by possessing both activation and repression domains and is found to undergo partial proteolytic processing. However, it is not clear that phosphorylation by PKA directly promotes this event (Ruiz i Altaba, 1999; Sasaki, 1999). Nonetheless, since dominant

negative PKA induces ectopic ventral floor plate neurons, cell types that fail to form in Gli2-null mice, it is likely that Gli2-mediated transcriptional activation of Shh target genes *in vivo* is antagonized by PKA (Ding et al., 1998; Epstein et al., 1996; Matise et al., 1998). Thus, control of Hh-signaling by PKA in mammals appears to be mediated via all Gli proteins and is context- and structure-dependent, indicative of a higher degree of regulatory complexity than that seen in invertebrates.

In contrast to our understanding in PKA-mediated regulation of Hh-signaling and its apparent cross-species conservation, less is known about the transcriptional regulation of *Hh* or its target genes. In the developing *Drosophila* embryo, both the ectodermal segments and the imaginal discs of the appendages and eye are subdivided into compartments of anterior (A) and posterior (P) cells. It is observed that *hh* expression is restricted to the P compartment cells, congruently with the homeoprotein transcription factor *engrailed* (*en*), which directs hh secretion while simultaneously rendering P cells refractory to the hh signal (Kornberg et al., 1985; Lee et al., 1992; Tabata et al., 1992; Zecca et al., 1995). Although En is not necessary for initiation of *hh* expression, it appears to play a role in its maintenance during later stages (Tabata et al., 1992). Defining the anterior compartment, hh-signaling induces A cells to express the genes *wingless* (*wg*), a member of the Wnt family of secreted morphogens, in the ectoderm or *decapentaplegic* (*dpp*), a member of the TGF-β family, in the imaginal discs while en is necessary for repressing their expression by adjacent P compartment cells. Exclusion of en from cells in the anterior compartment, thus permits them to express *Smo* and *Ci*, and thereby facilitates their ablity to receive and respond to hh-signaling emanating from P cells (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998). That en may mediate its effect by direct

modulation of hh-target genes, is supported by studies demonstrating en protein binding the promoters of the *Ci* and *dpp* genes, thus eliciting their transcriptional repression in cells of the posterior compartment (Sanicola et al., 1995; Schwartz et al., 1995).

Encoding for a homeodomain-containing transcription factor, En and its two mammalian homologues, En-1 and En-2, harbor five conserved structural domains designated EH1-EH5 (Logan et al., 1992). Whereas the EH4 homeodomain is responsible for DNA binding, EH2 was shown to mediate interactions with other homeodomain proteins and increase DNA-binding affinity and specificity (Peifer and Wieschaus, 1990; Peltenburg and Murre, 1997). The EH1 and EH5 regions have been shown to possess transcriptional repression activity that ascribe En proteins their characteristic properties as negative regulators of transcription (Han K, 1993; Smith and Jaynes, 1996). The EH1 domain was also shown to associate with the transcriptional repressor groucho (Gro), further suggesting that En synergizes with other factors to direct transcriptional outputs (Jimenez et al., 1997).

In contrast to *Drosophila*, there is no clear evidence for conservation in the epistatic relationship between *En* and *Hh* in mammals. En-1 and En-2 are both expressed in the developing mid-hindbrain while En-1 is also expressed in the limb, spinal cord, and developing vertebral column (Davis and Joyner, 1988; Joyner and Martin, 1987). Despite their similar expression patterns in the brain En-1 knockout mice die at birth and lack most mid-hindbrain structures, whereas En-2-null counterparts are viable and suffer only mild cerebellar structural alterations (Millen et al., 1994; Wurst et al., 1994). Furthermore, En-1 and Wnt-1 expression is mutually dependent during mid-hindbrain development. However, differing from invertebrate systems, these are expressed by the same cells

(Danielian and McMahon, 1996; McMahon et al., 1992). In addition, *Hh*-signaling is apparently not required for anterior-posterior patterning of this structure. In other contexts, however, the mutual exclusion between En-1 and Wnt7a expression appears to play an important role in ventral-dorsal patterning of the limb and pointstoward some level of mechanistic conservation (Cygan et al., 1997; Loomis et al., 1996). Despite this, there is no evidence that Shh signals emanating from the *zone of polarizing activity* (ZPA), a specialized region of posterior mesenchyme that functions in determining antero-posterior patterning and in maintenance of proximo-distal skeletal outgrowth in the developing limb, has any epistatic relationship with En-1 (Laufer et al., 1994; Niswander et al., 1994).

Interestingly, characterizing the En-1-null mutants are impaired caudal outgrowth of the sternum accompanied by its delayed ossification, truncation of the 13^{th} ribs, and reduced ossification in the phalanges, all which suggest a role in endochondral ossification (Wurst et al., 1994). Despite these features, En-1 expression and function within the endochondral skeleton has not been studied in detail.

5.1 Ihh in chondrocyte biology: coordination of proliferation and differentiation with PTHrP

A function for Ihh in chondrocyte biology was first suggested from observations on its tissue expression distribution during embryogenesis. Although Ihh is expressed in the developing gut (Bitgood and McMahon, 1995), kidney (Valentini et al., 1997), heart (Zhang et al., 2001), and primitive endoderm (Dyer et al., 2001), its prominent expression in cartilage during endochondral bone formation suggested that it plays an important part in this process (Bitgood and McMahon, 1995). Several studies have since described Ihh

expression beginning at stage 12.5 dpc in the mouse (St-Jacques et al., 1999) and at stage 26/27 of the chick (Vortkamp et al., 1996), during which expression is restricted to the early cartilaginous condensations where it concentrates at the centers of proliferating chondrocytes. As the primordial cartilage elements begin to differentiate, initiating *Col10a1* expression at stage 12.5/13.5, Ihh is excluded from hypertrophic chondrocytes but remains in an adjacent zone that is later defined as prehypertrophic, separating proliferating from terminally differentiated chondrocytes. In parallel and during all phases of cartilage development, cells expressing Ihh are surrounded by cells expressing its receptors *Ptc-1* and *Smo*. Thus, in addition to being expressed by proliferating chondrocytes above the Ihh expression domain, they are also expressed by cells in the perichondrium/periosteum and the primary spongiosa, suggesting also a role for Ihh in regions of osteogenesis (Akiyama et al., 1999; St-Jacques et al., 1999; Vortkamp et al., 1996; Vortkamp et al., 1998).

That Ihh, in conjunction with PTHrP, plays a major role in coordinating differentiation and proliferation of growth plate chondrocytes first became evident from overexpression studies in the chick limb (Vortkamp et al., 1996). Misexpression of Ihh through a replication-competent retroviral vector (RCAS) in the limb of stage 22 chick embryo wing buds resulted in disruption of endochondral bone formation, most prominently illustrated by strong delay in the expression of hypertrophic markers (*Col10a1*) and in ossification. Furthermore, it was noted that PTHrP expression by cells in the perichondrium of the joint (periarticular cells) was enhanced following Ihh transduction, suggesting that PTHrP may be a transcriptional target of Ihh. That Ihh coordinates the delay in chondrocyte hypertrophy with PTHrP was further substantiated

by the fact that limb explants from PTHrP (-/-) mice that responded to exogenous PTHrP by downregulating *Col10a1*, remained unresponsive to exogenous Shh.Moreover, limb explants from PTH1R-null mice were unresponsive to both PTHrP and Shh indicating that HH signals through PTHrP which, through activation of its receptor, mediates a delay in chondrocytic hypertrophy (Lanske et al., 1996). Based on these seminal experiments, a model was proposed by which Ihh, expressed within the prehypertrophic zone of the growth plate, induces PTHrP expression in periarticular chondrocytes which in turn acts primarily on maturing, PTH1R-expressing, chondrocytes thereby preventing their final progression into hypertrophy.

The generation of Ihh-null mice provided even further information regarding the role of Ihh in endochondral bone formation, suggesting extensive functions in chondrocyte proliferation and differentiation, as well as in osteoblast differentiation (St-Jacques et al., 1999). These animals displayed a form of skeletal dysplasia even more severe than that observed in their PTHrP-null or PTH1R-null counterparts where chondrocyte proliferation was clearly reduced and this was accompanied by accelerated hypertrophic differentiation. Interestingly, during the developmental stages of 14.5 and 16.5 dpc *Col10a1*-expressing cells were inappropriately dispersed throughout the growth plate, overlapping with *Col2a1* expressing cells, and at stage 18.5 dpc these concentrated appositionally to the perichondrium of the cartilage element. Moreover, in agreement with Ihh lying upstream of PTHrP in the negative regulatory loop, absence of PTHrP expression in the articular perichondrium was observed although expression of PTH1R was maintained. However, the severity of the phenotype by comparison to that of the PTHrP(-/-) or PTH1R(-/-)

animals and the absence of Ptc-1 expression in chondrocytes from Ihh-null mice suggested that Ihh may elicit direct effects that are not mediated by PTHrP.

Evidence for PTHrP-independent functions of Ihh on chondrocytes came from the attempted rescue of the Ihh-null phenotype by the chondrocyte-specific overexpression of the constitutively active PTH1R (H223R) (Karp et al., 2000). Whereas constitutive activation of PTH1R within an Ihh-null background was sufficient for restoring the number of non-hypertrophic cells and delaying *Col10a1* expression, it had no apparent effect on the proliferative rate of these cells. In further support of a direct role for Ihh in chondrocyte proliferation came from the *Cre-loxP*-mediated ablation of *Smo* from growth plate chondrocytes resulted in a considerable reduction of their proliferative capacity while not affecting their differentiation program (Long et al., 2001). These results corroborate the hypothesis that Ihh enhances chondrocyte proliferation directly through interaction and activation of the hedgehog receptor complex, *Ptc-1/Smo*, while mediating inhibitory effects on differentiation through signaling to the perichondrium and indirectly activating PTHrP.

Understanding the mechanism by which Ihh, expressed in prehypertrophic cells, can transverse its signal across a multitude of cell diameters to activate PTHrP transcription in periarticular cells has been subject to ongoing debate. However, several studies have recently demonstrated that Ihh probably does not activate PTHrP directly but rather through a secondary signal emanating from the perichondrium. As overexpression of a constitutively active form of the BMP receptor 1A (BMPR1A) in the developing cartilage resulted in inhibition of hypertrophy, it was hypothesized that BMPs may be the secondary mediators in relaying signals from Ihh to PTHrP (Zou et al., 1997). However, arguing

against this epistatic relationship are findings showing that treatment of bone explants with Noggin, a potent antagonist of BMP signaling, had no effect on hypertrophic inhibition or PTHrP upregulation by Ihh (Minina et al., 2001). That BMP was incapable of rescuing perturbation in Ihh signaling following cyclopamine treatment, further indicated that these pathways may act in parallel, rather than interactively, to regulate different aspects of terminal chondrocyte differentiation.

Alternatively, increasing evidence has emerged implicating TGFβ as the intermediary factor downstream of Ihh and upstream of PTHrP in the regulation of chondrocyte differentiation. Preliminary findings demonstrated that expression of a dominant-negative form of the TGFβ type II receptor (DNIIR) in skeletal tissue of transgenic mice resulted in increased hypertrophic differentiation, suggesting a role for TGFβ in regulating terminal differentiation *in vivo* (Serra et al., 1997). Conversely, treatment of bone rudiments with TGFβ resulted in an opposite effect where hypertrophic differentiation was inhibited concomitantly with and upregulation of PTHrP expression (Serra et al., 1999). Since explants from PTHrP-null mice were unresponsive to TGFβ, this further suggested that PTHrP is required for TGFβ-mediated effects and is situated downstream in its signaling pathway. Finally, cultured bone explants treated with recombinant Shh, a functional substitute for Ihh, resulted in delayed chondrocytic hypertrophy and a strong increase in TGFβ2 expression in the perichondrium (Alvarez et al., 2002). That Ihh requires TGFβ2 for mediating its inhibitory effects was demonstrated by Shh treatment of rudiments retrovirally infected with DNIIR or those derived from *Tgfb2*-null mice; in both cases HH-mediated inhibition of *Col10a1* and upregulation of PTHrP was not observed, suggesting that Ihh lies upstream of TGFβ, and specifically TGFβ2, in this signaling cascade (Alvarez

et al., 2002). Thus, these findings illustrate how Ihh can indirectly mediate a delay in hypertrophic differentiation by stimulating TGFβ2 in the perichondrium, which then acts on the periarticular cells to stimulate PTHrP expression. This in turn, directly affects the progression of PTH1R-expressing prehypertrophic cells to their terminal differentiated state.

5.2 Ihh and Chondrocyte biology: Direct induction of Chondrogenesis

In contrast to the indirect inhibitory actions of Ihh on chondrocyte differentiation, other studies indicate that Ihh can directly promote this process. During somitic chondrogenesis, signals emanating from the notochord and floor plate induce the ventral regions of the adjacent somites to develop into the sclerotome, a specialized mesenchymal tissue that gives rise to the vertebrae and the medial part of the ribs. In this context Shh, expressed by the notochord and the floor plate cells of the neural tube, has been shown to be sufficient for chondrogenic potentiation of presomitic cells (Murtaugh et al., 1999). Functioning at this early stage of mesenchymal-chondrocytic conversion, Shh increases the responsiveness of subsequently developing sclerotomal cells to BMP; increased cellular response to BMP at this stage results in a chondrogenic-specific induction.

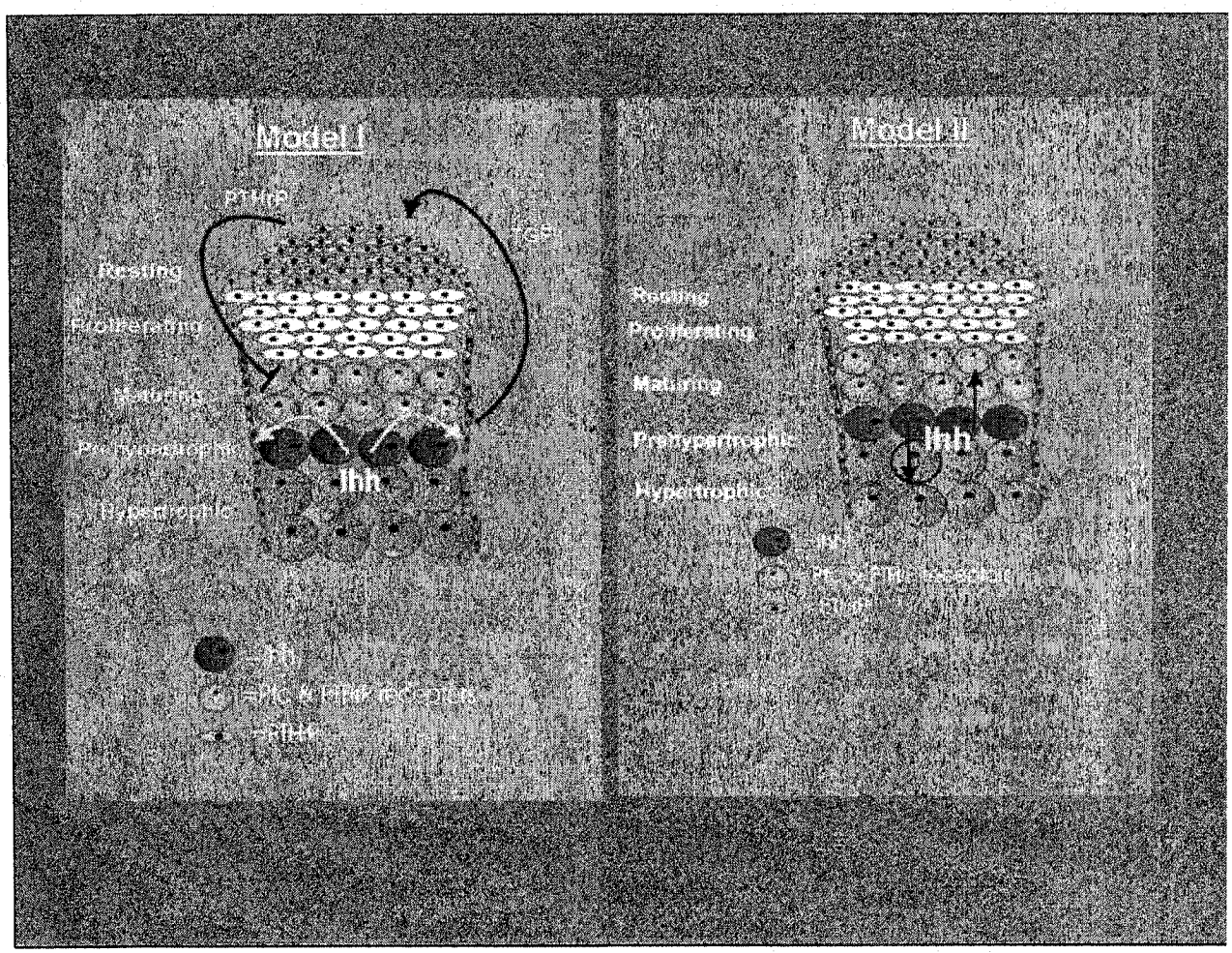
That hedgehog signaling can promote the chondrogenic program was further reiterated by studies on limb micromass cultures retrovirally transfected for Shh overexpression (Stott and Chuong, 1997). Unexpectedly, Shh overexpression induced cartilage-like nodule formation that was positive for ALP and *Col10a1*, suggesting that chondrocytic hypertrophy was promoted, rather than inhibited, by HH-signaling. Interestingly, PTHrP, although expressed diffusely in these cultures, was not induced by

Shh overexpression, indicating that HH-induced hypertrophy may be conditional and dependant on the absence of opposing PTHrP-mediated signals. In addition, studies employing the embryonic carcinoma cell line, ATDC5, have also implicated Ihh as a positive regulator in their differentiation toward the chondrogenic lineage. These cells can be induced to undergo chondrogenic differentiation through a temporal multiphase process culminating in cartilage nodule formation that is positive for *Col2a1*, *Col10a1* and *Pth1r* expression (Akiyama et al., 1997; Shukunami et al., 1996). Along this process, Ihh expression initiates during the middle phase of chondrogenic differentiation and slightly precedes the appearance of *Col10a1* (Akiyama et al., 1997). Furthermore, treatment of ATDC5 cells with exogenous Shh peptide resulted in enhanced hypertrophic differentiation, as evidenced by stimulated *Col10a1* expression and sulfated proteoglycan synthesis (Akiyama et al., 1999; Enomoto-Iwamoto et al., 2000).

In addition to evidence indicating that Ihh promotes chondrocytic differentiation *in vitro*, other findings suggest that this may also occur *in vivo*. Thus, a sequential appearance of ectopic cartilage and bone was observed following intramuscular transplantation of fibroblasts retrovirally overexpressing Shh or Ihh in nude mice (Enomoto-Iwamoto et al., 2000). That this ectopic endochondral bone was initiated after a decline in Ihh expression by the transplanted cells, suggested that Ihh acted at an earlier stage to induce host mesenchymal cells to undergo chondrogenic and osteogenic differentiation. However, the most compelling evidence for a chondrogenic inductive effect by Ihh is the observation that initiation of hypertrophic differentiation, concomitant with *Col10a1* expression, at stage 14.5 is clearly delayed in Ihh-null mice (Long et al., 2001; St-Jacques et al., 1999). Nonetheless, it remains unclear how Ihh may mediate this

early phase of differentiation, as chondrocyte-specific *Smo*-ablated animals do not display this phenotype (Long et al., 2001). Thus, the mechanisms by which Ihh enhances chondrocytic differentiation and whether these occur *in vivo* remain to be further studied and are central to the aims of the present study.

Figure 6. Proposed models for the coordinated regulation of growth plate chondrocyte differentiation by Ihh and PTHrP


Model I
PTHrP
Resting
Proliferating
Maturing
Prehypertrophic
Hypertrophic
Ihh
Model II
Resting
Proliferating
Maturing
Prehypertrophic
Hypertrophic
Ihh

Rational and specific aims

The objective of the present study was to further the understanding of mechanisms governing chondrocytic differentiation and how Ihh, a key mediator in this process, exerts its actions through interplay with other regulatory pathways. The four specific aims were:

1. To assess the effects of Ihh signaling on chondrocytic differentiation and to specifically define, through the use of an *in vitro* system, its capacity to either promote or delay chondrocyte differentiation.

2. To evaluate the possible interplay between the Ihh and PTHrP signaling pathways and to develop a mechanistic basis by which these two developmental factors coordinate chondrocytic differentiation.

3. To examine a possible role for En-1 in cartilage development and to assess its capacity to modulate Hh-signaling in chondrocytic cells.

4. To determine if heparan sulfate glycosaminoglycans play a role in mediating Hh-signals and how they influence chondrocytic differentiation.

Chapter 2

Ihh enhances differentiation of CFK-2 chondrocytic cells and antagonizes PTHrP-mediated activation of PKA

Abstract

Indian Hedgehog (Ihh), a member of the hedgehog (HH) family of secreted morphogens, and parathyroid hormone-related peptide (PTHrP) are key regulators of cartilage cell (chondrocyte) differentiation. We have investigated, *in vitro*, the actions of HH signaling and its possible interplay with PTHrP using rat CFK-2 chondrocytic cells. Markers of chondrocyte differentiation [alkaline phosphatase (ALP) activity, and type II (*Col2a1*) and type X collagen (*Col10a1*) expression] were enhanced by overexpression of Ihh or its NH_2-terminal domain (N-Ihh), effects mimicked by exogenous administration of recombinant NH_2-terminal HH peptide. Moreover, a missense mutation mapping to the NH_2-terminal domain of Ihh (W160G) reduces the capacity of N-Ihh to induce differentiation. Prolonged exposure of CFK-2 cells to exogenous N-Shh (5×10^{-9} M) in the presence of PTHrP (10^{-8} M) or forskolin (10^{-7} M) resulted in perturbation of HH-mediated differentiation. In addition, overexpression of a constitutively active form of the PTHrP receptor (PTH1R H223R) inhibited Ihh-mediated differentiation, implicating activation of protein kinase A (PKA) by PTH1R as a probable mediator of PTHrP's antagonistic effects. Conversely, overexpression of Ihh/N-Ihh or exogenous treatment with N-Shh led to dampening of PTHrP-mediated activation of PKA. Taken together, our data suggest that Ihh harbors the capacity to induce rather than inhibit chondrogenic differentiation, that PTHrP antagonizes HH-mediated differentiation through a PKA-dependent mechanism and that HH signaling, in turn, modulates PTHrP action through functional inhibition of signaling by PTH1R to PKA.

Introduction

Endochondral ossification, a complex process responsible for the formation of the appendicular and axial skeleton in vertebrates, begins with the condensation of mesenchymal cells, which subsequently differentiate into chondrocytes. These then follow a sequential program of differentiation where temporal and spatial mechanisms are involved in regulating their progression from a proliferative to a terminally differentiated, non-proliferative, or hypertrophic state. This process, restricted to sections of the bone's extremities known as the *epiphyseal cartilage* or the growth plate, is responsible for its longitudinal growth. The epiphysis is generally divided into zones of resting, proliferating, and hypertrophic chondrocytes that are defined according to proliferative capacity and extracellular matrix protein expression. Exemplifying this is the transition from *Col2a1* to *Col10a1* expression observed when proliferating chondrocytes progress to their hypertrophic stage.

The Hedgehog (HH) proteins, a family of secreted morphogens, have been implicated in a multitude of developmental processes (Nusslein-Volhard et al., 1980; Riddle et. al, 1993; Roberts et al., 1995; Bitgood et al., 1996). All known HH members are proteolytically processed through an autocatalytic mechanism to generate secreted peptides corresponding to the NH_2- and COOH-terminal domains of the native protein (Porter et al., 1996A; Lee et al., 1994; Bumcrot et al., 1995; Valentini et al., 1997). The COOH-terminal domain is believed to possess the catalytic properties required for HH cleavage, which occurs at a conserved Gly-Cys site. Cholesterol, participating as a nucleophile in the autocatalytic process, attaches to the COOH-terminal end of the nascent NH_2-terminal domain and enhances its lipophilic properties (Porter et al., 1996A; Porter et al., 1996B). The NH_2-terminal domain is believed to possess all the known biological activities of HH proteins and

is highly conserved and interchangeable amongst HH family members (Vortkamp et al., 1996). This domain can bind its cognate receptor Patched (Ptc), a 12-transmembrane (TM) protein that otherwise interacts with, and thereby inhibits, the 7-TM receptor protein Smoothened (Smo) (Stone et al., 1996; Carpenter et al., 1998). The ligand-induced release of Smo from its interaction with Ptc results in intracellular signal transduction.

Indian hedgehog (Ihh) has been shown to be a key regulator of chondrocyte differentiation. In addition to its expression in kidney, gut and osteoblasts (Valentini et al., 1997; Bitgood and McMahon, 1995), its restricted expression by a discrete layer of chondrocytes in the early hypertrophic zone of the epiphyseal cartilage has suggested a role for Ihh in directing these cells to their final differentiated state (Vortkamp et al., 1996). Retroviral-mediated overexpression of Ihh in chick limbs resulted in inhibition of chondrocyte differentiation, as exhibited by reduced type X collagen (*Col10a1*) expression (Vortkamp et al., 1996). Subsequently, it was proposed that the effects of Ihh on chondrocyte differentiation are indirect and occur via parathyroid hormone-related peptide (PTHrP), a potent inhibitor of chondrocyte differentiation, expressed in the resting zone cartilage (periarticular layer) (Karaplis et al., 1994). Treatment of bone explants from wild-type mice with Sonic Hedgehog (Shh) protein mimicked the ability of PTHrP to inhibit *Col10a1* expression (Vortkamp et al., 1996; Lanske et al., 1996). These findings, and the observation that HH protein did not affect *Col10a1* expression in bone explants from PTHrP-null mice, led to the postulate that PTHrP may act as a downstream mediator of Ihh action. The proposed model suggests that Ihh acts, in a paracrine fashion on cells of the perichondrium, to indirectly increase PTHrP expression in the periarticular cartilage; PTHrP

in turn, via activation of the PTHrP receptor (PTH1R), would then inhibit differentiation in the growth plate.

Several lines of evidence, however, have indicated that Ihh may also have direct effects on chondrocyte differentiation. Consistent with the general observation that *Ptc* expression is upregulated in response to HH signaling (Hooper and Scott, 1989; Phillips et al., 1990; Tabata and Kornberg, 1994; Goodrich et al., 1996), *Ptc* transcripts have been reported in epiphysial chondrocytes adjacent to the Ihh expression domain (Vortkamp et al., 1998). Furthermore, similar patterns of expression were described for *Smo* and *Gli1*, a member of the Gli family of transcription factors that mediates gene expression in response to HH (Vortkamp et al., 1998; Akiyama et al., 1999). Expression of these genes by growth plate chondrocytes suggests that these cells may be directly responsive to Ihh.

Moreover, a number of *in vitro* studies have indicated that the NH_2-terminal domain of HH proteins can promote chondrogenesis. Thus, retrovirally overexpressed Shh in limb bud micromass cultures resulted in induction of cartilaginous nodules that were strongly positive for *Col10a1* expression and alkaline phosphatase (ALP) activity in the absence of PTHrP upregulation (Stott and Chuong, 1997). Additionally, recombinant NH_2-terminal-Ihh induced *Col10a1* and *Ptc* expression in ATDC5 embryonic carcinoma cells undergoing chondrocytic differentiation, suggesting that this domain of Ihh harbors the ability to induce hypertrophy (Akiyama et al., 1999).

In this study, we have utilized the rat CFK-2 chondrocytic cell line to investigate the role of Ihh and its interplay with the PTHrP signaling pathway in chondrocyte differentiation. We present evidence indicating that Ihh or its NH_2-terminal domain harbors the capacity to induce chondrogenic differentiation, an effect mimicked by

recombinant NH_2-terminal HH protein. We also show that PTHrP, through a protein kinase A (PKA)-dependant mechanism, inhibits Ihh-mediated differentiation and that Ihh in turn impedes PKA stimulation by PTHrP.

Materials and methods

Construction of mammalian expression plasmids- the full-length *Ihh* cDNA (2103 bp) inserted in the pcDNA3 mammalian expression vector (Ihh-pcDNA3) was obtained as a gift from L. Holzman (University of Michigan). A *myc*-epitope tag was inserted at the carboxyl terminus of Ihh by PCR using oligonucleotides: 5'-[CGCATGTGCTTTCCTGCCGGAGCCCAGG]-3' as the sense primer and 5'-[CCTTCAGTTGTTCAGGTCCTCTTCGCTAATCAGCTTTTGTTCCATAGAGCTTCCT GCCCCAG]-3' as the antisense primer. The 676-bp fragment corresponding to the COOH-terminal end of Ihh was cloned into a TA cloning vector (pCR3.1, Invitrogen) and subsequently into the pcDNA3 mammalian expression vector. Generation of Ihh-*myc* was finalized by ligating the 911-bp *Bam*HI fragment from the Ihh-pcDNA3 to the previously cloned *myc*-tagged fragment. To express the amino terminal domain of Ihh (N-Ihh), a 720-bp fragment corresponding to this region of the protein was amplified using 5'[GCCCCCGCATGGAAGTCCCC]-3' as the sense primer and 5'-[TCAGCCACCTGTCTTGGCAGC]-3' as the antisense primer. This fragment was cloned into pcDNA3 for mammalian cell expression.

A PCR-based method for site directed mutagenesis (QuikChange, Stratagene) was used to generate the W160G mutation where the following primers altered nucleotide 661 of Ihh from a T to a G nucleotide (resulting in a tryptophan to glycine substitution): 5'-[CTCTGTCATGAACCAGGGGCCCGGTGTG]-3' as the sense primer and 5'-[CAGTTTCACACCGGGCCCCTGGTTCATGACAGAG]-3' as the antisense primer.

The cDNA encoding the constitutively active *PTH1R* (PTH1R H223R) was generously provided by H. Juppner (Massachusetts General Hospital and Harvard Medical School,

Boston, MA). This was subsequently subcloned into the pcDNA3.1/Zeo mammalian expression vector (Invitrogen). The cDNA encoding for full-length rat PTHrP was also subcloned into pcDNA3.1/Zeo.

Cell culture and transfections- CFK-2 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco BRL) supplemented with 10% fetal bovine serum (Wisent). To induce differentiation, cells were grown to confluence in 10% FBS after which serum was gradually reduced by 2% decrements every two days, and cells were maintained at 2% FBS thereafter until 16 days of post confluent growth. For some experiments medium was supplemented with 1×10^{-7} M forskolin, 1×10^{-8} M human PTHrP (1-34) or varying concentrations of recombinant N-Shh (Curis, Inc., Cambridge, MA). N-Shh was modified by the addition of a hydrophobic eight-carbon chain (octyl group) to the NH2-terminal cysteine and this is reported by the supplier to increase the biological activity of the native peptide by up to 10 fold. N-Shh protein was suspended in "Octyl" buffer (PBS pH 7.2, 50 μM DTT conjugated to N-octylmaleimide, 350 μM free DTT and 0.5% DMSO) and this buffer was also used as vehicle control, where indicated.

Generation of stably transfected CFK-2 cell populations was performed by electroporation. Cells were grown on 10 cm dishes and upon reaching 60% confluence were washed once in PBS and trypsinized. Cells were then suspended in HBS (20 mM HEPES pH 7.4, 0.14 M NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 25 mM glucose, 1 mM $CaCl_2$) at a density of 5×10^5 cells/ml, supplemented with plasmid DNA (2 μg), and electroporated at 240V/ 500μF. Following 48 hours of recovery, cells were subjected to selection by the addition of G418 at a final concentration of 500 μg/ml. Selection proceeded for ten days

and subsequently cells were maintained in G418 (500 μg/ml). Coexpression of PTHrP or PTH1R H223R with Ihh was achieved by subjecting stable populations of Ihh-transfected CFK-2 cells to a secondary transfection with PTHrP-pcDNA3.1/Zeo or PTH1R H223R-pcDNA3.1/Zeo. These were selected by growth in media containing 300 μg/ml of zeocin and 500 μg/ml of G418.

Transient transfection of COS-1 cells was performed by the calcium phosphate precipitation method. Briefly, DNA (2 μg/7×10^5 cells) was added to a solution of 2.5 M $CaCl_2$ and precipitated by the addition of HeBs solution (0.28 M NaCl, 0.05 M HEPES pH 7.05, 1.5 mM Na_2HPO_4). Precipitated DNA was layered onto cells and incubated for an 8-hour period. Cells were then washed 5 times in PBS and medium replaced for a recovery period of 48 hours. Alternatively, cells were transfected using Fugene™ 6 (Roche) according to the manufacturer's specifications.

Northern blot analysis- Total RNA was obtained from cell monolayers by guanidium isothiocyanate (GTC)/CsCl extraction. Briefly, cells were washed once with PBS and homogenized with 0.5 ml of GTC (4 M guanidium isothiocyanate, 0.1 M Tris-HCl pH 7.5, 1% β-mercaptoethanol) and passaged through a 25-gauge needle. Homogenates were layered on top of a 5.7 M CsCl / 0.01 M EDTA cushion and ultracentrifuged overnight at 32000 rpm in a SW40 rotor. Pellets were washed with 70% ethanol and suspended in DEPC-treated water. Alternatively, RNA was extracted by Trizol, as specified by the manufacturer (Gibco BRL).

Typically, aliquots (20 μg) of total RNA were size fractionated on a 1.5% agarose/formaldehyde gel and transferred overnight onto a supported nitrocellulose

membrane using 20 X SSC buffer (3 M NaCl, 0.003 M Na citrate, pH 7.0). Hybridization of membranes to ^{32}P-labeled probes was performed in a buffer containing 40% formamide, 10% dextran SO_4, 4 X SSC, and 1X Denhardt's blocking solution with 0.1 mg/ml salmon sperm DNA. Membranes were washed once in 2 X SSC/0.1% SDS at room temperature and once in 0.1 X SSC/0.1% SDS for 15 minutes at 58°C before exposing to film.

All probes were radiolabeled by the random priming method (Roche). Probes corresponding to the NH_2 and COOH terminus of Ihh were generated by PCR using the primers described above. A 390-bp probe verified by DNA sequencing corresponding to *Ptc* cDNA was generated by RT-PCR from total RNA extracted from CFK-2 cells. The oligonucleotides used to obtain this fragment were 5'-[GGACTTCCAGGATGCCATTTGACAGTG]-3' as the sense primer and 5'-[GCCGTTGAGGTAGAAAGGGAACTG]-3' as the antisense primer and were based on the mouse *Ptc* cDNA sequence. The cDNA probe for rat *Pth1r* was generously provided by H. Juppner (Endocrine Unit, Massachusetts General Hospital, Boston, MA); the cDNA probe for rat *Col2a1* was a gift from Y. Yamada (National Institute of Dental Research, Bathesda, MD); the probe for mouse *Col10a1* was kindly provided by K. Lee (Massachusetts General Hospital, Boston, MA). Northern blots were assessed quantitatively by videodensitometric analysis (Scion Image).

Western blotting- For western blot analysis of proteins, transiently transfected COS-1 cells were lysed 48 hours following transfection by scraping monolayers into 300 µl of ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na orthovanadate, 10 mM Na glycerophosphate, 50 mM NaF, 1% (v/v) Triton X-100, 0.1%

β-mercaptoethanol, 10 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin). Upon one freeze-thaw cycle at -70°C, debris was removed by centrifugation of lysates for 10 min at 12000×g / 4°C. Approximately 25 μl of cleared lysates were analyzed by SDS-PAGE. For preparation of protein from conditioned media, medium was centrifuged briefly to remove cellular debris, and proteins were precipitated by the addition of 5 volumes of acetone and incubation on ice for 1 hr. Centrifugation at 10000 × g for 15 min generated a pellet that was suspended in 100 μl of lysis buffer of which 20 μl aliquots were used for analysis.

Protein samples were loaded onto a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were blocked in TBST buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20) and 5% milk for one hour, and then incubated with primary antibodies that were diluted in TBST/0.5% milk [1:300 for α-N-Ihh (Santa Cruz, CA), 1:500 for α-myc] for 1 hr. Secondary antibodies [horseradish peroxidase-conjugated anti-goat IgG or anti-mouse IgG (Santa Cruz and Sigma, respectively), 1:2000 with TBST/0.5% milk] were incubated with membranes for 1 hr and bands were visualized with the BM chemiluminescence blotting substrate (Roche), according to manufacturer's instructions.

ALP activity assay- Cell monolayers were washed with PBS, lysed in 300 μl ALP lysis buffer (0.15 M Tris pH 9.0, 0.1 mM $ZnCl_2$, 0.1 mM $MgCl_2$), and subjected to one freeze-thaw cycle at -70°C. Lysates were cleared by a 10-min centrifugation at 10000 × g and 50 μl aliquots were analyzed spectrophotometrically at 410 nm with ALP assay solution (7.5 mM p-nitrophenyl phosphate (Sigma reagent 104), 1.5 M Tris pH 9.0, 1 mM $ZnCl_2$, 1 mM

$MgCl_2$). Protein concentrations were determined by the method of Lowry using the Bio-Rad DC protein assay kit (Bio-Rad).

PKA assay- CFK-2 cells were grown to 3 days post-confluence in the presence of 10% FBS, 500 μg/ml G418, 50 μg/ml ascorbate and 10 mM β-glycerophosphate. Following serum starvation for 24 hours, cells were then stimulated for 20 min in the presence of 10^{-8} M PTHrP 1-34 and 300 μM isobutylmethylxanthine (IBMX) or with IBMX alone. Cells were rinsed with PBS, placed on ice and lysed in PKA assay buffer (25 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.5% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.5 mM PMSF). Measurement of PKA activity in cell lysates was performed using a PKA assay kit (Upstate Biotech.). Briefly, reactions were performed in the presence of 5 μl of lysate, 83 μM kemptide substrate, 0.33 μM PKC inhibitor peptide, 3.33 μM CaMK inhibitor (R24571) and 83.3 μM ATP/ 16.67 μCi [γ-^{32}P] ATP. To demonstrate specificity for phosphorylation by PKA, reactions were also performed in the presence of both kemptide substrate and 1 μM of PKA inhibitor peptide. After an incubation of 10 min at 30°C, 15 μl samples were blotted onto phosphocellulose P81 paper, washed 4 times in 0.75% phosphoric acid, once in acetone, and radioactivity was measured in a scintillation counter. Values were normalized to protein concentrations of the lysates and to specific radioactivity (cpm/pmol) of the reaction mix.

Statistics - Statistical analysis of results was performed using one-way analysis of variance (ANOVA). Sample values were determined to be significantly different between groups when P values were ≤ 0.05.

Results

Expression of Ihh and its variants in COS-1 and CFK-2 cells

We first assessed the expression of recombinant Ihh and its NH_2-terminal domain in a mammalian cell system using COS-1 cells. Constructs derived from the pcDNA3 expression vector designed to express Ihh or N-Ihh (Fig. 1A), were used to transiently transfect COS-1 cells and western blot analysis was subsequently performed on lysate and conditioned medium samples from transfected cells. To facilitate the detection of Ihh and its processed forms, an antibody recognizing the NH_2-terminus of Ihh (α-Ihh-N) was utilized. In addition, we added a *myc*-epitope tag to the COOH-terminus of Ihh (generating Ihh-*myc*-pcDNA3) to enable detection of this domain subsequent to autoproteolysis (Fig. 1A). As observed in lysates from COS-1 cells transfected with Ihh-pcDNA3 or Ihh-*myc*-pcDNA3, two peptide species (45-kD and 19-kD) that were immunoreactive to α-Ihh-N were present, corresponding to the full-length and NH_2-terminal domain of Ihh, respectively (Fig. 1B, lanes 2 and 3). Lysates of cells expressing N-Ihh displayed a peptide that was immunoreactive to the same antibody and co-migrated with the 19-kD fragment generated from the native Ihh protein (Fig. 1B, lane 4). This peptide was secreted into the conditioned medium of cells transfected with N-Ihh-pcDNA3 but not of those transfected with Ihh-pcDNA3 or Ihh-*myc*-pcDNA3 (Fig. 1B, lanes 6-8). In addition, an antibody reactive to the *myc*-epitope detected a 26-kD protein corresponding to the COOH-terminal domain of Ihh (C-Ihh) (Fig. 1B, lane 10), in agreement with previous observations using other *hedgehog* family members, that the carboxyl-terminal domain is readily secreted into the conditioned medium of transfected cells (Fig. 1B, lane 12). These results are consistent with the capacity of Ihh to undergo

autocatalytic processing and generate a secreted COOH-terminal domain and a less diffusible NH_2-terminal peptide.

Next, an N-Ihh variant harboring a single base pair substitution that alters a tryptophan to a glycine residue at position 160 of the Ihh peptide was constructed (N-Ihh W160G; Fig. A). This mutation was first described in human Shh in patients afflicted with the autosomal dominant form of holoprosencephaly (Roessler et al., 1996). It was therefore deduced that this residue, conserved in all mammalian HH proteins, may be critical for the protein's bioactivity and that its alteration results in loss of function. Expression of N-Ihh W160G in transfected COS-1 cells was comparable to that of its wild type counterpart, as observed from western blot analysis, indicative of appropriate translation and maintenance of immunoreactivity to α-Ihh-N (Fig. 1C).

Ihh and N-Ihh induce *Ptc* expression in CFK-2 cells

We then utilized an *in vitro* system, the CFK-2 chondrocytic cell line, to examine the actions of Ihh on chondrocyte biology. CFK-2 cells have been shown previously to undergo progressive differentiation manifested by expression of chondrocytic markers such as type II collagen (*Col2a1*), type X collagen (*Col10a1*), link protein, and *Pth1r* (Bernier et al., 1990; Bernier and Goltzman, 1993; Henderson et al., 1996; Wang et al., 2001). To examine the effects of Ihh on CFK-2 differentiation, we generated stably transfected populations of these cells expressing Ihh, N-Ihh or N-Ihh W160G (Fig. 1A). As control, CFK-2 cell populations transfected with pcDNA3 vector were also generated. Stably transfected CFK-2 cells were then subjected to a 16-day postconfluent culture period with gradual serum withdrawal (as

described in *Material and Methods*) during which samples of total cellular RNA were obtained intermittently.

To verify the status of HH-signaling activity, the expression of *Ptc*-receptor was examined in stably transfected CFK-2 cells that underwent postconfluent culture (Fig. 2A). Since *Ptc* has been described as a downstream target gene of HH-signaling whose levels are presumably dependant on the amount of HH ligand present (Hooper and Scott, 1989; Phillips et al., 1990; Tabata and Kornberg, 1994; Goodrich et al., 1996), we also examined the expression levels of the transgene in each cell population using a probe encoding the NH_2-terminal domain of Ihh. Whereas pcDNA3 transfected cells expressed low constitutive levels of *Ptc*, Ihh- and N-Ihh-transfectants demonstrated a robust elevation in its expression levels (Fig. 2A, B, C). In comparison to pcDNA3-transfected cells, N-Ihh W160G was capable of inducing *Ptc* expression, albeit to a lesser degree than its wild type counterpart, suggesting that the W160G mutation only partially impedes the activity of Ihh (Fig. 2B). In addition, we found that the relative *Ptc* expression (*Ptc*/transgene/*GAPDH*) was higher in Ihh-transfected cells than in N-Ihh-transfectants, suggesting that the native molecule may harbour more potent biological activity (Fig. 2C).

Since the NH_2-terminal domains of HH proteins have been shown to be interchangeable with respect to biological action (Vortkamp et al., 1996), we further verified our findings by incubating naive CFK-2 cells with increasing concentrations of recombinant NH_2-terminal Shh peptide (N-Shh) in the culture medium. Induced *Ptc* expression was initially detected in response to N-Shh concentrations as low as 10^{-10} M and reached maximal levels at 10^{-8} M of the peptide (Fig. 2B). These results verified that N-Shh, similarly

to Ihh, induces *Ptc* expression in CFK-2 cells and that this is dependant on ligand concentration.

Ihh and N-Ihh induce chondrogenic differentiation in CFK-2 cells

Since Ihh expression has been localized *in situ* predominantly to pre-hypertrophic and hypertrophic growth plate chondrocytes, we assessed whether CFK-2 cells could differentiate under the influence of Ihh overexpression or in response to exogenous N-Shh. Differentiation of CFK-2 cells was assessed initially by measuring changes in ALP activity over a 16-day postconfluent culture period (Fig. 3A; left panel). Whereas control pcDNA3 transfected cells displayed low levels of ALP activity, Ihh- and to a lesser extent N-Ihh-expressing cells displayed increasing levels of ALP over time. In a separate experiment, ALP induction was also compared between N-Ihh and its mutant variant, N-Ihh W160G (Fig. 3A; right panel). Whereas N-Ihh induced ALP activity in CFK-2 cells subjected to an 8-day postconfluent period, N-Ihh W160G failed to do so. Similarly, Ihh and N-Ihh, but not N-Ihh W160G, induced the expression of the chondrogenic marker *Col2a1* whose levels increased during postconfluent culture (Fig. 3B). We then examined *Col10a1* expression as a definitive marker of the hypertrophic stage of differentiation. Whereas *Col10a1* expression was detected at low levels in control pcDNA3 transfectants, Ihh and N-Ihh induced *Col10a1* to a much greater extent (Fig. 3B). Surprisingly, cells transfected with N-Ihh W160G exhibited similar *Col10a1* levels to those observed in Ihh or N-Ihh transfectants. This suggests that N-Ihh W160G may be capable of transmitting sufficient levels of HH-signaling to selectively induce certain markers of differentiation (*Col10a1*) but not others (ALP, *Col2a1*) (Fig. 3B).

To examine the effects of recombinant N-Shh on chondrocytic differentiation, CFK-2 cells were subjected to increasing concentrations of this peptide over a postconfluent culture period of 8 days. Dose-dependant increases in ALP activity and *Col2a1* and *Col10a1* expression were observed, that were maximal in response to 10^{-8} M N-Shh. Taken together, these observations suggest that Ihh and N-Ihh can induce CFK-2 cells to attain chondrogenic properties exemplified by elevated ALP activity, *Col2a1* and *Col10a1* expression, and that this effect is mimicked by recombinant N-Shh.

PTHrP antagonizes HH-mediated chondrogenic differentiation through a PKA-dependant pathway

In bone cells, PTHrP mediates most of its biological actions through activation of its cognate G-protein coupled receptor, PTH1R, leading to stimulation of adenylate cyclase and consequent PKA activation (Shigeno et al., 1988; Capehart and Biddulph, 1991). PKA has been widely described as an inhibitor of HH-signaling in multiple systems (Hammerschmidt et al., 1996; Chen et al., 1999; Wang et al., 1999). We therefore examined the effects of induced PKA activity on HH-mediated differentiation in CFK-2 cells. Naive CFK-2 cells were subjected to treatment with N-Shh (5×10^{-9} M) in the presence or absence of PTHrP (1×10^{-8} M) or forskolin (10^{-6} M), a potent activator of adenylate cyclase, and their differentiation state was assessed after an 8-day culture period. Whereas N-Shh treatment promoted high ALP activity in CFK-2 cells, this was strongly impeded by co-treatment of the cells with PTHrP (Fig. 4A). Similarly, PTHrP led to severe dampening of HH-induced *Col2a1* and *Col10a1* mRNA expression (Fig. 4B). In contrast, PTHrP had no effect on N-Shh-induced *Ptc* expression. Moreover, all

the antagonistic effects exerted by PTHrP on HH-action were also mimicked by forskolin suggesting that this phenomenon may be attributed to PKA activation (Fig 4B).

To test this hypothesis further, we generated stably transfected CFK-2 cell populations expressing Ihh alone or in combination with PTHrP or PTH1R (H223R), a mutant variant of PTH1R known to selectively and constitutively activate the PKA pathway (Schipani and Juppner, 1995). Upon examination of these cells during postconfluent growth, it was observed that Ihh induced ALP enzymatic activity, but that this was strongly perturbed by co-expressing Ihh in conjunction with PTHrP or PTH1R (H223R) (Fig. 4C). These findings, showing that PTHrP action and constitutive activation of adenylate cyclase through PTH1R similarly abrogate Ihh function, implicate PKA as the mediator of PTHrP-mediated antagonism of signaling of HH-signaling.

Ihh dampens PTHrP responsiveness of CFK-2 cells

In the growth plate, PTH1R expression is observed in proliferating chondrocytes but is strongest in the prehypertrophic layer adjacent to and overlapping with Ihh-expressing cells (Valentini et al. 1997). One possibility for modulation of HH action by PTHrP may therefore be through differential PTH1R expression. To examine this possibility, northern blot analysis for *Pth1r* mRNA expression was performed in CFK-2 cells treated with PTHrP (10^{-8} M) or N-Shh (5×10^{-9} M) for 7 days during postconfluent growth and compared with cells that were treated with vehicle alone. In contrast to vehicle-treated cells, N-Shh treatment resulted in strong upregulation of *Pth1r* mRNA expression, whereas PTHrP treatment alone had no effect (Fig. 5A, left panel). Furthermore, upregulation of *Pth1r* expression was antagonized by concomitant treatment of cells with

PTHrP or forskolin, indicative of an HH-specific effect that can be antagonized by PTHrP-mediated activation of PKA (Fig. 5A, right panel).

To examine whether increased *Pth1r* mRNA expression also results in amplified PTHrP responsiveness, we measured PKA activity directly in CFK-2 cells following transient treatment with PTHrP (1-34). As HH-mediated chondrocytic differentiation can be modulated by PKA activation, measuring its activity serves as an assessment of the cells' responsiveness to HH-inhibitory signals. Examination of PKA activity in pcDNA3-transfected CFK-2 cells that underwent 2 days of post confluent growth and were then treated with PTHrP (IBMX + PTHrP) showed a nearly ten-fold increase in activity over cells treated with IBMX alone (Fig. 5B, left panel). In contrast, an approximate 50% reduction in PTHrP-mediated PKA activity was consistently apparent in cells transfected with either Ihh or N-Ihh. As control, both wild type and transfected cells showed similar PKA responses to forskolin (data not shown). Furthermore, western blot analysis for the catalytic subunit isoforms of PKA showed no significant difference between treatment groups (Fig. 5B, right panel), suggesting that reduced PTHrP stimulation of PKA likely did not arise from differences in PKA protein expression levels. To further confirm these results, naive CFK-2 cells were treated with vehicle or increasing concentrations of N-Shh for 7 days after which PKA activity was measured upon transient treatment with PTHrP. In agreement with the previous experiment, HH-treated cells displayed a significantly dampened response to PTHrP that was apparent even at the lowest concentration of N-Shh (Fig. 5C). This implies that HH may negatively regulate signaling through PTH1R ultimately leading to impeded PKA activity.

Discussion

In this study, the biological effects of HH-signaling on chondrocytic differentiation were examined by employing, as an *in vitro* model, rat CFK-2 chondrocytic cells. These cells were previously shown to undergo a progressive program of differentiation characterized by temporal increases in ALP activity, *Col2a1*, and *Col10a1* expression (Wang et al., 2001). We demonstrate that Ihh and its NH_2-terminal domain specifically enhance chondrogenic differentiation, as typified by upregulation in the expression of these molecular markers, effects that are mimicked by administration of recombinant amino terminal HH protein. Moreover, the data presented have led us to propose that the PTHrP and HH-signaling pathways interact directly to modulate the rate of chondrocytic differentiation. Thus, chondrogenic genes activated by HH-signaling are downregulated by exogenously administered PTHrP, an effect mimicked by forskolin. Furthermore, we show that HH-signaling leads to upregulation of *Pth1r* mRNA expression while paradoxically diminishing responsiveness to PTHrP.

Our initial monitoring of HH action in CFK-2 cells was performed by examination of *Ptc* mRNA expression, a transcriptional target of HH-signaling (Goodrich et al., 1996). Preceding the activation of chondrogenic markers, a robust upregulation of *Ptc*-receptor expression was observed in both Ihh and N-Ihh transfected cells, indicative of strong HH-responsiveness in CFK-2 cells. Consistently, recombinant N-Shh also induced *Ptc* expression in a dose-dependent fashion. Whereas Ihh/N-Ihh transfectants showed little variation in *Ptc* expression over the period of culture, it was noted that Ihh was a stronger inducer of *Ptc* than N-Ihh when transgene expression levels were considered (Fig. 2). This difference may be attributed to the cholesterol moiety present on the NH_2-terminal peptide

generated from the wild-type form of Ihh, inherently absent from its recombinant truncated counterpart. Indeed, other observations have indicated that lipid-modified forms of NH_2-terminal Shh have increased potency in ALP activation in pluripotent mesenchymal C3H10T1/2 cells, despite unaltered receptor binding capacity (Pepinsky et al., 1998). The existence of sterol sensing domains (SSD) in both the hedgehog receptor *Ptc* and in the *hedgehog*-releasing protein, *Dispatched*, would also suggest an important biological role for cholesterol modification of the NH_2-terminal domain (Burke et al., 1999).

The inherent capacity of the NH_2-terminal domain of Ihh to mediate chondrogenic differentiation in CFK-2 cells is further confirmed by the ability of a specific missense mutation introduced in this protein domain to partially abrogate this function. The W160G mutation has been described in an individual case of holoprosencephaly ascribing a presumptive loss of function to Shh, although this finding was not confirmed *in vitro* (Roessler et al., 1996). The corresponding residue in mouse *Shh*, Trp^{117}, was shown to localize adjacent to the first α-helix of the peptide (Hall et al., 1995). Since at least one residue (Asp^{115}) residing within this α-helix was shown to be involved in *Ptc* binding and activation of HH-signaling (Pepinsky et al., 2000), it is likely that Trp^{117}/Trp^{160} may also be crucial for this function. Here, we report that the W160G mutation reduces the capacity of NH_2-teminal Ihh to stimulate *Ptc* expression, indicative of a partial loss in HH-signaling activity. That N-Ihh W160G is unable to induce ALP activity and *Col2a1* expression, but yet capable of *Col10a1* induction, further corroborates the observation that this protein may act as a partial agonist of HH-signaling. Moreover, in comparison to other mutations mapping to the NH_2-terminal of Shh, mutations at Trp^{117} are associated with a milder form

of holoprosencephaly and this may be attributed to the partial signaling capacity of this variant (Roessler et al., 1996).

In this study we show that HH-signaling can promote chondrogenic differentiation. In contrast, previous *in vivo* studies have indicated that Ihh promotes chondrocyte proliferation, in part through a PTHrP-independent mechanism, while mediating PTHrP-dependant actions that result in delay of chondrocytic hypertrophy (Karp et al., 2000). That Ihh mediates its hypertrophic-inhibitory actions through transcriptional activation of PTHrP at the periarticular layer was suggested from observations of chick limbs retrovirally overexpressing Ihh or murine bone explants treated with recombinant N-Shh (Vortkamp et al., 1996; Lanske et al., 1996). However, further studies have demonstrated that signaling by TGF-β, but not BMPs, can also elicit PTHrP activation in the periarticular cartilage, suggesting that Ihh may act indirectly through a relay mechanism. (Serra et al., 1999; Minina et al., 2001). From these studies, and others (St.-Jacques et al., 1999), it was concluded that the propensity to activate PTHrP was indispensable for achieving HH-mediated inhibition of hypertrophy and that ablation of PTHrP completely abrogated this effect. In apparent contrast to these studies, we demonstrate that HH-signaling activates, rather than inhibits, chondrogenic differentiation in CFK-2 cells. Thus, activation of *Col10a1* by HH is indicative of the potential that this peptide has in driving CFK-2 cells toward a progressive state of hypertrophic differentiation. This observation is consistent with other *in vitro* reports describing the propensity of Shh or Ihh to induce hypertrophic marker expression in micromass cultures and in embryonic carcinoma cells (Akiyama et al., 1999; Stott and Chuong, 1997). It is also consistent with the initial delay in chondrocyte maturation and *Col10a1* expression described in *Ihh*-null mice (St-Jacques

et al., 1999). This phenomenon was initially attributed to a perturbation of chondrocytic proliferation (Karp et al., 2000), however later studies have indicated that ablation of HH-signaling in growth plate chondrocytes selectively interfered with their proliferative capacity but did not affect their differentiation programme (Long et al., 2001). Alternatively, a delay in *Col10a1* expression can indicate that Ihh may have direct inductive influences on chondrocytic differentiation. Ihh may play a temporal role in promoting early chondrocyte differentiation, analogous to the role Shh has in promoting somitic chondrogenesis (Murtaugh et al., 1999). The fact that *Ihh*-null mice eventually display an increase in hypertrophic chondrocytes indicates that factors other than Ihh are required for this process. These factors may include members of the bone morphogenetic proteins (BMPs) family, as these were shown to induce chondrocytic hypertrophy (Enomoto-Iwamoto et al., 1998; Grimsrud et al., 1999; Terkeltaub et al., 1998). The fact that it is possible to observe the intrinsic HH differentiating capacity in CFK-2 cells *in vitro* may be due to the fact that PTHrP, which is normally induced through the negative regulatory response observed *in vivo*, is not stimulated by Ihh in this system. Addition of exogenous PTHrP, however, mimicked this *in vivo* effect.

PTHrP-induced inhibition of chondrogenic differentiation mediated directly through activation of PTH1R is well described (Schipani et al., 1997; Chung et al., 1998). Here, we demonstrate for the first time that PTHrP action impedes HH-mediated differentiation in CFK-2 cells. Thus, treatment of CFK-2 cells with N-Shh in the presence of PTHrP or forskolin resulted in complete abrogation of ALP activity and *Col2a1* and *Col10a1* expression, suggesting that this effect was mediated through a PKA-dependent pathway. In further agreement, Ihh-mediated ALP activity was prevented by overexpressing PTH1R

H223R, a variant of PTH1R described in patients with Jansen-type metaphyseal chondrodysplasia, and known to selectively and constitutively activate the PKA pathway (Schipani and Juppner, 1995). The *in vivo* observation that overexpression of PTH1R H223R1 in the growth plate leads to a delay in chondrocytic hypertrophy is also consistent with our results (Schipani et al., 1997). PKA has been widely described as a negative regulator of the HH-signaling pathway and appears to exert its function through direct phosphorylation of specific consensus sites present in Gli family members and their *Drosophila* homologue, Ci (Hammerschmidt et al., 1996; Chen et al., 1999; Wang et al., 1999). Thus, an attractive scenario emerges in that PTHrP signaling modulates, via PKA, one or more of the Gli factors (Fig. 6). The fact that *Ptc* induction by HH was refractory to PTHrP or forskolin treatment indicates the inhibition is rather selective. Such selectivity may reflect divergent functions of different Gli factors that are context-dependent (Ruiz, 1999). Thus, phosphorylation of Gli3, but not Gli1, by PKA results in its proteolysis and formation of an alternate repressor form that downregulates transcription of certain HH target genes (Chen et al., 1999; Chen et al., 1998; Wang et al., 2000). We have observed the expression of Gli2 and Gli3 expression in CFK-2 cells (data not shown) and speculate that complex regulation of HH target genes may be regulated by relative levels of repressor and activator forms of these proteins.

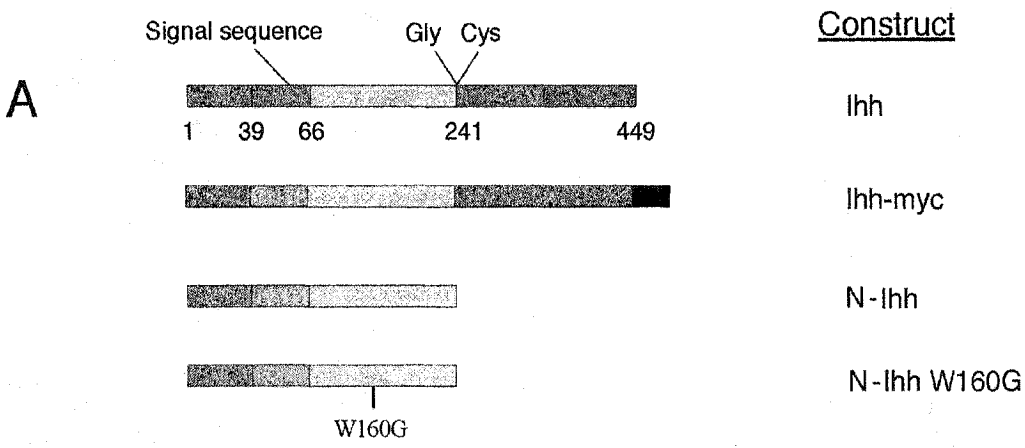
PTH1R has previously been shown to mediate the inhibitory effects of PTHrP on differentiation (Chung et al., 1998; Chung et al., 2001), a process involving activation of the cAMP but not the phospholipase C-dependant pathway (Guo et al., 2001). Interestingly, our data demonstrate an interaction between the HH and PTHrP signaling pathways at the level of PTH1R regulation. This is evidenced by HH-dependent

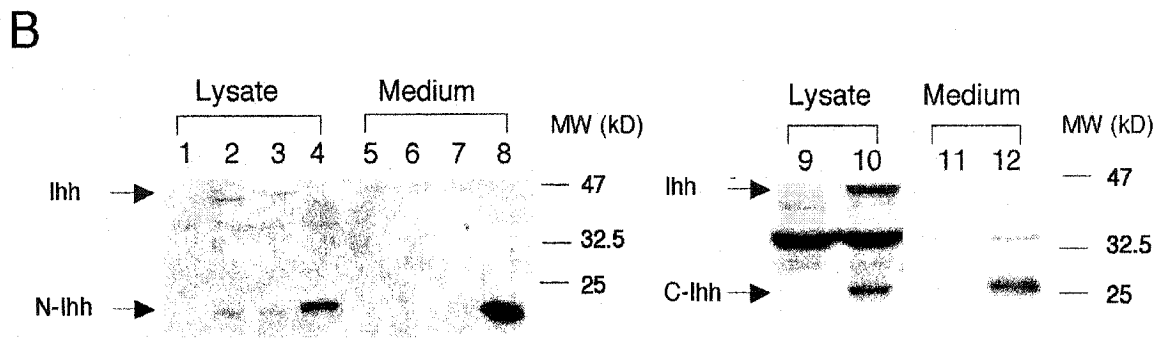
transcriptional upregulation of *Pth1r* mRNA, an effect that is antagonized by PTHrP or forskolin. Paradoxically, HH also renders cells less responsive to PTHrP and this is indicative of a functional inhibition of PTH1R by HH action. These finding have several implications that could converge with *in vivo* observations. First, the fact that *Pth1r* mRNA expression appears to be highest in the prehypertrophic cells that lie adjacent to, and overlap with, cells expressing Ihh may suggest the possibility of *Pth1r* being a transcriptional target of HH-signaling (St-Jacques et al., 1999). Second, *Pth1r* expression in proliferating chondrocytes is associated with enhanced responsiveness to PTHrP resulting in suppression of the hypertrophic markers ALP and *Col10a1*, a response that is diminished in hypertrophic chondrocytes (Iwamoto et al., 1994). Thus, signaling by Ihh may be required for inhibition of signaling via PTH1R, and consequently of PTHrP action, in order to allow prehypertrophic chondrocytes to proceed to their final differentiated state. However, HH signaling does not appear to completely inhibit PTHrP action, as cells treated with HH peptide remained responsive to transient and long-term PTHrP treatment. This suggests rather, that signaling by HH and PTHrP have interactive feedback mechanisms that allow for the appropriate pace of differentiation to occur (Fig. 6).

Figure 1. Expression of Ihh variants in COS-1 and CFK-2 cells

(A) Schematic representation of the cDNAs for Ihh, its *myc*-tagged variant, and its NH_2-terminal domain (N-Ihh), utilized by insertion into the pcDNA3 mammalian expression vector. Indicated are the internal signal sequence and the conserved cleavage site between Gly241 and Cys242. (B) Western blot analysis of lysates (lanes 1-4; 9-10) or conditioned media (lanes 5-8; 11-12) from COS-1 cells transiently transfected with pcDNA3 (lanes 1,5,9 and 11), Ihh (lanes 2 and 6), Ihh-*myc* (lanes 3, 7, 10 and 12) or N-Ihh (lanes 4 and 8). The detection of a 46kD and 19kD species in lysates from Ihh or Ihh-myc transfectants was facilitated by the use of α-Ihh-N antibody specific to the NH_2-terminal domain of Ihh (lanes 1-8). The 26kD COOH-terminal domain of Ihh was identified with the use of an anti-myc antibody (lanes 9-12). (C) Western blot analysis of COS-1 cell lysates following transient transfection with pcDNA3, N-Ihh-pcDNA3 or N-Ihh W160G-pcDNA3. The 19 kD peptide species attributed to the NH_2-terminal domain of Ihh in its normal and mutant forms was detected by the α-Ihh-N antibody.

Figure 1


A
Signal sequence
Gly
Cys
Construct
1
39
66
241
449
Ihh
Ihh-myc
N-Ihh
N-Ihh W160G
W160G


B
Lysate
Medium
MW (kD)
1 2 3 4
5 6 7 8
Ihh
N-Ihh
47
32.5
25
Lysate
Medium
MW (kD)
9 10
11 12
Ihh
C-Ihh
47
32.5
25

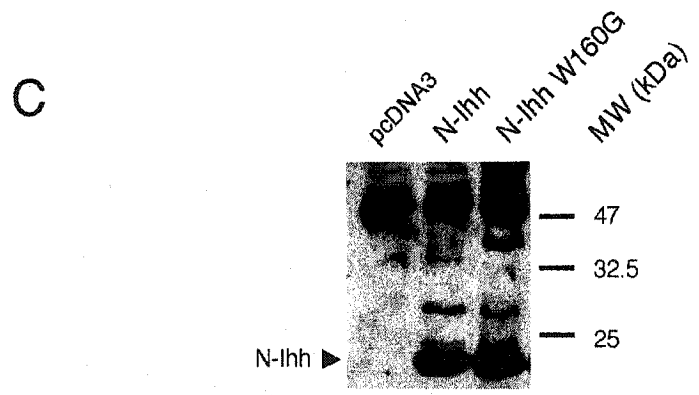

C
pcDNA3
N-Ihh
N-Ihh W160G
MW (kDa)
47
32.5
25
N-Ihh

Figure 2. Induction of *Ptc* receptor expression by Ihh, N-Ihh, N-Ihh W160G or exogenous N-Shh in CFK-2 cells

(A) Expression of *Ptc* in stably transfected CFK-2 cells. CFK-2 cells that were stably transfected with pcDNA3, Ihh-pCDNA3, N-Ihh-pcDNA3 or N-Ihh W160G-pcDNA3 were subjected to postconfluent growth conditions for a period of 16 days and total RNA was extracted intermittently at the indicated times. *Ptc* mRNA expression was assessed by northern blot analysis (left panel) and compared to the respective transgene level. (B) A graphic depiction of the results shown in (A) comparing *Ptc* expression to the levels of *Gapdh* expression where bars represent average levels at the three time points (*= $P<0.01$). (C) Comparison of *Ptc* expression to transgene levels in Ihh-, N-Ihh- and N-Ihh W160G-transfected cells after these were normalized to *Gapdh* mRNA. (D) Dose response induction of *Ptc* expression by exogenous N-Shh. CFK-2 cells were subjected to exogenous treatment with increasing concentrations of recombinant N-Shh or vehicle for a period of 6 days in postconfluent culture, after which total cellular RNA was extracted and subjected to northern blot analysis.

Figure 2

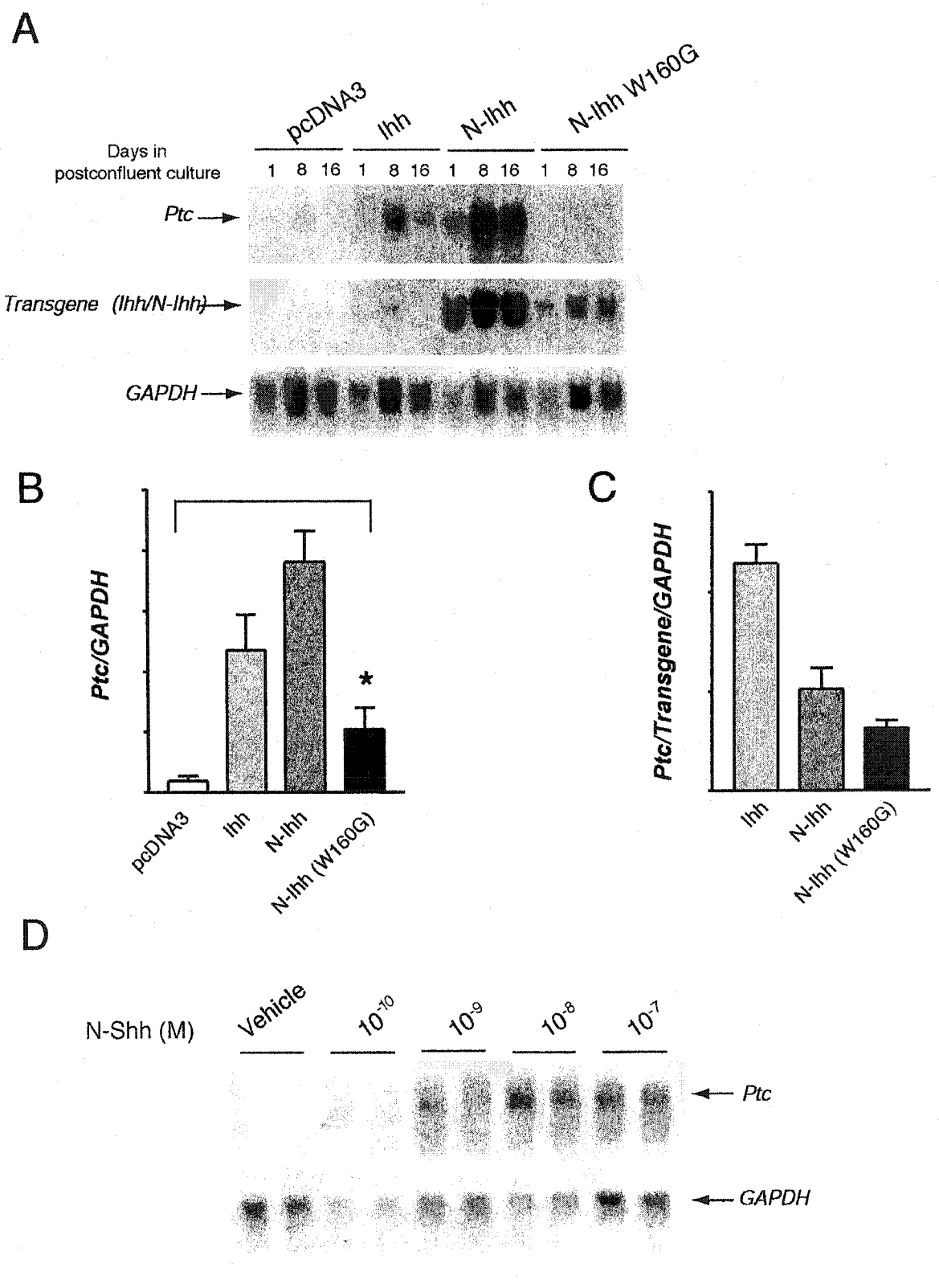

A
pcDNA3
Ihh
N-Ihh
N-Ihh W160G
Days in postconfluent culture
1 8 16 1 8 16 1 8 16 1 8 16
Ptc
Transgene (Ihh/N-Ihh)
GAPDH
B
Ptc/GAPDH
*
pcDNA3
Ihh
N-Ihh
N-Ihh (W160G)
C
Ptc/Transgene/GAPDH
Ihh
N-Ihh
N-Ihh (W160G)
D
N-Shh (M)
Vehicle
10-10
10-9
10-8
10-7
Ptc
GAPDH

Figure 3. Induction of chondrogenic markers in CFK-2 cells by Ihh is mimicked by recombinant N-Shh

(A) Alkaline phosphatase (ALP) activity is enhanced by Ihh or N-Ihh, but not by N-Ihh W160G. CFK-2 cells stably transfected with pcDNA3, Ihh or N-Ihh underwent postconfluent growth and were analyzed for ALP enzymatic activity during the indicated time periods (left panel). ALP activity was also compared between stably transfected CFK-2 cell populations expressing N-Ihh or its mutant variant form, N-Ihh W160G (right panel). (B) Induction of *Col2a1* and *Col10a1* collagens by Ihh or N-Ihh. Stably transfected CFK-2 cell populations expressing Ihh, N-Ihh, N-Ihh W160G, or vector alone were subjected to postconfluent growth conditions and total RNA was extracted at the indicated time periods. Northern blot analysis was utilized to evaluate expression levels of the chondrogenic markers *Col2a1* and *Col10a1*. (C) Dose-response induction of ALP, *Col2a1* and *Col10a1* by exogenous N-Shh. Naive CFK-2 cells grown to postconfluence were subjected to treatment by vehicle or exogenous N-Shh peptide at the indicated concentrations. Subsequent to 6 days of treatment, ALP enzymatic activity in cell extracts was measured (left panel) and total RNA was analyzed for *Col2a1* and *Col10a1* expression (right panel). A maximal response was observed at a concentration of 10^{-8} M N-Shh.

Figure 3

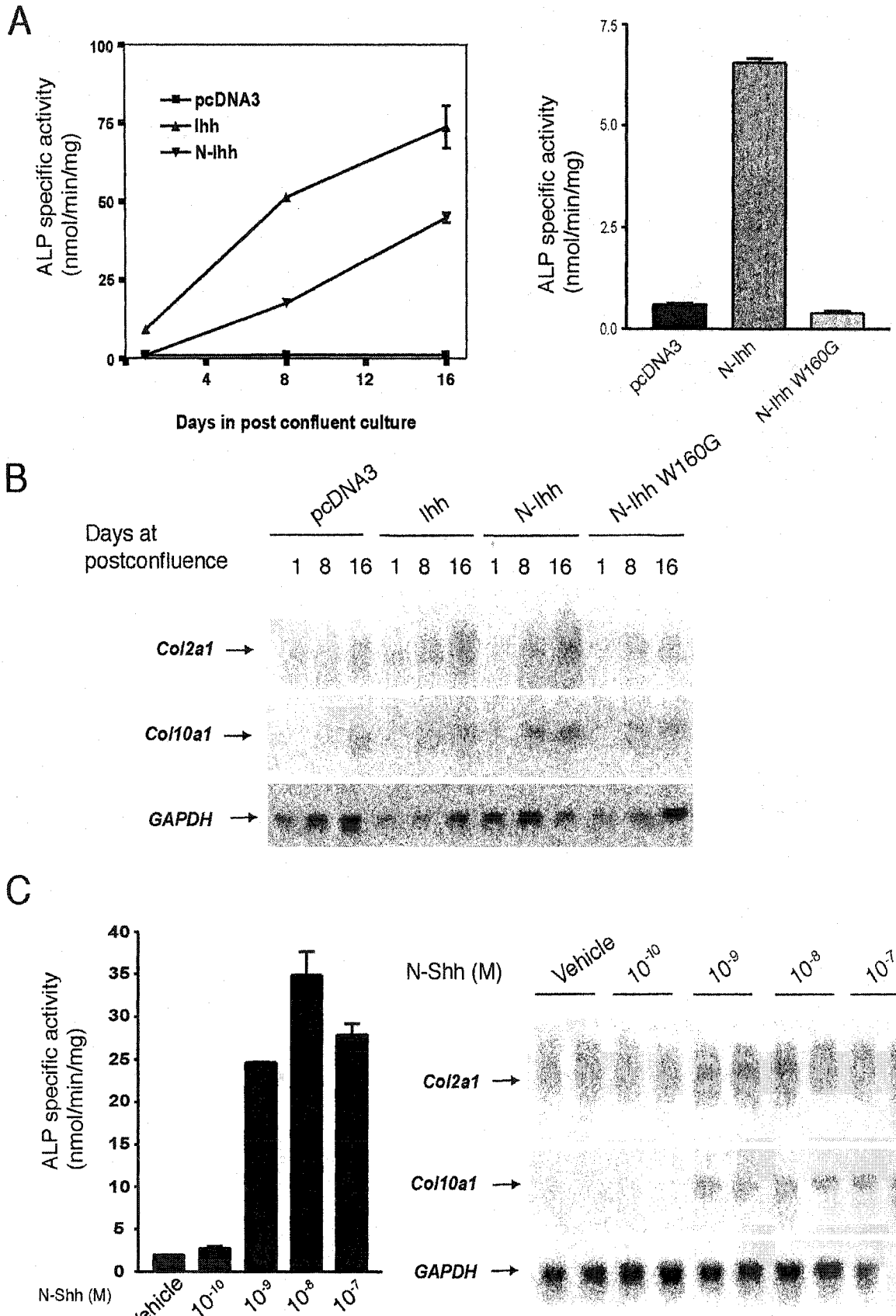
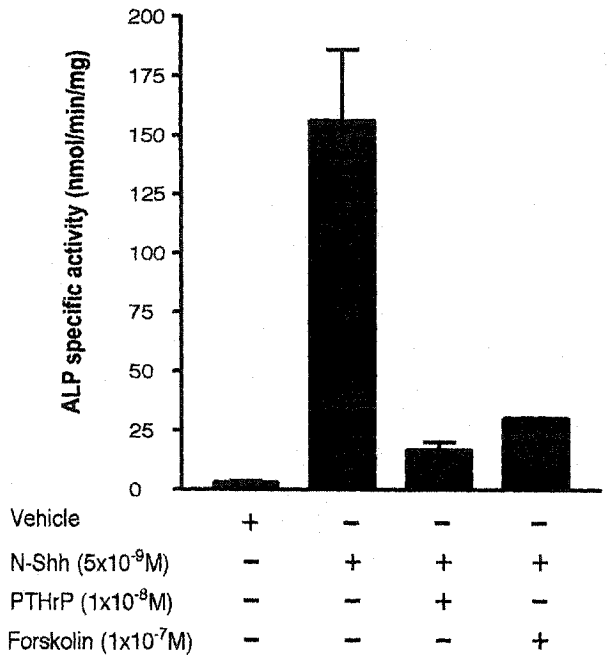

A
ALP specific activity (nmol/min/mg)
pcDNA3
Ihh
N-Ihh
Days in post confluent culture
N-Ihh W160G
B
Days at postconfluence
1 8 16 1 8 16 1 8 16 1 8 16
Col2a1
Col10a1
GAPDH
C
N-Shh (M)
Vehicle
GAPDH

Figure 4. PTHrP antagonizes *hedgehog*-induced chondrogenic differentiation through a PKA-dependant mechanism

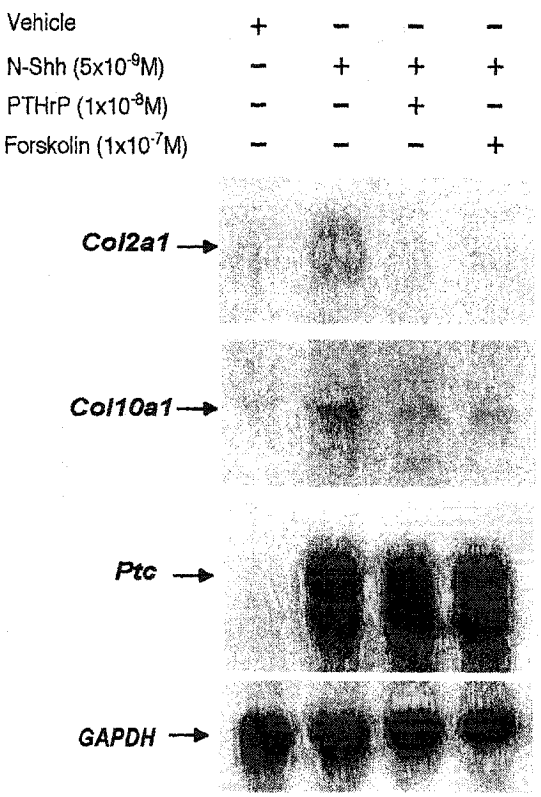
(A) PTHrP and forskolin impede HH-induced ALP activity. Naive CFK-2 cells grown to confluence were subjected to treatment with vehicle or N-Shh (5×10^{-9} M) in the absence or presence of PTHrP 1-34 (10^{-8} M) or forskolin (10^{-7} M) for a period of 8 days. Cell extracts were used to measure ALP enzymatic activity. (B) Selective inhibition of HH-induced gene expression by PTHrP. Northern blot analysis for the assessment of *Col2a1*, *Col10a1*, and Ptc expression was performed on total RNA extracted from postconfluent CFK-2 cells that were subjected to 8-day treatment with vehicle or N-Shh (5×10^{-9} M) in the absence or presence of PTHrP 1-34 (10^{-8} M) or forskolin (10^{-7} M). (C) Constitutive activation of PKA via PTH1R H223R interferes with HH-induced ALP activity. CFK-2 cell populations were generated following double stable transfection with Ihh-pcDNA3/pcDNA3.1, Ihh-pcDNA3/PTHrP-pcDNA3.1 or Ihh-pcDNA3/PTH1R (H223R)-pcDNA3.1. ALP specific activity was measured following 10 days of postconfluent culture.

Figure 4

A


ALP specific activity (nmol/min/mg)
200
175
150
125
100
75
50
25
0
Vehicle + − − −
N-Shh (5x10-9M) − + + +
PTHrP (1x10-8M) − − + −
Forskolin (1x10-7M) − − − +

B


Vehicle + − − −
N-Shh (5x10-9M) − + + +
PTHrP (1x10-8M) − − + −
Forskolin (1x10-7M) − − − +
Col2a1
Col10a1
Ptc
GAPDH

C

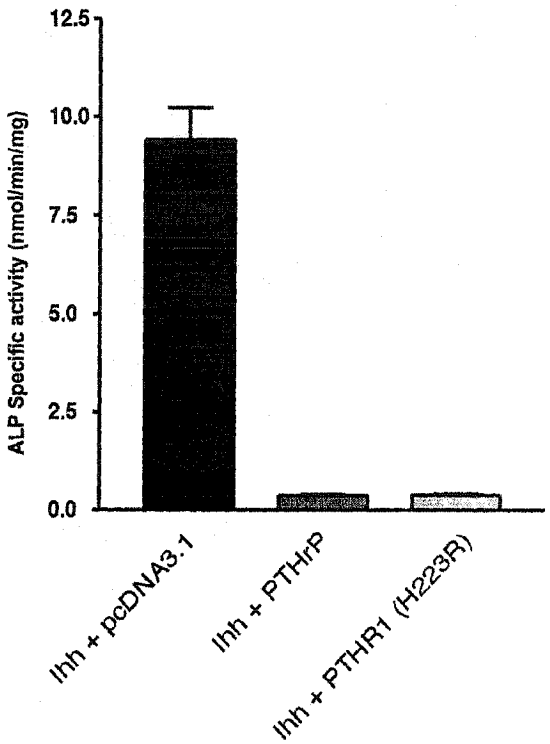

ALP Specific activity (nmol/min/mg)
12.5
10.0
7.5
5.0
2.5
0.0
Ihh + pcDNA3.1
Ihh + PTHrP
Ihh + PTHR1 (H223R)

Figure 5. HH-signaling upregulates PTHR1 expression while dampening PTHrP-mediated cAMP-dependent protein kinase A (PKA) activity

(A) Northern blot analysis for PTH1R mRNA expression in CFK-2 cells treated with vehicle, PTHrP (10^{-8} M) or N-Shh peptide (10^{-9} M) for 7 days during postconfluent growth (left panel). This experiment was also repeated with CFK-2 cells undergoing treatment with N-Shh in the absence or presence of PTHrP (10^{-8} M) or forskolin (10^{-7} M) (right panel). (B) Response to PTHrP (1-34) was assessed by measuring PKA activity in stable populations of CFK-2 cells following a 24-hour period of serum starvation. Cells were treated with 300 μM isobutylmethylxanthine (IBMX) in the presence or absence of 10^{-8} M PTHrP (1-34) for a period of 20 minutes. Lysates were then used to measure PKA activity and results are depicted as pmol of phosphate transferred to the kemptide substrate per unit time per mg of protein in the lysate. Activity in PTHrP-treated cells was also measured in the presence of a specific inhibitor to the catalytic subunit of PKA as control (PTHrP + PKI). Results from three independent experiments where samples were assayed in triplicates are depicted (**= $P<0.001$, *= $P<0.05$; left panel). Duplicate protein samples from cell lysates used for PKA activity measurement were subjected to western blot analysis for the detection of the catalytic subunit of PKA. Two catalytic subunits representing the C_α and C_β forms of PKA are shown (right panel). (C) Naive CFK-2 cells were treated for 7 days during postconfluent growth with the indicated concentrations of recombinant N-Shh. Following 24 hours of serum starvation cells were then transiently treated with PTHrP (10^{-8} M) and PKA activity was measured (**= $P<0.001$, *= $P<0.01$).

Figure 5

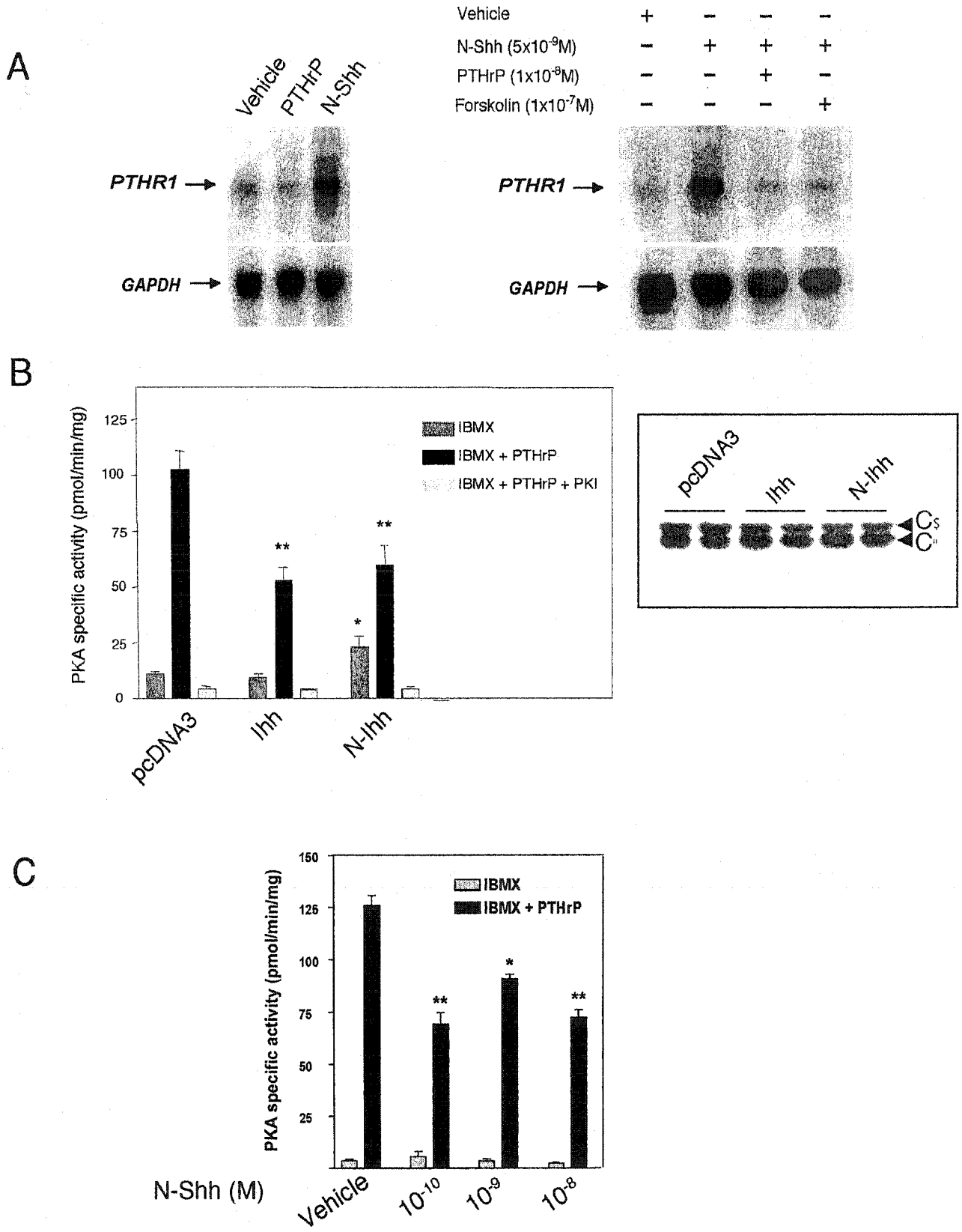
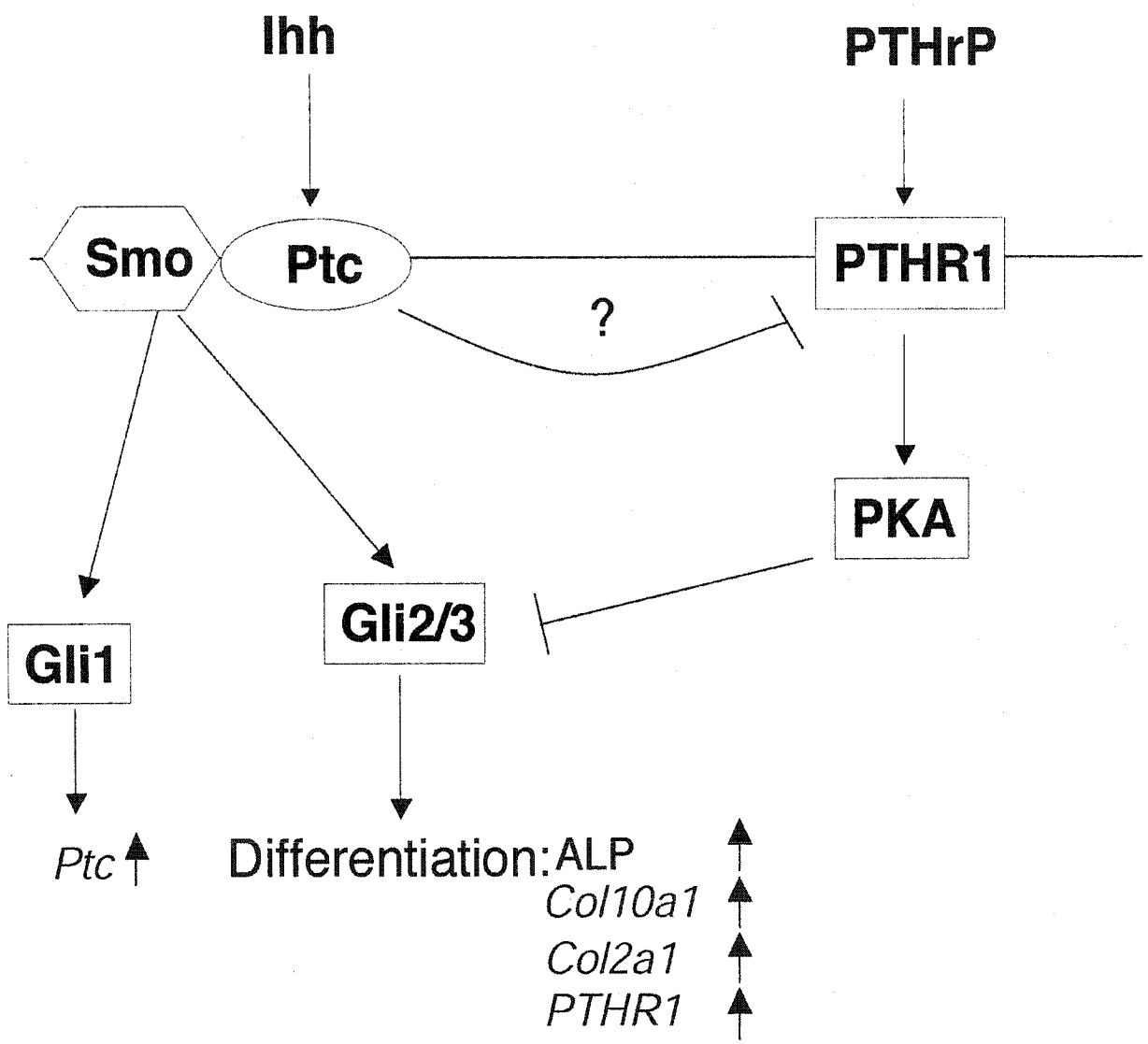

A
Vehicle
PTHrP
N-Shh
PTHR1
GAPDH
Vehicle + − − −
N-Shh (5x10-9M) − + + +
PTHrP (1x10-8M) − − + −
Forskolin (1x10-7M) − − − +
PTHR1
GAPDH
B
PKA specific activity (pmol/min/mg)
125
100
75
50
25
0
IBMX
IBMX + PTHrP
IBMX + PTHrP + PKI
**
**
*
pcDNA3
Ihh
N-Ihh
pcDNA3
Ihh
N-Ihh
Cs
C
C
PKA specific activity (pmol/min/mg)
150
125
100
75
50
25
IBMX
IBMX + PTHrP
**
*
**
N-Shh (M)
Vehicle
10-10
10-9
10-8

Figure 6. Proposed model for interaction between the PTHrP and HH signaling Pathways

Signaling by all mammalian HH proteins is mediated by the Gli family of transcription factors that consist of three members: Gli1, Gli2 and Gli3. Although all Gli proteins harbour consensus PKA phosphorylation sites, only Gli2 and Gli3 are known to be functionally converted to transcriptional repressors following phosphorylation by PKA. A plausible scenario for PTHrP to antagonize HH would be through activation of PKA via PTH1R. PKA may differentially phosphorylate Gli2 or Gli3, consequently converting them to repressors and causing downregulation of some HH target genes (ALP, *Col2a1*, *Col10a1*, *PTH1R*) while not affecting others (*Ptc*). HH in turn, may antagonize PKA activation, possibly through functional inhibition of PTH1R, thereby ensuring its own continued signaling capability.

Figure 6


Ihh
PTHrP
Smo
Ptc
PTHR1
?
PKA
Gli1
Gli2/3
Ptc
Differentiation:
ALP
Col10a1
Col2a1
PTHR1

Chapter 3

Evidence for functional interaction between En-1 and Ihh in the regulation of chondrocyte differentiation

Abstract

During Drosophila embryonic development, the segment polarity genes hedgehog (HH) and engrailed (en) synergize in establishing antero-posterior identity across the ectodermal parasegments and imaginal discs. Parallel functions have yet to be described in vertebrates. The present study examines the potential role of mammalian En-1 during chondrocyte differentiation and the possibility that it interacts functionally with the Hh-signaling pathway in regulating this process. Constitutive expression of En-1 in CFK-2 rat chondrocytic cells enhances the expression of alkaline phosphatase (ALP) and sulfated glycosaminoglycan (GAG) synthesis while not affecting expression of the hypertrophic marker, Collagen type X (*Col10a1*). In addition, En-1 suppresses the activity of the rat bone sialoprotein (BSP) promoter in CFK-2, and osteoblastic MC3T3 and ROS-17 cells, demonstrating a potential function for En-1 in chondrogenic/osteogenic differentiation. Furthermore, constitutively expressed En-1 in CFK-2 cells impairs HH-mediated induction of Patched (*Ptc*) mRNA and ALP activity. Immunohistochemical examination of growth plate cartilage from tibia and vertebrae of newborn mice revealed localized En-1 expression in prehypertrophic chondrocytes, congruent with Indian hedgehog (Ihh) expression, suggesting a function in chondrocytes *in vivo*. Taken together, these findings identify a novel function for En-1 in regulating chondrocytic differentiation and demonstrate, for the first time in a mammalian system, a role in the negative regulation HH-signaling.

Introduction

The development of the animal body plan employs a multitude of complex mechanisms some of which are based on cell to cell inductive interactions that have remained partially conserved throughout evolution. Exemplifying this, are cellular interactions mediated by temporally and spatially restricted expression of morphogens and transcription factors, which determine anterior-posterior (A/P) polarity in the ectodermal parasegments of the developing Drosophila embryo. Thus, critical to posterior parasegmental compartment identity is the co-expression of the homeodomain transcription factor *engrailed* (*en*), cell-autonomously conferring posterior fate, and the secreted morphogen *hedgehog* (*Hh*), which by a non cell-autonomous mechanism induces cells on the anterior side of the A/P boundary to express a second secreted morphogen, wingless (*wg*), that in turn is critical to *en* expression (Garcia-Bellido et al., 1972; Lee et al., 1992; DiNardo et al., 1988; Martinez-Ariaz et al., 1988). Further functional studies have indicated that *en* activates *Hh* expression in the posterior compartment while mediating repression of *Hh*-target genes in congruent cells (Tabata, 1992; Schwartz, 1995; Sanicola, 1995). Thus, En protein was shown to bind the promoters of the cubitus interruptus (*Ci*) gene and decapentaplegic (*dpp*), both Hh target genes in the anterior compartments, and elicits their repression (Schwartz, 1995; Sanicola, 1995).

Despite the evolutionary conservation of certain functional aspects of these peptide determinants in vertebrates, many others have been shown to diverge considerably. Thus, the two vertebrate homologues of Drosophila en, En-1 and En-2, are coexpressed in the mid-hindbrain and reciprocate with the *wg* homolog, Wnt-1, in ensuring the proper development of these structures (Joyner and Martin, 1987; Claytus and Joyner, 1988;

Danielian and McMahon, 1996). The absence of Hh expression in these tissues suggests a non-overlapping function between these molecules. Further divergence is also implicated by the exclusive expression patterns of En-1 in the limb bud ectoderm (apical ectodermal ridge or AER) and sclerotomal-derived pre-vertebral structures, in which both tissues are devoid of En-2 (Claytus and Joyner, 1988). In the AER, En-1 expression is ventrally restricted and through repressive mechanisms, it limits the expression of *Wnt7a* and *Lmxb1* to the dorsal ectoderm and mesenchyme, respectively. This information is pertinent to normal ventral patterning of the limb and when lost, as in En-1-null mice, dorsal transformation of ventral paw structures occurs (Loomis, 1996; Loomis, 1998). In contrast, Shh signaling emanating from the *zone of polarizing activity* (ZPA), a specialized region of posterior mesenchyme, functions in determining antero-posterior patterning in the developing limb and in maintenance of proximo-distal skeletal outgrowth by activating FGF4 expression in the apical ectoderm (Yang, 1997; Lewis, 2001; Laufer, 1994; Niswander, 1994). However, thus far no direct interaction between the Hh-signaling pathway and En has been described in vertebrates.

Intriguingly, several phenotypic aspects characterizing the *En-1*-null mutants indicate that this protein may function during skeletal development. Specifically, impaired caudal outgrowth of the sternum accompanied by its delayed ossification, truncation of the 13th ribs and reduced ossification in the phalanges indicate a role in endochondral ossification (Wurst, 1994). Despite these features, En-1 expression and function within the endochondral skeleton has not been studied in further detail. The fact that these animals die shortly after birth perturbs the possibility of studying En-1 function during postnatal skeletal development.

One of the central problems pertaining to endochondral ossification is the elucidation of mechanisms that regulate the transition of growth plate chondrocytes toward their terminal differentiated, or hypertrophic state. It is these cells that initiate the first stage of ossification by promoting vascular and concomitant osteoblast invasion into this region of the growth plate, while undergoing clearance by apoptosis. *In vivo* experiments have clearly demonstrated that Ihh is central to this process and through its restricted expression in a subpopulation of hypertrophic chondrocytes (prehypertrophic), activates parathyroid hormone-related peptide (PTHrP) expression in periarticular cells (Vortkamp, 1996; St-Jacques, 1999). PTHrP, in turn, delays the chondrocytic transition into the hypertrophic state by acting on prehypertrophic cells that express its cognate receptor, the type 1 PTH/PTHrP-receptor (PTH1R) (Lanske, 1996). However, experimental evidence suggests that Hh-signaling may also act directly on chondrocytes to promote, rather than inhibit, their differentiation (Stott, 1997).

This study was set up to examine the possibility that En-1 functions during chondrocytic differentiation, and to evaluate the possible interplay between En-1 and Hh-signaling during this process. We show that constitutive expression of En-1 in CFK-2 chondrocytic cells led to alterations in alkaline phosphatase (ALP) expression and sulfated glycosaminoglycan synthesis. These findings, further corroborated by the observation that En-1 can suppress bone sialoprotein (BSP) promoter activity, indicate a potential capacity for En-1 altering the expression of bone-specific genes. Finally, immunohistochemical analysis of growth plate chondrocytes, showing En-1 expression by prehypertrophic cells, is corroborated by *in vitro* studies that suggest that En-1 might regulate chondrocyte

differentiation by impeding Hh-signaling. This demonstrates for the first time a conserved functional interaction between these two proteins in a mammalian system.

Materials and Methods

Construction of mammalian expression plasmids- The cDNA encoding mouse En-1 was kindly provided by Alexandra Joyner (Skirball Institute, New York University, NY). This was subsequently subcloned into the pcDNA3 mammalian expression vector (Invitrogen). To express the amino terminal part of Ihh (N-IHh), a 720-bp fragment corresponding to this region of the protein was amplified using 5'-[GCCCCCGCATGGAAGTCCCC]-3' as the sense primer and 5'-[TCAGCCACCTGTCTTGGCAGC]-3' as the antisense primer. This fragment was cloned into pcDNA3 for mammalian cell expression.

Cell culture and transfections- CFK-2 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco BRL) supplemented with 10% fetal bovine serum (Wisent). To induce differentiation, cells were grown to confluence in 10% FBS after which serum was gradually reduced by 2% decrements every two days, and cells were maintained at 2% FBS thereafter until 16 days of post confluent growth. For some experiments medium was supplemented with 1×10^{-8} M human PTHrP (1-34) or varying concentrations of recombinant N-SHh (Curis, Inc., Cambridge, MA). N-SHh was modified by the addition of a hydrophobic eight-carbon chain (octyl group) to the NH_2-terminal cysteine and this is reported by the supplier to increase the biological potency of the native peptide by up to 10 fold. N-SHh protein was suspended in "Octyl" buffer (PBS pH 7.2, 50 μM DTT conjugated to N-octylmaleimide, 350 μM free DTT and 0.5% DMSO) and this buffer was also used as vehicle control, where indicated.

Generation of stably transfected CFK-2 cell populations was performed by electroporation. Cells were grown on 10 cm dishes and upon reaching 60% confluence were washed once in PBS and trypsinized. Cells were then suspended in HBS (20 mM

HEPES pH 7.4, 0.14 M NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 25 mM glucose, 1 mM $CaCl_2$) at a density of 5×10^5 cells/ml, supplemented with plasmid DNA (25 μg), and electroporated at 240V/ 500μF. Following 48 hours of recovery, cells were subjected to selection by the addition of G418 at a final concentration of 500 μg/ml. Selection proceeded for ten days and subsequently cells were maintained in G418 (500 μg/ml).

Transient transfection of COS-1 cells was performed by the calcium phosphate precipitation method. Briefly, DNA (2 μg/7×10^5 cells) was added to a solution of 2.5 M $CaCl_2$ and precipitated by the addition of HeBs solution (0.28 M NaCl, 0.05 M HEPES pH 7.05, 1.5 mM Na_2HPO_4). Precipitated DNA was layered onto cells and incubated for an 8-hour period. Cells were then washed 5 times in PBS and medium replaced for a recovery period of 48 hours. Alternatively, cells were transfected using Fugene™ 6 (Roche) according to the manufacturer's specifications.

Northern blot analysis- Total RNA was extracted at the indicated time points by Trizol reagent, as specified by the manufacturer (Gibco BRL). Aliquots (20 μg) were then size fractionated on a 1.5% agarose/formaldehyde gel and transferred overnight onto a supported nitrocellulose membrane using 20 X SSC buffer (3 M NaCl, 0.003 M Na citrate, pH 7.0). Hybridization of membranes to ^{32}P-labeled probes was performed in a buffer containing 40% formamide, 10% dextran SO_4, 4 X SSC, and 1X Denhardt's blocking solution with 0.1 mg/ml salmon sperm DNA. Membranes were washed once in 2 X SSC/0.1% SDS at room temperature and once in 0.1 X SSC/0.1% SDS for 15 minutes at 58°C before exposing to film.

All probes were radiolabeled by the random priming method (Roche). A 390-bp probe verified by DNA sequencing to correspond to *Ptc* cDNA was generated by RT-PCR from total RNA extracted from CFK-2 cells. The oligonucleotides used to obtain this fragment were 5'-[GGACTTCCAGGATGCCATTTGACAGTG]-3' as the sense primer and 5'-[GCCGTTGAGGTAGAAAGGGAACTG]-3' as the antisense primer and were based on the mouse *Ptc* cDNA sequence. The cDNA probe for rat *Pthr1* was generously provided by H. Juppner (Endocrine Unit, Massachusetts General Hospital, Boston, MA). The Gli1 probe, encompassing the first two exons of the murine *Gli1* gene, was excised by a BamH1 and HindIII restriction digest from the *Gli1* promoter construct, pHR-Luc (Provided by Shunsuke Ishii, Tsukuba Life Science Center, Japan; used by permission from Alexandra Joyner, NYU). Northern blots were assessed quantitatively by videodensitometric analysis (Scion Image).

Subcellular fractionation and Western blotting- COS-1 cells transiently transfected with En-1-pcDNA3 or pCDNA3 were subjected to rapid subcellular fractionation (Andrews and Failer, 1991). Cells were washed twice in ice cold PBS and scraped into 400 µl of hypotonic buffer A (10mM HEPES-KOH, pH 7.9, 1.5mM MgCl2, 10mM KCl, 0.5mM DTT, 1mM PMSF, 5 µg/ml leupeptine, 5 µg/ml aprotinin). Cells were allowed to swell for 20 minutes on ice, and following brief vortexing nuclear and cytosolic fractions were separated by brief centrifugation. Nuclei were resuspended in 40 µl of buffer B (20mM HEPES-KOH, pH 7.9, 25% glycerol, 0.42M NaCl, 1.5 mM MgCl2, 0.2mM EDTA, 0.5mM DTT, 1mM PMSF, 5 µg/ml leupeptine, 5 µg/ml aprotinin) and incubated on ice for 20 minutes. Nuclear extracts were obtained by brief centrifugation.

A monoclonal antibody reactive against murine En-1 was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. For Western blot analysis, approximately 50 µl of cytosolic and 15 µl of nuclear fractions were analyzed by SDS-PAGE. Protein samples were loaded onto a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were blocked in TBST buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20) and 5% milk for one hour, and then incubated with primary antibodies that were diluted in TBST/0.5% milk [1:500 for anti-En-1, 1:500 for anti-α-tubulin (ICN)] for 1 hr. Secondary antibodies [horseradish peroxidase-conjugated anti-mouse IgG (Sigma), 1:2000 with TBST/0.5% milk] were incubated with membranes for 1 hr and bands were visualized with the BM chemiluminescence blotting substrate (Roche), according to manufacturer's instructions.

Immunohistochemistry and immunofluorescence- For *in situ* detection of En-1 and IHh protein expression, paraffin embedded sections of tibia and vertebrae from newborn mice were de-waxed and rehydrated by successive treatment with xylene, ethanol (100%, 90%, 75%), and water. Following digestion by 0.5% hyaluronidase or trypsin for 30 minutes at 37°C, tissues were blocked with 10% normal goat serum and then incubated overnight with primary antibodies, α-En-1 (1:50) or α-Ihh (1:100)/ 0.5% BSA. Following several washings with TBST tissues were subjected to a biotinylated anti-mouse (for En-1) or α-goat (for IHh) secondary antibodies for 1 hour. Signal detection was obtained using the ABC-alkaline phosphatase kit (Vector Laboratories).

Immunofluorescent detection of En-1 or IHh was achieved following transient transfection of COS-1 cells with En-1-pcDNA3 or IHh-myc-pcDNA3. Transfected cells were plated

on glass coverslips, washed twice in PBS, and fixed in paraformaldehyde (pH 7.0) for 10 minutes. Following three washes with PBS cells were permeabilized for 10minutes with 0.2% Triton. Following several washings with PBS, cells were incubated for 20minutes in the presence of 10% fetal bovine serum / PBS. Cells were incubated in the presence of primary antibody for En-1 (1:200/ 1.5% serum) or IHh (anti-myc, 1:250) for 1 hour at room temperature. Following five washes with PBS cells were incubated for 45 minutes with FITC-conjugated anti-mouse (1.5% serum) which enabled visualization of En-1 or Ihh by fluorescence microscopy.

Measurement of sulfated glycosaminoglycans (GAG)- Quantitative assessment of cell surface GAG expression was determined spectrophotometrically according to the method of Farndale et. al., 1982. Briefly, cells grown to confluence in 6-well plates were scraped into 300µl of 50mM phosphate buffer (pH 6.5) containing 2mM N-acetyl cysteine and 2mM EDTA and subjected to papain digestion (300 µg/ml) for 1 hour at 65°C. Following the removal of cellular debris by brief centrifugation, 100µl aliquots were analyzed by mixing into 400µl of DMB solution (prepared as a stock of 16 mg 1,9-dimethylmethylene blue in 5ml ethanol, 2g sodium formate, and 2ml of formic acid brought to 1L), and immediately measuring absorbance at 535nm. Absorption values were plotted against a standard curve generated from incremental concentrations of bovine chondroitin sulfate (Sigma).

ALP activity assay- Cell monolayers were washed with PBS, lysed in 300 µl ALP lysis buffer (0.15 M Tris pH 9.0, 0.1 mM $ZnCl_2$, 0.1 mM $MgCl_2$), and subjected to one freeze-thaw cycle at -70°C. Lysates were cleared by a 10-min centrifugation at 10000 × g and 50

µl aliquots were analyzed spectrophotometrically at 410 nm with ALP assay solution (7.5 mM p-nitrophenyl phosphate (Sigma reagent 104), 1.5 M Tris pH 9.0, 1 mM $ZnCl_2$, 1 mM $MgCl_2$). Protein concentrations were determined by the method of Lowry using the Bio-Rad DC protein assay kit (Bio-Rad).

Promoter activity assays- A 1 kb fragment of the bone sialoprotein promoter was cloned by RT-PCR using rat genomic DNA as template. Primers used were 5'-AACATTCAGCCTGCCAACAT-3' in the sense direction and 5'-TTACGTTACCCAGCCTGGTC-3' in the antisense direction. This fragment was subsequently verified by DNA sequencing and subcloned into the PXP-2 luciferase reporter vector, termed BSP-Prom-PXP-2 (a gift from J. White, McGill). A 3.5 kb genomic fragment containing the *Gli1* promoter was kindly provided by Shunsuke Ishii (Tsukuba Life Science Center, Japan). This fragment was subcloned into the PXP-2 luciferase reporter vector, termed *Gli1*-Prom-PXP-2.

For BSP promoter activity measurements, cells were transiently transfected with 1 µg of BSP-pro-PXP-2 / well (6-well plate) with the addition of 1 µg of pCDNA3 or En-1-pcDNA3. Transfection efficiencies were assessed by cotransfecting 0.2 µg of ß-galactosidase expressing vector. Cells were lysed in 200 µl of Passive Lysis Buffer (PLB, Promega) 3 days following transfection, of which 20 µl were mixed with 100 µl of luciferase substrate and light emission was measured by a luminometer. Transfection efficiencies were assessed by contransfecting 0.2 µg of pCMVβ (Clontech), expressing β–galactosidase (β-gal). Assessment of β-gal activity was measured in the presence of O-Nitrophenyl-β-D-galactopyranoside (ONPG), as described (Sambrook, 1989).

PKA assay- CFK-2 cells (En-1 or pcDNA3, stably transfected) were grown to 7 days post-confluence in the presence of 5% FBS, 500 μg/ml G418, in the presence or absence of 10^{-8} M N-SHh peptide. Following serum starvation for 24 hours, cells were then stimulated for 20 min with 10^{-8} M PTHrP 1-34 in the presence of 300 μM isobutylmethylxanthine (IBMX) or with IBMX alone. Cells were rinsed twice with PBS, placed on ice and lysed in PKA assay buffer (25 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.5% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.5 mM PMSF). Measurement of PKA activity in cell lysates was performed using a PKA assay kit (Upstate Biotech.). Briefly, reactions were performed in the presence of 5 μl of lysate, 83 μM kemptide substrate, 0.33 μM PKC inhibitor peptide, 3.33 μM CaMK inhibitor (R24571) and 83.3 μM ATP/ 16.67 μCi [γ-^{32}P] ATP. To demonstrate specificity for phosphorylation by PKA, reactions were also performed in the presence of both kemptide substrate and 1 μM of PKA inhibitor peptide. After an incubation of 10 min at 30°C, 15 μl samples were blotted onto phosphocellulose P81 paper, washed 4 times in 0.75% phosphoric acid, once in acetone, and radioactivity was measured in a scintillation counter. Values were normalized to protein concentrations of the lysates and to specific radioactivity (cpm/nmol) of the reaction mix.

Results

In vitro expression and subcellular localization of En-1

This study aimed at examining the possibility that En-1 may function during endochondral bone formation. To address this question *in vitro,* we employed rat CFK-2 cells that have been shown to recapitulate the chondrogenic differentiation program and to be highly responsive to signals by PTH/PTHrP and HH (Bernier, 1990; Bernier and Goltzman, 1993; Henderson, 1996; Deckelbaum, 2002). En-1 is classified as a homeodomain transcription factor harboring five functional domains that are conserved between *Drosophila* and vertebrates (Fig. 1A; Logan, 1992). To examine En-1 expression, we analyzed its subcellular localization by immunofluorescence in COS-1 cells following transient transfection with En-1-pcDNA3, a mammalian expression vector harboring the murine *En-1* cDNA. As shown in Fig. 1 B1, En-1 protein localized entirely to the nucleus and this is in contrast to overexpressed Ihh which localized to the cytosol (Fig. 1 B2). A similar distribution for En-1 protein was observed by western blot analysis comparing nuclear and cytosolic fractions of transfected COS-1 cells (Fig. 1C). We next generated a stable population of CFK-2 cells expressing En-1-pcDNA3, or as control, pcDNA3 vector alone.

En-1 expression alters the chondrogenic phenotype of CFK-2 cells

CFK-2 cells harbor the potential to undergo differentiation along the chondrocytic lineage when grown to postconfluence under reduced serum levels (Bernier, 1992; Wang, 2001; Deckelbaum, 2002). Compared to pcDNA3-transfected cells, En-1 expressing cells subjected to this growth environment assumed an enlarged and more round cellular morphology reminiscent of hypertrophic cells. As ALP is a characteristic marker of

hypertrophic chondrocytic differentiation (Stott, 1996), we compared its activity in En-1-pcDNA3-transfected CFK-2 cells to that in vector control counterparts over a 16-day postconfluent growth period (Fig. 2A). Whereas no appreciable activation of ALP was observed in pcDNA3-transfected cells over time, En-1 expressing cells displayed a five fold enhancement in its enzymatic activity that remained stable over the culture period. As shown by our previous studies, CFK-2 cells constitutively expressing Ihh or its NH_2-terminal fragment, N-Ihh, display a temporal increase in ALP activity (Deckelbaum, 2002). By comparison to En-1-transfectants, ALP activity in N-Ihh-expressing cells is increased at a faster rate. Furthermore, examination for *Col10a1* mRNA, the *bona fide* marker of hypertrophic differentiation, revealed that while vector control and N-Ihh-expressing cells displayed a temporal increase in its expression, this was inhibited in cells transfected with *En-1* (Fig. 1B). By comparison, En-1 expressing did not modulate significantly the temporal increase in levels of *PTH1R* mRNA (Fig. 1B).

Sulfated glycosaminoglycan (GAG) synthesis, commonly associated with heparan sulfate proteoglycans (HSPG), is usually enhanced over the course of chondrocytic differentiation. Therefore, we assessed its levels over a 16-day postconfluent period. By comparison to pcDNA3- and N-Ihh-transfectants, En-1 expressing cells exhibited a strong enhancement in GAG levels that increased in a temporal fashion over the culture period (Fig. 1C). These findings suggest that En-1 harbors the capacity to modulate chondrocytic differentiation in a fashion that is distinct from that elicited by Ihh.

En-1 represses bone sialoprotein promoter activity

To examine the possibility that En-1 may be involved in modulating the expression of chondrocytic genes at the transcriptional level we searched for bone-related gene

promoters that harbor an engrailed-homeobox regulatory element. Bone sialoprotein (BSP), characterized by its high glutamate and glycosylated sialic acid content, is synthesized hypertrophic chondrocytes and localizes to areas of mineralized cartilage (Bianco, 1993; Chen, 1993). The BSP proximal promoter contains a consensus homeobox sequence which can be potentially utilized by En-1 (Yang and Gerstenfeld, 1997). The fact that this potential En-1 binding site is conserved amongst species further indicates toward its likely functional importance (Li and Sodek, 1993; Yang and Gerstenfeld, 1997). We therefore cloned a 1.1 kb fragment corresponding to the rat BSP proximal promoter, using CFK-2 genomic DNA as template, and subsequently subcloned this into the PXP-2 luciferase reporter vector. We first tested BSP promoter activity in CFK-2 cells stably transfected with either pcDNA3, N-terminal Ihh (N-Ihh) or En-1. As we anticipated, its promoter activity was altered by the differentiation state of the cells (Fig. 3A). N-Ihh and En-1 expressing cells displayed a two fold increase in promoter activity by comparison to pcDNA3-transfected cells. However, BSP promoter activity was strongly and similarly repressed in all CFK-2 cell populations following transient co-transfection with En-1. As BSP expression was associated with osteoblastic cell phenotype we also examined its promoter activity in mouse osteogenic MC3T3 and rat osteosarcoma ROS-17 cells (Fig. 3B). Similarly to CFK-2 cells, BSP promoter activity was strongly repressed following transient expression of En-1 in both cell types. Taken together, these findings demonstrate that En-1 can potentially downregulate the activity of a chondrogenic/osteoblastic gene, presumably through its interaction with the homeobox sequence element in the BSP promoter.

En-1 is expressed by prehypertrophic chondrocytes

To eamine the possibility that En-1 may also function during chondrocytic differentiation *in vivo* we used immuhistochemistry to examine for expression of this protein in tibial and vertebral growth plates from newborn mice. As *En-1* expression was described to occur in the developing mid-hindbrain junction, where it is required for its proper formation (Claytus and Joyner, 1988; Wurst, 1994), we first examined sections from brains of newborn mice. To validate the suitability for α-En-1 for immunohistochemistry, En-1 positive cells were detected in the mid-hindbrain region (Fig. 4A) but not in the negative control (Fig. 4B). Examination of bone sections from newborn mice revealed a novel expression domain for En-1 in chondrocytes. Specifically, En-1 protein expression in growth plate chondrocytes was restricted to the prehypertrophic zone of both tibia and vertebrae although, to a lesser extent, some presumptive hypertrophic cells in the vertebrae were also En-1-positive (Fig. 4C-E). Notably, En-1 expression was excluded from proliferating and most hypertrophic chondrocytes. As Ihh expression has also been reported to localize to prehypertrophic cells (St. Jacques, 1999) we examined its protein expression in parallel bone sections. In both tibia and vertebrae Ihh localized to prehypertrophic and, to a lesser extent, hypertrophic chondrocytes (Fig. 4F, G). The overlapping expression domain of En-1 and Ihh by growth plate chondrocytes suggests an interplay between these two factors.

Inhibition of Hh-signaling by En-1 in CFK-2 cells

Despite ample data demonstrating a role for En protein in regulating HH-signaling in *Drosophila*, functional interactions between these factors has never been shown to occur in a mammalian context. As our *in situ* data demonstrated an overlap in Ihh and En-1

expression in the growth plate, we asked whether this may also extend to a functional level. As CFK-2 cells are highly sensitive to recombinant N-Shh, exhibiting activation of *Ptc* expression and ALP activity, we assessed how cells constitutively expressing En-1 may alter their response to treatment by this protein. Stably transfected CFK-2 cells expressing En-1 or pcDNA3 were grown to postclonfluence in the presence or absence of 10^{-8}M or 10^{-9}M N-Shh for a period of 8 days following which ALP activity and *Ptc* expression were examined. We observed a declined sensitivity to increased N-SHh dosage in En-1 transfected cells, as assessed by *Ptc1* mRNA expression in response to N-Shh treatment (Fig. 5A, B).Furthermore, whereas pcDNA3-transfectants displayed a >3 fold induction in ALP activity following 10^{-9}M N-Shh treatment, En-1 expressing cells exhibited a much lower stimulation under similar conditions (Fig. 5C). These findings indicate toward the existence of functional interactions between Hh and En-1 during chondrocyte differentiation and suggest that En-1 suppresses Hh-responsiveness.

En-1 does not affect PTHrP signaling in CFK-2 cells

Since Hh-signaling and its chondrogenic properties were significantly downregulated by En-1 and that signaling by PTHrP was shown to antagonize Hh activity in CFK-2 cells (Deckelbaum, 2002), we also examined the possibility that PTHrP-responsiveness may be modulated in En-1-expressing CFK-2 populations. Previously we have showed that PTHrP-stimulated PKA activity is downregulated by Hh-signaling, and therefore examined the possibility that En-1 activity may be altered by Hh stimulation. Postconfluent CFK-2 cultures, En-1 or pcDNA3-transfected, were grown for 7 days in the presence or absence of 10^{-8}M recombinant N-Shh peptide (5% FBS) and serum starved for 24 hours prior to transient stimulation by 10^{-8}M PTHrP (1-34). Cell lysates were then assayed for PKA

activity (Fig. 6A). PTHrP stimulated PKA to a similar degree in both pcDNA3 and En-1 transfectants although a higher basal activity was noted in pcDNA3 control cells. As observed previously, Shh significantly diminished PTHrP-mediated PKA activity and to a similar extent in both En-1- and pcDNA3-transfected cells. Thus, En-1 does not affect signaling by PTH1R, at least in its capacity to activate the cAMP/PKA pathway, and does not interfere with the capability of HH to antagonize its stimulation by PTHrP.

Paradoxically, we previously described the upregulated transcriptional activity of PTH1R in response to Shh treatment. As this phenomenon was negatively correlated to the functional profile of PTH1R and interpreted as a possible HH-mediated compensatory mechanism, we examined its mRNA expression in SHh-stimulated En-1-transfectants. In contrast to pcDNA3 transfected cells, which displayed an upregulation in PTH1R mRNA in response to increasing levels of Shh, En-1-transfectants maintained low message levels that were not upregulated following HH stimulation (Fig. 6B). Taken together, En-1 appears not to affect PTH1R activity but yet negatively influences a HH-dependent mechanism for upregulating its mRNA.

Discussion

Mice lacking En-1 display multiple defects implicating its functions in mid-hindbrain development, limb patterning, sternum outgrowth, and rib formation (Wurst, 1994). The present study investigated potential roles for En-1 during skeletal development by examining its influence on chondrocytic differentiation. Specifically, *in vitro* data presented here suggests that En-1 modulates this process both directly, via transcriptional repression of a bone-specific gene promoter, and indirectly through negative regulation of the HH-signaling pathway. Further establishing the physiological relevance of these findings to chondrogenesis *in vivo* is the demonstration of localized En-1 protein expression in growth plate chondrocytes of the both long bones and vertebrae.

That En-1 may participate in particular processes pertaining to skeletogenesis, is in conformity with that of other homeoproteins. Thus, members of the Hox gene family, required for pattern formation of the vertebrae and limb skeleton (Krumlauf, 1994), also participate in the regulation of chondrocyte proliferation and differentiation (Yueh, 1998). Other homeoproteins involved in regulating chondrogenesis and osteogenesis in distinct elements of the appendicular and axial skeleton include Dlx5/Dlx6 (Robledo, 2002), Bapx1 (Tribioli, 1999), and Pbx1 (Selleri, 2001). The previous description of localized En-1 expression to sclerotome-derived cells of the pericordal tube suggested its possible role in vertebral bone formation. However, the phenotypic consequence of En-1 ablation was not evident in this tissue, at least at the gross morphological level (Davis and Joyner, 1988; Davidson, 1988). The present study shows that En-1 localizes to prehypertrophic and early hypertrophic vertebral growth plate chondrocytes in post-natal mice, indicative of a function in later phases of somitic chondrogenesis. In parallel, its detection in

appendicular skeletal chondrocytes suggests that in the limb En-1 is not restricted to ectodermal tissues but is also present in cells of mesenchymal origin. The precise temporal expression pattern of En-1 in these tissues remains to be clarified, as it is possible that it occurs at later stages of embryonic development than those previously examined.

That En-1 expression is restricted to prehypertrophic chondrocytes in both types of skeletal elements, indicates a conserved function for this protein in these locations. Prehypertrophic chondrocytes are essentially in a transient state and their progression toward the terminal differentiated stage is under strict control by the Ihh/PTHrP regulatory cascade (Vortkamp, 1996; St.Jacques, 1999; Karp, 2000). It is therefore intriguing to postulate that En-1, a transcription factor previously not described to associate with these pathways, may participate in regulating this critical step in chondrocytic differentiation. Data presented here indicates two postulated mechanisms by which En-1 may influence this process; direct transcriptional repression of bone-specific genes and negative input on HH-signaling. We demonstrate that En-1 mediates repression of the BSP promoter in transient transfection experiments in both chondrocytic and osteoblastic cell types. Engrailed proteins are known to elicit active transcriptional repression through the interaction of the conserved N-terminal EH1 and C-terminal EH5 domains with other transcriptional activators (Han, 1993; Jaynes, 1996). Furthermore, active repression can be either dependant or independent of homeodomain (EH4)-mediated DNA-binding of En to specific homeobox elements (Jaynes, 1991; Plaza, 1997). In some contexts, En proteins may also elicit repression by competing with TFIID for DNA binding to the TATA box (Ohkuma, 1998). Alternatively, En proteins were shown to interact with and enhance the repressive activity of *Groucho*, thereby demonstrating the

ability to recruit additional co-repressors in regulating transcription (Tolkunova, 1998). Therefore, the exact mechanism by which En-1 mediates repression of the BSP promoter remains unclear. However, the presence of a homeobox binding site in its promoter suggests toward its possible interaction with the En-1 homeodomain. The observation that constitutive En-1 expression results in increased BSP promoter activity in CFK-2 cells, which was abrogated following transient transfection by *En-1*, suggests that the En/DNA ratio may be important for observing active repression. As only a fraction of stably transfected CFK-2 cells did express En-1 protein, the elevated basal BSP promoter activity may reflect on other, possibly non cell-autonomous, effects mediated by En-1 on this population. Interestingly, the osteoblastic gene promoters of osteocalcin and osteopontin also harbor En-1 binding sites, rendering them susceptible to potential negative regulation by this homeoprotein (Yang, 1997). Thus, the repression of BSP promoter activity by En-1 may be indicative of a general function in preventing premature osteoblast-specific gene expression by chondrocytes, however this was not yet examined.

In *Drosophila*, En has been shown to affect several aspects of HH-signaling and expression. Expressed in posterior compartment cells of embryonic ectoderm and the imaginal disc, en was shown determine their identity and induce the expression and secretion of Hh (Tabata, 1992; Lee, 1992; Dahmann, 2000). Concomitantly, en mediates negative regulation of *Ptc* and *Ci,* which are essential for mediating the Hh signal, thus ensuring that cells in the posterior compartment remain refractory to Hh (Schwartz, 1995; Sanicola, 1995). Evidence presented here indicates that several of these aspects are conserved in mammals, at least in particular contexts. First, the congruent expression of En-1 and Ihh in a subset of prehypertrophic cells suggests that En-1 may participate in

regulating Ihh expression in the growth plate. The magnitude of this function, however, may be to a smaller degree since *En-1*-/- and *Ihh*-/- animals display different phenotypes, suggesting that En-1 does not lie directly downstream of Ihh or that the expression of other homeodomain proteins may be compensating for its function. Second, *in vitro* data demonstrates that constitutive expression of En-1 hampers Hh-responsiveness as evidenced by inhibited *Ptc* and *Gli1* expression and consequent reduction in Hh-induced ALP activity. This suggests that En-1 maintains the capacity to inhibit Hh signaling and that it may execute these functions by utilizing similar mechanisms to those found in *Drosophila*. It is clear, however, that mammalian forms of En acquired divergent functions as *Drosophila* en can only partially rescue the *En-1*-null phenotype (Hanks, 1998). Interestingly, En-1 appeared to not affect PTHrP-mediated stimulating of PKA or the ability of Hh to diminish this response, although it did inhibit Hh-mediated upregulation of PTH1R mRNA. This indicates that cells expressing En-1 remain susceptible to the inhibitory actions of PTHrP on differentiation and Hh-signaling, and in fact may imply a synergistic relationship between En-1 and PTHrP. Further studies will be required to determine if En-1 enhances the inhibitory effects of PTHrP over the course of chondrocytic differentiation.

The fact that En-1-ablated mice were not described to present with skeletal defects that would indicate an obvious En-1 function in regulating endochondral bone growth, does not exclude the possibility that it may execute such activities in association with other factors. Thus, En-1 was shown to associate with and enhance the DNA binding activities of the Groucho/TLE family of transcriptional repressors where, interestingly, both are involved in inhibiting Wnt signaling (Tolkunova, 1998; Roose, 1998; Loomis, 1996).

Moreover, En-1 was shown to interact with the homeodomain factor Pbx1 through its EH2 domain resulting in increased DNA-binding affinity and specificity (Peltenburg, 1996). Since Pbx1-defficient mice display clear defects in endochondral ossification attributed to decreased chondrocyte proliferation and increased hypertrophy, it is likely that En-1 acts in conjunction with Pbx1 to regulate chondrogenesis (Selleri, 2001). An examination of Pbx1/En-1 compound mutants would be informative in elucidating this possibility.

In summary, we propose a model for the regulation of chondrocyte differentiation in which En-1 participates in hampering local differentiation-promoting signals that emanate from Ihh-expressing cells. By reducing Hh-responsiveness while permitting PTHrP signaling, En-1 may negatively regulate the rate of prehypertrophic to hypertrophic conversion and thus ensure the timely progression of chondrocytes into their terminal state. In addition, En-1 may affect the chondrogenic phenotype in an Ihh-independent manner.

Figure 1. Nuclear localization and expression of En-1 protein. (A) A schematic representation of the cDNA encoding the En-1 protein that was subcloned into the pcDNA3 mammalian expression vector. EH1-EH5 represent the engrailed homology domains where the EH4 homeodomain (HD) is in black (B) Immunofluorescent detection of En-1 protein in COS-1 cells that were transiently transfected with En-1-pcDNA3 and compared to Cells expressing Ihh-*myc*. Whereas En-1 was localized to the nuclei of transfected cells, Ihh was detected primarily in the cytoplasm and perinuclear endoplasmic reticulum. (C) Western blot analysis following subcellular fractionation of En-1-transfected COS-1 cells. En-1 protein separated primarily to the nuclear fraction (**N**). The low levels of En-1 in the cytosolic fraction (**C**) are probably due to cross contamination as indicated by the profile of the cytoplasmic α-tubulin.

Figure 1

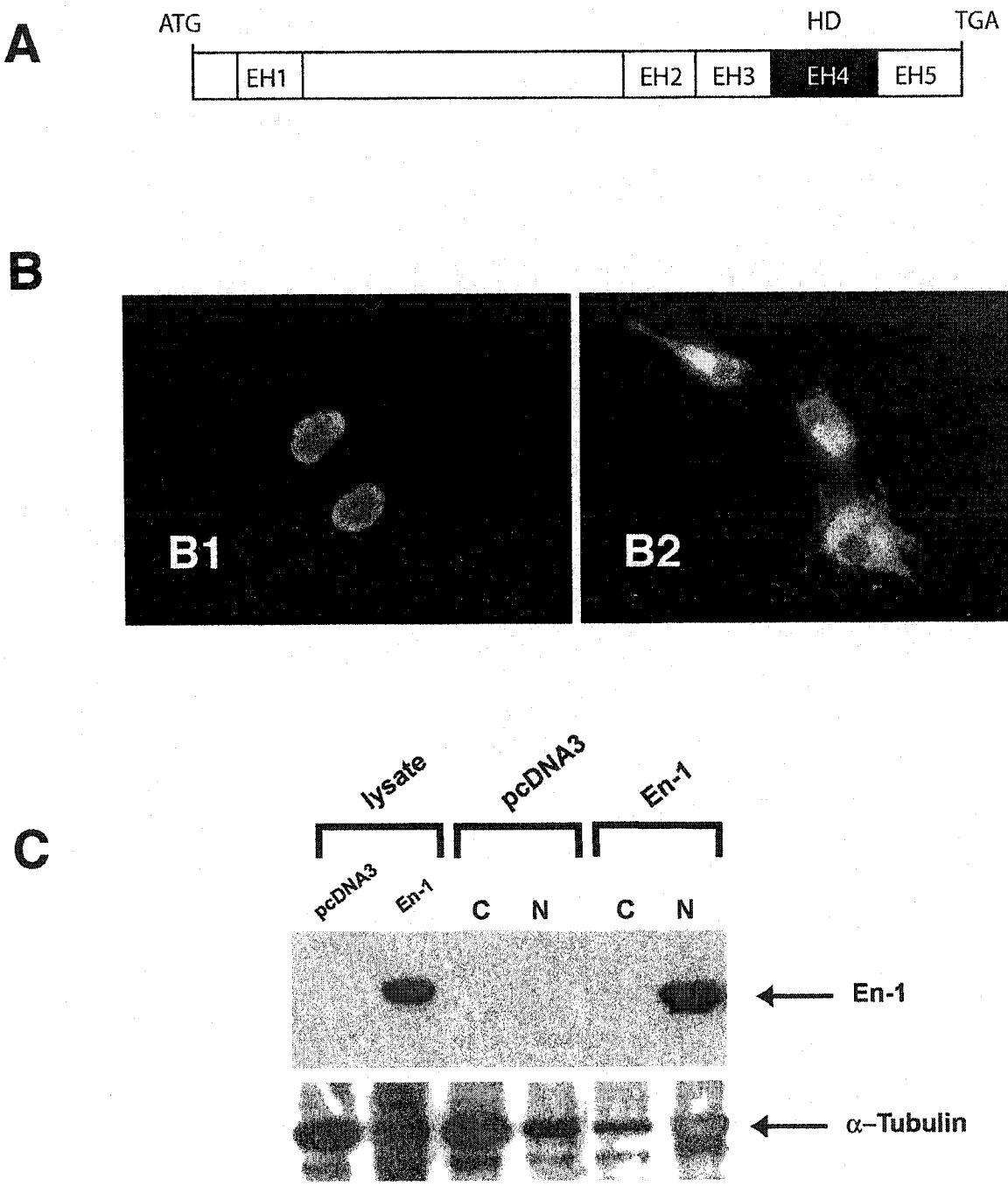

A
ATG
HD
TGA
EH1
EH2
EH3
EH4
EH5
B
B1
B2
C
lysate
pcDNA3
En-1
pcDNA3
En-1
C
N
C
N
En-1
α–Tubulin

Figure 2. En-1 alters chondrocytic differentiation in CFK-2 cells. Stably transfected CFK-2 cells expressing En-1 were compared to cells expressing N-Ihh or those transfected with vector control. (A) Comparison of alkaline phosphatase activity between stably trasfected CFK-2 cell populations expressing En-1, N-Ihh, or pcDNA3 vector, was assessed at days 1, 8, and 16 of postconfluent growth where cells were suscepted to gradual serum reduction (* $P \leq 0.05$; ** $P \leq 0.01$; n=3). (B) Northern blot analysis of *PTH1R* (upper panel) and *Col10a1* (lower panel) mRNA expression in En-1, N-Ihh, or pcDNA3-transfected CFK-2 cells over a 16 day postconfluent growth period. (C) Spectrophotometric measurement of sulfated glycosaminoglycan levels in stably trasfected CFK-2 cell populations expressing En-1, N-Ihh, or pcDNA3 vector, under similar growth conditions as in (A).

Figure 2

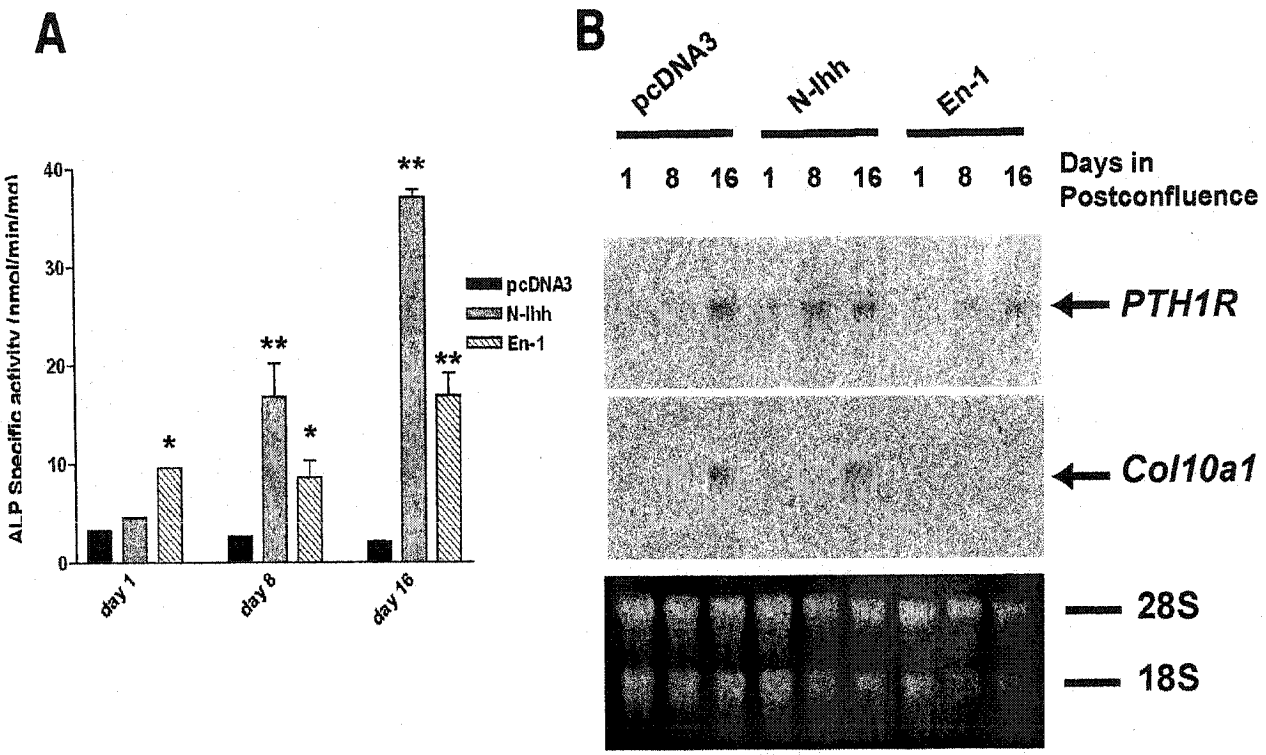

A
B
pcDNA3
N-Ihh
En-1
1 8 16 1 8 16 1 8 16
Days in Postconfluence
PTH1R
Col10a1
28S
18S
ALP Specific activity (nmol/min/mg)
40
30
20
10
0
pcDNA3
N-Ihh
En-1
day 1
day 8
day 16
*
**
C
HSPG (ng/μg protein)
125
100
75
50
25
0
5
10
15
20
pcDNA3
N-Ihh
En-1
Days in postconfluent culture

Figure 3. Repression of BSP promoter activity in CFK-2, MC3T3 and ROS-17 cells. (A) CFK-2 cells were transiently transfected with BSPPr-PXP-2 alone or co-transfected with En-1-pcDNA3. Luciferase activity was measured two (CFK-2, ROS-17) or seven (MC3T3) days following transfection and results are standardized against β-galactosidase expression (internal transfection control) and are depicted as fold stimulation over basal activity generated by PXP-2 vector alone. (B) BSP promoter activity in the presence or absence of En-1 expression in ROS-17 and MC3T3osteoblastic cells was measured 4 and 7 days following transfection, respectively.

Figure 3

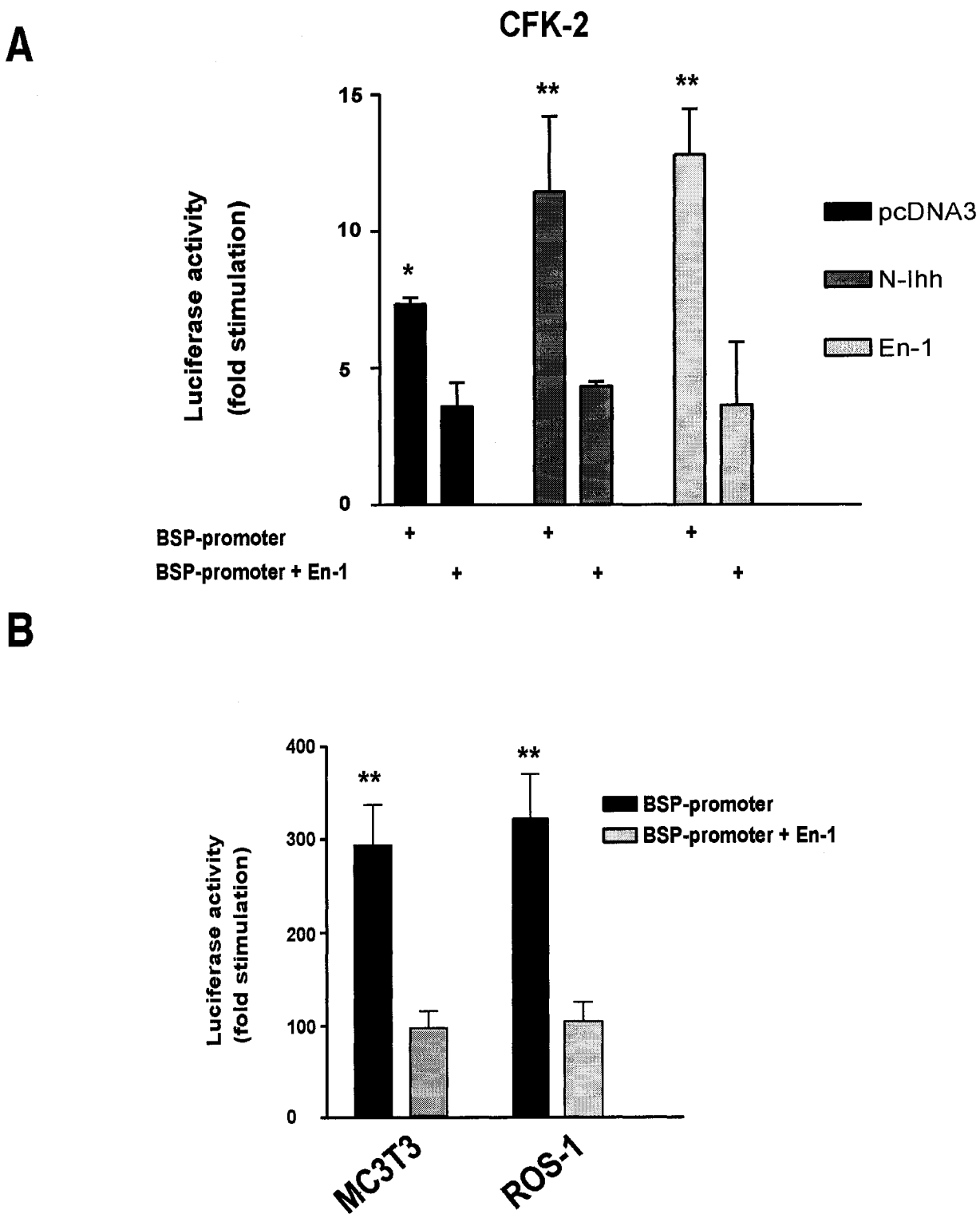

A
CFK-2
Luciferase activity
(fold stimulation)
15
10
5
0
*
**
**
pcDNA3
N-Ihh
En-1
BSP-promoter
BSP-promoter + En-1
+
+
+
+
+
+
B
Luciferase activity
(fold stimulation)
400
300
200
100
0
**
**
BSP-promoter
BSP-promoter + En-1
MC3T3
ROS-1

Figure 4. Immunohistochemical detection of En-1 protein in growth plate chondrocytes of newborn mice. En-1 protein expression was detected by immunohistochemistry in cells within the mid-hindbrain junction (A- negative control, B). Examination of growth plate chondrocytes from tibia (C-negative control, D) and vertebrae (E) revealed that En-1 localizes to prehypertrophic and some hypertrophic cells. By comparison, Ihh expression in the tibial (F) and vertebral (G) growth plate was primarily localized to prehypertrophic cells and partially overlapped with En-1 expressing cells.

Figure 4

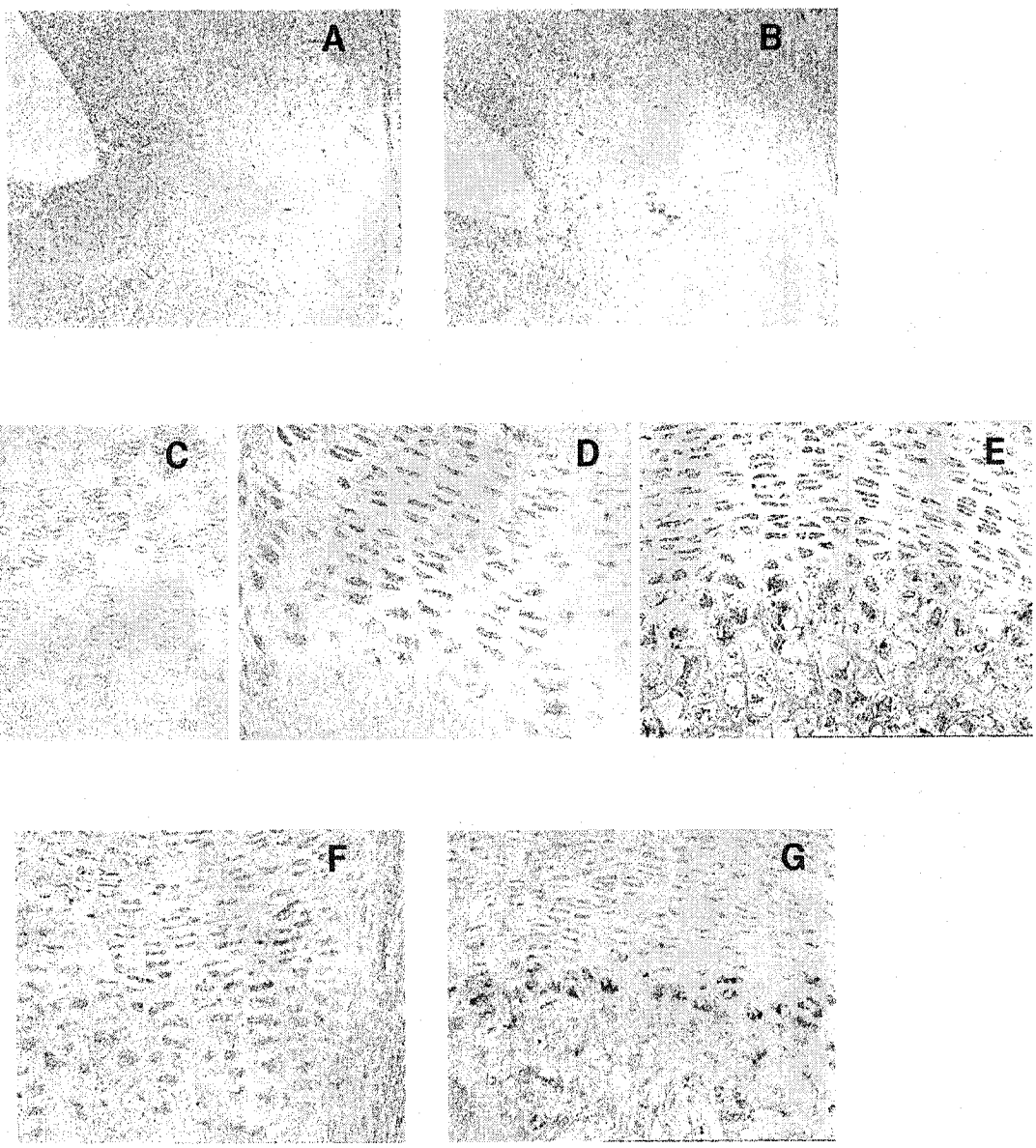
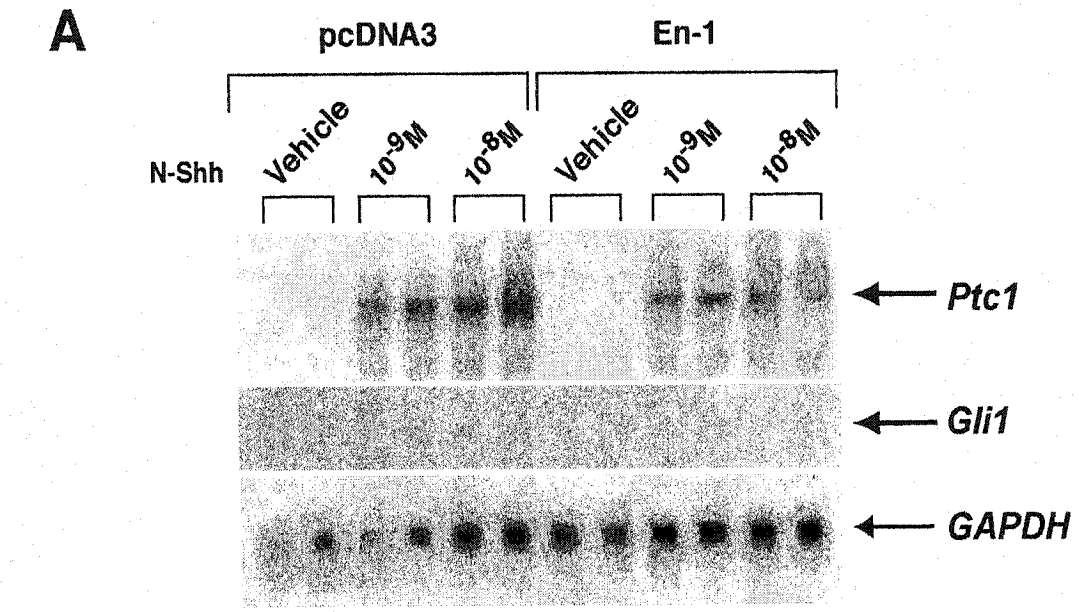
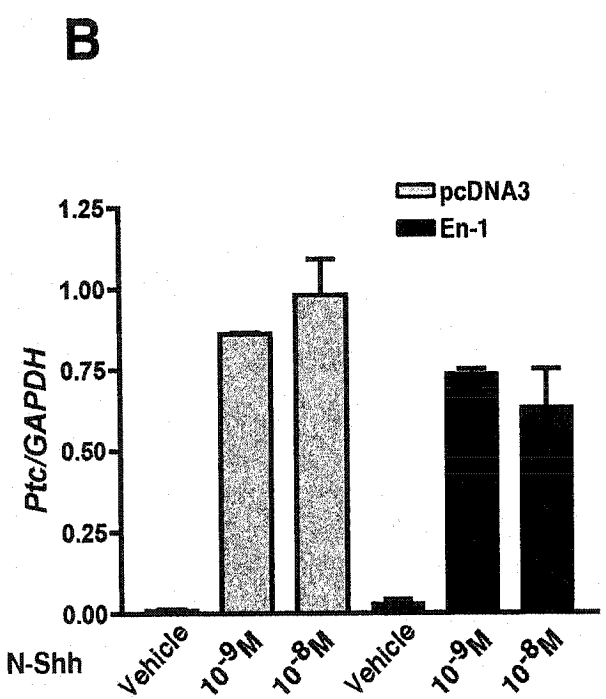


Figure 5. En-1 antagonizes HH-signaling in CFK-2 cells. CFK-2 cells stably transfected with pcDNA3 or En-1-pcDNA3 were subjected to exogenous treatment by recombinant N-Shh peptide at 10^{-9}M, 10^{-8}M or with vehicle alone over a period of 8 days during postconfluent growth. (A) Northern blot analysis of *Ptc1* mRNA expression in En-1- or pcDNA3-transfected CFK-2 cells was assessed following the regimen described above. (B) Quantification of *Ptc1* mRNA expression shown in (A).(C) ALP activity was assessed in En-1- or pcDNA3-transfected CFK-2 cells in response to N-Shh treatment and is expressed as fold stimulation over the activity in vehicle-treated cell.

Figure 5


A
pcDNA3
En-1
N-Shh
Vehicle
10-9M
10-8M
Vehicle
10-9M
10-8M
Ptc1
Gli1
GAPDH


B
pcDNA3
En-1
Ptc/GAPDH
1.25
1.00
0.75
0.50
0.25
0.00
N-Shh
Vehicle
10-9M
10-8M
Vehicle
10-9M
10-8M

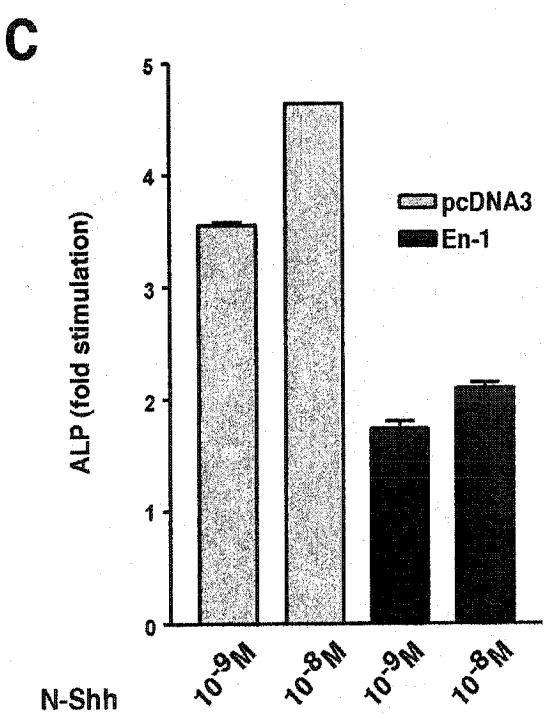
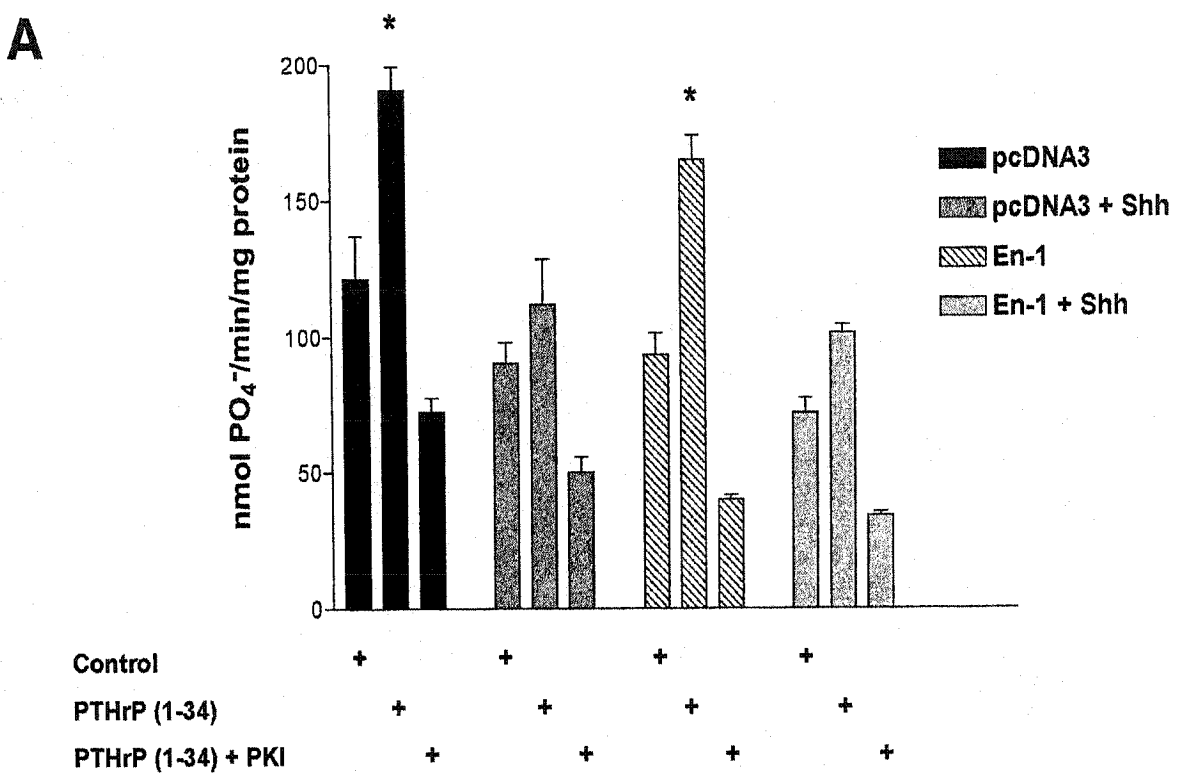

C
ALP (fold stimulation)
5
4
3
2
1
0
pcDNA3
En-1
N-Shh
10-9M
10-8M
10-9M
10-8M

Figure 6. En-1 does not affect PTHrP-responsiveness in CFK-2 cells. Stable populations of En-1 or pcDNA3-expressing CFK-2 cells were grown for 7 days in the presence or absence of 10^{-8}M N-Shh peptide. Following 24 hours of serum starvation cells were suscepted to treatment with 300μM IBMX (control) in the presence or absence of 10^{-8}M PTHrP (1-34) for a period of 20 minutes. Protein kinase A (PKA) activity, depicted as nmol phosphate transferred to the kemptide substrate per unit time and protein, was assessed in crude lysates of treated cells. As a control for kinase specificity, the activity in PTHrP-treated cells was assessed in the presence of a PKA inhibitor peptide (PKI). (B) Northern blot analysis of *PTH1R* expression in En-1- or pcDNA3-transfected cells in response to increasing concentrations (10^{-9}M and 10^{-8}M) of N-Shh peptide. En-1 appears to inhibit HH-mediated induction of *PTH1R* mRNA.

Figure 6


A
*
*
200
150
100
50
0
nmol PO4-/min/mg protein
pcDNA3
pcDNA3 + Shh
En-1
En-1 + Shh
Control
PTHrP (1-34)
PTHrP (1-34) + PKI
B
pCDNA3
En-1
N-Shh
Vehicle
10-9M
10-8M
Vehicle
10-9M
10-8M
PTH1R
GAPDH

Chapter 4

Inhibition of glycosaminoglycan sulfation impedes hedgehog signaling and alters differentiation of CFK-2 chondrocytic cells

Abstract
Heparan sulfate proteoglycans (HSPG) play an important role in maintaining the structural integrity of cartilage and in mediating crucial signal transduction pathways pertaining to chondrocyte proliferation and differentiation. In this study, we assessed the functional role of glycosaminoglycans (GAG) sulfation in regulating signaling by the developmental morphogen *Hedgehog* (Hh) in cultured CFK-2 rat chondrocytic cells. Inhibition of GAG sulfation by chlorate treatment in cells constitutively expressing Indian hedgehog (Ihh) or its NH_2-terminal domain resulted in strong inhibition of Hh-signaling as characterized by decreased alkaline phosphatase (ALP) activity and *Ptc1* receptor mRNA expression. These effects were mimicked by the anionic compound heparin and also recapitulated by subjecting naive cells to exogenous recombinant Hh protein in the presence or absence of chlorate, in agreement with a role for sulfated GAG moieties in enhancing Hh signaling. Constitutive expression of the heparan sulfate synthesis genes, *EXT1* and *EXT2* (*exostosins*), observed in CFK-2 cells and in growth plate chondrocytes adds further support to the suggestion that HSPGs, modified by exostosins, participate in mediating Hh -signaling during endochondral ossification.

Introduction

Heparin sulfate (HS) polysaccharides modify the structure of cell-surface proteoglycans and ascribe biochemical properties that render these proteins critical for a plethora of biological events ranging from cell adhesion to proliferation and differentiation (Bernfield, 1999). In cartilage, heparan sulfate proteoglycans (HSPG) are crucial for the structural integrity of this tissue, and in addition, participate in modulating receptor-ligand interactions in a number of developmental signaling pathways. Illustrating their importance is the HSPG perlecan, expressed abundantly in cartilage, and whose ablation in mice or humans results in a severe form of chondrodyplasia (Costell, 1999; Arikawa-Hirasawa, 2001).

Recent findings have demonstrated that signaling by the hedgehog (HH) family of secreted morphogens is modulated by HSPGs. In *Drosophila*, the gene *tout velu* (*ttv*) that encodes for a glucoronic acid (GlcA)/N-acetyl-glucosamine (GlcNAc) glycosyltransferase (HS polymerase, henceforth) is essential for HH ligand distribution (Bellaiche, 1998; The, 1999; Toyoda, 2000). In mammals, *ttv* has three known homologues that are members of the *EXT* gene family (*EXT1*, *EXT2* and *EXT3*, encoding for exostosin proteins) and human mutations are associated with the multiple exostoses syndrome (HME), characterized by bone outgrowths (exostoses) and a higher risk for chondrosarcomas and osteosarcomas (Ludecke, 1995; Wuyts, 1995; Le Merrer, 1994). Of these, EXT1 and EXT2 have been cloned and shown to possess GlcA- and GlcNAc-transferase activity (Stickens, 1996; Lind, 1998; Ahn, 1995). Interestingly, EXT1 and EXT2 physically associate and accumulate within the Golgi apparatus where they comprise part of a HS polymerase multienzyme complex (McCormick, 2000).

During the course of development, EXT1 and EXT2 are expressed virtually in every tissue with particularly high levels in the developing limb and skeleton where they specifically localize to chondrocytes and osteoblasts (Lin, 1998; Stickens, 2000). The wide distribution of *EXT* gene expression and the ubiquitous abundance of HSPGs in many cell types reflect the severe phenotype resulting from *Ext1* ablation in mice (Lin, 2000). *Ext*1-/- animals present with early embryonic lethality resulting from perturbed gastrulation and lack of mesoderm and extraembryonic tissues. Heterozygous mice, although they do not recapitulate the human haploinsufficient condition observed in HME, display a 10% decrease in bone growth associated with a 50% reduction in HS synthesis. Interestingly, chondrocytes harboring mutations in EXT1 or EXT2 are deficient in HS synthesis and display inappropriate cytoskeletal accumulation of actin filaments, high levels of the hypertrophic marker *Col10a1*, and premature calcification (Bernard 2000; Legeai-Mallet, 2000; Hecht, 2002).

During endochondral bone formation Indian hedgehog (Ihh) plays a critical role in coordinating the rate of chondrocyte proliferation and differentiation. Expressed by a subpopulation of prehypertrophic growth plate chondrocytes, Ihh upregulates the expression of parathyroid hormone-related peptide (PTHrP) in the articular perichondrium, which in turn acts upon and retards further differentiation of maturing chondrocytes to the hypertrophic state (Vortkamp, 1996; St-Jacques, 1999; Karp, 2000). The mechanism by which Ihh relays signaling to articular chondrocytes separated by several cell diameters from the prehypertrophic layer, is not fully understood but appears to partly involve the activation of TGFß in the perichondrium (Alvarez, 2002). The possibility that EXT1 and EXT2 may serve functions parallel to ttv in regulating the

distribution of Ihh protein in the growth plate has not been explored. Analogous to the situation in *Drosophila*, EXT1 is necessary for HH binding to the surface of target cells, indicating that mammalian hedgehog proteins may require HSPGs for receptor interactions as well as for movement across multicellular fields (Lin, 2000).

In this study, we addressed the question of whether sulfated proteoglycans are essential for HH-signaling during chondrocytic differentiation. Employing the rat CFK-2 chondrocytic cell line, shown previously to be responsive to both HH and PTHrP ligands, we demonstrate that inhibition of proteoglycan sulfation by chlorate ion ($NaClO_3$) treatment results in perturbed HH-responsiveness. In addition, we observed that chondrogenic markers of differentiation (alkaline phosphatase, *Col10a1*) that are induced by Ihh overexpression or by exogenous HH were altered in chlorate treated cells. Moreover, *in situ* data presented here demonstrates *EXT1* and *EXT2* transcripts in growth plate chondrocytes. Taken together, these findings indicate that Ihh may utilize particular proteoglycans whose heparan sulfate modification by EXT1 and EXT2 is required for appropriate signaling and for regulation of differentiation.

Materials and Methods

Construction of mammalian expression plasmids- Full-length *Ihh* cDNA (2103 bp) inserted in the pcDNA3 mammalian expression vector (Ihh-pcDNA3) was a gift from L. Holzman (University of Michigan). To express the amino terminal portion of Ihh (N-Ihh), a 720-bp fragment encoding this region of the protein was amplified by PCR using 5'[GCCCCCGCATGGAAGTCCCC]-3' as the sense primer and 5'-[TCAGCCACCTGTCTTGGCAGC]-3' as the antisense primer. This fragment was cloned into pcDNA3 for mammalian cell expression.

Cell culture and transfections- CFK-2 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco BRL) supplemented with 10% fetal bovine serum (Wisent). To induce differentiation, cells were grown to confluence in 10% FBS after which serum was gradually reduced by 2% decrements every two days, and were maintained at 2% FBS thereafter for post confluent growth. In some experiments, medium was supplemented with 5 mM or 50 mM $NaClO_3$ (Sigma), heparin (10mg/ml), or varying concentrations of recombinant N-Shh (Curis, Inc., Cambridge, MA). N-Shh was modified by an addition of a hydrophobic eight-carbon chain (octyl group) to the NH2-terminal cysteine and this alteration is reported to increase the biological activity of the native peptide by up to 10-fold. N-Shh protein was suspended in "Octyl" buffer (PBS pH 7.2, 50 μM DTT conjugated to N-octylmaleimide, 350 μM free DTT and 0.5% DMSO) and this buffer was also used as vehicle control, where indicated.

Generation of stably transfected CFK-2 cell populations was performed by electroporation. Cells were grown on 10 cm dishes and upon reaching 60% confluence were washed once in PBS and trypsinized. Cells were then suspended in HBS (20 mM HEPES

pH 7.4, 0.14 M NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 25 mM glucose, 1 mM $CaCl_2$) at a density of 5×10^5 cells/ml, supplemented with plasmid DNA (2 μg), and electroporated at 240V/ 500μF. Following 48 hours of recovery, cells were subjected to selection by the addition of G418 at a final concentration of 500 μg/ml. Selection proceeded for ten days and subsequently cells were maintained in G418 (500 μg/ml).

Northern blot analysis- Total RNA was obtained from cell monolayers by guanidium isothiocyanate (GTC)/CsCl extraction. Briefly, cells were washed once with PBS,homogenized with 0.5 ml of GTC (4 M guanidium isothiocyanate, 0.1 M Tris-HCl pH 7.5, 1% β-mercaptoethanol), and passaged through a 25-gauge needle. Homogenates were layered on top of a 5.7 M CsCl / 0.01 M EDTA cushion and ultracentrifuged overnight at 32 000 rpm in a SW40 rotor. Pellets were washed with 70% ethanol and suspended in DEPC-treated water. Alternatively, RNA was extracted by Trizol, as specified by the manufacturer (Gibco BRL).

Typically, aliquots (20 μg) of total RNA were size fractionated on a 1.5% agarose/formaldehyde gel and transferred overnight onto a supported nitrocellulose membrane using 20 X SSC buffer (3 M NaCl, 0.003 M Na citrate, pH 7.0). Hybridization of membranes to ^{32}P-labeled probes was performed in a buffer containing 40% formamide, 10% dextran SO_4, 4 X SSC, and 1X Denhardt's blocking solution with 0.1 mg/ml salmon sperm DNA. Membranes were washed once in 2 X SSC/0.1% SDS at room temperature and once in 0.1 X SSC/0.1% SDS for 15 minutes at 58°C before exposing to film.

All probes were radiolabeled by the random priming method (Roche). Probes corresponding to the NH_2 and COOH terminus of Ihh were generated by PCR using the

primers described in Chapter 2. A 390-bp probe corresponding to *Ptc1* cDNA and verified by direct DNA sequencing was generated by RT-PCR from total RNA extracted from CFK-2 cells. The oligonucleotides used to obtain this fragment were 5'-[GGACTTCCAGGATGCCATTTGACAGTG]-3' as the sense primer and 5'-[GCCGTTGAGGTAGAAAGGGAACTG]-3' as the antisense primer and were based on the mouse *Ptc1* cDNA sequence. A 587-bp and 452-bp probes corresponding to EXT1 and EXT2 cDNAs, respectively, and verified by DNA sequencing were generated by RT-PCR from total RNA extracted from CFK-2 cells. The oligonucleotides used were 5'-[GGAAGTGGATTTTGCCTTCA]-3' and 5'-[CAAAATGCCTGGAGACATCA]-3' as the sense primers, and 5'-[TATTCATGCAGCTCTGTCGC]-3' and 5'-[TGTTGGGGAAGCTCTTCAGT]-3' as the antisnense primer for EXT1 and EXT2, respectively.

In situ hybridization for EXT1 and EXT2 expression in bone - cDNA probes for rat EXT1 and EXT2 were subcloned into pcDNA3 in the sense and anti-sense orientation and verified by direct DNA sequencing. Following plasmid linearization with SacI or ApaI (for T7 and SP6 promoter usage, respectively), RNA probes were synthesized using the SP6/T7 MAXIscript kit (Ambion). Labeling of the RNA probes was performed by including biotin-16-UTP in the reaction mix. Probes were subsequently size fractionated on a 5% polyacrylamide denaturing (urea) gel and extracted at 37°C by overnight incubation in probe elution buffer (0.5M NH_4-acetate, 1mM EDTA, 0.2% SDS). RNA probes were precipitated with EtOH and suspended in RNAse-free water. *In situ* hybridization was performed on deparafinized sections of long bone from newborn mice using the mRNA *locator* kit (Ambion).

Measurement of sulfated glycosaminoglycans (GAG)- Quantitative assessment of cell surface GAG expression was determined spectrophotometrically, as previously described (Farndale, 1982). Briefly, cells grown to confluence in 6-well plates were scraped into 300 µl of 50 mM phosphate buffer (pH 6.5) containing 2 mM N-acetyl cysteine and 2 mM EDTA and subjected to papain digestion (300 µg/ml) for 1 hour at 65°C. Following the removal of cellular debris by brief centrifugation, 100 µl aliquots were analyzed by mixing with 400µl of DMB solution (prepared as a stock of 16 mg 1,9-dimethylmethylene blue in 5 ml ethanol, 2 g sodium formate, and 2 ml of formic acid brought to1L with water), and immediately measuring absorbance at 535 nm. Absorption values were plotted against a standard curve generated using incremental concentrations of bovine chondroitin sulfate (Sigma).

ALP activity assay- Cell monolayers were washed with PBS, lysed in 300 µl ALP lysis buffer (0.15 M Tris pH 9.0, 0.1 mM $ZnCl_2$, 0.1 mM $MgCl_2$), and subjected to one freeze-thaw cycle at -70°C. Lysates were cleared by a 10-min centrifugation at 10 000 × g and 50 µl aliquots were analyzed spectrophotometrically at 410 nm with ALP assay solution (7.5 mM p-nitrophenyl phosphate (Sigma reagent 104), 1.5 M Tris pH 9.0, 1 mM $ZnCl_2$, 1 mM $MgCl_2$). Protein concentrations were determined by the method of Lowry using the Bio-Rad DC protein assay kit (Bio-Rad).

Results

Inhibition of sulfation reduces HH activity in Ihh- and N-Ihh-expressing CFK-2 cells

To address the functional relationship between HSPGs and HH-signaling during chondrocytic differentiation we first examined the effect of chlorate ($NaClO_3$) treatment on populations of CFK-2 cells stably transfected with pcDNA3, Ihh, or N-Ihh. As varying concentrations of chlorate were shown to affect 2-*O*-sulfation, 6-*O*-sulfation, or N-sulfation differentially (Safaiyan, 1999), we first subjected CFK-2 cells to either 5 mM or 50 mM $NaClO_3$ for a period of 7 days in post confluent culture and evaluated the levels of sulfated GAG expressed by comparison to untreated cells (Fig 1A). Using a spectrophotometric assay for measuring sulfated GAGs, we observed an appreciable decrease in their expression in all stably transfected CFK-2 populations that underwent treatment with 50 mM $NaClO_3$. On the other hand, subjecting these cells to 5 mM had no detectable effect (not shown). To evaluate the status of Hh-responsiveness under these conditions we examined the extent of *Ptc1* mRNA upregulation (Fig 1B). We observed that *Ptc1* mRNA in Ihh or N-Ihh transfectants was strongly inhibited following 50 mM chlorate treatment but to a much lesser extent after 5 mM treatment. Taken together, these results suggest that sulfated moieties on HSPGs play an integral part in mediating full Hh signal activation. As alkaline phosphatase (ALP) activity was previously shown to be activated in Ihh- or N-Ihh- stable CFK-2 transfectants, we next examined the effects of chlorate on its Hh-mediated induction (Fig 1C). Whereas treatment of cells with 5 mM chlorate had little effect on ALP induction following the 7 day postconfluence period, subjecting cells to 50 mM levels diminished ALP activity by at least 50% in Ihh and N-Ihh

transfectants. Interestingly, the polyanionic compound heparin which sequesters the Hh ligand from binding to the extracellular matrix (Bumcrot, 1995) also resulted in strong inhibition of ALP activity when added to culture medium of CFK-2 cells stably transfected with N-Ihh (Fig 1D). By comparison, En-1-transfected cells exhibiting elevated ALP activity were not susceptible to inhibition by heparin. As ALP activity depicts an indirect consequence of Ihh expression, we also examined *Ptc1* receptor mRNA expression as a direct indicator for HH-signaling (Fig. 1D).

Inhibition of sulfation perturbs activation of ALP and *Ptc1* expression by exogenous N-Shh

Studies in *Drosophila* suggest that HSPGs function in regulating Hh ligand distribution but may not necessarily be required for receptor binding, a distinction that is difficult to make using stably transfected cells. In order to evaluate if sulfated GAGs are involved directly in modulating HH signaling, we subjected cultured naive CFK-2 cells to increasing concentrations of recombinant NH_2-terminal Shh in the presence or absence of 50 mM chlorate. Similar to stably transfected cells, naive cells exhibited a 50-60% reduction in sulfated GAG levels following chlorate treatment (Fig. 2A). We also noted an increase in GAG expression as a result of Shh treatment but this has not been consistently confirmed. In correlation with sulfation levels, the dose-responsive upregulation of ALP activity by Shh was strongly abrogated by chlorate treatment, demonstrating a consistent inhibition of Hh-induced differentiation (Fig. 2B). Indicative of a direct inhibition of HH-signaling, we observed that chlorate-mediated inhibition of GAG sulfation diminished the capacity of Shh peptide to upregulate *Ptc1* mRNA expression. As activation of Hh-

signaling by exogenous Shh peptide appeared to be similarly susceptible to inhibition by chlorate, these findings are in agreement with the proposed role of sulfated glycosaminoglycan moieties in the mediation of the Hh signal and may actually participate in receptor-ligand interactions.

HSPGs may also mediate negative regulation on HH signaling

Our previous studies have shown that HH signaling in CFK-2 cells induces the expression of chondrogenic markers such as ALP, *Col2a1*, and *Col10a1*, signifying a tendency toward the hypertrophic state of differentiation (Deckelbaum *et al.*, Cell Sci 2002 Jul 15). Since inhibition of GAG sulfation by chlorate led to a decrease in *Ptc1* expression and ALP activity, we anticipated that, other markers of differentiation would be similarly affected. Surprisingly, we observed that HH-upregulation of *Col10a1* mRNA expression was augmented rather than suppressed following chlorate treatment of cells in the presence of Shh peptide (Fig. 3). Thus, it appears that in addition to promoting HH-signaling, sulfated GAGs or HSPGs may play a role in actively suppressing particular downstream effects of HH activity.

Expression of endogenous *Ext1* and *Ext2* in CFK-2 cells and growth plate chondrocytes

As chlorate treatment of cells results in global reduction in GAG sulfation levels, it is possible that signaling pathways other than Hh, which utilize HSPGs, may have consequently been affected (i.e. FGF, Wnt). To address this issue, we hypothesized that since *ttv* synthesizes a particular heparan sulfated moiety required specifically for Hh

signaling (The, 1999), a parallel function might be mediated by EXT1 and/or EXT2 in mammals. Studies have yet to describe, however, a direct functional relationship between Hh action and EXT family members. Therefore, we first examined mRNA expression profile of *EXT1* and *EXT2* in CFK-2 cells and assessed how these change in response to Hh-signaling. Northern analysis revealed abundant expression of both *EXT1* and *EXT2* in stably transfected CFK-2 cells with no significant variation between pcDNA3, Ihh, or N-Ihh-transfectants (Fig. 4A).Furthermore, treatment of naive CFK-2 cells with increasing concentrations of Shh peptide had no appreciable effect on *EXT1/EXT2* transcript levels(Fig 4B). In addition, supplementing the medium with 50 mM chlorate had no effect on *EXT1/EXT2* expression. Thus, CFK-2 cells express high constitutive levels of *EXT1* and *EXT2* mRNA, which are not modulated by Hh-signaling or inhibition of GAG sulfation.

Due to the unavailability of antibodies to EXT1 or EXT2, we assessed their mRNA expression in the growth plate of newborn mice by *in situ* hybridization using anti-sense probes. By comparison to cells probed with sense-control RNA (Fig 5 A and B, lower panels), expression of *Ext1* and *Ext2* localized in an overlapping fashion to prehypertrophic and hypertrophic chondrocytes (Fig 5A and B, upper panels). Furthermore, proliferating epiphyseal chondrocytes stained extensively for both *Ext1* and *Ext2* mRNA. In addition to growth plate chondrocytes, examination of the trabecular bone revealed that *Ext1* and *Ext2* transcripts also localized to osteoblasts of the primary spongiosa as well as to osteoclasts (Fig 5A and B, data not shown). These findings are in agreement with previous descriptions of *Ext1* and *Ext2* expression in the growth plate and

provide preliminary information on the potential modulation they may exert on Hh-signaling particularly, as mediated by Ihh.

Discussion

In this study, we provide insights on the functional importance of HSPGs to Hh-signaling and how these may collaborate to regulate chondrocytic differentiation. The chlorate ion is a competitive inhibitor of ATP sufurylase, the enzyme catalyzing the formation of the high energy sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS), required for the sulfation of heparan polysaccharides in the Golgi (Farley, 1978). Achieving effective inhibition of glycosaminoglycan sulfation without affecting vital cellular processes, chlorate has been widely used in studies examining sulfate-dependent ligand receptor interactions and cell adhesion (Bansal and Pfeiffer, 1994; Fannon and Nugent, 1996). We show that global inhibition of glycosaminoglycan sulfation *in vitro*, and consequently that of proteoglycans, leads to diminished Hh-signal responsiveness. This effect, evidenced by reduced Hh-mediated induction of ALP and *Ptc1*, was observed in both cells constitutively expressing Ihh or N-Ihh and in cells treated with exogenous Shh peptide. As heparin similarly impedes Hh-induced ALP, our findings provide evidence in support of a model in which heparan sulfated glycosaminoglycans participate in binding the Hh ligand and promoting, at least partially, its signaling capacity.

That HSPGs participate in binding growth and morphogenic factors, in some cases serving as co-receptors, was previously illustrated by studies on signaling by fibroblast growth factors (FGFs) and Wg/Wnt (reviewed by Ornitz, 2000; Baeg and Perrimon, 2000). Thus, heparan sulfated proteoglycans are required for regulating the distribution and local concentrations of FGFs and, through physical interactions with FGF ligands, they also increase their binding specificity and affinity toward their receptors (FGFRs). These biochemical properties are paralleled by *in vivo* data that demonstrate perturbed

FGF-mediated biological activity consequent to mutations in enzymes of the HS synthetic pathway (Lin, 1999). Similarly, Wg signaling in *Drosophila* requires the HSPG *Dally* for cooperative interaction and activation of its receptor *frizzled*. Moreover, this activity is disrupted by mutations in *sugarless*, the homologue of HS *N*-deacetylase/*N*-sulfotransferase required for modification of HS GAG (Lin and Perrimon, 1999).In contrast, signaling by Hh, but not Wg or FGF, is specifically affected by mutations to *ttv*, the activity of which is required for the movement of Hh across cell boundaries (The, 1999).

Results provided here, however, support the hypothesis that in a mammalian context, Hh activities are dependent in part on HSPGs, presumably synthesized by EXT1/2, for actual binding to cells and possibly the receptor *Ptc1*. Thus, under conditions of evenly distributed Hh ligand (exogenous addition of recombinant protein), inhibition of GAG sulfation resulted in perturbed Hh-induction of *Ptc1*. This is in agreement with immunohistochemical observations of hampered Ihh binding to cells from E6.5 embryos harboring an *EXT1*-ablation or to wild type counterparts following heparitinase treatment (Lin, 2000). In parallel, activation of ALP activity was similarly impeded by chlorate treatment or the addition of heparin to the medium, further substantiating interference in Hh-signaling. Interestingly, the apparent induction of *Col10a1* by Hh in chlorate-treated cells is in agreement with reports of increased and inappropriate distribution of *Col10a1* in chondrocytes from exostoses harboring *EXT1* or *EXT2* mutations (Legeai-Mallet, 2000). That this phenotypic aspect is recapitulated by chlorate treatment further supports the model by which inhibition of GAG sulfation mimics EXT1/EXT2 deficiency. One plausible explanation for this phenomenon is that Ihh, secreted by prehypertrophic chondrocytes,

has the potential to induce *Col10a1* expression in adjacent maturing cells and that an EXT1/2-synthesized HS may modulate its signaling capacity or magnitude to downregulate this effect. It is interesting that the regulation of ALP and *Col10a1* by Hh and HS are differentially coordinated, further suggesting that genes activated downstream of Ihh are regulated by distinct branches of its signaling pathway.

Despite the accumulating circumstantial evidence in support of an EXT1/2 function in the Hh-signaling pathway, there is a lack of direct experimental data to demonstrate this conclusively. That EXT1 and EXT2 are expressed in cells localizing to both proliferating and maturing growth plate chondrocytes, is in agreement with their characterization as components of a multi-enzyme glycosyltransferase (McCormick, 2000). The distribution in expression along the growth plate suggests that, similarly to *ttv*, EXT1/2 may also play a role in mediating active Ihh diffusion over a multitude of cell diameters thereby enabling induction of PTHrP in periarticular chondrocytes. As osteoblasts lining the trabecular bone also express EXT1/2 it is possible that their function may also be required for Ihh-dependent differentiation along this lineage (St-Jacques, 1999).

If EXT1/EXT2 mediate actions that exclusively affect the Hh-signaling pathway then identification of the involved HSPGs would be crucial. One likely candidate is perlecan, whose synthesis is reduced and its distribution is abnormal in exostoses derived from *EXT1/EXT2*-mutants (Hecht, 2002). In humans, frameshift mutations in the *perlecan* gene that result in premature truncation of the core protein are the cause of dyssegmental, Silverman-Handmaker, chondrodysplasia while mutations that affect its structure less severely are associated with a milder form of the disease (Arikawa-Hirasawa, 2002; Arikawa-Hirasawa, 2001). Mice homozygous for a deletion in the *perlecan* gene

recapitulate the human condition but also reveal the importance of this proteoglycan in the structural integrity of the growth plate (Costell, 1999). Since *Ext1*-null mice present with early embryonic lethality, this prevents the possibility of obtaining Ext1-non-expressing chondrocytes. With the objective of demonstrating a direct role for EXT1/EXT2 in Ihh-signaling we are currently testing various gene knock-down methods for obtaining chondrocytes with compromised EXT1/EXT2 expression.

Figure 1. Repression of hedgehog signaling in stably transfected CFK-2 cells by ClO_3^- ion treatment. Stably transfected CFK-2 cells expressing pcDNA3, N-Ihh or Ihh were cultured for 8 days postconfluence in the presence or absence of 5 mM or 50 mM $NaClO_3$. (A) Quantitative assessment of sulfated GAG expression in stable CFK-2 transfectants. Whereas at a concentration of 5 mM $NaClO_3$ there was no significant decrease in GAG levels (not shown), these were reduced by treatment at a concentration of 50 mM. (B) Expression of *Ptc1* receptor mRNA in pcDNA3, Ihh, and N-Ihh stable transfectants was examined following continuous ClO_3^- treatment. By comparison to pcDNA3-transfected cells, Ihh/N-Ihh induces *Ptc1* mRNA that is repressed by increasing concentrations of $NaClO_3$. (C) ALP activity in CFK-2 stable transfectants that were subjected to similar $NaClO_3$ treatment. Results indicate that $NaClO_3$ concentrations of 50 mM, but not 5 mM, are effective in reducing Ihh/N-Ihh-mediated ALP activity. (D) Hh-induction of ALP is inhibited following treatment by heparin (10 mg/ml) for 7 days of postconfluent growth. Note that cells transfected with En-1 did not exhibit a similar sensitivity to heparin.

Figure 1

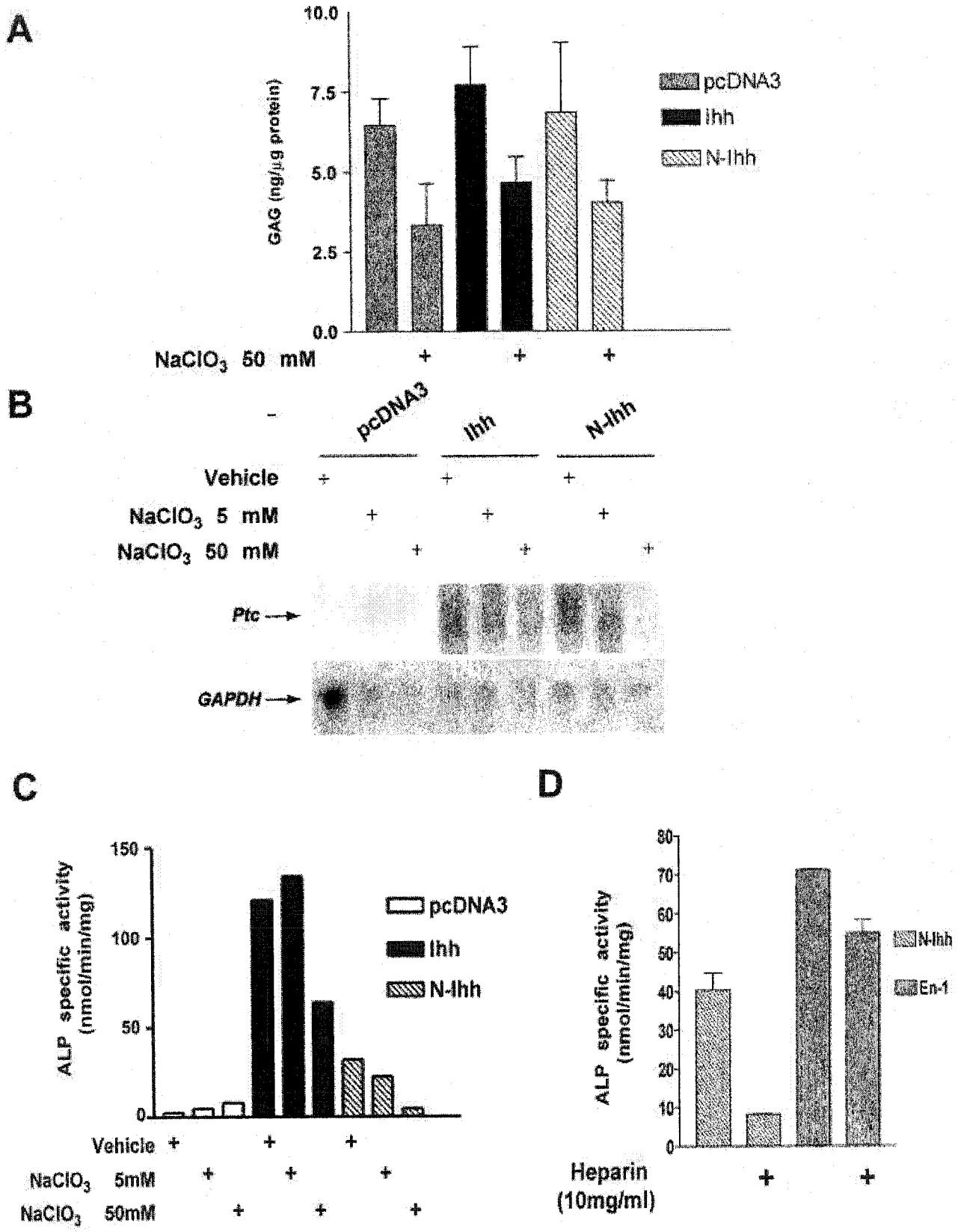
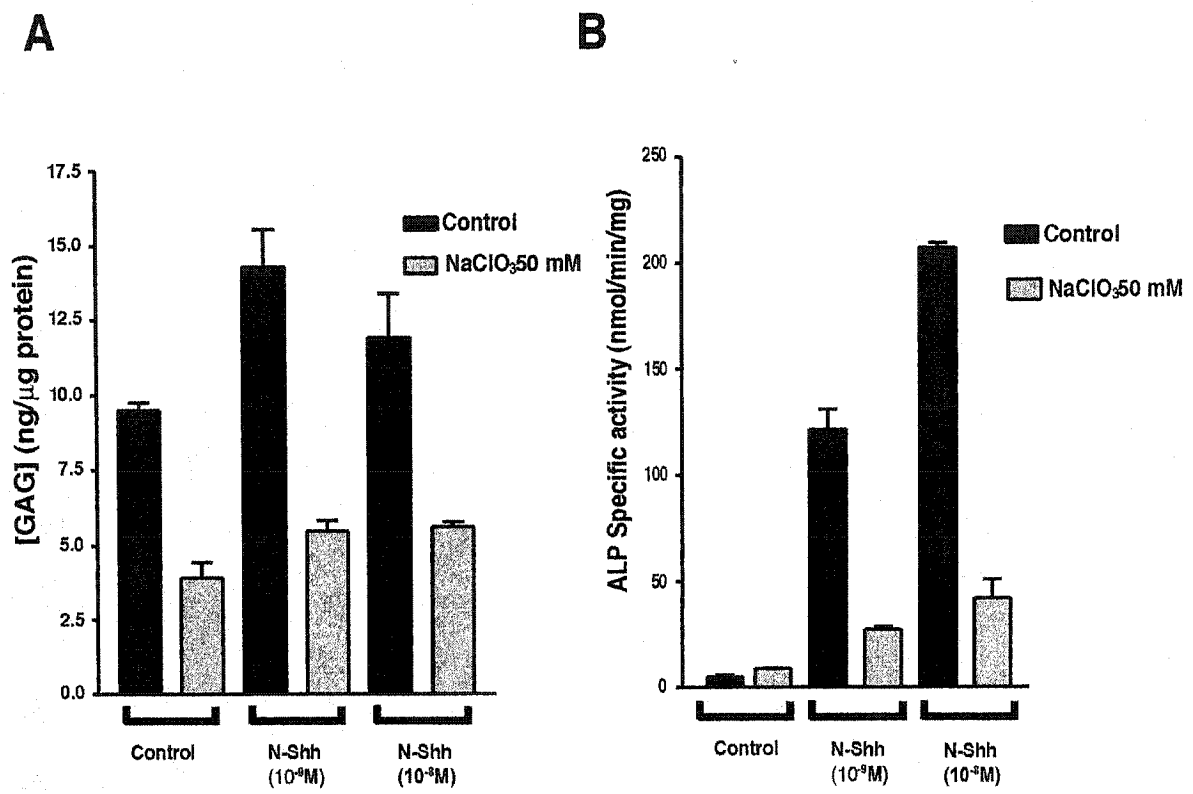

A
10.0
7.5
5.0
2.5
0.0
GAG (mg/μg protein)
pcDNA3
Ihh
N-Ihh
NaClO3 50 mM
+
+
+
B
pcDNA3
Ihh
N-Ihh
Vehicle
NaClO3 5 mM
NaClO3 50 mM
Ptc
GAPDH
C
150
100
50
0
ALP specific activity (nmol/min/mg)
pcDNA3
Ihh
N-Ihh
Vehicle
NaClO3 5mM
NaClO3 50mM
D
80
70
60
50
40
30
20
10
0
ALP specific activity (nmol/min/mg)
N-Ihh
En-1
Heparin (10mg/ml)

Figure 2. Inhibition of glycosaminoglycan sulfation in CFK-2 cells impedes signaling by exogenous hedgehog. Naive CFK-2 cells were subjected to 8 days of postconfluent growth in the presence of recombinant N-Shh (10^{-9} M or 10^{-8} M) with or without the addition of 50 mM ClO_3^- ion and were tested for HH-responsiveness by assessing ALP activity and *Ptc1* receptor expression. (A) Spectrophotometric quantification of sulfated GAG levels in CFK-2 cells following the above described treatment. As depicted, the addition 50 mM ClO_3^- into the culture medium led to an approximate 50% reduction in sulfated GAG levels in both N-Shh-treated and non-treated cells. The apparent increase in GAG levels following N-Shh treatment (shown here in the absence of ClO_3^-) was not always consistent. (B) N-Shh-mediated activation of ALP activity in CFK-2 cells was assessed following the described treatment. The dose-dependent increase in ALP following N-Shh treatment was abolished when cells were grown in the presence of 50 mM ClO_3^-. (C) Northern blot analysis of *Ptc1* receptor expression in cells subjected to the above treatment. When grown in the absence of ClO_3^- ion, cellular levels of *Ptc1* mRNA increase in a dose-dependent fashion upon exposure to increasing concentrations of recombinant N-Shh. A significant, yet incomplete, downregulation of *Ptc1* mRNA levels was observed in ClO_3^--treated cells.

Figure 2


A
B
[GAG] (ng/μg protein)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Control
NaClO3 50 mM
Control
N-Shh (10-9M)
N-Shh (10-8M)
ALP Specific activity (nmol/min/mg)
250
200
150
100
50
0
Control
NaClO3 50 mM
Control
N-Shh (10-9M)
N-Shh (10-8M)

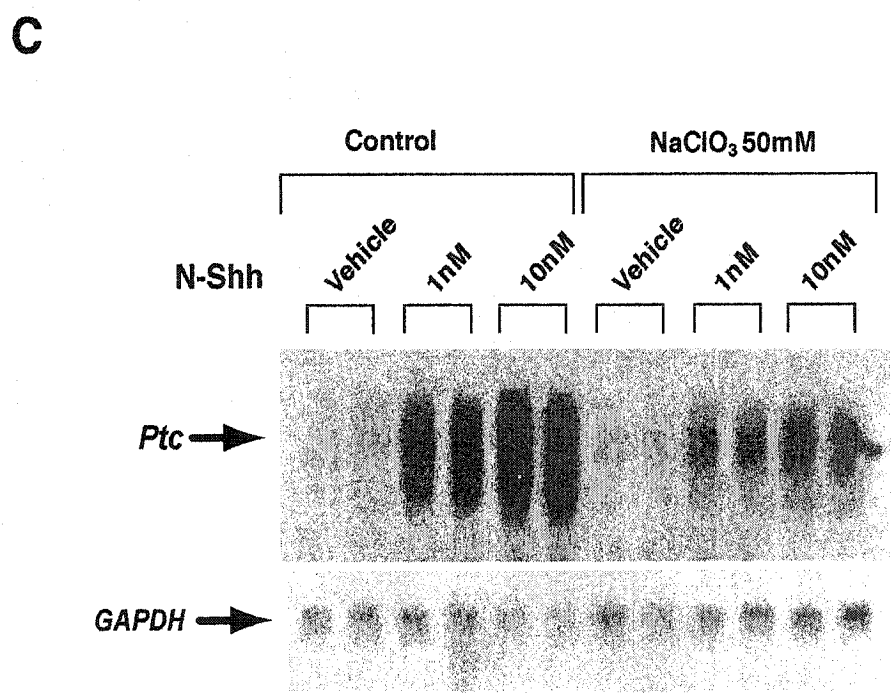

C
Control
NaClO3 50mM
N-Shh
Vehicle
1nM
10nM
Vehicle
1nM
10nM
Ptc
GAPDH

Figure 3. Induction of *Col10a1* mRNA by hedgehog is enhanced by inhibition of GAG sulfation. CFK-2 cells subjected to increasing concentrations of Shh recombinant peptide in the presence or absence of 50 mM chlorate were examined for *Col10a1* mRNA expression. Elevated *Col10a1* levels following chlorate treatment indicate a role for HSPGs in downregulating hypertrophic differentiation.

Figure 3

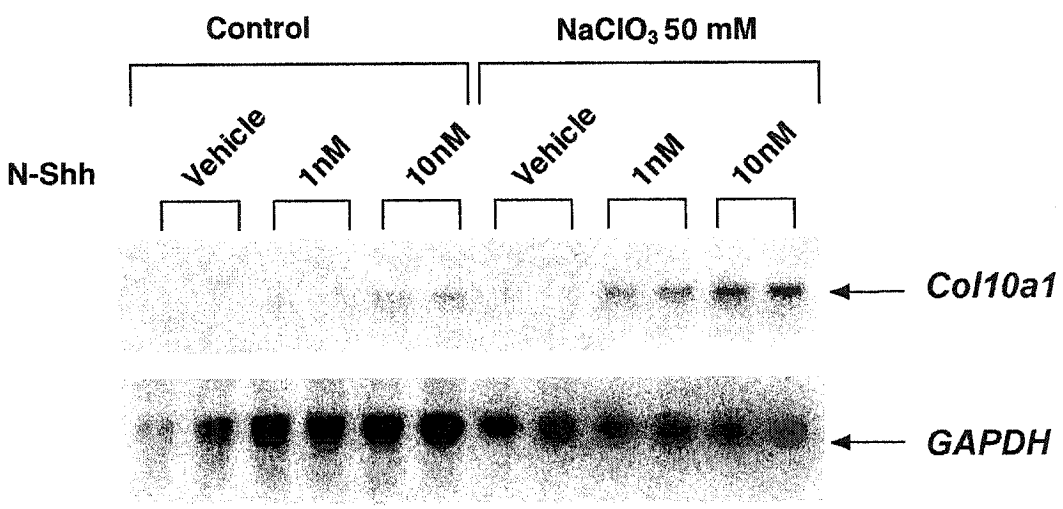

Control
NaClO3 50 mM
N-Shh
Vehicle
1nM
10nM
Vehicle
1nM
10nM
Col10a1
GAPDH

Figure 4. Endogenous expression of *Ext1* and *Ext2* in CFK-2 cells. (A) Northern blot analysis for the expression of *Ext1* and *Ext2* transcripts was performed in CFK-2 cells stably transfected with pcDNA3 empty vector Ihh, or N-Ihh over a 16-day postconfluency culture period . (B) *Ext1* and *Ext2* mRNA expression in naïve CFK-2 cells treated for 7 days with increasing concentrations of Shh in the presence or absence of chlorate.

Figure 4

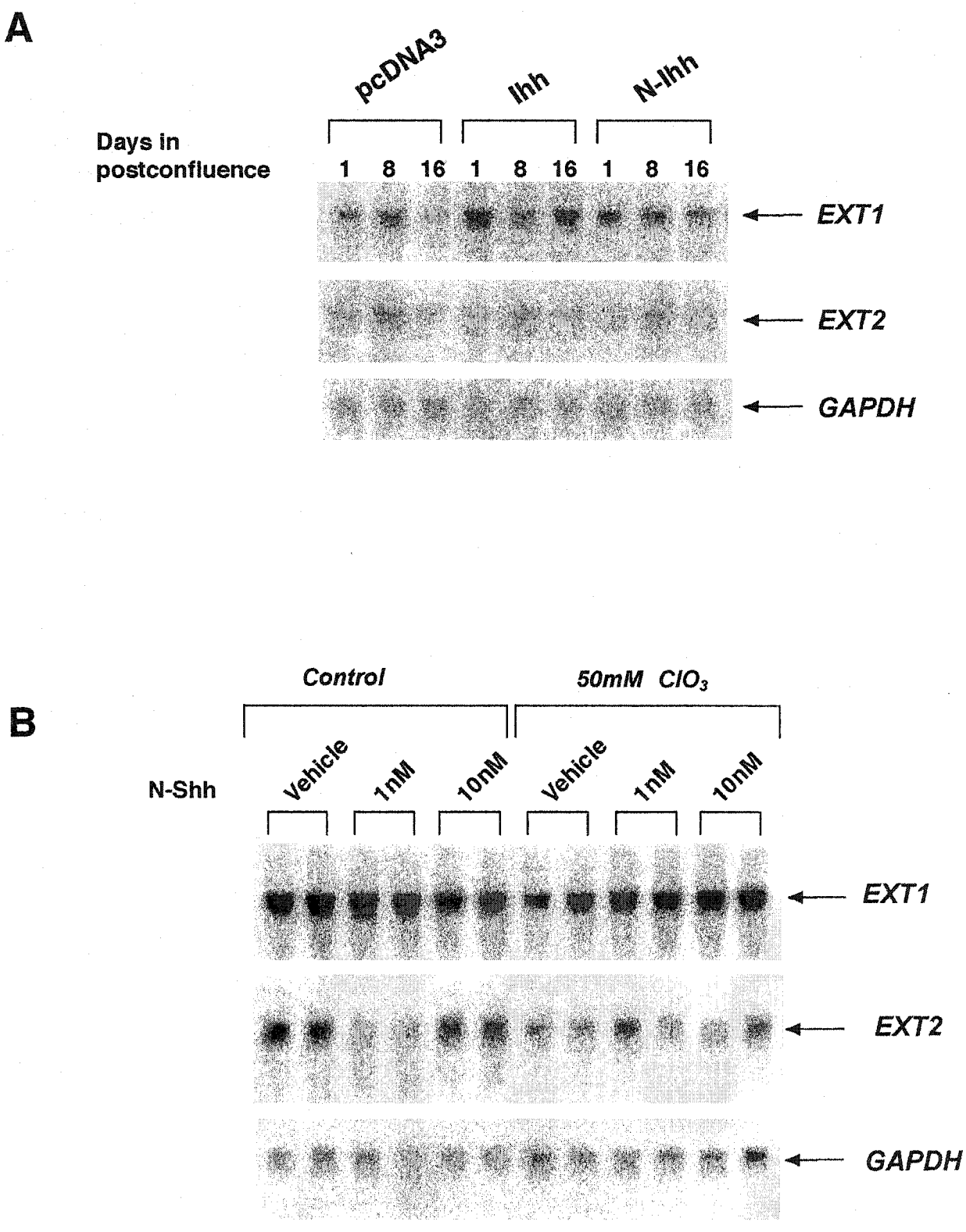
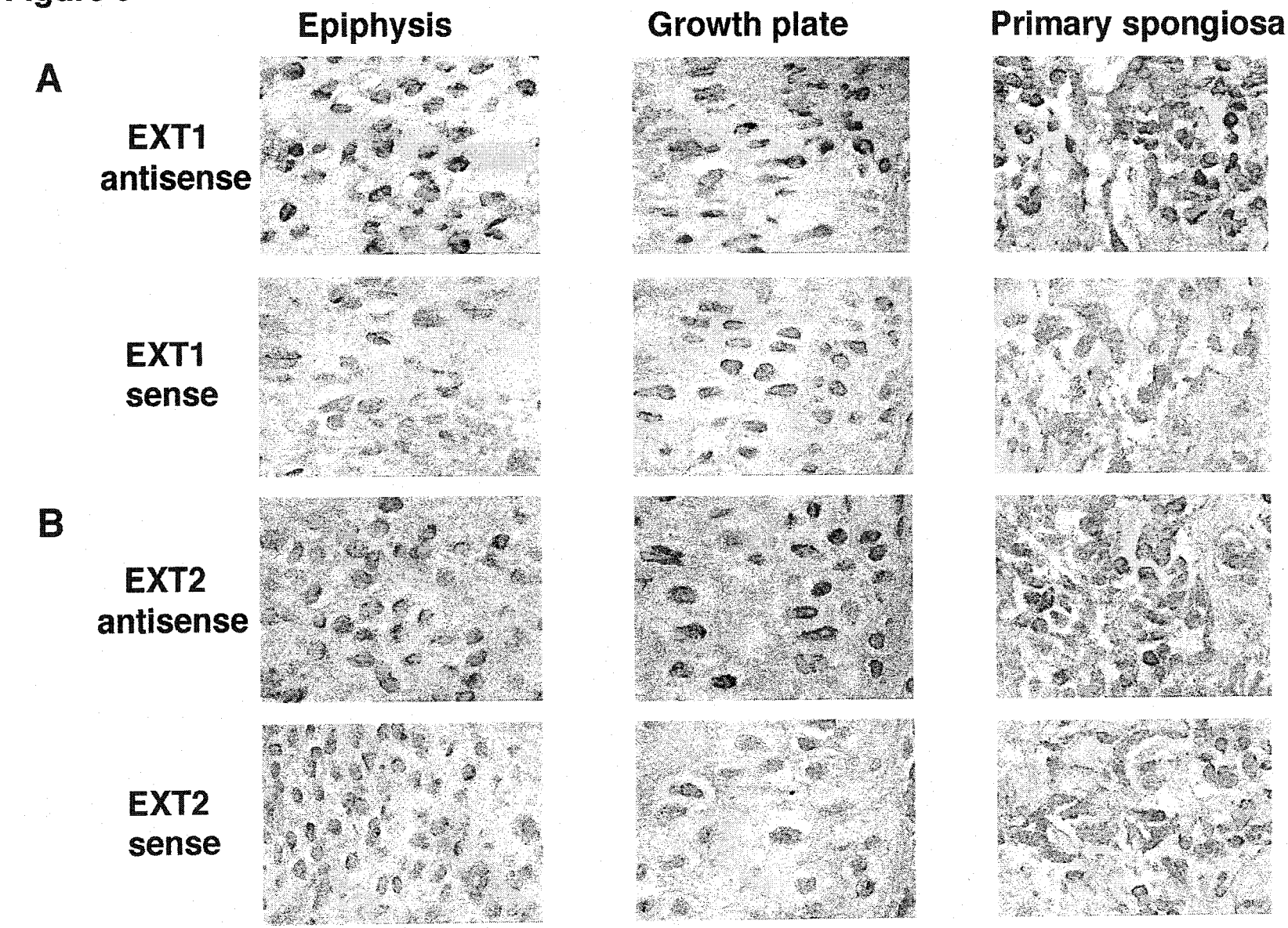

A
pcDNA3
Ihh
N-Ihh
Days in postconfluence
1 8 16 1 8 16 1 8 16
EXT1
EXT2
GAPDH
B
Control
50mM ClO3
N-Shh
Vehicle
1nM
10nM
Vehicle
1nM
10nM
EXT1
EXT2
GAPDH

Figure 5. Detection of *Ext1* and *Ext2* expression by *In situ* hybridization. Sections of bones from newborn mice were analyzed by *in situ* hybridization for the mRNA expression of *EXT1* (A) and *EXT2* (B). Positive signal was detected in cells treated with antisense probe (Upper panel) but not with the sense control probe (lower panel). As shown, EXT1 and EXT2 expression is observed to overlap and localizes to proliferating and prehypertrophic growth plate chondrocytes as well as in osteoblasts of the primary ossification centers. Hypertrophic chondrocytes display expression to a much lesser extent than that observed in the proliferating or prehypertrophic layers.

Figure 5


Epiphysis
Growth plate
Primary spongiosa
A
EXT1 antisense
EXT1 sense
B
EXT2 antisense
EXT2 sense

Chapter 5

Discussion

Discussion

The appropriate rate of conversion of proliferating columnar chondrocytes to hypertrophic chondrocytes is of critical importance to the proper formation of skeletal elements undergoing endochondral ossification. Illustrating the significance of this process, animals harboring ablations to *Ptrp* (Karaplis et al., 1994), *Pth1r* (Lanske et al., 1996), or *Ihh* (St-Jacques et al., 1999) all display phenotypes characterized by premature hypertrophic differentiation accompanied by varying degrees of perturbed chondrocyte proliferation. This is further reiterated by human diseases that are manifested by skeletal abnormalities due to hampered PTHrP/PTH1R signaling: Jansen's metaphyseal chondrodysplasia, caused by constitutive activation of PTH1R (Schipani E, 1995), and Blomstrand chondrodysplasia resulting from lack of a functional PTH1R (Jobert et al., 1998; Karaplis et al., 1998). The present study aimed to address issues pertaining to the mechanism by which Ihh regulates chondrocytic differentiation and to analyze its functional interactions with the PTHrP/PTH1R signaling pathway. To further expand the understanding of how additional signaling pathways may influence this process, the roles of En-1 and heparan sulfate glycosaminoglycans as modulators of Ihh signaling and chondrocytic differentiation were also investigated. Most *in vivo* studies employing genetic manipulations in mice, have come to support a model in which Ihh retards the progression of columnar chondrocytes to their terminally differentiated, hypertrophic state through a relay signaling system, involving TGFβ2, that results in upregulated PTHrP expression in the periarticular cartilage. In turn, PTHrP acts upon PTH1R-expressing prehypertrophic chondrocytes to slow their hypertrophic differentiation (Alvarez et al., 2002; Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996). On the other

hand, *in vitro* models have demonstrated the capacity of Ihh to induce, rather than inhibit, hypertrophic differentiation suggesting the possibility that this protein may exert direct actions on chondrocytes (Akiyama et al., 1999; Enomoto-Iwamoto M and Nohno T, 2000; Murtaugh et al., 1999; Stott and Chuong, 1997). Interestingly, a direct action for Ihh in promoting chondrocyte proliferation, independently of PTHrP, has been demonstrated *in vivo* (Karp et al., 2000; Long et al., 2001). To reconcile between these contrasting findings, we designed an *in vitro* model based on the CFK-2 chondrocytic cell line in which either overexpression of Ihh, or treatment of these cells with exogenous Shh peptide, was assessed for its capacity to alter their differentiation program.

As discussed in Chapter 2, it was demonstrated that Ihh overexpression, and Hh-signaling in general, promotes chondrogenic differentiation in CFK-2 cells, as characterized by increased ALP activity and *Col2a1* and *Col10a1* expression. That most of these phenotypic changes are abrogated by an inactivating missense mutation (W160G) in the amino terminal end of Ihh, further substantiates the notion that Hh-signaling harbors the capacity to induce, rather than inhibit, chondrocytic differentiation. In further support of these findings, are the several indications that this may also be the case *in vivo*. First, the delay in chondrocytic hypertrophy observed in Ihh-ablated animals suggests that the onset of *Col10a1* expression might be Ihh-dependent (Long et al., 2001; St-Jacques et al., 1999). Second, and perhaps more convincing for a direct role for Ihh in promoting chondrocyte maturation, are recent studies on mice harboring a chondrocyte-specific ablation of *Pth1r* (termed *Col2-Cre* $Pth1r^{fl/fl}$) (Kobayashi et al., 2002). In addition to phenocopying many of the alterations previously observed in growth plates of *Pth1r*-null animals, namely the premature hypertrophic differentiation of chondrocytes, it was

observed that *Col2-Cre Pth1r*$^{fl/fl}$ mice exhibited an overexpression of Ihh in columnar chondrocytes that was associated with accelerated maturation of periarticular chondrocytes. Furthermore, narrowing the domain of *Pth1r* ablation to columnar chondrocytes through usage of a fragment from the osteocalcin promoter that directs *Cre* expression to this region, it was demonstrated that ectopic Ihh expression by these cells accelerates the differentiation of overlying periarticular chondrocytes independently of PTHrP. Thus, it appears that Ihh may induce chondrogenic differentiation *in vivo* and that this might be restricted to an early stage (periarticular to columnar) of their development. Our data is in agreement with these observations but also suggests that, in the absence of PTHrP signaling, Ihh maintains the potential to induce chondrocytes to differentiate further toward hypertrophy. In addition, since the delay in *Col10a1* expression, observed in *Ihh*$^{-/-}$-animals, is corrected in mice harboring a chondrocyte-specific deletion of *Smo*, it is possible that Ihh may mediate *Col10a1* induction indirectly via signaling to the perichondrium (Long et al., 2001).

That PTHrP, through its activation of PTH1R and PKA, elicits an inhibitory effect on chondrocytic hypertrophy is well established (Chung et al., 1998; Farquharson C, 2001; Lanske et al., 1996; Schipani E and Kronenberg HM, 1997). The present study shows that PTHrP inhibits Ihh-mediated chondrogenic differentiation by activating PKA. A logical way to follow these observations would be the examination of downstream effectors converging the Hh and PTHrP signaling pathways, of which the most obvious candidates would be members of the Gli protein family, all shown to be susceptible to phosphorylation by PKA (Ruiz i Altaba, 1999). As both *Gli2* and *Gli3* mutant mice display abnormalities that implicate their function both in patterning of the skeletal

elements as well as in regulating their growth, it is likely that they govern at least part of the activities mediated by Ihh in chondrocytes (Mo et al., 1997; Henderson and Miao, unpublished data). Of these, Gli3 appears the most likely candidate for mediating the negative effects on Ihh-signaling for a number of reasons: (1) in most contexts examined so far, Gli3 appears to act as a repressor, not activator, of Hh-target genes (Ruiz i Altaba, 1998,1999); (2) Gli3 undergoes proteolytic cleavage when misexpressed in *Drosophila* suggesting that it has a conserved function with Ci (Aza-Blanc et al., 2000); (3) inhibition of Hh-target genes in the embryonic limb is mediated by an NH_2-terminal repressor form of Gli3 generated by its proteolytic processing (Wang et al., 2000a); (4) loss of this repressor form, or its unregulated activity, underlies the skeletal abnormalities observed in humans harboring *GLI3* mutations causing Greig cephalopolysyndactyly syndrome (GCPS) or Pallister-Hall and post-axial polyadatyly type A syndromes (PHS; PAP-A), respectively (Kang et al., 1997; Radhakrishna et al., 1997; Vortkamp et al., 1991). Thus, it is likely that PTHrP-mediated activation of PKA would result in the phosphorylation of Gli3 and the subsequent conversion to the NH_2-terminal repressor form, which would then act directly by inhibiting Hh-target gene expression. Although considerable effort has been made to examine this hypothesis- testing of several antibodies for their ability to detect Gli3 and an unsuccessful attempt to express it as a tagged protein- we were unable to make any progress in this direction. Key to understanding the roles of Gli2 and Gli3 in chondrocyte biology would be the delineation of their *in situ* expression domains; information that is currently lacking from the epiphysis and growth plate cartilage but which appeared crucially informative in understanding Shh-Gli function in the developing neural tube and limb (Lee et al., 1997; Ruiz i Altaba, 1998; Wang et al., 2000a).

Intriguingly, we present novel evidence indicative of Ihh downregulating PTH1R-mediated PKA activity. It has been previously speculated that Smo, through coupling to an unknown G-protein, may modulate PKA activity (Alcedo et al., 1996), however, other studies provided evidence that Hh-signaling has no effect on this kinase (Jiang and Struhl, 1995). That Hh-signaling impedes PTHrP-mediated PKA activity, suggests the existence of a functional antagonistic interplay between these pathways that may be unique to chondrocytes. One explanation is the possible coupling of Smo to a G-protein that is shared by PTH1R (G_s or G_q), thereby competitively sequestering it from coupling to PTH1R. Arguing against this proposal is the fact that, unless this is a phenomenon unique to chondrocytes, there is no evidence in support of Smo interacting with such proteins. Alternatively, Smo may affect PKA activity downstream of PTH1R by coupling to a pertussis toxin (Ptx)-sensitive G_i protein. In zebrafish, ectopic expression of Ptx results in phenotypic features resembling those observed following constitutive activation of PKA or inhibition of Hh signaling, suggesting that in certain contexts, Hh might employ a G_i-protein to augment its own signaling output (Hammerschmidt and McMahon, 1998). Although not tested in this study, it would be of merit to evaluate the effects of Hh on PTHrP-responsiveness in the presence or absence of Ptx. These potential antagonistic effects of Ptx on Hh inhibition of PKA should be hampered in cells misexpressing a dominant-negative form of this kinase (dnPKA).

An additional facet pertaining to the interplay between Ihh and PTHrP is the demonstration of Hh-dependent upregulation of PTH1R mRNA. This effect appears to be dose-dependent (Chapter 3, figure 6B) and is impeded by PTHrP administration (Chapter 2, Figure 5). The inverse relationship between PTH1R expression and activity is indicative

of a potential compensatory mechanism that is induced by Hh-signaling. Thus, cells receiving opposing PTHrP and Ihh signals may try to compensate for Hh-mediated PKA inhibition by increasing PTH1R mRNA expression, and presumably cell-surface receptor number. One way to test this hypothesis would be through PTH1R receptor-ligand binding studies, performed in the presence or absence of Hh-stimulation, that would enable the determination of receptor numbers and thus exclude the possibility that Ihh may in any way elicit a negative effect on this parameter. These finding are of relevance to the *in vivo* situation, as the highest levels of PTH1R expression are observed in prehypertrophic chondrocytes lying adjacent to, or overlapping with, Ihh-expressing cells (St-Jacques et al., 1999; Vortkamp et al., 1996), indicating that this regulatory loop may occur in cells receiving strong signals from both Ihh and PTHrP. One experimental approach for addressing this question *in vivo* was used by Chung and associates who generated chimeric mice harboring individual *Pth1r*$^{-/-}$ or *Ihh*$^{-/-}$/*Pth1r*$^{-/-}$ cells thereby creating a mosaic with wild-type growth plate chondrocytes (Chung et al., 1998; Chung et al., 2001). These authors showed that, when placed within the epiphysis, *Pth1r*$^{-/-}$-chondrocytes underwent ectopic hypertrophy while expressing higher levels of Ihh. In turn, this caused an increase in wild-type cell proliferation that was associated with PTHrP-induction in periarticular chondrocytes. While still undergoing ectopic hypertrophy, the effects on proliferation were abolished in chimeras harboring *Ihh*$^{-/-}$/*Pth1r*$^{-/-}$ chondrocytes, suggesting that Ihh is the mediator of this process. Using this approach to generate *Ihh*$^{-/-}$ chimeras would enable a closer examination of *Ihh*$^{-/-}$ chondrocytes, particularly within the prehypertrophic layer. If Ihh is involved in upregulating *PTH1R* mRNA, then one would expect to see a decrease in its levels in cells surrounding Ihh-null chondrocytes.

An additional potential regulator of Ihh signaling in chondrocytes is proposed to be En-1, a homeodomain-containing transcription factor whose function was primarily described in mid-hindbrain development and dorso-ventral patterning of the limb (Loomis et al., 1996; Wurst et al., 1994). Here, we demonstrate that constitutive expression of En-1 in chondrocytic cells alters their differentiation in a manner that is different from that of Ihh. Although ALP is induced in En-1-expressing cells, by comparison to Ihh-mediated ALP activation, its levels remain stable over long culture periods. En-1 also enhances expression levels of sulfated GAG, an additional characteristic not observed in Ihh-expressing cells. Furthermore, *bona fide* hypertrophic differentiation is inhibited by En-1 whereas Ihh enhances it. Moreover, its detection in prehypertrophic and hypertrophic chondrocytes of the growth plate suggests that En-1 functions in these cells *in vivo*. The previous descriptions of En-1 expression in sclerotome-derived cells of the prevertebral column are supportive of some of our observations (Davidson et al., 1988; Davis and Joyner, 1988). Finally, we show that En-1 negatively modulates Hh-signaling *in vitro* suggesting that such a function may be relevant to its regulation of Ihh in the growth plate.

The En-1 and En-2 pattern of expression, by comparison to that of genes within the Hh pathway, appears to have diverged considerably from that desribed in *Drosophila*. Thus, in contrast to invertebrates, mammalian *En-1/En-2* expression does not overlap with Shh or its downstream genes, *Ptc1* and *Gli*, during brain development. Shh is required for dorsal-ventral patterning of the neural tube whereas En-1/En-2 act more anteriorly in mid-hindbrain and cerebellar specification (Chiang et al., 1996; Ericson et al., 1996; Joyner and Martin, 1987; Millen et al., 1994; Wurst et al., 1994). However, in other aspects, a limited conserved relationship between En/en and Wnt1/wg does occur in both *Drosophila* and

mammals. Thus, similar to wg maintenance of en expression in neighboring cells, Wnt1 appears to regulate En-1 expression, although this occurs in congruent neurons of the mid-hindbrain (Danielian and McMahon, 1996; Ingham and Martinez Arias, 1992). Other structure-function studies have illustrated that En-1 possesses biological properties differing from those of en or En-2. Thus, *Drosophila* en can substitute for En-1 in proper brain development but not limb patterning (Hanks et al., 1995), and En-2 can replace En-1 functions in most tissues except for dorsal-ventral patterning of the limb dermis (Loomis et al., 1996). These findings illustrate the cross species and intra-species divergence that has occurred to En over the course of evolution. Thus, it is possible that En-1 acquired a specialized function for regulating chondrogenic differentiation in the growth plate.

Ultimately, a detailed examination of long bone and vertebral growth plates from En-1-null mice would be necessary to determine if any pre-natal or post-natal alterations occur that would indicate a function for this protein in chondrogenesis. Alternatively, the generation of a transgenic animal overexpressing En-1 under the control of the *Col2a1*-promoter would be informative for demonstrating its potential capacity to modulate chondrocyte differentiation and/or proliferation. This would also enable the analysis of its interactions with the Ihh and other pathways that may be influenced by En-1. Further information could be gained from analysis of compound mutants of En-1 and other homeodomain transcription factors shown to play a role in skeletal development. One good candidate would be pbx1. Shown to physically interact with En-1, it also affects chondrocyte proliferation and hypertrophy and therefore might synergize with En-1 in regulating these processes (Peltenburg and Murre, 1997; Selleri et al., 2001). Other candidate partners for En-1 may be Dlx5/Dlx6, as combinatorial ablation of these genes in

mice leads to defects in endochondral bone as well as in AER formation (apical ectodermal ridge), the latter structure also requiring En-1 for its proper development (Loomis et al., 1998; Robledo et al., 2002). Interestingly, En-1 and pbx1 may coordinately regulate the expression of *fgf8*, an essential factor for limb development that is expressed in the AER and is important for its maintenance (Gemel et al., 1999; Lewandoski et al., 2000).

The overlapping expression domains of Ihh and En-1 in the vertebral and tibial growth plates suggest a functional conservation in this tissue between *Drosophila* and mammals. Ihh expression was recently described to occur at the tips of the developing digits (E12.5) and later, in the developing phalanges, and metatarsals, and was proposed to maintain *Gli1* and *Ptc1* expression in these regions in the absence of *Shh* (Kraus *et al.*, 2001). Interestingly, En-1 mutants display distinct abnormalities in these skeletal elements (Wurst *et al.*, 1994). It is possible that early initiation of *Ihh* expression in these domains, by analogy to *en* and *hh*, requires the activity of En-1. Moreover, that En-1 may elicit its activity in a non cell-autonomous manner, distant from its origin of transcription, is suggested by studies showing the capacity of En proteins to be secreted and internalized by neighboring cells (Joliot *et al.*, 1998; Maizel *et al.*, 1999).

An additional aspect of evolutionary conservation is implied by the ability of En-1 to repress endogenous induction of *Ptc1* in response to exogenous Hh-signaling. Although not strongly induced by Hh, basal *Gli1* expression in CFK-2 cells was also suppressed by En-1. Together with the fact that this was accompanied by downregulating at least some Hh-mediated biological outcomes, it is likely that En-1 maintains the capacity to modulate Hh-activity through possible transcriptional control of its downstream target genes. In

support of this hypothesis, *Drosophila* en was shown to directly bind and suppress the transcriptional activity of Ci and dpp (Sanicola et al., 1995; Schwartz et al., 1995). The possibility that En-1 may also regulate Gli2 and Gli3 expression remains to be investigated. These findings indicate that En-1 might downregulate Hh-responsiveness in prehypertrophic, En-1/Ihh-expressing, chondrocytes and thereby desensitize them to Hh-signaling. That signaling by PTH1R appears to be unaltered in En-1-expressing cells further suggests that these remain susceptible to PTHrP signals that result in their delayed differentiation. Thus, from this perspective, En-1 and PTHrP synergize in inhibiting hypertrophic differentiation.

The third study focused on the role of HSPGs in regulating Hh-mediated effects on chondrocyte differentiation. The data provided suggests several roles for HS in this context: (1) heparan sulfated glycosaminoglycans play a role in mediating Hh-signaling, possibly through direct interactions with the Hh-ligand or its receptor *Ptc1*; (2) inhibition of GAG sulfation reveals that HS may influence Hh both positively and negatively to affect diverse aspects of chondrocytic differentiation.

The first conclusion is based primarily on the fact that Hh-signaling, as evaluated by the extent of *Ptc1* induction, is diminished following inhibition of GAG sulfation. This effect was observed both in cells constitutively expressing Ihh and in cells exposed to exogenous Hh, suggesting that perturbation of Hh-signaling was occurring at the cell surface. One way to further assess the possibility that HS participates in Hh-Ptc1 interactions would be through immunocytochemical staining of Hh-treated cells under sulfation-inhibiting conditions. This method was employed to demonstrate that EXT1-null tissues were unable to bind Ihh (Lin et al., 2000).

Secondly, our data suggests that HS may differentially regulate the biological outcomes of Hh signaling. Thus, in an apparent discrepancy, Hh-induced ALP and *Col10a1* are oppositely affected by chlorate treatment. Surprisingly, Hh induction of *Col10a1* expression was exacerbated, rather than diminished, by disruption of GAG sulfation. This result implies that HSPG and Ihh might specifically modulate the regulation of *Col10a1* transcription in a coordinated fashion. Although type X collagen appears dispensable for gross endochondral bone formation (Rosati et al., 1994), other studies demonstrated that *Col10a1*-null animals present with abnormalities in both cartilage and trabecular bone architecture (Kwan et al., 1997). Moreover, HSPG distribution in the ECM of prehypertrophic and hypertrophic chondrocytes is altered in both *Col10a1*-null mice or in transgenic animals expressing a mutant variant of this gene (Jacenko et al., 2001). As type X collagen was shown to physically associate with proteoglycans through its NC1 (non-collagenous) domain, it is possible that, coordinately with HSPGs, it may impact on the structural integrity and influence cell signaling within the hypertrophic zone. That Ihh may signal through HSGPs to modulate *Col10a1* expression is implicated by studies on exostoses resulting from mutations in *EXT1*, which display abnormally high levels of type X collagen (Legeai-Mallet et al., 2000). It is possible therefore, that Hh-signaling is negatively regulated by HSPGs to reduce its capacity to induce *Col10a1* expression. Interestingly, an uncoupling between ALP activation and *Col10a1* expression was noted in cells expressing N-Ihh (W160G) (see Chapter 2). It would be of interest to study the possibility that this mutation interferes with aspects of Hh-Ptc1 interactions that might involve their association with HSPGs.

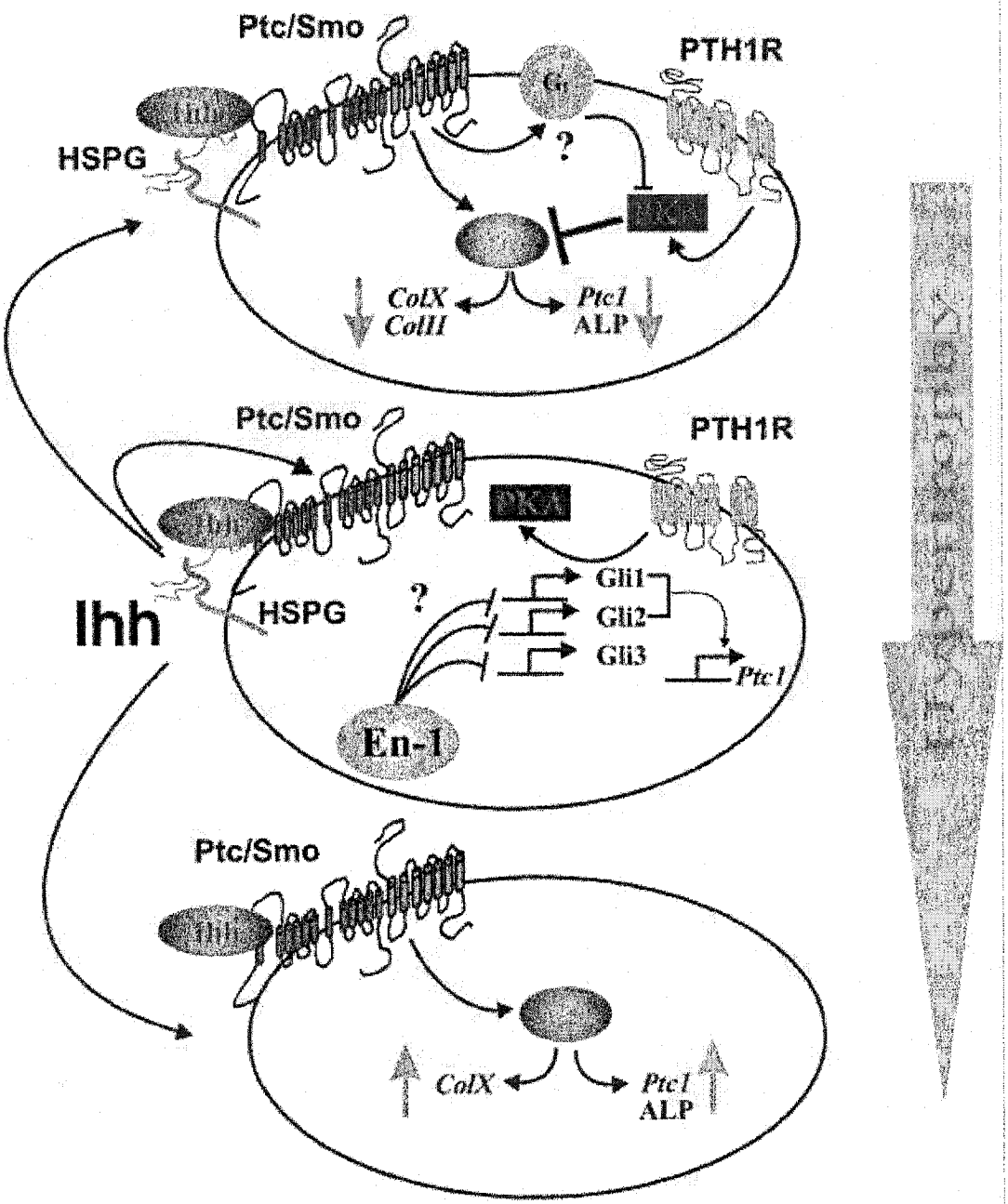
The question that remains to be addressed is whether or not EXT1 and EXT2 are in fact the genes responsible for the synthesis of an HS moiety that participates in regulating Hh-signaling, particularly in the context of cartilage biology. Thus far, the knowledge pertaining to their function in Hh-signaling is circumstantial and there is no direct evidence of perturbed Hh-signaling in EXT1/EXT2-deficient chondrocytes. We have shown the expression of *EXT1/EXT2* in both growth plate/ epiphyseal chondrocytes and in CFK-2 cells, implicating their potential for regulating HSPG expression and Hh-signaling. In an attempt to study their direct involvement in these processes we are currently investigating methods for sequestering *EXT1/EXT2* expression by stable anti-sense RNA expression in CFK-2 cells.

In summary, this study contributes information pertaining to key regulatory pathways in chondrocytic differentiation. An integrated model encompassing data presented here with that of previous studies can be proposed as follows (see Figure 1): During early mesenchymal condensations and cartilage formation, En-1 may participate, in collaboration with other factors, in establishing Ihh expression in the developing limb buds. At later stages of endochondral ossification En-1, expressed together with Ihh, modulates Hh signaling in prehypertrophic cells and thereby prevents these from entering the hypertrophic state prematurely. Concomitantly, Ihh induces PTHrP expression in periarticular chondrocytes which downregulate the differentiation of PTH1R-expressing prehypertrophic cells. Locally, Ihh signals to both PTH1R-expressing cells and non-expressing cells. Thus, PTH1R-expressing cells oppose differentiation-promoting signal by responding to PTHrP and thereby remaining non-hypertrophic, while non-PTH1R-expressing cells remain fully responsive to Ihh and undergo terminal differentiation. In

addition, HSPGs expressed differentially on the cell surface of non-hypertrophic chondrocytes, may modulate the signal strength and distribution of Ihh and thereby contribute to its regulation of chondrocytic hypertrophy.

Figure 1: Integrated model for the coordinated actions of Ihh, PTHrP, En-1, and HSPG in the regulation of chondrocyte differentiation

The cell in the center represents an Ihh/En-1-expressing chondrocyte that is inhibited from receiving Hh-signals by both En-1 and PTHrP. The top cell represents a less mature chondrocyte that is strongly inhibited by PTHrP signaling and possibly modulated by HSPG, affecting Hh-signal reception. In contrast, the bottom cell does not express PTH1R or EXT1/2-produced HS GAGs, and is therefore fully responsive to the Hh-signaling and undergoes hypertrophy.


Ptc/Smo
PTH1R
Ihh
HSPG
?
PKA
ColX
ColII
Ptc1
ALP
Ptc/Smo
PTH1R
PKA
Ihh
HSPG
?
Gli1
Gli2
Gli3
Ptc1
En-1
Ptc/Smo
Ihh
ColX
Ptc1
ALP

Original Contributions to Knowledge

This study has provided novel insights and new information pertaining to the regulation of chondrocyte differentiation by Indian Hedgehog (Ihh) and Parathyroid Hormone-related Peptide (PTHrP), which are summarized as follows:

1. Evidence is presented to show that Ihh can directly induce cartilage cell differentiation in vitro. This finding is novel and adds information to previous studies pertaining to its indirect role in mediating inhibition of chondrocyte differentiation.
2. This study demonstrates that PTHrP may play a negative regulatory role in inhibiting Ihh signaling and consequently, chondrocyte differentiation.
3. Data presented in this study indicates toward a novel functional regulation of PTHrP-activated PKA activity by Ihh-signaling. This is the first time that evidence is shown to demonstrate inhibition of PKA by Hh-signaling
4. Novel data presented here suggests a role for the homeobox transcription factor En-1 in regulating chondrocytic differentiation. Furthermore, it is shown, for the first time in a mammalian system that En-1 and Ihh may functionally interact.
5. Contributing information presented here indicates toward a function for heparan sulfate proteoglycans in mediating Hh-signaling and modulating chondrocytic differentiation.

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