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

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Summary

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 signalling 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 N-terminal domain (N-Ihh), effects mimicked by exogenous administration of recombinant N-terminal HH peptide. Moreover, a missense mutation mapping to the N-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 (PTHR1 H223R) inhibited Ihh-mediated differentiation, implicating activation of protein kinase A (PKA) by PTHR1 as a probable mediator of the antagonistic effects of PTHrP. 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 signalling, in turn, modulates PTHrP action through functional inhibition of signalling by PTHR1 to PKA.

Key words: Indian hedgehog, Parathyroid hormone-related peptide, Chondrocytes, Protein kinase A

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 N- and C-terminal domains of the native protein (Porter et al., 1996a; Lee et al., 1994; Bumcrot et al., 1995; Valentini et al., 1997). The C-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 C-terminal end of the nascent N-terminal domain and enhances its lipophilic properties (Porter et al., 1996a; Porter et al., 1996b). The N-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 (PTHR1), would then inhibit differentiation in the growth plate.

However, several lines of evidence 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 signalling (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 N-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 N-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 used the rat CFK-2 chondrocytic cell line to investigate the role of Ihh and its interplay with the PTHrP signalling pathway in chondrocyte differentiation. We present evidence indicating that Ihh or its N-terminal domain harbors the capacity to induce chondrogenic differentiation, an effect mimicked by recombinant N-terminal HH protein. We also show that PTHrP, through a protein kinase A (PKA)-dependent 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 C-terminus of Ihh by PCR using oligonucleotides: 5′-CGCATGTGCTTTCCTGCCGGAGCCCAGG-3′ as the sense primer and 5′-CCTTCAGTTGTTCAGGTCCTCTTCGCTAATCAGC-TTTTGTTCCATAGAGCTTCCTGCCCCAG-3′ as the antisense primer. The 676-bp fragment corresponding to the C-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 N-terminal 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 *PTHR1* (PTHR1 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 2 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, Cambridge, MA). N-Shh was modified by an addition of a hydrophobic eight-carbon chain (octyl group) to the N-terminal cysteine and this is reported by the suplier 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 were 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 10 days and subsequently cells were maintained in G418 (500 μg/ml). Coexpression of PTHrP or PTHR1 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 PTHR1 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 five 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 120,000 ***g*** 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× 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× SSC, and 1× Denhardt's blocking solution with 0.1 mg/ml salmon sperm DNA. Membranes were washed once in 2× SSC/0.1% SDS at room temperature and once in 0.1× 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 N and C 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 *Pthr1* 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 sodium orthovanadate, 10 mM sodium 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 minutes at 12,000 ***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 five volumes of acetone and incubation on ice for 1 hour. Centrifugation at 10,000 ***g*** for 15 minutes 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 1 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 hour. 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 hour 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 minute 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 minutes in the presence of 10^{-8} M PTHrP 1-34 and 300 µM isobutylmethylxanhine (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 minutes at 30°C, 15 µl samples were blotted onto phosphocellulose P81 paper, washed four 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 in the order of $P \leq 0.05$.

Results

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

We first assessed the expression of recombinant Ihh and its N-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 N-terminus of Ihh (α-Ihh-N) was used. In addition, we added a *myc*-epitope tag to the C-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 kDa and 19 kDa) that were immunoreactive to α-Ihh-N were present, corresponding to the full-length and N-terminal domain of Ihh, respectively (Fig. 1B, lanes 2,3). Lysates of cells expressing N-Ihh displayed a peptide that was immunoreactive to the same antibody and co-migrated with the 19 kDa 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 kDa protein corresponding to the C-terminal domain of Ihh (C-Ihh) (Fig. 1B, lane 10), in agreement with previous observations using other *hedgehog* family members, that the C-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 C-terminal domain and a less diffusible N-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. 4A). 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 with 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 used 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 *Pthr1* (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 Materials and Methods) during which samples of total cellular RNA were obtained intermittently.

To verify the status of HH-signalling 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-signalling, whose levels are presumably dependent 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 N-terminal domain of Ihh. Whereas pcDNA3-transfected cells expressed low constitutive levels of *Ptc*, Ihh- and N-Ihh-transfectants demonstrated a robust elevation in *Ptc* expression levels (Fig. 2A-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 N-terminal domains of HH proteins have been

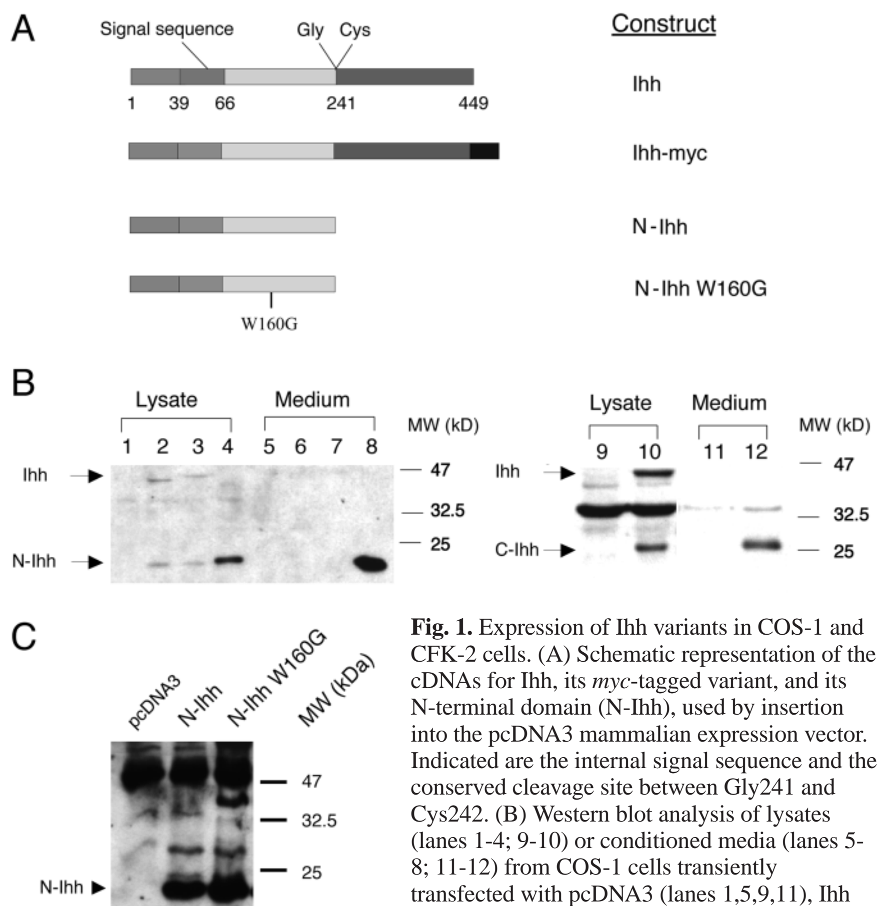

Fig. 1. Expression of Ihh variants in COS-1 and CFK-2 cells. (A) Schematic representation of the cDNAs for Ihh, its *myc*-tagged variant, and its N-terminal domain (N-Ihh), used by insertion into the pcDNA3 mammalian expression vector. Indicated are the internal signal sequence and the conserved cleavage 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 1,5,9,11), Ihh (lanes 2,6), Ihh-*myc* (lanes 3,7,10,12) or N-Ihh (lanes 4,8). The detection of a 46 kDa and 19 kDa species in lysates from Ihh or Ihh-myc transfectants was facilitated by the use of α-Ihh-N antibody specific to the N-terminal domain of Ihh (lanes 1-8). The 26 kDa C-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 kDa peptide species attributed to the N-terminal domain of Ihh in its normal and mutant forms was detected by the α-Ihh-N antibody.

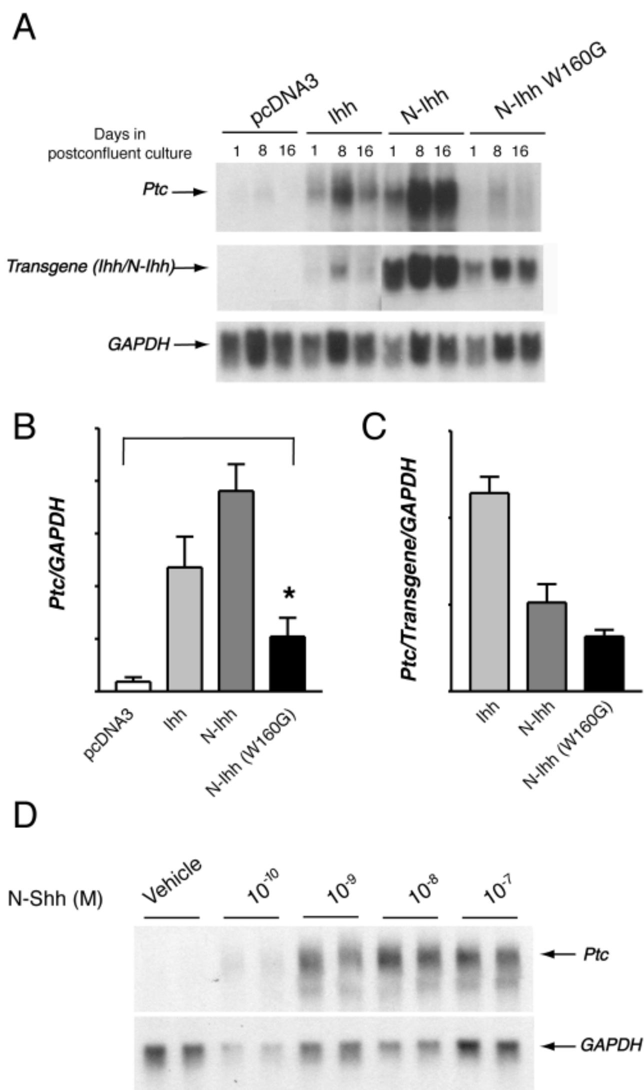

Fig. 2. Induction of *Ptc* receptor expression by Ihh, N-Ihh, N-Ihh W160G or exogenous N-Shh in CFK-2 cells. (A) Expression of *Ptc* in stably transfected CFK-2 cells. CFK-2 cells that were stably transfected with pcDNA3, Ihh-pCDNA3, N-Ihh-pcDNA3 or N-Ihh W160G-pcDNA3 were subjected to postconfluent 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 and compared to the respective transgene level. (B) A graphic depiction of the results shown in A, comparing *Ptc* expression with 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.

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 N-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. 2D). These results verified that N-Shh, similarly to Ihh, induces *Ptc* expression in CFK-2 cells and that this is dependent 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). 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 in N-Ihh and its mutant variant, N-Ihh W160G (Fig. 3A, right). 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-signalling 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 (Fig. 3C). Dose-dependent 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-dependent pathway

In bone cells, PTHrP mediates most of its biological actions through activation of its cognate G-protein-coupled receptor, PTHR1, 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-signalling 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}

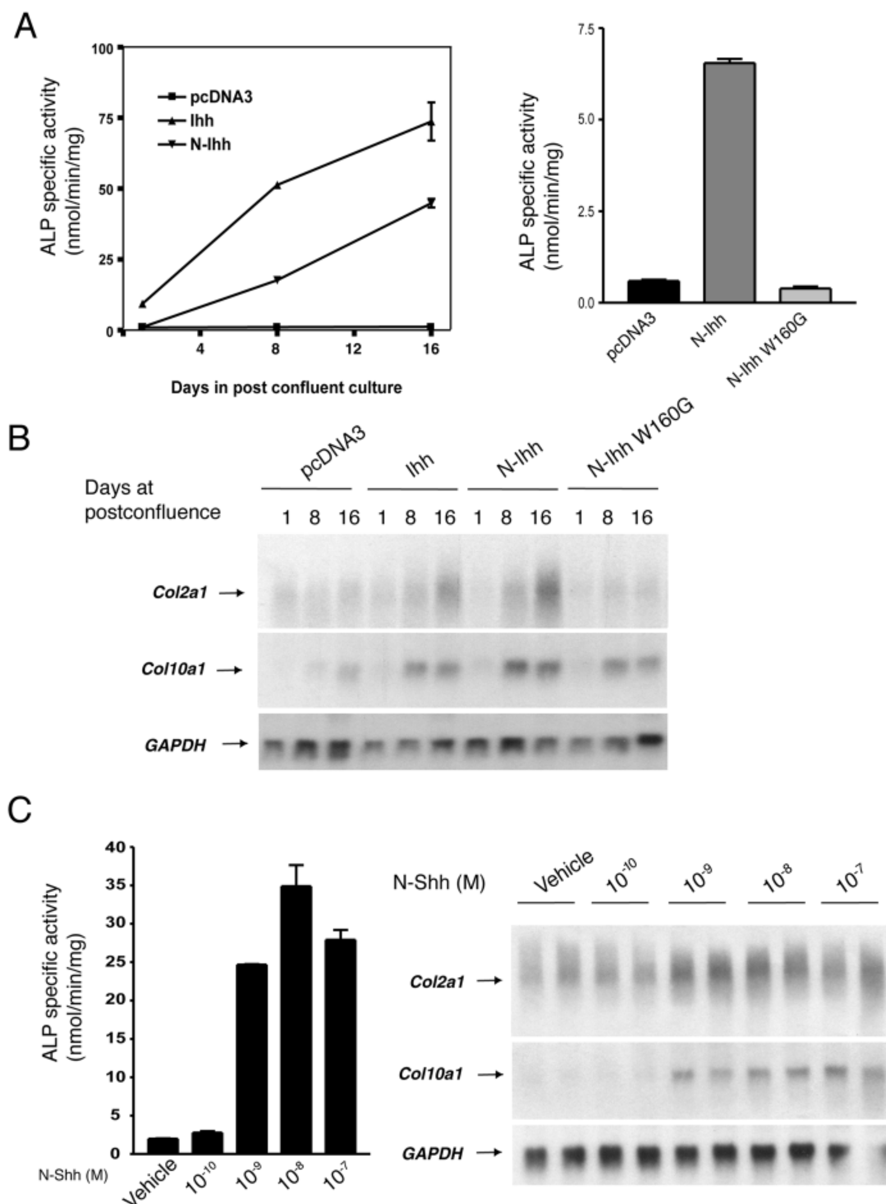

Fig. 3. Induction of chondrogenic markers in CFK-2 cells by Ihh is mimicked by recombinant N-Shh. (A) Alkaline phosphatase (ALP) activity is enhanced by Ihh or N-Ihh, but not by N-Ihh W160G. CFK-2 cells stably transfected with pcDNA3, Ihh or N-Ihh underwent postconfluent growth and were analyzed for ALP enzymatic activity during the indicated time periods (left). ALP activity was also compared in stably transfected CFK-2 cell populations expressing N-Ihh or its mutant variant form, N-Ihh W160G (right). (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 used 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. After 6 days of treatment, ALP enzymatic activity in cell extracts was measured (left) and total RNA was analyzed for *Col2a1* and *Col10a1* expression (right). A maximal response was observed at a concentration of 10^{-8} M N-Shh.

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 PTHR1 (H223R), a mutant variant of PTHR1 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 PTHR1 (H223R) (Fig. 4C). These findings, showing that PTHrP action and constitutive activation of adenylate cyclase through PTHR1 similarly abrogate Ihh function, implicate PKA as the effector of PTHrP-mediated antagonism of HH-signalling.

Ihh dampens PTHrP responsiveness of CFK-2 cells

In the growth plate, PTHR1 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 PTHR1 expression. To examine this possibility, northern blot analysis for *Pthr1* 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 *Pthr1* mRNA expression, whereas PTHrP treatment alone had no effect (Fig. 5A, left). Furthermore, upregulation of *Pthr1* 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).

To examine whether increased *Pthr1* 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 tenfold increase in activity over cells treated with IBMX alone (Fig. 5B, left). In contrast, an approximately 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), 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 signalling through PTHR1, ultimately leading to impeded PKA activity.

Discussion

In this study, the biological effects of HH-signalling 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 N-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 N-terminal HH protein. Moreover, data presented has led us to propose that the PTHrP and HH-signalling pathways interact directly to modulate the rate of chondrocytic differentiation. Thus, chondrogenic genes activated by HH-signalling are downregulated by exogenously administered PTHrP, an effect mimicked by forskolin. Furthermore, we show that HH-signalling leads to upregulation of *Pthr1* mRNA expression, while paradoxically diminishing responsiveness to PTHrP.

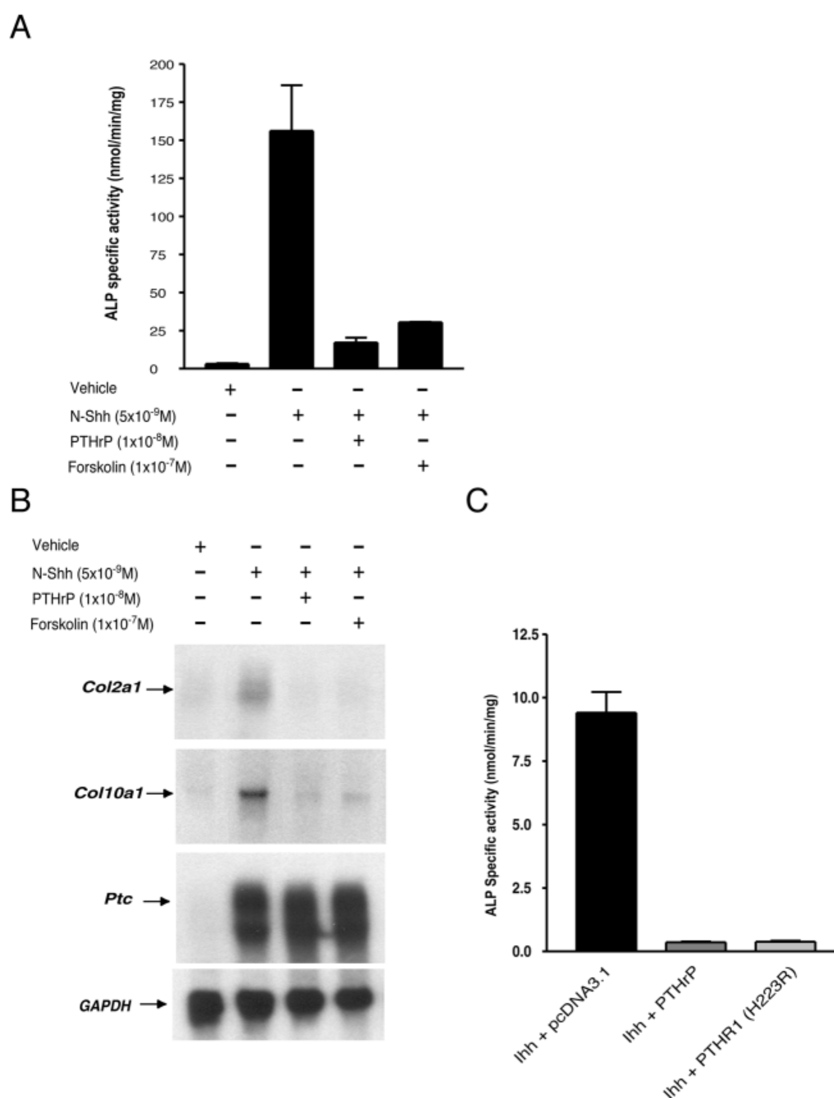

Fig. 4. PTHrP antagonizes *hedgehog*-induced chondrogenic differentiation through a PKA-dependent mechanism. (A) PTHrP and forskolin impede HH-induced ALP activity. Naive CFK-2 cells grown to confluence were subjected to treatment with vehicle or N-Shh (5×10^{-9} M) in the absence or presence of PTHrP 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 PTHR1 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/PTHR1 (H223R)-pcDNA3.1. ALP specific activity was measured following 10 days of postconfluent culture.

Our initial monitoring of HH action in CFK-2 cells was performed by examination of *Ptc* mRNA expression, a transcriptional target of HH-signalling (Goodrich et al., 1996). Preceding the activation of chondrogenic markers, a robust upregulation of *Ptc*-receptor expression was observed in both Ihh/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 N-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 N-terminal Shh have increased potency in ALP activation in 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 N-terminal domain (Burke et al., 1999).

The inherent capacity of the N-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*, Trp117, was shown to localize adjacent to the first α-helix of the peptide (Hall et al., 1995). Since at least one residue (Asp115) residing within this α-helix was shown to be involved in *Ptc* binding and activation of HH-signalling (Pepinsky et al., 2000), it is likely that Trp117/Trp160 may also be crucial for this function. Here, we report that the W160G mutation reduces the capacity of N-teminal Ihh to stimulate *Ptc* expression, indicative of a partial loss in HH-signalling 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-signalling. Moreover, in comparison to other mutations mapping to the N-terminal of Shh, mutations at Trp117 are associated with a milder form of holoprosencephaly and this may be attributed to the partial signalling capacity of this variant (Roessler et al., 1996).

In this study we show that HH-signalling 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-dependent actions that result in delay of chondrocytic hypertrophy (Karp et al., 2000).

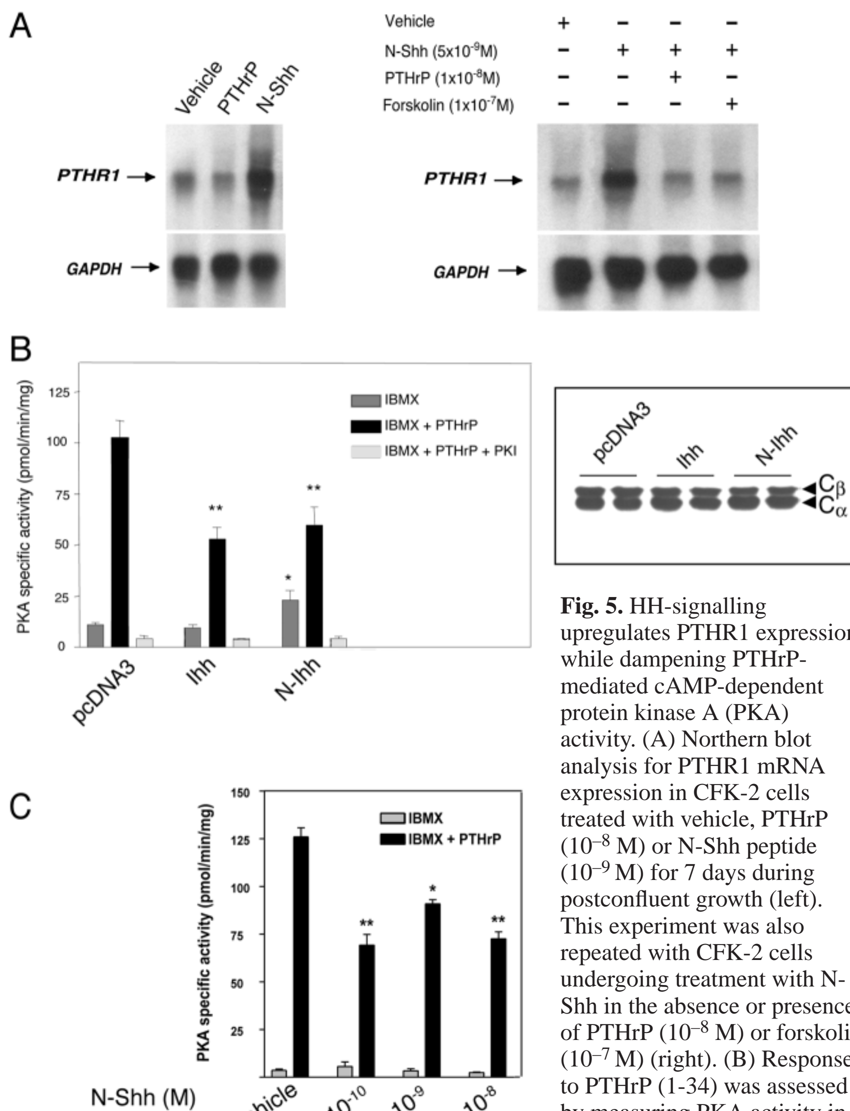

Fig. 5. HH-signalling upregulates PTHR1 expression while dampening PTHrP-mediated cAMP-dependent protein kinase A (PKA) activity. (A) Northern blot analysis for PTHR1 mRNA expression in CFK-2 cells treated with vehicle, PTHrP (10^{-8} M) or N-Shh peptide (10^{-9} M) for 7 days during postconfluent growth (left). 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). (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<0.05; **P<0.001; 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). (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<0.01; **P<0.001).

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 signalling 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-signalling 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-signalling 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 protein (BMP) 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. However, addition of exogenous PTHrP mimicked this in vivo effect.

PTHrP-induced inhibition of chondrogenic differentiation mediated directly through activation of PTHR1 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 PTHR1 H223R, a variant of PTHR1 described in patients with Jansen-type metaphyseal chondrodysplasia, and known to selectively and constitutively activate the PKA pathway (Schipani and Juppner,

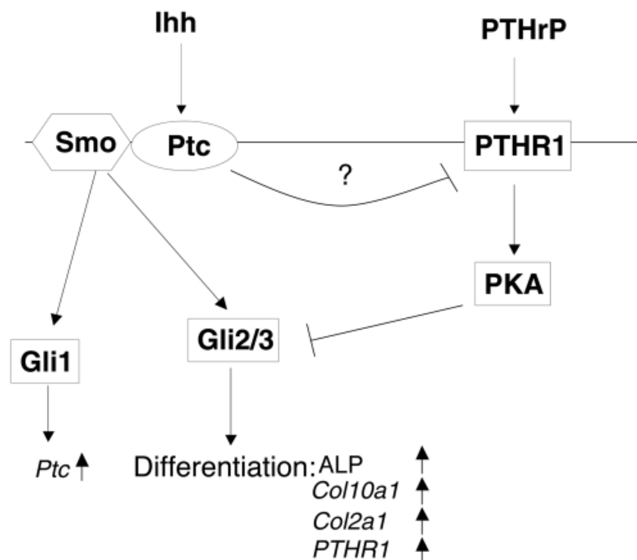

Fig. 6. Proposed model for interaction between the PTHrP and HH signalling pathways. Signalling by all mammalian HH proteins is mediated by the Gli family of transcription factors that consist of three members: Gli1, Gli2 and Gli3. Although all Gli proteins harbour consensus PKA phosphorylation sites, 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 PTHR1. PKA may differentially phosphorylate Gli2 or Gli3, consequently converting them to repressors and causing downregulation of some HH target genes (ALP, *Col2a1*, *Col10a1*, *PTHR1*) while not affecting others (*Ptc*). HH, in turn, may antagonize PKA activation, possibly through functional inhibition of PTHR1, thereby ensuring its own continued signalling capability.

1995). The in vivo observation that overexpression of PTHR1 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-signalling 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 where PTHrP signalling 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 i. Altaba, 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.

PTHR1 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-dependent pathway (Guo et al., 2001).

Interestingly, our data demonstrates an interaction between the HH and PTHrP signalling pathways at the level of PTHR1 regulation. This is evidenced by HH-dependent transcriptional upregulation of *Pthr1* 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 PTHR1 by HH action. These finding have several implications that could converge with in vivo observations. First, the fact that *Pthr1* 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 *Pthr1* being a transcriptional target of HH-signalling (St-Jacques et al., 1999). Second, *Pthr1* 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, signalling by Ihh may be required for inhibition of signalling via PTHR1, and consequently of PTHrP action, in order to allow prehypertrophic chondrocytes to proceed to their final differentiated state. However, HH signalling 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 signalling by HH and PTHrP have interactive feedback mechanisms that allow for the appropriate pace of differentiation to occur (Fig. 6).

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