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HiPER1, a phosphatase of the endoplasmic reticulum with a role in chondrocyte maturation

Paul R. Romano[1], Jun Wang, Regis J. O'Keefe, J. Edward Puzas, Randy N. Rosier and Paul R. Reynolds*

Department of Orthopaedics, Department of Pathology and Laboratory Medicine[1] and Eastman Dental Center[1], University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642, USA

*Author for correspondence at Department of Orthopaedics, University of Rochester, School of Medicine and Dentistry, 601 Elmwood Avenue, Box 665, Rochester, NY 14642, USA (e-mail: preynolds@orthomail.ortho.rochester.edu)

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SUMMARY

We have previously identified and partially cloned *Band 17*, a gene expressed in growth plate chondrocytes transiting from proliferation to hypertrophy. We now rename this gene *HiPER1*, Histidine Phosphatase of the Endoplasmic Reticulum-1, based on the results reported here. *HiPER1* encodes two proteins of 318 (HiPER1^{318}) and 449 (HiPER1^{449}) amino acids, which are 20-21% identical to a group of yeast acid phosphatases that are in the histidine phosphatase family. HiPER1^{449} is significantly more abundant than HiPER1^{318}, correlating with the abundance of the alternatively spliced messages encoding HiPER^{449} and HiPER^{318}. Anti-HiPER1 antibodies detect two proteins of 53 and 55 kDa in growth plate chondrocytes that are absent in articular chondrocytes. We confirm that the 53 and 55 kDa proteins are HiPER1^{449} by heterologous expression of the HiPER1^{449} coding sequence in chick embryo fibroblasts. The 53 and 55 kDa proteins are glycosylated forms of HiPER1^{449}, as N-glycosidase F digestion reduces these proteins to 48 kDa, the predicted size of HiPER1^{449} without the N-terminal signal sequence. Immunocytochemistry demonstrates that HiPER1^{449} is found in chondrocytes maturing from proliferation to hypertrophy, but is not detectable in resting zone, deep hypertrophic zone or articular chondrocytes, a distribution that is consistent with the message distribution. HiPER1^{449} was predicted to localize to the lumen of endoplasmic reticulum by an N-terminal signal sequence and by the C-terminal sequence Ala-Asp-Glu-Leu, which closely matches the consensus signal for ER retention, Lys-Asp-Glu-Leu. We confirm this prediction by demonstrating colocalization of HiPER1^{449} with the ER protein HSP47 using dual-label immunofluorescence. PTHrP, a peptide that prevents hypertrophy in chondrocytes, suppressed *HiPER1* and HiPER1^{449} expression in vitro, an observation that further supports a role for HiPER1 in chondrocyte maturation. The yeast phosphatase homology, localization to the endoplasmic reticulum and pattern of expression suggest that *HiPER1* represents a previously unrecognized intracellular pathway, involved in differentiation of chondrocytes.

Key words: Endoplasmic reticulum, Phosphatase, Chondrocyte differentiation

INTRODUCTION

Endochondral ossification, the process by which bones grow and repair, depends on chondrocyte maturation, a series of phenotypic changes in chondrocytes that are reflected in a gradual shift in matrix composition and morphology. In the growth plate, the most organized example of endochondral ossification, the spatial arrangement of the chondrocytes in columns correlates with their maturation from resting, to proliferating, then to hypertrophic states. The increase in cell number and swelling of the cells both contribute to longitudinal bone growth (reviewed in Hunziker, 1994). As growth plate cells differentiate the relative amounts of types II, IX and XI collagens and large proteoglycans decrease, while expression of type X collagen, lower molecular mass proteoglycans and alkaline phosphatase increase. The differentiation of growth plate chondrocytes culminates in hypertrophic cells synthesizing mineralized cartilage, a matrix that serves as a scaffold for vascular invasion and subsequent bone deposition.

The maturational sequence of growth plate chondrocytes is effected by systemic and local controls. Systemic factors such as vitamins A, C and D, thyroid hormones and growth hormone play a role in growth plate chondrocyte development (Schwartz et al., 1989; Leboy et al., 1989; Gerstenfeld et al., 1990; Iwamoto et al., 1993; Sullivan et al., 1994; Alini et al., 1996; Klaus et al., 1996). Rates of growth in different bones depend on cell numbers and different rates of proliferation and hypertrophy (Wilsman et al., 1996a,b), suggesting that growth plate progression is strongly influenced by local factors. There is evidence that once growth plate chondrocytes have initiated their developmental program, they may progress independently

of systemic factors (Farnum and Wilsman, 1993; Ishizaki et al., 1994; Bohme et al., 1995). This hypothesis has strong experimental support, as chondrocyte differentiation has been shown to depend upon the activities of a number of paracrine and autocrine peptide growth factors such as TGFβ, bFGF and IGF-1 (Kato and Iwamoto, 1990; Crabb et al., 1990; Hill and Logan, 1992; Bohme et al., 1995).

In particular, PTHrP (parathyroid hormone-related peptide) has an essential role in the transition of growth plate chondrocytes from proliferation to hypertrophy. Homozygous deletion of the gene from the mouse chromosome is lethal due to premature hypertrophy of growth plate chondrocytes (Karaplis et al., 1994). Constitutive overproduction of PTHrP in cartilage and haploinsufficiency of the *PTHrP* gene also have deleterious effects on the growth plate (Weir et al., 1996; Amizuka et al., 1996a). PTHrP is mitogenic for chondrocytes in vitro (Loveys et al., 1993; Henderson et al., 1996), and appears to suppress hypertrophy, mineralization and apoptosis in vivo (Lee et al., 1996; Amizuka et al., 1996b; Amling et al., 1997). PTHrP acts in a negative feedback loop with Indian Hedgehog (Ihh), a morphogen that is expressed in the lower proliferative/upper hypertrophic zone of the growth plate (Vortkamp et al., 1996).

The mechanism for transition from proliferation to hypertrophy in chondrocytes is not well characterized, as few proteins other than PTHrP and Indian hedgehog have been identified with defined roles in this maturational step. The genes for the PTHrP receptor and Cartilage Matrix Protein are also expressed in the lower proliferative/upper hypertrophic zone (Lee et al., 1994; Chen et al., 1995), and we have reported a similar pattern for *Band 17* (Reynolds et al., 1996).

Band 17 was identified as specific to growth plate chondrocytes by a differential display of cDNAs expressed in articular and growth plate chondrocytes. In situ hybridization demonstrated expression of *Band 17* in the lower proliferative/upper hypertrophic zone, and experiments in vitro indicated that *Band 17* expression is associated with the induction, but not the maintenance, of the hypertrophic phenotype. Cloning and sequence analysis of the 3′ end of *Band 17* predicted two gene products due to alternative splicing of the messages, and the longer, more abundant product was predicted to be retained in the lumen of the endoplasmic reticulum (ER). The partial *Band 17* cDNA sequence showed homology with two human cDNA clones in the GenBank, but no significant homology with other cDNAs was found that would predict an activity for the Band 17 gene products (Reynolds et al., 1996).

Our goal is to identify the function of *Band 17* in the growth plate, and we present data that confirm and extend our original observations. We have completed the cloning and sequence analysis of the coding sequences for the two *Band 17* gene products. This analysis has uncovered a limited but clear homology with a certain class of phosphatase, which has led to *Band 17* being renamed *HiPER1* (Histidine Phosphatase of the Endoplasmic Reticulum-1). We have also developed antibodies and used them to study the *HiPER1* gene products, and have found that *HiPER1* expression is regulated by PTHrP in vitro. The data presented here enhance the possibility that *HiPER1* represents a previously uncharacterized cellular pathway active during chondrocyte maturation.

MATERIALS AND METHODS

Chondrocyte cell culture, RNA and protein analysis

Embryonic cephalic sternal chondrocytes (day 13) were prepared and cultured as described (Leboy et al., 1989). After isolation and primary culture for 5-7 days, the day of transfer of the floating cells was counted as day 0. On day 1 the medium was supplemented either with 10 μg/ml ascorbate, or with ascorbate plus 10^{-7} M PTHrP (amino acids 1-34, Bachem) or with ascorbate plus 25 ng/ml bFGF (Gibco/BRL). Ascorbate was freshly made and replenished daily, and was increased to 25 μg/ml on day 4 and 50 μg/ml on day 7. The peptide factors were withdrawn after the day 6 samples were taken. Mitogenicity of the two peptides was verified by increased incorporation of tritiated thymidine during treatment (Crabb et al., 1990) and increased cell numbers compared to untreated controls. Suppression of alkaline phosphatase activity by PTHrP was also confirmed (Loveys et al., 1993).

RNA was isolated and analyzed on northern blots as described (Reynolds et al., 1996). Protein extracts were obtained by washing the culture dish twice with ice-cold PBS, then scraping the cell layer into polyacrylamide gel loading buffer. The protein samples were heated to 100°C for 2 minutes before loading on the gel. The protein from approximately 10^6 cells was loaded into one gel lane. Coomassie Blue staining of a pilot gel was used to correct for discrepancies in loading, in order to balance the protein amounts for western blot analysis. Protein analyses of juvenile chick growth plate and articular chondrocytes were performed on cells isolated as described (Crabb et al., 1990).

For western blots, proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose. For use of 4-chloronapthol as a chromogen, blots were probed with affinity-purified anti-HiPER1 antibodies as described (Towbin et al., 1979). For use of chemiluminescence to detect $HiPER1^{449}$, the blots were blocked in 5% nonfat dry milk in PBT (PBS/0.1% Tween-20) for 2-12 hours, washed with PBT, incubated for 1 hour with affinity-purified antibody diluted 1:2000 in PBT/10% NGS, washed, then incubated with HRP-goat anti-rabbit IgG diluted 1:10,000 for 30 minutes. The blots were washed, developed with the Enhanced Chemiluminescence kit (Amersham) according to the manufacturer's directions, and the signal displayed on Kodak AR film.

Analysis of the 5′ end of HiPER1

Screening of the Stratagene growth plate cDNA and chick genomic libraries, and sequence analysis, was as described (Reynolds et al., 1996). The last two rounds of screening of the cDNA library did not identify clones that contained more upstream sequences. RACE (Rapid Identification of cDNA Ends) was then used to identify additional 5′ cDNA sequences (Gibco/BRL kit). The furthest 5′ end of one of the RACE clones is designated as the 5′ end of the cDNA (nucleotide 1 in Fig. 1B).

$HiPER1^{449}$ expression in chick embryo fibroblasts

Two expression vectors were used to produce $HiPER1^{449}$ in chick embryo fibroblasts (CEFs). The first was the retroviral vector RCASBP(A) (Hughes et al., 1987; Petropoulos et al., 1992). As the start codon of $HiPER1^{449}$ is contained within a *Nco*I site, a 1.6 kb *Nco*I-*Eco*RI cDNA fragment containing the entire reading frame plus 0.25 kb of 3′ UTR was cloned into identical sites in the adapter plasmid Cla12NCO. A 1.65 kb *Cla*I fragment was then cloned out of the adapter plasmid construction into the RCASBP(A) in the appropriate orientation for expression.

The second expression vector, pcDNA 3.1(+) (Invitrogen), uses a constitutive cytomegalovirus (CMV) promoter to express inserted cDNAs. A *Bam*HI site in the 5′ UTR 60 bp upstream of the start codon was used as the 5′ end of the inserted cDNA. A 1.65 kb *Bam*HI-*Eco*RI

fragment containing the HiPER1[449] reading frame was cloned into identical sites in the vector.

CEFs were prepared from fertilized eggs, free of endogenous subgroup A Rous sarcoma virus and susceptible to subgroup A infection (SPAFAS, Inc.), then cultured (Morgan and Fekete, 1996). Subconfluent CEFs in 60 mm plates were transfected using LipofectAMINE (Gibco/BRL) according to the manufacturer's directions. For cells transfected with pcDNA 3.1(+) with or without HiPER1[449] reading frame, the cells were incubated without antibiotic for 48 hours before adding G418 at 800 μg/ml. After 8 days the cells were passaged, and after an additional 48 hours protein samples from cell layers were analyzed as described for chondrocytes. For cells transfected with the retroviral expression vector, cells were passaged every 2-3 days, dividing 1:4 to maintain the cells in a proliferative state. After 3 passages, the cell layers were analyzed for HiPER1[449] expression.

Development of anti-HiPER1 antibodies

The *HiPER1* reading frame from Arg 123 (Fig. 1B) to the stop codon was fused to a 5′ $(His)_6$-tag in the *E. coli* expression vector PRO-EX (Gibco-BRL). The fusion protein was induced with IPTG for 4 hours, the cells were collected by centrifugation at 10,000 ***g*** for 10 minutes at 4°C, then lysed at 4°C in a French Press in 0.05 M Tris-HCl, pH 7.5, 0.01 M EDTA, 0.5 M NaCl, 10% sucrose and 0.002 M phenymethylsulfonyl fluoride (PMSF). The insoluble material was collected by centrifugation and dissolved in 6 M guanidine-HCl, 0.1 M Na_3PO_4, 0.01 M Tris HCl, pH 8.0, recentrifuged, then loaded onto a 10×1 cm column of Nickel-NTA affinity resin (Qiagen) and purified according to the manufacturer's directions. The purified fusion protein was precipitated during dialysis against PBS, collected by centrifugation, and was resuspended in 8 M urea, 0.01 M Tris HCl, pH 7.5, at 7 mg/ml. Approximately 20 mg of protein was purified from 2 l of cultured cells.

Polyclonal antisera were produced in rabbits by Berkley Antibody Co. (BABCO, Richmond, CA) using the purified fusion protein as an antigen. One rabbit produced antiserum that interacted only with the 53/55 kDa proteins in growth plate extracts (Fig. 3). This antiserum was used to affinity-purify antibodies as described (Gu et al., 1994).

Immunocytochemistry

Tibial and femoral epiphyseal cartilage were dissected free of soft tissue, fixed sequentially in 10% formalin for 2 hours, then demineralized in Decal (Decal Chemical Co., Congress, NY) for 24 hours. Tissue was processed, embedded via the automated Surgical Pathology system at the University of Rochester, and cut into 5 μm sections. Affinity-purified anti-HiPER1 antibodies were used at 1:200 dilution, followed by biotin-conjugated goat anti-rabbit IgG, HRP-strepavidin and color development with 3-amino-9-ethylcarbazole as described (Rosier et al., 1997). The section in Fig. 4A,B was treated with 1 mg/ml trypsin in PBS for 20 minutes at 37°C before blocking and application of the primary antibody.

Immunofluorescence analysis of subcellular distribution

Sternal chondrocytes were cultured with ascorbate for 8 days on glass coverslips, fixed for 30 minutes in 4% paraformaldehyde in PBS, permeabilized for 5 minutes in −20°C acetone/methanol (1:1), then incubated for 1 hour with either affinity-purified anti-Band 17 antibodies (polyclonal, rabbit) at 1:800 dilution in PBS/0.5% BSA and/or anti-HSP47 antibodies (monoclonal, StressGen Biotech. Corp.) at 1:800. Slides were washed three times in PBS. Secondary antibody incubation was for 1 hour in PBS/10% normal goat serum. Goat secondary antibodies (Zymed Laboratories) against rabbit IgG conjugated to fluorescein isothiocyanate (FITC, excitation wavelength 495 nm, emission wavelength 525 nm, green) were used at 1:40 dilution. Goat anti-mouse antibodies conjugated to tetramethyl rhodamine isothiocyanate (TRITC, excitation wavelength 552 nm, emission wavelength 570 nm, red) were used at 1:100 dilution. Cells were mounted in medium (Vectashield, Vector laboratories) containing 4,6-diamidino-2-phenyl-indole (DAPI) in order to visualize the nucleus. Individual cells were photographed at a magnification of ×1000 on a Zeiss microscope equipped with standard fluorescence filter sets. Photography and image analysis were as described (Olson et al., 1995).

Treatment of growth plate chondrocyte extracts with N-glycosidase F

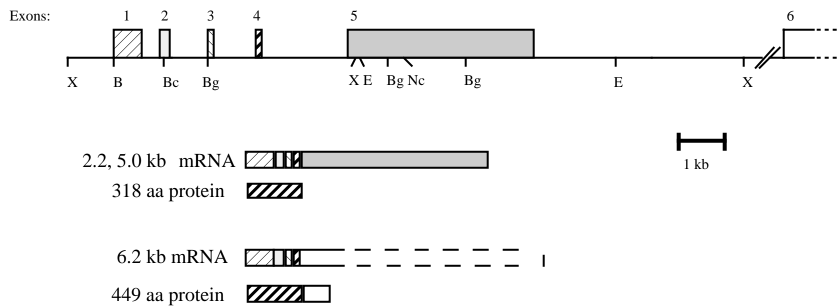
Growth plate chondrocytes were cultured at 10^7 cells/100 mm plate and incubated overnight in DMEM/10% FBS supplemented with 50 μg/ml ascorbate. The cell layer was rinsed in cold PBS/2 mM PMSF, 1 ml of lysis buffer (100 mM $NaPO_4$, pH 7.2, 25 mM EDTA, 1% SDS) was added to the plate, and the cells were scraped into a 15 ml tube. The cells were rocked for 60 minutes at 4°C, then centrifuged at 7500 ***g*** for 10 minutes at 4°C. 200 μl of the supernatant was diluted 1:6 into incubation buffer (100 mM $NaPO_4$, pH 7.2, 25 mM EDTA, 1% Triton X-100, 1% β-mercaptoethanol). 100 μl samples of the diluted cellular lysate were incubated for 16 hours at 37°C with 0, 1, 2 or 4 units of recombinant N-glycosidase F (Boehringer-Mannheim). Reaction products were analyzed by gel electrophoresis and immunoblotting.

RESULTS

HiPER1 shows homology to histidine phosphatases

We have reported the cloning and sequence analysis of the 3′ end of *HiPER1*, which was referred to as *Band 17* (Reynolds et al., 1996). The 5′ portion of *HiPER1* was cloned from a growth plate cDNA library and a chicken genomic library, and the 5′ end of the cDNA was mapped by RACE (see Materials and methods). The chick *HiPER1* gene structure is depicted in Fig. 1A. The 6.2 kb transcript, comprised of exons 1-4 plus exon 6, is significantly more abundant than the 2.2 and 5.0 kb transcripts, which comprise exons 1-5 (the 2.2 kb transcript includes approx. 1 kb of exon 5). The predicted gene product of the 6.2 kb transcript is 449 amino acids (HiPER1[449], Figs 1B, 2A), with a molecular mass of 50.2 kDa. Exon 5 is completely untranslated, and the predicted gene product of 318 amino acids from the 2.2 and 5.0 kb mRNAs is a truncated form of HiPER[449] (HiPER[318], Figs 1B, 2A) of 35.2 kDa. Analysis of the predicted peptide sequences shows four potential N-glycosylation sites, two of which are only in the longer protein (Fig. 1B). Amino acids 6-20 constitute the only significantly hydrophobic segment of both proteins, as predicted by a Kyte-Doolittle plot (boxed in Fig. 1B). Thus, both proteins contain a putative signal sequence at their N terminus that would enter the proteins into the secretory pathway. HiPER1[449] has a consensus sequence at the C terminus for retention of the protein in the luminal space of the endoplasmic reticulum. Fig. 5 demonstrates that this retention signal is functional (below), which strongly suggests that the signal sequence is functional.

Comparison of the HiPER1[449] sequence with the NCBI data bank using the BLAST sequence homology program (Altschul et al., 1990) indicated a significant match with a group of yeast acid phosphatases (probability of random match approx. e^{-6}). Individually matching the HiPER1[449] amino acid sequence with those of the yeast phosphatases using the program



B.

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   1  GGGGCGAAGGGAGGCGAGAGGATCCCGGAGCAGCTGGAGCAGGCGGCCGCGCCCGTCCTCCTCTTCCTGCAGCTG

  76  CCGCCATGGCGCCGTGCCGCGCTGCCTGTCTGCTGCCGCTTCTGGTAGCGGTGGCGAGCGCCGGGCTGGGCGGCT
          M  A  P  C  R  A  A  C  L  L  P  L  L  V  A  V  A  S  A  G  L  G  G  Y   24

 151  ACTTCGGCACCAAGTCCCGCTACGAGGAGGTGAACCCGCACCTGGCGGAGGACCCGCTGTCCCTCGGGCCGCACG
        F  G  T  K  S  R  Y  E  E  V  N  P  H  L  A  E  D  P  L  S  L  G  P  H  A   49

 226  CCGCCGCCGCCCGGCTGCCCGCCGCCTGCGCCCCGCTGCAGCTCCGCCGCGTCGTCCGCCACGGCACCCGCTACC
        A  A  A  R  L  P  A  A  C  A  P  L  Q  L  R  R  V  V  R  H  G  T  R  Y  P   74

 301  CCACGGCCGGGCAAATCCGCCGCCTGGCCGAGCTGCACGGCCGCCTCCGCCGCGCCGCCGCCCCGTCCTGCCCCG
        T  A  G  Q  I  R  R  L  A  E  L  H  G  R  L  R  R  A  A  A  P  S  C  P  A   99

 375  CCGCCGCCGCGCTGGCCGCCTGGCCGATGTGGTACGAGGAGAGCCTCGACGGGCGGCTGGCGCCGCGGGGCCGCC
        A  A  A  L  A  A  W  P  M  W  Y  E  E  S  L  D  G  R  L  A  P  ℝ  G  R  R  124

 451  GCGACATGGAACACCTGGCGCGCCGCCTGGCCGCCCGCTTCCCCGCGCTCTTCGCCGCCCGCCGCCGCCTGGCGC
        D  M  E  H  L  A  R  R  L  A  A  R  F  P  A  L  F  A  A  R  R  R  L  A  L  149

 526  TGGCCAGCAGCTCCAAGCACCGCTGCCTGCAGAGCGGCGCGGCCTTCCGGCGCGGCCTCGGGCCCTCCCTCAGCC
        A  S  S  S  K  H  R  C  L  Q  S  G  A  A  F  R  R  G  L  G  P  S  L  S  L  174
                Exon 1/Exon2
 601  TCGGCGCCGACG AGACGGAGATCGAAGTGAACGACGCGCTGATGAGGTTTTTGATCACTGCGACAAGTTCGTGG
        G  A  D  E   T  E  I  E  V  N  D  A  L  M  R  F  F  D  H  C  D  K  F  V  A  199

 676  CCTTCGTGGAGGACAACGACACAGCCATGTACCAAGTGAACGCCTTCAAAGAGGGCCCGGAGATGAGGAAGGTGT
        F  V  E  D  N  D  T  A  M  Y  Q  V  N  A  F  K  E  G  P  E  M  R  K  V  L  224
                                                   Exon 2/Exon 3
 751  TGGAGAAGGTGGCGAGTGCCCTGTGTCTGCCGGCCAGCGAGCTGAACGCAG ATCTCGTTCAAGTGGCTTTCCTCA
        E  K  V  A  S  A  L  C  L  P  A  S  E  L  N  A  D   L  V  Q  V  A  F  L  T  249
                                                                 Exon 3/Exon4
 826  CTTGCTCGTATGAGTTGGCTATAAAAAATGTGACCTCCCCGTGGTGTTCGCTCTTCAGTGAAGAAGATGCTAAG G
        C  S  Y  E  L  A  I  K  N  V  T  S  P  W  C  S  L  F  S  E  E  D  A  K   V  274

 901  TACTGGAGTACCTGAATGACCTGAAGCAATACTGGAAGAGAGGATATGGCTATGACATCAATAGTCGCTCCAGCT
        L  E  Y  L  N  D  L  K  Q  Y  W  K  R  G  Y  G  Y  D  I  N  S  R  S  S  C  299
                                                            Exon 4/Exon 6
 976  GCATTTTATTCCAGGATATCTTCCAGCAGTTGGACAAAGCAGTGGATGAGAGCAGAAG TTCAAAACCCATTTCTT
        I  L  F  Q  D  I  F  Q  Q  L  D  K  A  V  D  E  S  R  S   S  K  P  I  S  S  324

1051  CACCTTTGATTGTACAAGTTGGACATGCAGAAACACTTCAGCCACTTCTTGCTCTTATGGGCTACTTCAAAGATG
        P  L  I  V  Q  V  G  H  A  E  T  L  Q  P  L  L  A  L  M  G  Y  F  K  D  A  349

1126  CTGAGCCTCTCCAGGCCAACAATTACATCCGCCAGGCGCATCGGAAGTTCCGCAGCGGCCGGATAGTGCCTTATG
        E  P  L  Q  A  N  N  Y  I  R  Q  A  H  R  K  F  R  S  G  R  I  V  P  Y  A  374

1201  CAGCCAACCTGGTGTTTGTGCTGTACCACTGTGAGCAGAAGACCTCTAAGGAGGAGTACCAAGTGCAGATGTTGC
        A  N  L  V  F  V  L  Y  H  C  E  Q  K  T  S  K  E  E  Y  Q  V  Q  M  L  L  399

1276  TGAATGAAAAGCCAATGCTCTTTCATCACTCGAATGAAACCATCTCCACGTATGCAGACCTCAAGAGCTATTACA
        N  E  K  P  M  L  F  H  H  S  N  E  T  I  S  T  Y  A  D  L  K  S  Y  Y  K  424

1351  AGGACATCCTTCAAAACTGTCACTTCGAAGAAGTGTGTGAATTGCCCAAAGTCAATGGTACCGTTGCTGACGAAC
        D  I  L  Q  N  C  H  F  E  E  V  C  E  L  P  K  V  N  G  T  V  A  D  E  L  449

1426  TTTGAGGGAATGAAATGGAGTGGCCGATTTGGAAACCGATCTCAGTTTTCTTCAACAGATGTTGTGAACGAGCAC

1501  TTTGGATGCAATGCTGCTGCTGTGCCGACTCTCTAAGCTCGCAGATTTGACGGCCGTTATTTACCTGGGTTGTCT

1575  CTGTCAGCTCAA  1586
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Fig. 1. Structure of *HiPER1* and predicted gene products.
(A) Diagram of the *HiPER1* gene. The cDNA for the 5.0 kb transcript is composed of exons 1-5, the 2.2 kb transcript is composed of exons 1-4 plus a truncated version of exon 5, and the 6.2 kb transcript is composed of exons 1-4 plus 6. B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; Nc, *Nco*I; X, *Xba*I. In the previous publication identifying *HiPER1* (*Band 17*) (Reynolds et al., 1996), exons 2-6 were referred to as exons A-E. The segments not sequenced are approx. 0.5 kb near exon 6, which is denoted by slashes, and the 3′ untranslated region of the 6.2 kb transcript, which is denoted by dashed lines.
(B) *HiPER1* cDNA and gene product. Nucleotide numbering is on the left and the start of transcription is labeled 1. The initiation ATG is at 81, and amino acid numbering is on the right. Exon junctions are labeled above the nucleotide sequence. The putative signal sequence is boxed, and the N-glycosylation sites are underlined. The Arginine that is the HiPER1 N-terminal of the *E.* coli fusion protein used as an antigen is outlined (ℝ). Accession number for the cDNA sequence is U59421.

A.

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MAPCRAACLLPLLVAVASAGLGGYFGTKSRYEEVNPHLAEDPLSLGPHAAAARLPAACAPLQLRRV
VRHGTRYPTAGQIRRLAELHGRLRRAAAPSCPAAAALAAWPMWYEESLDGRLAPRGRRDMEHLAR*
RLAARFPALFAARRRLALASSSKHRCLQSGAAFRRGLGPSLSLGADETEIEVNDALMRFFDHCDKF
VAFVEDNDTAMYQVNAFKEGPEMRKVLEKVASALCLPASELNADLVQVAFLTCSYELAIKNVTSPW
CSLFSEEDAKVLEYLNDLKQYWKRGYGYDINSRS★
SCILFQDIFQQLDKAVDESRSSKPISSPLIVQVGH*AETLQPLLALMGYFKDAEPLQANNYIRQAH
RKFRSGRIVPYAANLVFVLYHCEQKTSKEEYQVQMLLNEKPMLFHHSNETISTYADLKSYYKDIL
QNCHFEEVCELPKVNGTVADEL
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B.

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                              PZ1,PZ4...RHGSRNPT...
  PHO3,PHO5,PHO11, PHO12,Hyp,Kl5...RHGERYPT...
                              Pp1,PhyB...RHGERYPS...
                                  PhyA...RHGARYPT...
                     Ch/hu/mu HPER1...RHGTRYPT...
                                  EcAP...RHGVRAPT...
                                  HPAP...RHGDRSPI...
                             HLAP,RLAP...RHGDRSPV...
                                  LLAP...RHGARSPL...
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Fig. 2. HiPER1 is in the histidine phosphatase family. (A) Conserved residues among HiPER1[449] and yeast acid phosphatases. This analysis was done using the program PILEUP (Genetics Computer Group, 1994). Bold lettering designates identical or highly conserved residues in the HiPER1 sequence that are matched between HiPER1 and at least 8/10 of the following yeast proteins (given here with their organism, accession numbers, and abbreviations used in B): *Schizosaccharomyces pombe*, P08091 (PZ1) and Q01682 (PZ4); *Saccharomyces cerevisiae*, P24031 (PHO3), P00635 (PHO5), P35842(PHO11), P38693(PHO12), and S52495(Hyp); *Kluyveromyces lactis*, Z33995 (Kl5); *Aspergillus nidans*, P34754 (PhyB); *Pichia pastoris*, U28658 (Pp1). The highly conserved active site is underlined, and Arg[131] and His[332] are denoted by asterisks. The C-terminal of the 318 amino acid protein is a star (★). (B) Homology of Band 17 family members at the active site histidine. Ten yeast acid phosphatases (abbreviations as in A), *E. coli* acid phosphatase (EcAP, Accession number L03370), human prostatic acid phosphatase (HPAP, Accession number M24902), and human, rat and protozoan lysosomal acid phosphatases (HLAP, RLAP and LLAP, Accession numbers X12548, M27893 and Z46971) all share the same sequence at the active site histidine. Bold lettering denotes the consensus sequence found in all family members.

BESTFIT (Genetics Computer Group, 1994) shows that HiPER1[449] is 20-21% identical with each phosphatase. The homology with HiPER1[449] and this group of enzymes is comparable with the lowest homology within the group. For example, the *S. cerevisiae* gene products PHO11 and PHO12 are 99% identical, but PHO11 is only 26% identical with the PhyA protein of *Aspergillus ficuum* and the PHO1 protein of *Schizosaccharomyces pombe*. The *A. ficuum* and *S. pombe* enzymes are also 26% identical. The highly conserved residues among the yeast enzymes and HiPER1[449] are shown in bold lettering in Fig. 2A. The highest conservation is clearly at the Arg-His-Gly-X-Arg-X-Pro (RHGxRxP) motif, which is the predicted active site of the enzyme.

Phosphatases have been classified into groups based on catalytic mechanism and substrates (Guan and Dixon, 1991; Vincent et al., 1992). The yeast acid phosphatases and HiPER1 are in a family of enzymes that includes *E. coli*, lysosomal and human prostatic acid phosphatases. These enzymes utilize a phosphohistidine as an intermediate at the active site during removal of the phosphate group from the substrate (Van Etten, 1982; Vincent et al., 1992; Ullah and Dischinger, 1993). The active site histidine is within an invariant Arg-His-Gly tripeptide that is part of the RHGxRxP consensus (Fig. 2B). The essential nature of the RHGxRxP and certain other residues for phosphatase activity in this group of enzymes has been established by in vitro mutagenesis and biophysical studies of the *E. coli* (EcAP) and human prostatic (HPAP) acid phosphatases (Van Etten et al., 1991; Ostanin et al., 1992, 1994). In addition to the RHGxRxP, there are a highly conserved arginine (Arg[131]) and histidine (His[332]) in HiPER[449] and the yeast phosphatases (Fig. 2A, asterisks) that correspond to the required Arg[92] and His[303] residues of EcAP (Ostanin et al., 1992) and Arg[79] and His[257] of HPAP (Ostanin et al., 1994).

The HiPER[318] protein does not have the His[332] residue, making the classification of the short gene product as a phosphatase unsettled. HiPER[318] also does not have a luminal retention sequence and, based on the signal sequence, might be an extracellular protein. We detect very little extracellular staining with anti-HiPER1 antibodies (Fig. 4), and we have not detected HiPER1[318] protein in concentrated extracellular medium from sternal chondrocyte cultures. We have focused our current studies on HiPER1[449].

HiPER1[449] codes for two N-glycosylated proteins of 53 and 55 kDa in growth plate chondrocytes

In growing long bones, the *HiPER1* (*Band 17*) mRNAs were detectable in growth plate chondrocytes undergoing a transition from proliferation to hypertrophy, and were not found in articular, resting zone or hypertrophic chondrocytes (Reynolds et al., 1996). We have confirmed an identical pattern of expression for HiPER1 protein with anti-HiPER1 antibodies raised against a fusion protein produced in *E. coli*. The antiserum and affinity-purified antibodies recognize a protein doublet of 53 and 55 kDa that is present in growth plate chondrocyte protein extracts but is not detectable in articular chondrocyte extracts (Fig. 3A). The antiserum faintly detects a protein of 37 kDa, which may correspond to the HiPER[318] protein (arrowhead, Fig. 3A).

We have established that the HiPER1[449] cDNA codes for the 53/55 kDa doublet by expression of the HiPER1[449] reading frame in CEFs. The HiPER1[449] cDNA expression was effected through a retroviral (RCASBP(A)) or CMV promoter (pcDNA 3.1(+)). CEFs without either expression vector, or with pcDNA 3.1(+) alone, do not make detectable HiPER1[449] (Fig. 3B, lanes 1 and 2). CEFs transfected with either retroviral-driven (Fig. 3B, lane 3) or CMV-driven (lane 4) expression synthesize the 53/55 kDa doublet and a 51 kDa protein, which may be another glycosylated form (see below).

The molecular mass of the 53/55 kDa doublet is slightly larger than the 50.2 kDa predicted by the primary sequence. The endoplasmic reticulum is a site for protein glycosylation (Abejion and Hirschberg, 1992). Therefore, we investigated whether HiPER1 may be glycosylated on an asparagine (N-glycosylation) by treatment of growth plate chondrocyte protein extracts with N-glycosidase F (NGF). The 53 and 55 kDa proteins are decreased in molecular mass by NGF (Fig. 3C, compare lanes 2-4 with lane 1). A single protein of 48

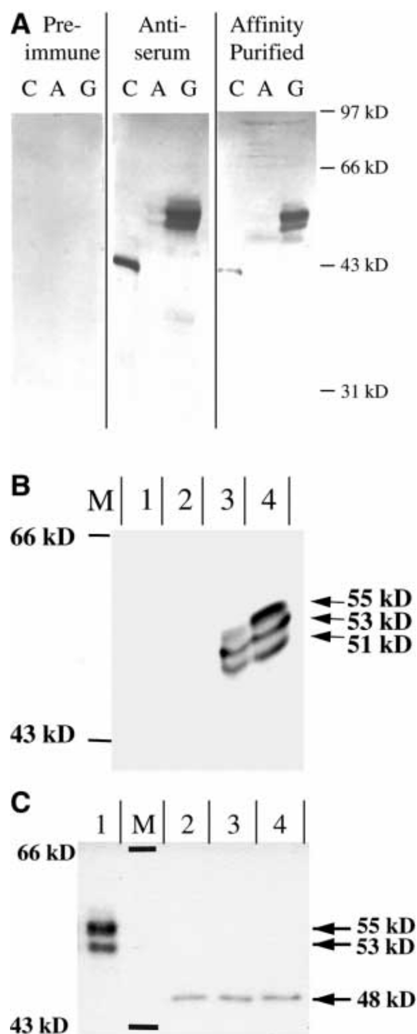

Fig. 3. HiPER1 protein analysis. (A) Anti-HiPER1 antibodies interact with growth plate chondocyte proteins. Protein extracts were prepared from isolated growth plate (lanes G) and articular chondrocytes (lanes A). Equal amounts of protein from each cell type were separated on a polyacrylamide gel, blotted to nitrocellulose, and probed with either preimmune serum, antiserum at 1:3000 dilution, or affinity-purified anti-HiPER1 antibody at 1:3000 dilution. 5 ng of antigen used to raise the antibody was used as a control (lanes C). 4-chloronapthol was used as a chromogen. (B) Expression of HiPER1^{449} in chick embryo fibroblasts. Chick embryo fibroblasts (CEFs) were transfected with various expression vectors, and cellular protein extracts were analyzed as described in Materials and methods. Blots were developed using chemiluminescence. Lane M, standards (sizes given on left). CEF sample lanes: lane 1, CEF protein from untransfected cells; lane 2, CEF cells transfected with pcDNA 3.1(+)CMV vector (Invitrogen); lane 3, pcDNA 3.1(+) with the HiPER1^{449} reading frame downstream of the CMV promoter; lane 4, CEFs transfected with RCASBP(A) retroviral vector expressing HiPER1^{449}. Sizes of the expressed proteins are given on the right. (C) HiPER1^{449} is N-glycosylated. Protein extracts from growth plate chondrocytes were treated with N-glycosidase F (NGF), then analyzed by gel electrophoresis, western blotting and probing with affinity-purified antibody. Lane M, standards (sizes given on left). Lane 1, GPC extract without NGF treatment; lanes 2-4, GPC extract digested with 1, 2 or 4 units of NGF. Blots were developed using chemiluminescence. Sizes of the untreated and treated protein are given on the right.

kDa appears after NGF treatment, the size of which agrees with the predicted molecular mass of 48.4 kDa of the HiPER^{449} protein after cleavage of the putative signal sequence boxed in Fig. 1B. There are four potential N-glycosylation sites in HiPER^{449}, and the doublet of protein may reflect variable numbers of those sites being glycosylated, or variable glycosylation at one site.

HiPER1^{449} is enriched in the lower proliferative/upper hypertrophic zone

In the growth plate, HiPER1 protein is detectable in chondrocytes (red staining, Fig. 4) in the lower portion of the proliferative zone (P, Fig. 4A,B) and upper portion of the hypertrophic zone (H, Fig. 4A,B), an area of the growth plate referred to as the zone of maturation (Chen et al., 1995). No HiPER1 is detectable in chondrocytes within articular cartilage (AC, Fig. 4A,B) and very few chondrocytes of the lower hypertrophic zone contain detectable HiPER1 protein. Perichondrium cells (PC, Fig. 4C) contain HiPER1, but most of the hypertrophic cells (H, Fig. 4C) derived from the perichondrium do not contain detectable HiPER1. All protein that is detected is intracellular, and a closer examination of the intracellular localization in cells just beginning to express HiPER1 shows a perinuclear staining (Fig. 4D, arrowheads) that is similar to that found for proteins associated with the endoplasmic reticulum (Bult et al., 1996). A very faint amount of staining external to a few cells of the lower proliferative zone may indicate the presence of HiPER1^{318} or a limited amount of HiPER1^{449} that 'escapes' the retrieval process. In vitro sternal chondrocytes do not secrete HiPER1^{449} (P. R. Romano and P. R. Reynolds, unpublished data). Thus, we believe that growth plate chondrocytes retain nearly all of the HiPER1^{449} protein, which is consistent with the retrieval signal at the C terminus (see next section).

HiPER^{449} localizes to the endoplasmic reticulum

The C terminus of the HiPER1^{449} protein is Ala-Asp-Glu-Leu (ADEL), which closely resembles the KDEL sequence that is necessary and sufficient for retention of a protein in the ER (Munro and Pelham, 1987). Therefore, we have examined the intracellular distribution of HiPER1^{449} by immunofluorescence, colocalizing HiPER^{449} with HSP47. HSP47 is a luminal ER protein that functions as a collagen chaperone in a number of cell types, including chondrocytes (Kambe et al., 1994; Nagata, 1996). In cultured embryonic sternal chondrocytes, the intracellular distribution of HSP47 (Fig. 5A) is identical with the distribution of HiPER1^{449} (Fig. 5B). Computer alignment of the images shows a complete overlap of the two proteins (yellow, Fig. 5C).

HiPER1 and HiPER1^{449} are regulated by PTHrP

PTHrP has a strong influence on chondrocyte maturation, inhibiting the transition from proliferation to hypertrophy (Amizuka et al., 1994). *HiPER1* (*Band 17*) message expression correlates with this transition, but is not maintained during hypertrophy (Reynolds et al., 1996), and Fig. 4 corroborates this pattern for HiPER1^{449}. Therefore we investigated the effect that PTHrP would have on *HiPER1* expression. The experiments were performed in cultured embryonic sternal chondrocytes, which increase *HiPER1* expression when

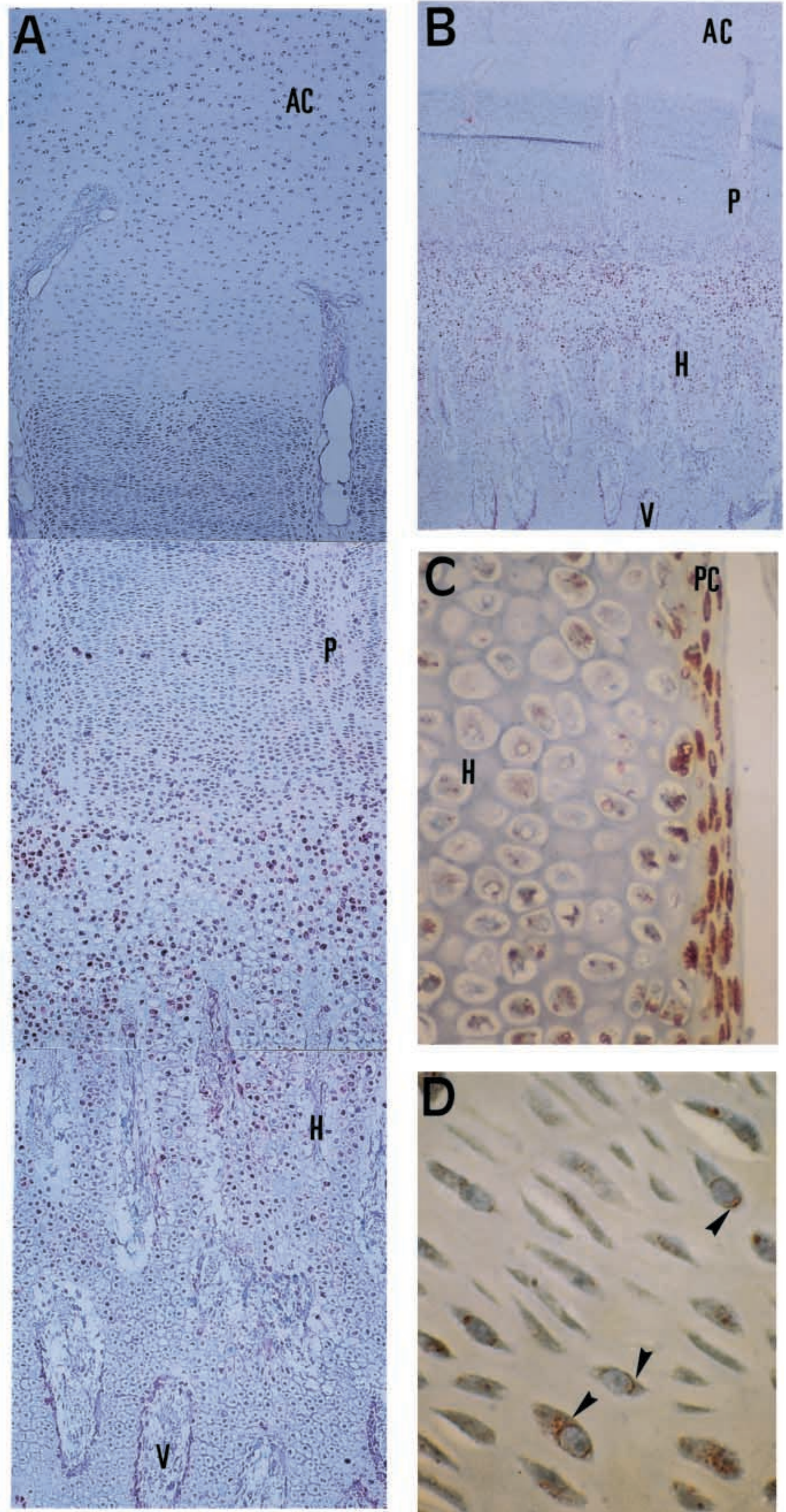

Fig. 4. HiPER1 is expressed transiently in epiphyseal chondrocytes. Immunocytochemistry was performed on a paraffin-embedded thin section of a juvenile chick epiphysis. (A) A composite of three photographs taken sequentially along the same line parallel to the long axis of the bone. Chondrocytes in the lower proliferative (P) and upper hypertrophic zone (H) are enriched for HiPER1, as shown by red staining. Articular cartilage (AC) cells and chondrocytes of the upper proliferative and lower hypertrophic zones do not contain detectable HiPER1. ×100. (B) A lower power view of the composite section in A, ×40. (C) Cells in the perichondrium (PC) express HiPER1, but hypertrophic cells (H) derived from the perichondrium do not. ×400. (D) HiPER1 is initially expressed in a perinuclear pattern (arrowheads). ×1000.

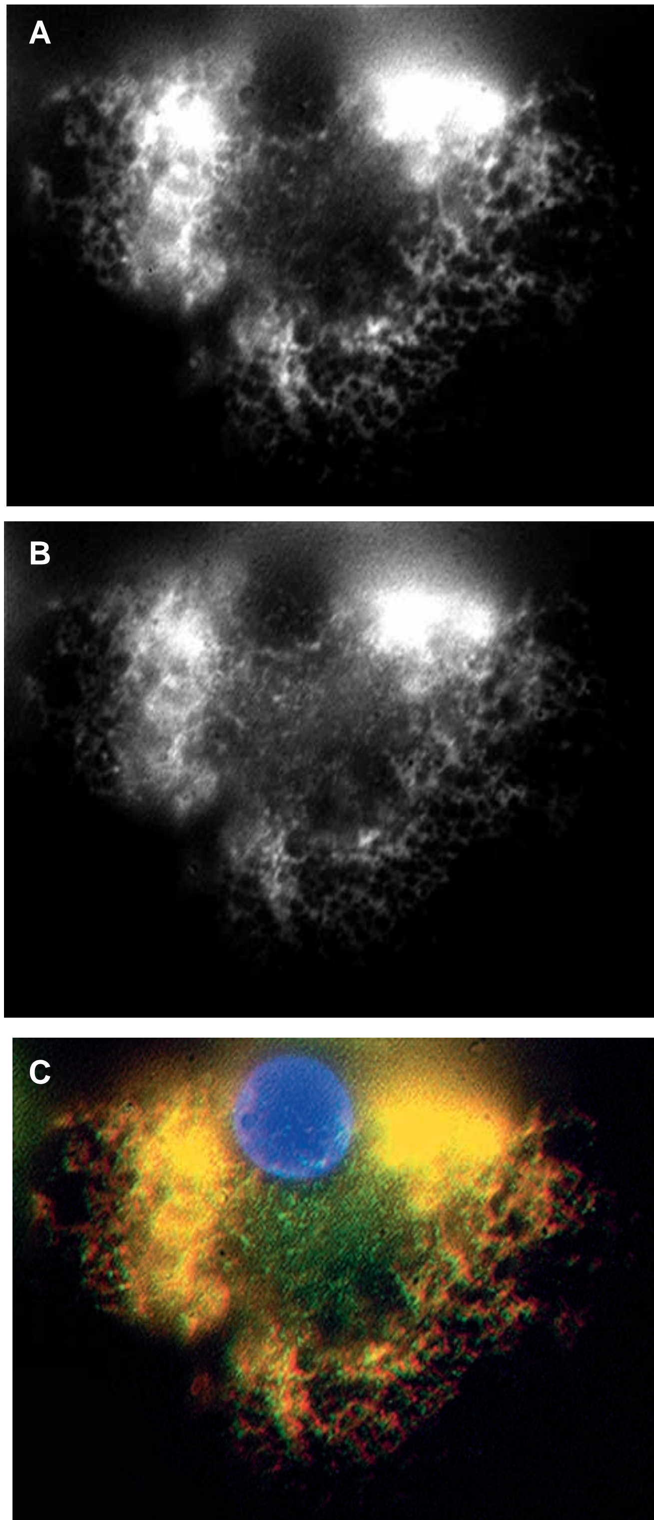

Fig. 5. HiPER1^{449} colocalizes to the endoplasmic reticulum with HSP47. Embryonic sternal chondrocytes were probed with either affinity-purified anti-HiPER1 antibodies or anti-HSP47 antibodies, as described in Materials and Methods. Individual cells were photographed at ×1000. (A) Intracellular distribution of HSP47 (red emission, photographed in black and white). (B) Intracellular distribution of HiPER1^{449} (green emission, photographed in black and white). (C) Overlapping expression of HiPER1^{449} and HSP47 (yellow). Nucleus is stained with DAPI and appears blue. The green color below the nucleus in the picture may be due to greater signal intensity from HiPER1^{449} than HSP47, which is discernible by comparing that area of the cell in B (HiPER1^{449}) with A (HSP47).

incubated with ascorbate, a factor that induces the hypertrophic phenotype in these cells (Leboy et al., 1989; Sullivan et al., 1994). We also investigated the response of HiPER1 expression to bFGF, another chondrocyte mitogen (Kato and Iwamoto, 1990; Crabb et al., 1990). The responses of the *Indian hedgehog* (*Ihh*) and *Type X* collagen genes to PTHrP were used as controls, as the expression of these genes is decreased by PTHrP (Loveys et al., 1993; Vortkamp et al., 1996).

During culture, the sternal chondrocytes treated with ascorbate increase their expression of *HiPER1* significantly compared to the nontreated cells, with the expression peaking at day 6 (Fig. 6A, compare lanes 1 and 2, 5 and 6, 9 and 10, 13 and 14). The HiPER1^{449} protein follows a similar pattern, but the protein appears to be more stable than the message: expression of the protein in the non-treated cells compared to treated appears relatively higher than is found for the message through day 8 (Fig. 6B, same lane numbers). As expected, *Type X* message is increased in ascorbate treated versus non-treated chondrocytes; this effect is seen through day 8 (Fig. 6A). *Ihh* messages are not affected by ascorbate treatment (Fig. 6A).

PTHrP inhibits the expression of *HiPER1* nearly completely (Fig. 6A, lanes 3, 7 and 11) and suppresses the expression of HiPER1^{449} to undetectable levels (Fig. 6B, lanes 3, 7 and 11) during a 6-day treatment. Withdrawal of the PTHrP results in partial recovery of message (Fig. 6A, lane 15) and protein expression (Fig. 6B, lane 15). In contrast, bFGF treatment for 6 days effected only partial suppression of the *HiPER1* and *Type X* messages, and did not affect *Ihh* expression (Fig. 6A, Lanes 4, 8 and 12). bFGF had very little effect on HiPER^{449} expression (Fig. 6B, lanes 4, 8 and 12), which reflects the small effect that bFGF had on the message.

DISCUSSION

The acid phosphatase family includes a large number of enzymes, but the RHGxRxP sequence at the active site defines *HiPER1* as a histidine phosphatase (Van Etten, 1982; Vincent et al., 1992; Ullah and Dischinger, 1993). The *E. coli* acid phosphatase and the eukaryotic lysosomal and prostatic acid phosphatases are also histidine phosphatases. However, the homology of HiPER1^{449} with these phosphatases is limited to the active site residues such as the RHGxRxP and the Arg^{131} and His^{332} residues. Computer searches of GenBank for homologs of *HiPER1* listed only the homology with the yeast enzymes as significant, and individually matching of *HiPER1* with the histidine phosphatases using the computer alignment program BESTFIT (Genetics Computer Group, 1994) aligns the active site residues for matches with the yeast enzymes. In constrast, BESTFIT does not align the active sites of the other enzymes such as *E. coli* or human prostatic acid phosphatase, underscoring that the overall homology of these enzymes with *HiPER1* is not strong.

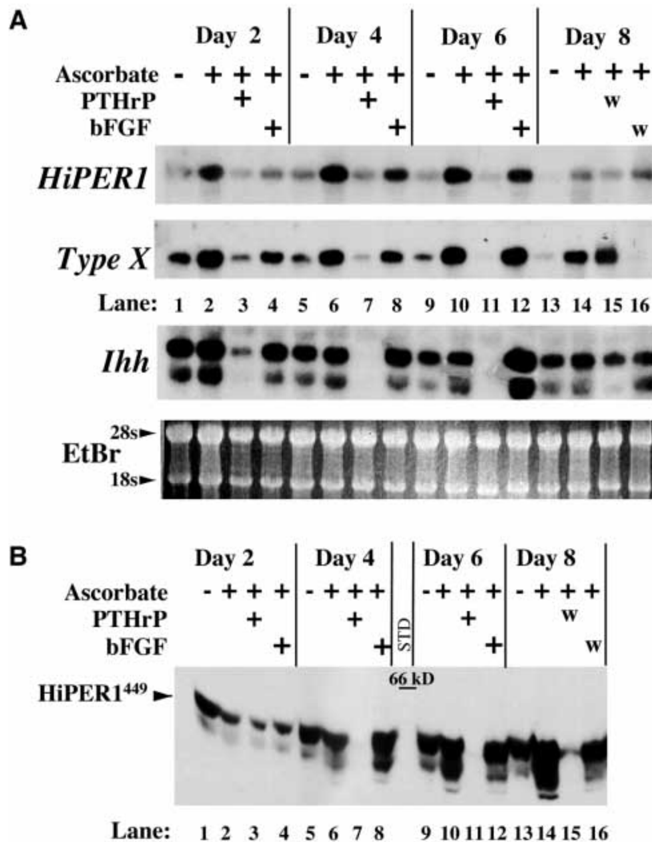

Fig. 6. Expression of *HiPER1* and HiPER1[449] is regulated by PTHrP. Cultured sternal chondrocytes were treated with ascorbate with or without PTHrP or bFGF, as described in Materials and Methods. Additions of various factors are indicated by plus signs. w, PTHrP or bFGF treatment withdrawn at day 6. (A) Northern blots were hybridized to random primed cDNA fragments for *HiPER1* or *Ihh* messages, or an end-labeled oligonucleotide for analysis of the *Type X* message. EtBr, ethidium bromide staining of the ribosomal RNAs on the samples probed for *HiPER1*. Identical loadings were used on the blots probed for *Type X* and *Ihh.* This experiment shows representative results from one of three experiments. (B) A western blot of protein samples from sternal chondrocytes cultured identically to those analyzed for mRNA expression in A. The line labeled 66 kD denotes the position of the bovine serum albumin molecular mass marker.

The function of the *HiPER1* gene products in chondrocyte physiology will depend on identification of substrates for the phosphatase activity. The yeast homologs are secreted or periplasmic enzymes that are part of the phosphate metabolic pathway, for example the *PHO3/PHO5* genes of *S. cerevisiae* (Bajwa et al., 1984) or the *phyA* gene of *Aspergillus* (Ullah and Dischinger, 1993). The phytase enzymes of *Aspergillus* cleave phosphate groups from phytic acid (*myo*-inositol hexaphosphate) and other inositol polyphosphates (Ullah and Phillippy, 1988). The homology of *HiPER1* with the phytase group of enzymes suggests that HiPER1[449] may have activity on polyphosphorylated *myo*-inositols or possibly phosphatidyl inositols. Various forms of these metabolites have a broad spectrum of activities within the cell (Liscovitch and Cantley, 1995; Majerus, 1992). Another broad set of substrates is suggested by a report that *S. cerevisiae* acid phosphatase has phosphotyrosine phosphatase activity (Donella-Deane et al., 1986). Clearly, at this juncture predicting the specific biochemical activity for HiPER1 is difficult.

Presumably the substrate(s) will be limited to the endoplasmic reticulum, as our immunofluorescence studies demonstrate the ER localization of HiPER1[449] (Fig. 5). The localization to the ER suggests a role for HiPER1[449] in post-translational modification or folding of proteins in the secretory pathway, or a more general role in protein trafficking (Nichitta and Blobel, 1993; Pelham and Munro, 1993). The latter possibility is indicated by the binding of inositol hexakisphosphate and tetrakisphosphate to a coatomer protein (Fleischer et al., 1994). A luminal ER inositol-3 phosphatase (MIPP) with activity on inositol polyphosphates has been identified in the rat. HiPER1[449] may be distinct from MIPP. MIPP has two reported molecular masses of 47 and 66 kDa (Ali et al., 1993; Nogimori et al., 1993; Craxton et al., 1995). Neither molecular mass matches a protein of 58 kDa that we detect in mouse tissues with anti-HiPER1 antibodies, and the 58 kDa size is consistent with the predicted size of the gene product from mouse *HiPER1* (H. Chi and P. R. Reynolds, unpublished).

HiPER1 may have phosphatase activity on inositol polyphosphates, but this activity could be limited to a specific cell type and/or a specific cellular state, e.g. the transition from proliferation to hypertrophy in chondrocytes. The suppression of *HiPER1* and HiPER[449] expression by PTHrP supports a role for *HiPER1* in the transition of chondrocytes from proliferation to differentiation, which had been strongly suggested by our previous findings (Reynolds et al., 1996), as well as the data presented in Fig. 4. Somewhat unexpectedly, bFGF does not have the same strong suppressive effect on *HiPER1*, *Type X* or *Ihh* expression. bFGF has been reported to suppress hypertrophy in rabbit costal chondrocyte pellet cultures (Kato and Iwamoto, 1990); the different result we present may be the result of different culture conditions. In sternal chondrocyte cultures, bFGF has been found to suppress chondrocyte differentiation in combination with TGFβ, but has significantly less effect alone (Bohme et al., 1995). Our results suggest that while PTHrP and bFGF are both mitogenic for chondrocytes, only PTHrP has the additional and distinct property of inhibiting differentiation.

HiPER1 presents a number of intriguing features: (1) the phosphatase homology; (2) the localization to the endoplasmic reticulum; and (3) the involvement of the gene product in differentiation of chondrocytes. Collectively, our data suggest that *HiPER1* has a role in chondrocyte maturation, and that the study of this gene may reveal one of the pathways that are employed to effect the change from proliferation to hypertophy.

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REFERENCES

Abejion, C. and Hirschberg, C. B. (1992). Topography of glycosylation reactions in the endoplasmic reticulum. *Trends Biochem. Sci.* **17**, 32-66.

Ali, N., Craxton, A. and Shears, S. B. (1993). Hepatic Ins(1, 3, 4, 5)P4 3-phosphatase is compartmentalized inside the endoplasmic reticulum. *J. Biol. Chem.* **268**, 6161-6167.

Alini, M., Kofsky, Y., Wu, W., Pidoux, I. and Poole, A. R. (1996). In serum-free culture thyroid hormones can induce full expression of chondrocyte hypertrophy leading to matrix calcification. *J. Bone Min. Res.* **11**, 105-113.

Altschul, S. F., Gish, W., Miller, W., Myers, W. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol*. **215**, 403-410.

Amizuka, N., Warshawsky, H., Henderson, J. E., Goltzman D. and Karaplis, A. C. (1994). Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J. Cell Biol.* **126**, 1611-1623.

Amizuka, N., Karaplis, A. C., Henderson, J. E., Warshawsky, H., Lipman, M. L., Matsuki, Y., Ejiri, S., Tanaka, M., Izumi, N., Ozawa, H. and Goltzman, D. (1996a). Haploinsufficiency of parathyroid hormone-related peptide (PTHrP) results in abnormal postnatal bone development. *Dev. Biol.* **175**, 166-176.

Amizuka, N., Henderson, J. E., Hoshi, K., Warshawsky, H., Ozawa, H., Goltzman, D. and Karaplis, A. C. (1996b). Programmed cell death of chondrocytes and aberrant chondrogenesis in mice homozygous for parathyroid hormone-related peptide gene deletion. *Endocrinol.* **137**, 5055-5067.

Amling, M., Neff, L., Tanaka, S., Inoue D., Kuida K., Weir E., Philbrick W. M., Broadus, A. E. and Baron, R. (1997). Bcl-2 lies downstream of parathyroid hormone-related peptide in a signaling pathway that regulates chondrocyte maturation during skeletal development. *J. Cell Biol.* **136**, 205-13.

Bajwa, W., Meyhack, B., Rudolph, H., Schweingruber, A. and Hinnen, A. (1984). Structural analysis of the two tandemly repeated acid phosphatases in yeast. *Nuc. Acids Res.* **12**, 7721-7739.

Bohme, K., Winterhalter K. H. and Bruckner, P. (1995). Terminal differentiation of chondrocytes in culture is a spontaneous process and is arrested by transforming growth factor beta 2 and basic fibroblast growth factor in synergy. *Exp. Cell Res.* **216**, 191-198.

Bult, A., Zhao, F., Dirkx, R., Sharma, E., Lukacsi, E., Solimena, M., Naegele, J. R. and Lombroso, P. J. (1996). $STEP_{61}$, A member of a family of brain-enriched PTPs is localized to the endoplasmic reticulum. *J. Neurosci.* **16**, 7821-7831.

Chen, Q., Johnson, D. M., Haudenschild, D. R. and Goetinck, P. F. (1995). Progression and recapitulation of the chondrocyte differentiation program, cartilage matrix protein is a marker for cartilage maturation. *Dev. Biol.* **172**, 293-306.

Crabb, I. D., O'Keefe, R. J., Puzas, J. E. and Rosier, R. N. (1990). Synergistic effect of transforming growth factor β and fibroblast growth factor on DNA synthesis in chick growth plate chondrocytes. *J. Bone Min. Res*. **5**, 1105-1112.

Craxton, A., Ali, N. and Shears, S. B. (1995). Comparison of the activities of a multiple inositol polyphosphate phosphatase obtained from several sources, a search for heterogeneity in this enzyme. *Biochem. J.* **305,** 491-498.

Donella-Deane, A., Lopandic, K., Barbaric, S. and Pinna, L. A. (1986). Distinct specificities of repressible acid phosphatase from yeast toward phosphoseryl and phosphotyrosyl phosphopeptides. *Biochem. Biophys. Res. Commun.* **139**, 1202-1209.

Farnum, C. E. and Wilsman, N. J. (1993). Determination of proliferative characteristics of growth plate chondrocytes by labeling with bromodeoxyuridine. *Calcif. Tiss. Int.* **52**, 110-119.

Fleischer, B., Xie, J., Mayrleitner, M., Shears, S. B., Palmer, D. and Fleischer, S. (1994). Golgi coatamer binds and forms K^+-selective channels gated by, inositol polyphosphates. *J. Biol. Chem.* **269**, 17826-17832.

Genetics Computer Group (1994). Program Manual for the GCG Package, Version 8. 575 Science Drive, Madison, Wisconsin 53711.

Gerstenfeld, L. C., Kelly, C. M., Von Deck, M. and Lian, J. B. (1990). Comparative morphological and biochemical analysis of hypertrophic, non-hypertrophic and $1,25(OH)_2D3$ treated non-hypertrophic chondrocytes. *Conn. Tiss. Res.* **24**, 29-39.

Gu, J., Stephenson, C. G. and Iadarola, M. J. (1994). Recombinant proteins attached to a Nickel-NTA column. Use in affinity purification of antibodies. *Biotechniques* **17**, 257-262.

Guan, K. and Dixon, J. E. (1991). Evidence for protein-tyrosine-phosphatase catalysis proceeding via a cysteine-phosphate intermediate. *J. Biol. Chem.* **266**, 17026-17030.

Henderson, J. E., He, B., Goltzman, D. and Karaplis, A. C. (1996). Constitutive expression of parathyroid hormone-related peptide (PTHrP) stimulates growth and inhibits differentiation of CFK2 chondrocytes. *J. Cell Physiol.* **169**, 33-41.

Hill, D. J. and Logan, A. (1992). Peptide growth factors and their interactions during chondrogenesis. *Prog. Growth Factor Res.* **4**, 45-68.

Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J. and Sutrave, P. (1987). Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* **61**, 3004-3012.

Hunziker, E. B. (1994). Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. *Microscopy Res. Tech.* **28**, 505-519.

Ishizaki, Y., Burne, J. F. and Raff, M. C. (1994). Autocrine signals enable chondrocytes to survive in culture. *J. Cell Biol.* **126**, 1069-1077.

Iwamoto, M., Golden, E. B., Adams, S. L., Noji, S. and Pacifici, M. (1993). Responsiveness to retanoic acid changes during chondrocyte maturation. *Exp. Cell Res.* **205**, 213-224.

Kambe, K., Yamamoto, A., Yoshimori, T., Hirayoshi, K., Ogawa, R. and Tashiro, Y. (1994). Preferential localization of heat shock protein 47 in dilated endoplasmic reticulum of chicken chondrocytes. *J. Histochem. Cytochem.* **42**, 833-841.

Karaplis, A. C., Luz, A., Glowacki J., Bronson R. T., Tybulesicz, V. L. J., Kronenberg H. M. and Mulligan R. C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* **8**, 277-289.

Kato, Y. and Iwamoto, M. (1990). Fibroblast growth factor is an inhibitor of chondrocyte terminal differentiation. *J. Biol. Chem.* **265**, 5903-5909.

Klaus, G., Jux, C., Leiber, K., Hugel, U. and Mehls, O. (1996). Interaction between insulin-like growth factor I, growth hormone, parathyroid hormone, 1-alpha, 25-dihydroxyvitamin D3 and steroids on epiphyseal chondrocytes. *Acta Paediatrica. (Suppl).* **417**, 69-71.

Leboy, P. S., Vaias, L., Uschmann, B., Golub, E., Adams, S. L. and Pacifici, M. (1989). Ascorbic acid induces alkaline phosphatase, type X collagen and calcium deposition in cultured chick chondrocytes. *J. Biol. Chem.* **264**, 17281-17286.

Lee, K., Deeds, J. D., Chiba, S., Un-No, M., Bond, A. T. and Segre, G. V. (1994). Parathyroid hormone induces sequential c-fos expression in bone cells in vivo, in situ localization of the its receptor and c-fos messenger ribonucleic acids. *Endocrinol.* **134**, 441-450.

Lee, K., Lanske, B., Karaplis, A. C., Deeds, J. D., Kohno, H., Nissenson, R. A., Kronenberg, H. M. and Segre, G. V. (1996). Parathyroid hormone-related peptide delays terminal differentiation of chondroyctes during endochondral bone formation. *Endocrinol.* **137**, 5109-5118.

Liscovitch, M. and Cantley, L. C. (1995). Signal transduction and membrane traffic: The PITP/phosphoinositide connection. *Cell* **81**, 659-662.

Loveys, L. S., Gelb, D., Hurwitz, S. R., Puzas, J. E. and Rosier, R. N. (1993). Effects of parathyroid hormone-related peptide on chick growth plate chondrocytes. *J. Orthop. Res.* **11**, 884-891.

Majerus, P. W. (1992). Inositol phosphate biochemistry. *Annu. Rev. Biochem.* **61**, 225-250.

Morgan, B. A. and Fekete, D. M. (1996). Manipulating gene expression with replication-competent retroviruses. *Meth. Cell Biol.* **51**, 185-218.

Munro, S. and Pelham, H. R. B. (1987). A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899-907.

Nagata, K. (1996). Hsp47, a collagen-specific molecular chaperone. *Trends Biochem. Sci.* **21**, 23-26.

Nicchitta, C. V. and Blobel, G. (1993). Lumenal proteins of the mammalian endoplasmic reticulum are required to complete protein translocation. *Cell* **73**, 989-998.

Nogimori, K., Highes, P. J., Blennon, M. C., Hodgson, M. E., Putney, J. W. and Shears, S. B. (1991). Purification of an inositol (1, 3, 4, 5)-tetrakisphosphate 3-phosphatase activity from rat liver and the evaluation of its substrate specificity. *J. Biol. Chem.* **266**, 16499-16506.

Olson, K. R., McIntosh, R. and Olmsted, J. B. (1995). Analysis of MAP 4 function in living cells using green fluorescent protein (GFP) chimeras. *J. Cell Biol.* **130**, 639-650.

Ostanin, K., Harms, E. H., Stevis, P. E., Kuciel, R., Zhou, M.-M. and VanEtten, R. L. (1992). Overexpression, site-directed mutagenesis and mechanism of *E. coli* acid phosphatase. *J. Biol. Chem.* **267**, 22830-22836.

Ostanin, K., Saeed, A. and VanEtten, R. L. (1994). Heterologous expression of human prostatic phosphatase and site-directed mutagenesis of the enzyme active site. *J. Biol. Chem.* **269**, 8971-8978.

Pelham, H. R. B. and Munro, S. (1993). Sorting of membrane proteins in the secretory pathway. *Cell* **75**, 603-605.

Petropoulos, C. J., Payen, W., Salter, D. W. and Hughes, S. H. (1992). Using avian retroviral vectors for gene transfer. *J. Virol.* **66**, 3391-3397.

Reynolds, S. D., Johnston, C., Leboy, P. S., O'Keefe, R. J., Puzas, J. E., Rosier, R. N. and Reynolds, P. R. (1996). Identification and characterization of a unique chondrocyte gene involved in transition to hypertrophy. *Exp. Cell Res.* **226**, 197-207.

Rosier, R. N., O'Keefe, R. J., Teot, L. A., Fox, E. J., Nester, T. A., Puzas, J. E., Reynolds, P. R. and Hicks, D. G. (1997). P-glycoprotein expression in cartilaginous tumors. *J. Surg. Oncol.* **65**, 95-105.

Schwartz, Z., Schlader, D. L., Ramirez, V., Kennedy, M. B. and Boyan, B. D. (1989). Effects of Vitamin D metabolites on collagen production and cell proliferation of growth zone and resting zone cartilage cells in vitro. *J. Bone Min. Res.* **4**, 199-207.

Sullivan, T. A., Uschmann, B., Hough, R. and Leboy, P. S. (1994). Ascorbate modulation of chondrocyte gene expression is independent of its role in collagen secretion. *J. Biol. Chem.* **269**, 22500-22506.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

Ullah, A. H. and Phillippy, B. Q. (1988). Immobilization of *Aspergillus ficuum* phytase: product characterization of the bioreactor. *Prep. Biochem.* **18**, 483-489.

Ullah, A. H. J. and Dischinger, H. C. (1993). Identification of active site residues in *Aspergillus ficum* extracellular pH 2.5 optimum acid phosphatase. *Biochem. Biophys. Res. Comm.* **192**, 754-759.

Van Etten, R. L. (1982). Human prostatic acid phosphatase, a histidine phosphatase. *Ann. NY Acad. Sci.* **390**, 27-51.

Van Etten, R. L., Davidson, R., Stevis, P. E., MacArthur, H. and Moore, D. L. (1991). Covalent structure, disulfide bonding and identification of reactive surface and active site residues of human prostatic acid phosphatatase. *J. Biol. Chem.* **266**, 2313-2319.

Vincent, J. T., Crowder, M. W. and Averill, B. A. (1992). Hydrolysis of phosphate monoesters, a biological problem with multiple solutions. *Trends. Biochem. Sci.* **17**, 105-110.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M. and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian Hedgehog and PTH-Related Protein. *Science* **273**, 613-622.

Weir, E. C., Philbrick, W. M., Amling, M., Neff, L. A., Baron, R. and Broadus, A. E. (1996). Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondroal bone formation. *Proc. Nat. Acad. Sci. USA* **93**, 10240-10245.

Wilsman, N. J., Farnum, C. E., Green. E, M., Lieferman, E. M. and Clayton, M. K. (1996a). Cell cycle analysis of proliferative zone chondrocytes in growth plates elongating at different rates. *J. Orthop. Res.* **14**, 562-572.

Wilsman, N. J., Farnum, C. E., Leiferman, E. M., Fry, M. and Barreto, C. (1996b). Differential growth by growth plates as a function of multiple parameters of chondrocytic kinetics. *J. Orthop. Res.* **14**, 927-936.

Note added in proof

Rat hepatic multiple inositol polyphosphate phosphatase (MIPP) is the homolog of HiPER1449 (Craxton, A., Caffrey, J. J., Burkhart, W., Safrany, S. T. and Shears, S. B. (1997) *Biochem. J.* **328**, 75-81). This homology confirms that HiPER1449 will have phosphatase activity, and strongly suggests that the in vivo substrates of HiPER1449 will be one or more of the inositol polyphosphates.