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The human and rat forms of multiple inositol polyphosphate phosphatase: functional homology with a histidine acid phosphatase up-regulated during endochondral ossification

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Abstract We have derived the full-length sequences of the human and rat forms of the multiple inositol polyphosphate phosphatase (MIPP); their structural and functional comparison with a chick histidine acid phosphatase (HiPER1) has revealed new information: (1) MIPP is approximately 50% identical to HiPER1, but the ER-targeting domains are divergent; (2) MIPP appears to share the catalytic requirement of histidine acid phosphatases, namely, a C-terminal His residue remote from the RHGxRxP catalytic motif; (3) rat MIPP mRNA is up-regulated during chondrocyte hypertrophy. The latter observation provides a context for proposing that MIPP may aid bone mineralization and salvage the inositol moiety prior to apoptosis.
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Key words: Chondrocyte; Inositol phosphate; Bone; Apoptosis; Multiple inositol polyphosphate phosphatase

1. Introduction

By determining the structural and functional relationships that exist between proteins from different organisms, we can gain insight into their evolutionary origins and physiological roles. This is the approach taken in the current study of the multiple inositol polyphosphate phosphatase (MIPP), as part of our overall goal of determining the significance to the cell of this enzyme's ability to dephosphorylate a number of inositol phosphates, including Ins(1,3,4,5)P_4, InsP_5 and InsP_6 [1,2].

We have previously obtained a partial cDNA clone that encodes a truncated rat hepatic MIPP (rMIPP) [1]. This protein possesses the RHGxRxP catalytic motif that defines members of the histidine acid phosphatase family [3–5]. Outside this motif, there is considerable variation in the sequence of individual histidine acid phosphatases, reflecting the fact that these enzymes serve a multitude of functions [6]. The member of this family that comes closest in sequence similarity to rMIPP (approx. 50%, see below) is a chick chondrocyte protein, HiPER1[449] (histidine acid phosphatase of the endoplasmic reticulum) [7,8]. rMIPP is also an ER-based protein [9]. Interestingly, the levels of avian HiPER1[449] mRNA are up-regulated in bone growth plates during chondrocyte hypertrophy [7,8]. The possibility that MIPP is functionally related to HiPER1[449] could reflect the importance to endochondral ossification of an accelerated metabolism of inositol phosphates. However, such speculation is premature; alternative splicing generates several variants of HiPER1, each of which may have specific functions [7]. It is not known if any of these HiPER1 proteins can hydrolyze inositol phosphates [8]. Thus, it was a goal of the current study to improve our understanding of the structural and functional relationship between the MIPP and HiPER1 families.

The procurement of additional structural information was facilitated by our sequencing a full-length cDNA of a human form of MIPP (hMIPP), and by extending the cDNA for rMIPP to include the predicted initiator codon. One new development to arise from this molecular study was the identification and comparison of the N-terminal ER-targeting domains of the MIPP and HiPER1 proteins. This is significant because ER-targeting regions are not all functionally equivalent, so there is considerable interest in understanding how their amino acid sequence determines which mechanisms of protein entry into the ER are utilized [10,11]. Another facet to our structural analysis relates to an emerging opinion that groups of histidine acid phosphatases may have similar tertiary structures despite their primary sequences being quite divergent [4,12]. To take one example, this family has the general catalytic requirement that the three-dimensional structure must permit the N-terminal RHGxRxP catalytic motif to be juxtaposed to a remote, C-terminal HD proton donor pair [3–5]. However, neither MIPP [1] nor HiPER1 [8] possesses an HD dipeptide; we therefore investigated how this unusual feature might be relevant to the relationship that MIPP and HiPER1 have to other histidine acid phosphatases.

In parallel with these structural goals, the key functional objective was to determine the pattern of expression of MIPP mRNA in developing rat chondrocytes, and to compare these data with earlier studies [8] of HiPER1 mRNA in chick chondrocytes. The results of these experiments provide direct evidence to indicate that changes in inositol phosphate metabolism may be an important aspect of bone development.

2. Materials and methods

2.1. Sequencing

EST cDNAs were obtained from ATCC. DNA was prepared by Qiagen Maxiprep. Both strands of each clone were completely sequenced, in-house and by Sequetech Corp. (Mountain View, CA), using a fluorescent dye-based chemistry.

2.2. 5′ RACE of rat MIPP cDNA

5′ RACE reactions were carried out using Marathon-Ready rat

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Abbreviations: ER, endoplasmic reticulum; HiPER1, histidine phosphatase of the endoplasmic reticulum-1; MIPP, multiple inositol polyphosphate phosphatase; MLP, MIPP-like protein; NTS, non-translated sequence

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liver and rat kidney cDNAs (Clontech). Initial PCR reactions were performed using a number of antisense primers to rMIPP, and the adaptor primer AP1 (Clontech). Aliquots of these primary PCR reactions were then used in PCR reactions using nested rMIPP antisense primers and AP2 (Clontech). Individual PCR products were gel-purified, cloned into the TA cloning vector pCR2.1 (Invitrogen), and sequenced. RACE products of varying lengths were obtained, the longest of which encoded the rMIPP predicted sequence from Arg-5 onward (see Section 3). This RACE sequence was confirmed and extended to the putative initiator Met by sequence analysis of a rat genomic clone which encodes the rMIPP cDNA (Caffrey and Shears, unpublished data).

2.3. Expression of recombinant proteins in Escherichia coli

We prepared nucleotide constructs to encode recombinant forms of hMIPP and hMLP (human MIPP-like protein); in both cases the sequence $MRGSH_6GS$ replaced the N-terminal 30 residues of the predicted ER-targeting domain of the native proteins. The appropriate cDNA coding regions were amplified by PCR, using a 5′ primer that contained a *Bam*HI site and a 3′ primer that contained a *Sal*I site. PCR products of the expected size were digested with *Bam*HI and *Sal*I, and cloned individually into these sites in the vector pQE30 (Qiagen). The His370Ala hMIPP mutant was generated by 'recombinant PCR': sense and antisense mutagenic primers were used in separate primary PCR reactions with, respectively, 3′ (TCATAGTTCATCAGATGTACTGTTAGC) and 5′ (GATATGGAGTTTGGACCTCC) external primers. Aliquots of the two PCR products generated were mixed, and amplified in a second round of PCR using the 5′ and 3′ external primers only. A single 840 bp PCR product was obtained, digested with *Eco*RV and *Nsi*I, and the desired 177 bp *Eco*RV-*Nsi*I fragment was gel-purified and used to replace the cognate fragment in recombinant hMIPP. The nucleotide sequence of each construct was verified by sequencing (see above), transformed into *E. coli* strain M15, and protein production was induced as follows: overnight 50 ml cultures were diluted five-fold into LB containing 100 μg/ml ampicillin and 25 μg/ml kanamycin, and grown at 37°C to an OD_{600} of 0.6–0.8. Cultures were induced with 0.1 mM IPTG for 2 h at 23°C. The cells were pelleted by centrifugation, and recombinant His-tagged proteins were purified as described below.

2.4. Affinity purification of recombinant hMIPP and hMLP

Purification was performed at 0–4°C. Cells were lysed for 15–30 min in 10 ml buffer A (50 mM Tris-HCl, pH 8.5; 300 mM KCl; 20 mM β-mercaptoethanol; 0.25 mM AEBSF; 10 μM E-64; 10 μg/ml leupeptin; 1 μg/ml pepstatin A; 10 mg/ml lysozyme). Next, 4 mM CHAPS was added, and samples were sonicated. Lysates were centrifuged at $17\,000\times g$ for 15 min, and the resulting supernatants (10 ml) were incubated with 0.2 ml Ni-NTA Agarose (50% slurry; Qiagen) with gentle rocking for 15–30 min. The beads were washed successively with 1 ml of each of the following buffers: B (20 mM Tris-HCl, pH 8.5; 300 mM KCl; 10% glycerol; 20 mM β-mercaptoethanol), C (as for B, but 2 M NaCl replaced the 300 mM KCl), D (buffer B plus 30% glycerol, 1% Triton X-100). Beads were then re-equilibrated in B. Proteins were eluted with 0.4 ml of 20 mM Tris-HCl, pH 8.5; 100 mM KCl; 10% glycerol; 200 mM imidazole, and analyzed by SDS-PAGE using 12% polyacrylamide Tris-glycine gels (Novex). Recombinant hMIPP was detected using an anti-$RSG(H)_4$ mouse monoclonal antibody (Qiagen). Recombinant hMLP was detected using an antibody raised against the unique hMLP C-terminus (CFLSWATSKTRNP). The appropriate HRG-conjugated, anti-IgG secondary antibodies were supplied by Amersham. Pierce Supersignal reagent was used for chemiluminescent detection. MIPP activity was assayed as previously described [1].

2.5. In situ hybridization and Northern blotting

To generate in situ hybridization riboprobes, two rMIPP cDNA subclones were constructed in pBluescript II SK^-. One construct contained the 730 bp *Spe*I-*Apa*I fragment (nt 1302–2031 from the rMIPP 3′-non-translates sequence (NTS), see [1]). The second construct contained a 232 bp *Bam*HI-*Xho*I cDNA fragment (nt 698–929 from rMIPP, see [1]) which was generated by PCR using the following PCR cloning primers: 5′-TAATCGCGGATCCACTTAATTCAGGTAGCCTTTTTC-3′ (forward), 5′-TAAGATACTCGAGCTTTGCTTCTGCTCAACTGC-3′ (reverse). Digoxigenin-labeled sense and antisense RNA probes were generated with T3 and T7 RNA polymerases, respectively, using an RNA synthesis kit (Nippon Gene, Toyama, Japan). The in situ hybridization was performed according to the method described previously [13] using the tibia from 6 day old rats.

Northern blots were performed using a 433 bp DIG-labeled DNA probe encompassing nt 1619–2051 of the hMIPP 3′-NTS. Blots were probed overnight at 50°C in DIG EasyHyb solution and washed two times in 0.1×SSC/0.1% SDS for 15 min each at 50°C. The hMIPP mRNA was detected by chemiluminescence using an alkaline phosphatase-conjugated anti-DIG antibody and CDP-STAR (Boehringer Mannheim), according to the manufacturer's protocol.

3. Results and discussion

3.1. Molecular cloning and expression of hMIPP; structural comparison with rMIPP and HiPER1

To improve our understanding of the structure-function relationship within the MIPP family, we have identified a full-length cDNA of hMIPP by sequencing an EST cDNA (GenBank accession number AA312011) that the BLAST algorithm indicated was similar to the nucleotide sequence of rMIPP [1]. The hMIPP cDNA, which originates from Jurkat T-cells, has an open reading frame encoding a predicted protein of 487 amino acids (Fig. 1). The 2412 bp nucleotide sequence contains an initiator codon within an adequate Kozak consensus [14], and an in-frame TGA stop codon. The 3′-NTS contains two polyadenylation signals and a polyA tail. The hMIPP sequence from nt 557 to 2412 was also found in an independent EST cDNA (GenBank accession number AA315402) isolated from human colon. A 2.5 kb hMIPP mRNA was seen in all tested tissues, except for peripheral blood leukocytes; the most prominent expression was in kidney, liver and placenta (Fig. 2).

Recombinant, His-tagged hMIPP was expressed in *E. coli* without its ER-targeting domain, since this is expected to be cleaved in vivo, once the protein has entered the ER [15]. The theoretical size of the expressed protein is 53.4 kDa. Western blotting identified a protein of approx. 51 kDa (Fig. 3, lane 2). This protein expressed the enzyme activity that characterizes the MIPP protein family, namely, a Mg^{2+}-independent hydrolysis of the 3-phosphate of $Ins(1,3,4,5)P_4$ (K_m of 1.6 ± 0.7 μM, $n=5$). HPLC was used to identify $Ins(1,4,5)P_3$ as the reaction product (data not shown). The rate of $Ins(1,3,4,5)P_4$ hydrolysis by the recombinant enzyme was typically 80-fold greater than the activity obtained from parallel preparations made from vector-transformed control cultures (Fig. 4).

In order to complete our comparison of hMIPP with rMIPP, we have added additional 5′-nucleotide sequence (see Section 2) to our previously truncated rMIPP cDNA, which now encodes a predicted full-length rMIPP protein (Fig. 1). The amino acid sequences of hMIPP and rMIPP are 84% identical; these proteins are less closely related to $HiPER1^{449}$ from chick chondrocytes (52–54% identical) (Fig. 1).

The N-termini of both MIPP proteins contain the three distinct domains ('c', 'h' and 'n', see Fig. 1) that typify an ER-targeting sequence [16]. Hydropathy analysis (see legend to Fig. 1) indicated that residues 14–24 in hMIPP, and residues 11–24 in rMIPP, each comprise the necessary hydrophobic core (the h region). The preceding n region has the required net positive charge (from two Arg in hMIPP, and two Arg and a His in rMIPP, Fig. 1). Von Heijne's rules [17] were satisfied by the six residues of the candidate c region (a typical length for this region [17]), which serves as a recognition site

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                    n               h          c
hMIPP   1    MLRAPGCLLRTSVAPAAALAAALLSSLARCSLLEPRDPVASSLSPYFGTK
rMIPP   1    MLRGARSHLSASVALAAVLAAALLSSFARCSLPGRGDPVASVLSPYFGTK
HiPER1  1    MAPCR........AACLLPLLVAVASAGLG..............GYFGTK

hMIPP   51   TRYEDVNPVLLSGPEA..PWRDPELLEGTCTPVQLVALIRHGTRYPTVKQ
rMIPP   51   TRYEDVNPWLLGDPVA..PRRDPELLAGTCTPVQLVALIRHGTRYPTTKQ
HiPER1  29   SRYEEVNPHLAEDPLSLGPHAAAARLPAACAPLQLRRVVRHGTRYPTAGQ

hMIPP   99   IRKLRQLHGLLQARGSRDGGASSTGSRDLGAALADWPLWYADWMDGQLVE
rMIPP   99   IRKLRQLQGLLQTRESVDGGSR......VAAALDQWPLWYDDWMDGQLVE
HiPER1  79   IRRLAELHGRLR........RAAAPSCPAAAALAAWPMWYEESLDGRLAP

hMIPP   149  KGRQDMRQLALRLASLFPALFSRENYGRLRLITSSKHRCMDSSAAFLQGL
rMIPP   143  KGRQDMRQLALRLAALFPDLFCRENYGRLRLITSSKHRCVDSSAAFLQGL
HiPER1  121  RGRRDMEHLARRLAARFPALFAARR..RLALASSSKHRCLQSGAAFRRGL

hMIPP   199  WQHYHPGLPPPDVADMEFGPPTVNDKLMRFFDHCEKFLTEVEKNATALYH
rMIPP   193  WQHYHPGLPPPDVSDMECDPPRVNDKLMRFFDHCEKFLTEVERNATALYH
HiPER1  169  GPSLSLGADETEI........EVNDALMRFFDHCDKFVAFVEDNDTAMYG

hMIPP   249  VEAFKTGPEMQNILKKVAATLQVPVNDLNADLIQVAFFTCSFDLAIKGVK
rMIPP   243  VEAFKTGPEMQTVLKKVAATLQVPVNNLNADLIQVAFFTCSFDLAIQGVH
HiPER1  211  VNAFKEGPEMRKVLEKVASALCLPASELNADLVQVAFLTCSYELAIKNVT

hMIPP   299  SPWCDVFDIDDAKVLEYLNDLKQYWKRGYGYTINSRSSCTLFQDIFQHLD
rMIPP   293  SPWCDVFDVDDAKVLEYLNDLKQYWKRSYGYAINSRSSCNLFQDIFLHLD
HiPER1  261  SPWCSLFSEEDAKVLEYLNDLKQYWKRGYGYDINSRSSCILFQDIFQQLD
                                                   ↑
                                  *
hMIPP   349  KAVEQKQRSQPISSPVILQFGHAETLLPLLSLMGYFKDKEPLTAYNYKKQ
rMIPP   343  KAVEQKQRSQPVSSSVILQFGHAETLLPLLSLMGYFKDKEPLTAYNFEEQ
HiPER1  311  KAVDESRSSKPISSPLIVQVGHAETLQPLLALMGYFKDAEPLQANNYIRQ

hMIPP   399  MHRKFRSGLIVPYASNLIFVLYHCENAKTPKEQFRVQMLLNEKVLPLAYS
rMIPP   393  VHREFRSGHIVPYASNLIFVLYHCEDAQTPQEKFQIQMLLNEKVLPLAHS
HiPER1  361  AHRKFRSGRIVPYAANLVFVLYHCEQ.KTSKEEYQVQMLLNEKPMLFHHS

hMIPP   449  QETVSFYEDLKNHYKDILQSCQTSEECELARANST.SDEL
rMIPP   443  QKTVALYEDLKNHYQDILQSCQTSKECNLPKVNIT.SDEL
HiPER1  410  NETISTYADLKSYYKDILQNCHFEEVCELPKVNGTVADEL
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Fig. 1. Comparison of the sequences of the MIPP and HiPER1 families. The amino acid sequence of hMIPP was deduced from the corresponding nucleotide sequence (GenBank accession number is AF084943). The rMIPP sequence is as previously published [1], plus the completed N-terminus deduced from the extra 5′-nucleotide sequence we have now obtained (see Section 2). The HiPER1[449] sequence is as published previously [8]. Residues that are identical to those of hMIPP are highlighted in bold type. The RHGxRxP catalytic motif is underlined, and an asterisk marks a conserved His residue with a proposed catalytic role (see text). The arrow marks the HiPER1[318] C-terminus [8]. The predicted ER targeting domains are boxed, and divided into the following subdomains: the c region (gray), h region (white) and n region (gray). These were identified using von Heijne's rules [17], and hydropathy analysis [24]. Mean hydropathy values (window = 8) were as follows: hMIPP, n = −0.6, h = −1.9, c = −0.9; rMIPP, n = +0.24, h = −2.2, c = −0.65; HiPER1, n = −0.5, h = −2.2, c = −1.1.

for the signal peptidases (Fig. 1). This new information provides us with the first opportunity to determine that the MIPP ER-targeting sequences are not conserved in HiPER1 (Fig. 1). Additionally, the putative n regions of the MIPP proteins (13 residues in hMIPP, 10 in rMIPP) are longer than that of HiPER1 (five residues). The latter observation is relevant to evidence that n regions of five residues or less occur in proteins for which delivery into the ER depends upon the translocating chain-associating membrane protein (TRAM) [11]. In contrast, TRAM-independent translocation may be a property of proteins where the n region is larger than nine residues [11]. Thus, MIPP and HiPER1 may be targeted into the ER by different mechanisms.

3.2. A catalytic requirement for the C-terminus of hMIPP

Sequencing of a human CFPAC-1 cell EST cDNA (GenBank accession number AA161161) with a 2165 bp insert revealed a putative coding region identical to that of hMIPP cDNA, except for a 232 nt deletion which also introduces a single base shift in the reading frame. Thus, the CFPAC-1 cDNA encodes a 312 residue hMLP; the N-terminal 278 residues are identical to those in hMIPP (Fig. 1), but the 34 residue carboxy terminus of hMLP (GLSQFLLQSSSSLVMQRLFFHCFLSWATSKTRNP) is unique. We failed to detect any hMLP upon Western analysis of extracts of CFPAC-1 cells (data not shown). Moreover, there was no evidence for an appropriately sized hMLP mRNA in human multiple tissue Northern blots (Fig. 2). A His-tagged construct of recombinant hMLP was expressed in *E. coli.* (Fig. 3, lane 4), but this did not exhibit any 3-phosphatase activity (data not shown). The GenBank accession number for the hMLP clone is AF084944.

It is possible that the hMLP cDNA arises as a consequence of a cloning artifact. Nevertheless, the fact that recombinant hMLP did not express 3-phosphatase activity suggests a catalytic requirement for the carboxy terminus of hMIPP, that is missing from hMLP. Indeed, the carboxy portion of histidine acid phosphatases typically contains an HD pair that is utilized as a proton donor during catalysis [3–5]. MIPP and HiPER1 are atypical, since they lack any HD dipeptides (Fig. 1). However, the His residue within the HD motif of other acid phosphatases has been aligned with His-332 of HiPER1[449] [8]. Multiple sequence analysis suggested that this same histidine residue is conserved in MIPP (His-370 in

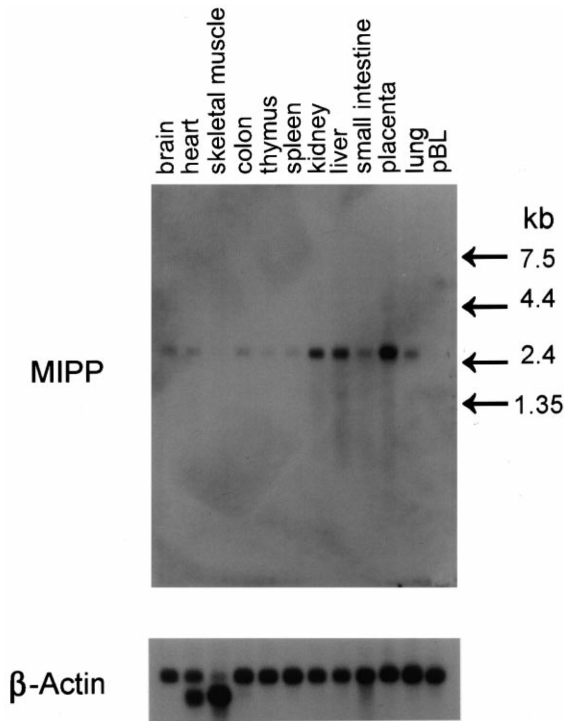

Fig. 2. Expression of hMIPP in human tissues. A Clontech human multiple tissue Northern blot was probed sequentially with digoxigen-labeled MIPP-specific and β-actin probes (see Section 2). PBL = peripheral blood leukocytes.

hMIPP, Fig. 1). Therefore, a His370Ala mutant of hMIPP was generated by site-directed mutagenesis; this protein expressed $<5\%$ of the activity of the wild-type enzyme (Fig. 4). The catalytic requirement for His-370, albeit in a novel context, is further evidence of MIPP sharing mechanistic and structural similarities with other histidine acid phosphatases. The absence of an equivalent His residue in hMLP may help account for why it is catalytically inactive. Our data also suggest that HiPER1[318], which also lacks this conserved His residue (Fig. 1), will not hydrolyze inositol phosphates.

3.3. The pattern of expression of MIPP in rat chondrocytes

The expression of HiPER1[449] mRNA is up-regulated during chick chondrocyte hypertrophy [7,8]. To determine if rMIPP is functionally similar to HiPER1, we used in situ hybridization to investigate the expression pattern of rMIPP in rat chondrocytes. Sense and antisense probes against rMIPP RNA were applied across a longitudinal section of the growth plate of 6 day old rat tibia (Fig. 5). It is possible to distinguish the three zones that contain the resting (R), proliferating (P) and hypertrophic (H) chondrocytes. The zone of hypertrophy contains the most intense signal from the antisense probes (Fig. 5c,d), and this signal is not evident when the sense probes were used (Fig. 5a,b).

This demonstration that rMIPP expression is up-regulated during rat chondrocyte hypertrophy represents the first direct experimental evidence that rMIPP has some functional similarity to HiPER1[449]. This observation provides a useful context for considering the physiological advantage of rMIPP's substrates (Ins(1,3,4,5)P_4, InsP_5 and InsP_6 [2]) being more actively dephosphorylated during chondrocyte hypertrophy.

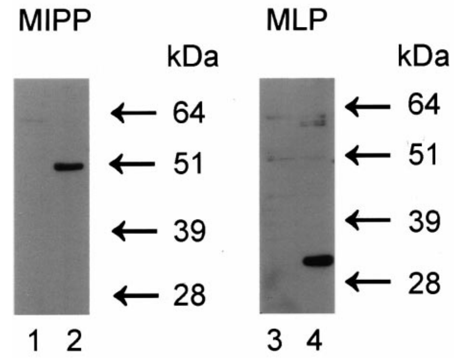

Fig. 3. Expression of recombinant hMIPP and hMLP in *E. coli*. Samples of affinity-purified, recombinant hMIPP (lane 2) and hMLP (lane 4) were analyzed by SDS-PAGE and Western blotting as described in Section 2. Lanes 1 and 3 represents an extract of affinity-purified proteins from cells transformed with vector alone.

At this time, vesicles that will eventually mineralize the extracellular matrix bud-off from the chondrocyte plasma membrane [18]. InsP_6, which binds to cellular membranes [19] and is present in cell cytosol [20], has the potential to become trapped inside the matrix vesicles. The ability of InsP_6 to inhibit calcium phosphate crystal growth [21] could, therefore, impede the mineralizing function of these vesicles. This situation may be avoided, prior to matrix vesicle formation, by increased InsP_6 hydrolysis as a result of up-regulation of MIPP. Another aspect of the hypertrophic phenotype is that it can commit chondrocytes to enter an apoptotic program [22]. Increased MIPP activity may also serve to salvage for other cells the important inositol moiety from the higher inositol phosphates that the apoptotic chondrocytes no longer

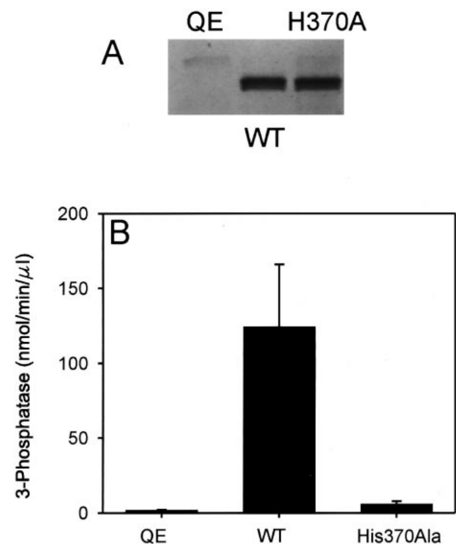

Fig. 4. Catalytic activity of wild type hMIPP and a His370Ala mutant. Recombinant wild-type hMIPP ($n = 3$), a His370Ala mutant ($n = 5$), and samples of QE vector controls ($n = 3$) were assayed for Ins(1,3,4,5)P_4 3-phosphatase activity (panel B). The units of activity (with S.E.M.s) are per μl of stock enzyme, adjusted for any differences in MIPP concentration, as determined by scanning densitometry of silver-stained gels (panel A).

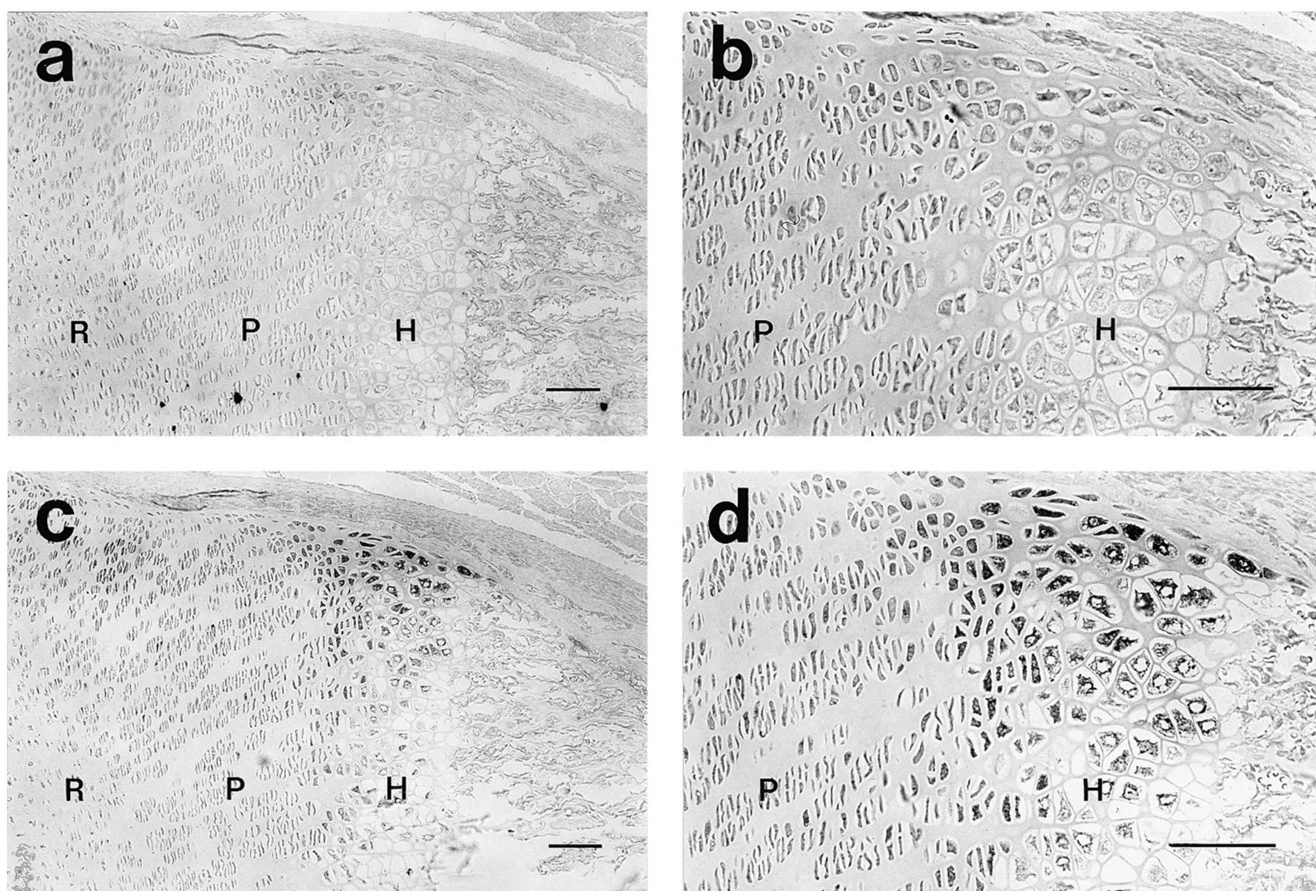

Fig. 5. Analysis of MIPP RNA in the growth plate of rat tibia using in situ hybridization. In situ hybridization using sense (panels a and b) or the corresponding antisense (panels c and d) rMIPP RNA probes was performed on longitudinal sections of 6 day old rat tibia. Three distinct developmental stages of chondrocytes are indicated: 'R', 'P' and 'H' for resting, proliferating and hypertrophic. The horizontal bars in the figures indicate 100 μm. The rMIPP nucleotide template that we used to prepare the RNA probes was almost entirely contained within the 3′-NTS, which shows no significant identity with any region of HiPER1 RNA. Similar results (not shown) were obtained with an RNA probe directed against the 232 nt region of rMIPP that is missing from hMLP.

require. This idea fits the general concept that the body has developed a number of means to conserve inositol [23].

The prediction that an increased rate of inositol phosphate turnover is important to endochondral ossification would have been premature without the new information generated by this study concerning the structural and functional relationships between MIPP and HiPER1. Additionally, the cloning of hMIPP may eventually be of clinical interest; increased knowledge of the genetic control of mineralizing chondrocytes is relevant to our understanding of normal bone growth, as well as pathological situations such as fracture healing and osteoarthritis [7].

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