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Parathyroid Hormone (hPTH 1–38) Stimulates the Expression of UBP41, an Ubiquitin-Specific Protease, in Bone

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Abstract Parathyroid hormone (PTH) stimulates bone formation in both animals and humans, and the expression of a number of genes has been implicated in the mediation of this effect. To discover new bone factors that initiate and support this phenomenon, we used differential display reverse transcription polymerase chain reaction (DDRT-PCR) and screened for genes, which are differentially expressed in osteoblast-enriched femoral metaphyseal primary spongiosa of young male rats after a single subcutaneous (s.c.) injection of hPTH (1–38) (8 μg/100 g). We found and cloned one full-length cDNA, which encodes a putative 348 amino acid protein. Sequence analysis of this protein demonstrates a 98, 93.7, and 82.5% identity with mouse, human, and chicken ubiquitin-specific protease UBP41, respectively. Northern blot analysis confirmed that a 3.8–4 kb UBP41 mRNA transcript was rapidly increased 1 h after acute hPTH (1–38) exposure in both metaphyseal (6- to 8-fold) and diaphyseal (3-fold) bone, but returned to control levels by 24 h after exposure. In contrast, continuous exposure to hPTH (1–38), resulted in a rapid and sustained elevation of UBP41 mRNA. PTH (1–31), which stimulates intracellular cAMP, and PTHrP (1–34) both induced UBP41 mRNA expression; whereas PTH analogs (3–34) and (7–34), that do not stimulate cAMP, had no effect on UBP41 expression. UBP41 mRNA expression was also rapidly induced 1 h after injection of PGE_2, but returned to the control level by 6 to 24 h. In vitro, UBP41 mRNA is expressed in primary osteoblasts (metaphyseal and diaphyseal derived) and in the osteoblast-like cell lines UMR106, ROS17/2.8, and BALC. PTH (1–38) treatment induced UPB41 expression (3.6- to 13-fold) in both primary cultures of osteoblasts and in UMR106 cells. Further analysis in UMR 106 cells demonstrated that PGE_2, forskolin and dibutyryl cAMP increased UBP41 mRNA expression 4-, 4.5-, and 2.4-fold, respectively. Tissue distribution analysis of UBP41 mRNA detected transcripts in brain, heart, skeletal muscle, kidney, liver, and testis. Together, these results demonstrate that UBP41, an ubiquitin-specific protease, is selectively upregulated in bone by the osteotropic agents PTH, PTHrP, and PGE_2, possibly via the PKA/cAMP pathway. We speculate that the rapid induction of UBP41 in response to these physiological regulators contributes to the mechanism by which either the structure, activity, half-life or localization of essential proteins are modified to maintain bone homeostasis. J. Cell. Biochem. 85: 229–242, 2002.

Key words: hPTH (1–38); hPTHrP (1–34); PGE_2; ubiquitin-specifc protease; UBP41; deubiquitinating enzyme; rat; bone

Parathyroid hormone (PTH) is a potent modulator of bone metabolism. In experimental animals and patients with osteoporosis, intermittent administration of PTH increases net bone mass by stimulating de novo bone formation to enhance trabecular thickness, connectivity, and strength [Reeve et al., 1980; Slovik et al., 1981; Hock et al., 1989a,b,c; Hock and Gera, 1992; Riggs and Melton, 1992; Dempster et al., 1993; Oxlund et al., 1993; Jerome et al., 1999]. On a cellular level, this increase in bone mass correlates with an increased number of osteoblasts [Hock et al., 1994; Schmidt et al.,

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1995]. We have recently demonstrated in young rats that PTH targets proliferating bone cells in the primary metaphyseal trabecular spongiosa and increases the number that differentiate into osteoblasts [Hock et al., 1994, 1995; Onyia et al., 1995, 1997]. We and others have demonstrated in vivo that the earliest molecular events leading to the effects of PTH in bone involve the expression of genes such as *c-fos*, *fra-2*, *c-jun*, *junB*, *c-myc*, *cbfa1*, interleukin-6 (IL-6), leukemia inhibitory factor (LIF), histone H4, regulator of G-protein signaling 2 (RGS-2), ADAMTS-1 (A disintegerin and metalloprotease with thrombospondin motifs-1), osteoprotegerin (OPG), and RANKL [Lee et al., 1994; Onyia et al., 1995, 2000; Pollock et al., 1996; Liang et al., 1999; Takeda et al., 1999; Miles et al., 2000a,b; Stanislaus et al., 2000a; Ma et al., 2001]. The changes in the expression of these genes following in vivo PTH treatment have been localized predominantly to cells of the osteoblast lineage that control de novo bone formation [Lee et al., 1994; Liang et al., 1999; Takeda et al., 1999; Onyia et al., 2000; Ma et al., 2001]. Although the metabolic outcomes of PTH involve the synthesis of these proteins, little is known about the role of protein degradation in controlling PTH activity. Furthermore, there is a gap in our understanding of the events occurring between the synthesis and degradation of immediate early response genes and the activation of the biochemical machinery that regulates bone metabolism.

The regulation of intracellular protein turnover by the ubiquitin-dependent proteasome pathway is a universal mechanism employed by cellular systems [Ciechanover et al., 2000; Kornitzer and Ciechanover, 2000]. In eukaryotes, the ubiquitin degradation pathway mediates extralysosomal intracellular protein degradation and is essential for the regulation of normal growth, proliferation, and differentiation [Zwickl et al., 1999; Ciechanover et al., 2000; Kornitzer and Ciechanover, 2000]. Proteins targeted for degradation are initially conjugated to the 76-amino acid ubiquitin (Ub) polypeptide tag. Following polyubiquitination by one or more classes of enzymes, targeted proteins are next recognized and degraded by the proteasome, a multisubunit protein degradation complex. A large superfamily of deubiquitinating enzymes known as ubiquitin-specific proteases (UBPs) has been identified [Wilkinson, 1997, 2000; Chung and Baek, 1999]. UBPs are ubiquitin-specific thio-proteases that cleave either linear ubiquitin precursor proteins or ubiquitinated protein substrates. Recent studies suggest that like protein phosphorylation, protein ubiquitination is a reversible process that is tightly regulated in cells [Wilkinson, 1997, 2000]. Although the structure, catalytic mechanism, and function of the ubiquitin-proteasome degradation pathway have been well studied in organisms ranging from yeast to mammals, less is known about its regulation in specialized mammalian tissues such as bone and cartilage under normal or hormone-stimulated conditions. It has been hypothesized that the regulation of intracellular protein turnover by the ubiquitin-dependent proteasome pathway is involved in PTH actions in vivo. Recent in vitro studies support this hypothesis and suggest that the ubiquitin-proteasome system is essential for osteoblast proliferation, differentiation, and survival under normal and PTH-stimulated conditions [Murray et al., 1998]. PTH mediates its metabolic effects on the osteoblast in part by enhancing ubiquitinylation of protein substrates and stimulation of proteasome activities [Murray et al., 1998]. Natural substrates of the ubiquitin proteolytic pathways include cyclin B, cyclin D, p27 Kip1, p53, c-myc, c-fos, c-jun, RGS2, OSF2; many of which we and others have demonstrated to be regulated by PTH [Krishnan et al. (accepted pending successful revision); Onyia et al., 1995; Onishi and Hruska, 1997; Liang et al., 1999; Miles et al., 2000b].

In view of the important role of the ubiquitin-proteasome system on protein metabolism in diverse intracellular processes, it seems likely that the expression and regulation of one or more genes in the ubiquitin-degradation pathway might contribute to the metabolic actions of PTH in bone. In an effort to elucidate some of the earliest molecular events involved in PTH actions in bone, we used differential display polymerase chain reaction (DDRT-PCR) to identify new early response genes that are differentially expressed in the distal femoral metaphysis of PTH-treated young male rats. In the present study, we show that a member of the UBP superfamily, UBP41, is rapidly and transiently upregulated in bone and in osteoblasts by PTH (1–38) treatment, as well as by other osteotropic agents such as parathyroid hormone related peptide (PTHrP) and prostaglandin E_2 (PGE_2). We speculate that UBP41,

and perhaps other related proteins, may be involved in the cascade of events mediating the metabolic effects of PTH in bone.

MATERIALS AND METHODS

Biochemicals

Synthetic human PTH (1–38), PTH (1–31), PTHrP (1–34), and bovine PTH (3–34) and (7–34) (Bachem Biochemicals, Torrance, CA) were prepared in a vehicle of acidified saline containing 2% heat-inactivated rat serum. PGE_2 (Sigma, St. Louis, MO) was first dissolved in 100% ethanol and further diluted in vehicle to a final ethanol concentration of 10%. IL-1α, M-CSF, and TGF-β1 (R&D Systems, Inc., Minneapolis, MN) were resuspended in PBS containing 0.1% bovine serum albumin.

Animals

Young virus-antibody-free male Sprague-Dawley rats, 60–75 g, (Harlan Laboratories, Indianapolis, IN) were housed with a 12 h light-dark cycle. Animals were fed Purina chow [(calcium 1%, phosphate 0.61%) PMI Feeds, Inc., St. Louis, MO] and water ad libitum. Animal protocols were approved by the Lilly Animal Care and Use Committee.

In Vivo Protocols

Rats were weighed and sorted into groups of comparable mean body weight (four rats per group). For acute treatment studies, rats were injected subcutaneously (s.c.) with either the various analogs of PTH (8 μg/100 g), PTHrP (1–34) (8 μg/100/g) or PGE_2 (6 mg/kg), and were euthanized using CO_2 at indicated time points. Control rats received an equal amount of the vehicle s.c. and were euthanized at the same time intervals. The doses of PTH and PGE_2 were chosen based on our previous work [Onyia et al., 1995; McClelland et al., 1998; Miles et al., 2000b] and reports from other laboratories [Jee et al., 1985; Weinreb et al., 1997] demonstrating an effect on bone and gene expression.

The catabolic effects of continuous PTH administration were evaluated in parathyroidectomized (PTX) rats using a well-characterized resorption model for PTH. PTX animals were used to reduce the confounding effects of endogenous PTH and to enhance the resorptive response to PTH. The studies were done under conditions that promoted optimal catabolic effects of PTH in PTX rats as we previously described [Ma et al., 2001]. For these studies, animals were parathyroidectomized by the vendor and delivered to our facility 2–3 days post surgery. They were maintained on a 12-h light-dark cycle at 22°C with ad lib access to tap water. To minimize the intestinal and renal effects on serum calcium, rats were maintained on a calcium-free diet containing 0.02% Ca, 0.3% P (TD 99171, Teklad, Madison, WI) during the experimental period. Synthetic human PTH (1–38) (20 μg/100 g/6 h) was administered by subcutaneous infusion for 1, 3, 6, and 24 h using an Alzet mini-pump (#20011, Durect-Corp, Palo Alto, CA). Following euthanasia, rat femora were resected and all connective tissues, including the periosteum, were completely removed. The distal epiphysis, including the growth plate, was removed and a subjacent 3 mm wide band of the metaphyseal primary spongiosa or diaphyseal middle third of the same femur was resected and frozen in liquid nitrogen until mRNA analyses were performed [Onyia et al., 1995; McClelland et al., 1998; Miles et al., 2000b]. For experiments involving PGE_2, the distal metaphysis (6 mm subjacent to the growth plate) was used for mRNA analysis.

Osteoblast Cell Cultures

The rat cell line, UMR 106, was maintained in DMEM/Ham's F-12 (3:1) (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT) plus 2 mM glutamine (Gibco BRL). ROS 17/2.8 cells were maintained in the growth medium F-12 nutrient mixture (Gibco BRL) containing 10% FBS plus 2 mM glutamine (Gibco BRL). BALC cells, a mouse calvaria-derived stromal/osteoblastic cell line [John et al., 1996], were grown in RPMI 1640 (Gibco BRL) supplemented with 5% FBS and 2 mM glutamine. Primary osteoblast cultures were derived from the rat femoral metaphysis and diaphysis as previously described [Alvarez et al., 1997; Onyia et al., 1997, 1998]. All cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C. For mRNA analysis, cultures (4-T150 flasks/group) of cells were grown (as described above) to 80–90% confluence, and then switched into medium containing 0.1% FBS overnight. The cells were then treated with the indicated concentrations of compound for the specified time periods.

Bone Marrow-Derived Osteoclast Cell Cultures

Osteoclasts were generated in vitro from bone marrow as previously described [Sells Galvin et al., 1999]. In brief, bone marrow was flushed from the femurs of male Balb/C mice (aged 6–12 weeks) with culture medium (RPMI with 5% FBS and 1% antibiotic/antimycotic, Gibco BRL). The bone marrow cells were seeded at a density of 2.5×10^5 mononuclear cells/cm^2 in 150 mm culture dishes (Falcon). The cultures were treated with the following factors: recombinant murine M-CSF (M-CSF 50 ng/ml, R & D systems), recombinant human soluble RANKL (RANKL 50 ng/ml, Chemicon, Temecula, CA), and recombinant human TGF-β1 (100 ng/ml, R & D Systems). The media and factors were replaced on day 3 and poly A$^+$ RNA was isolated on day 6. In this culture system, formation of osteoclasts is totally dependent on the presence of RANKL and M-CSF, while TGF-β1 was added to the cultures to enhance osteoclast formation. As we recently reported, these cells display the distinguishing characteristics of authentic osteoclasts, which include the ability to resorb bone, and the expression of lineage-specific markers including tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor (CTR) [Sells Galvin et al., 1999]. These cultures consist of osteoclasts and progenitor cells with very few contaminating stromal or osteoblastic cells [Sells Galvin et al., 1999].

Isolation of RNA and cDNA Synthesis

To ensure reproducibility and to reduce false positives, total RNA from three independent experiments was used in cDNA synthesis and differential display. For each experiment, RNA was extracted from the metaphyseal primary spongiosa of vehicle or PTH-treated rats at 1 and 24 h, as previously described [Onyia et al., 1995; McClelland et al., 1998]. With each experiment, samples were pooled into treated or control groups (four animals per group) for each indicated time point after treatment. Tissue samples were removed from the animals, snap frozen, and pooled for isolation of RNA. Total RNA was extracted by homogenization in Ultraspec-II^{TM} (BIOTECX, Houston, TX) using an LS 10–35 Polytron homogenizer (Brinkmann Instruments, Westbury, NY) as recommended by the manufacturer. Isolated RNA was quantitated using spectrophotometry by measuring the absorbance at 260 nm, and the 260/280 nm ratio was calculated to ensure the absence of protein contamination. To remove contaminating DNA from the RNA preparation, samples were incubated with RNase-free DNase I (Roche, Indianapolis, IN) for 15 min at room temperature and then extracted with phenol/chloroform. First strand cDNA was synthesized from 4 μg of total RNA by oligo dT priming, using the Superscript Preamplification kit (Gibco BRL) in a final volume of 40 μl.

PCR and Differential Display

Differential display was carried out using arbitrary primer sets as previously described [Liang and Pardee, 1992; McClelland et al., 1998; Miles et al., 2000b]. The upstream (arbitrary primer) and downstream (anchored) primers that detected UBP41 were 5′ TGTCA TCCCC 3′ and 5′ TTTTTTTTTTTTG 3′, respectively. Using cDNA (diluted 1:25) or a negative control containing no cDNA template, duplicate PCR reactions were assembled robotically (Tecan Genesis, Reading, UK) to a final concentration of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 2.0 mM dNTP's, 15 nM [^{33}P]-dATP (Amersham, Arlington Heights, IL), and 1 U AmpliTaq polymerase (Perkin-Elmer, Foster City, CA) in a final volume of 20 μl. Reactions were then subjected to the following PCR conditions on a DNA Engine PTC-225 thermal cycler (MJ Research, Inc., Watertown, MA): 1 cycle of 92°C for 2 min; 40 cycles of 92°C for 15 s, 40°C for 2 min, 72°C for 1 min; 1 cycle of 72°C for 5 min. Subsequently, PCR products were separated on a 6% TBE/urea sequencing gel (Sequagel, National Diagnostics, Atlanta, GA) for 3 h at 1700 V. Gels were dried and exposed to BIOMAX X-ray film (Eastman Kodak, Co., Rochester, NY). The negative controls with no cDNA template yielded no PCR products.

Reamplification, Cloning, and Sequencing of cDNA

Bands of interest representing differentially expressed genes were excised from the gel, boiled for 5 min in H_2O, and purified over a Centricon 50 column (Amicon, Beverly, MA). Samples were then reamplified to confirm the size and specificity of the primer sets used in the display. Reamplified products were ligated into pCR2.1 TA cloning vector (Invitrogen, San Diego, CA) and transformed into DH10B cells

(Gibco BRL). For each clone, ten colonies were picked, amplified in LB broth, and the plasmids isolated (Wizard Plus, Promega, Madison, WI). Clones that contained inserts were submitted for automated cycle sequencing (Lilly DNA Technology Group, Indianapolis, IN). All sequences were analyzed using BLAST2 against GenBank and EMBL databases to determine sequence identity.

Cloning Full-Length UBP41 cDNA

UBP41 cDNA was cloned by screening a metaphyseal bone cDNA library in Ziplox (Lilly DNA Technology Group). An 800 bp cDNA amplified from rat metaphyseal primary spongiosa RNA in a differential display analysis was labeled with α-[^{32}P]-dCTP (Amersham) using the random primer method (Gibco BRL). Free nucleotides were removed by centrifugation through a Centricon-50 column. The radiolabeled cDNA was used to screen approximately 1.0×10^6 phage of a rat metaphyseal bone cDNA library in Ziplox. Filters were hybridized overnight at 42°C in Northern Max hybridization buffer (Ambion, Austin, TX). Filters were washed with 2 × SSC, 0.1% SDS at 45°C for 15 min and then in 0.2 × SSC, 0.1% SDS at 55°C for an additional 60 min. A single unique clone was isolated and the plasmid rescued into pSPORT vector using the manufacturer's suggested protocol (Gibco BRL). The insert sequence was confirmed by the Lilly DNA Technology Group using automated cycle sequencing.

Generation of Radiolabeled Probes for Northern Analysis

To generate radioactive probes for Northern analysis, the inserts containing UBP41 cDNA were released from the plasmid by restriction digest. Rat GAPDH cDNA probes were cloned using PCR with specific primer pairs as published previously [Alvarez et al., 1997]. Twenty-five nanograms of cDNA was labeled by the random primer method (Gibco BRL,) using α-[^{32}P]-dCTP (Amersham). Free nucleotides were removed by centrifugation through a Centricon-50 column (Amicon).

Isolation of Poly A$^+$ RNA and Northern Blotting

The expression of UBP41 mRNA was analyzed by Northern blot. Bone and cell culture samples were pooled into treated or control groups for each indicated time point after treatment. Total RNA was extracted from bone by homogenization in Ultraspec-II (BIOTECX) using an LS 10–35 Polytron homogenizer (Brinkmann Instruments) as recommended by the manufacturer. Total RNA was extracted from the osteoblast or osteoclast cultures by adding Ultraspec-II directly to the culture flasks. The resulting cell lysates were passed several times through a 10 ml pipette before collection. Poly A$^+$ RNA was isolated from total RNA using Oligotex (Qiagen, Valencia, CA) according to the manufacturer's protocol and quantitated by spectrophotometry. The absorbance at 260 nm was determined and the 260/280 nm absorbance ratio was calculated to ensure the absence of protein contamination. Samples of poly A$^+$ RNA (2 μg) were denatured in 0.04 M 3-(N-morpholino) propanesulfonic acid, pH 7.0, 10 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, and 50% formamide at 60°C for 10 min, and size fractionated by electrophoresis through 1% agarose gels in 2.1 M formaldehyde and 1 × MOPS and transferred to nylon membranes (Brightstar-Plus™; Ambion Inc.). The nylon membranes were air dried and the RNA samples cross-linked to the membranes by UV irradiation in a Stratalinker (Stratagene, La Jolla, CA). Migration of 28 S and 18 S ribosomal RNA was determined by ethidium bromide staining. DNA probes were labeled by the random primer method (Gibco BRL) using α-[32P]-dCTP. Prehybridization and hybridization were carried out at 48°C in NorthernMax buffers (Ambion Inc.). After hybridization, membranes were washed for 30 min at room temperature in buffer containing 2 × SSC, and 0.1% SDS, then 30 min at 48°C in 0.2 × SSC and exposed to Biomax MS X-ray film (Eastman Kodak) at −70°C. Autoradiograms were quantitated by scanning laser densitometry (2400 Gel Scan XL, LKB, Piscataway, NJ). Labeled bands were quantitated as densitometric units and normalized to that of the GAPDH signals to correct for variations in RNA transfer and gel loading. The data were expressed as fold change versus untreated control samples. The experiments were repeated 2–4 times for each time point to confirm findings.

Multi-Tissue RNA Analysis

The tissues expressing UBP41 transcripts were determined by probing poly A$^+$ RNA from

rat tissues using Multiple Tissue Northern (MTN) blots (CLONTECH Laboratories, Palo Alto, CA). The MTN blot contained 2 μg/lane of poly A^+ RNA from heart, brain, kidney, spleen, lung, liver, skeletal muscle, and testis.

RESULTS

Identification and Cloning of UBP41 as a PTH-Regulated Gene in Rat Metaphyseal Bone

To identify novel PTH-responsive genes in bone, we screened for genes that are differentially regulated by acute administration of PTH in rat metaphyseal bone using differential display PCR (DDRT-PCR) [Liang and Pardee, 1992]. Total RNA isolated from hPTH (1–38)-treated and control femoral metaphyses at 1 and 24 h after treatment was reverse transcribed into cDNA and differentially displayed using primer sets as described in Materials and Methods. To enhance reproducibility and reduce the occurrence of false positives [Wang et al., 1996; Sung and Denman, 1997; Wang and Feuerstein, 1997; Ledakis et al., 1998; Miele et al., 1998], cDNA prepared from total RNA samples derived from three independent experiments was used to perform the DDRT-PCR. Parallel display of duplicate samples from control and treated bones showed an 800 bp band that was rapidly upregulated in 1 h, but returned to control levels by 24 h (Fig. 1). This band was excised from the gel, re-amplified by PCR, and the resulting PCR product was then cloned, sequenced, and used to screen a metaphyseal bone cDNA library. A single unique clone containing a 2.1 kb cDNA insert was isolated and sequenced. Sequence analysis revealed that this cDNA encodes an open reading frame of 348 amino acids and is 98, 93.7, and 82.5% identical with mouse (AF079565), human (AF079564), and chicken (AF016107) UBP41 proteins, respectively (data not shown). On the basis of sequence similarity, we have designated this clone as rat UBP41.

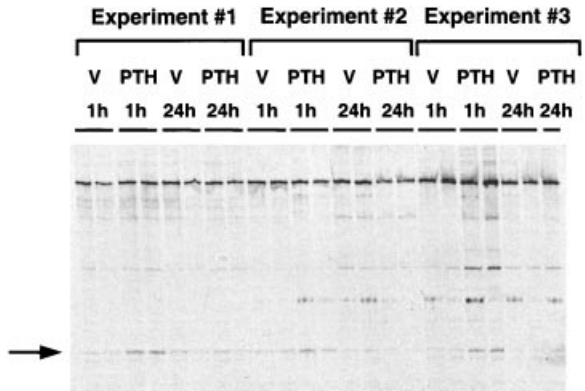


Fig. 1. DDRT-PCR identification of UBP41 as a PTH-regulated gene in rat metaphyseal bone. DDRT-PCR products amplified from cDNA derived from vehicle- and hPTH (1–38)-treated femoral bones (pooled, n = 4/group) were resolved on a 6% TBE/urea sequencing gel. To eliminate false positives, cDNAs derived from total RNA prepared from three independent experiments were analyzed simultaneously. A negative control omitting the cDNA template (no cDNA control) was also analyzed and yielded no bands (data not shown). Samples were run in duplicate for each time point examined. The band representing the candidate PTH-regulated gene is indicated by the arrow. This band was excised from the gel, reamplified by PCR, and cloned for sequence analysis.

Effect of hPTH (1–38) on UBP41 mRNA Expression in Rat Femoral Metaphysis and Diaphysis

We performed Northern blot analysis using poly A^+ RNA from control and PTH-treated metaphyseal bone to reconfirm the regulation of UBP41 observed via DDRT-PCR. Administration of hPTH (1–38) (8 μg/100 g) to rats resulted in a rapid and transient increase in a 3.8–4 kb UBP41 mRNA transcript (Fig. 2A). UBP41 mRNA levels were increased eightfold by 1 h, but returned to near control levels by 3–24 h. We next examined the basal and PTH-induced effect on UBP41 mRNA in both metaphyseal and diaphyseal bone taken from the same animals (Fig. 2B). In the control (vehicle treated), low levels of UBP41 mRNA were detected in the diaphyseal and metaphyseal bone samples. Following PTH treatment, UBP41 was rapidly increased in 1 h in both metaphyseal (6.6-fold) and diaphyseal (3.1-fold) bone, but returned to control levels by 24 h.

Effects of PTH Analogs, PTHrP and PGE_2 on UBP41 mRNA Expression in Rat Femur

As PTH independently activates two signaling pathways; one that stimulates adenylate cyclase/protein kinase A (cAMP/PKA) and one that stimulates phospholipase C/protein kinase C (PLC/PKC); we next evaluated whether the cAMP/PKA-mediated signal transduction was required for stimulation of UBP41 in vivo. Specifically, we compared the effect of PTH (1–38) to PTH (1–31) which activates primarily the cAMP/PKA pathway, and PTH (3–34) and (7–34) which have no effect on the PKA pathway [Fujimori et al., 1991, 1992;

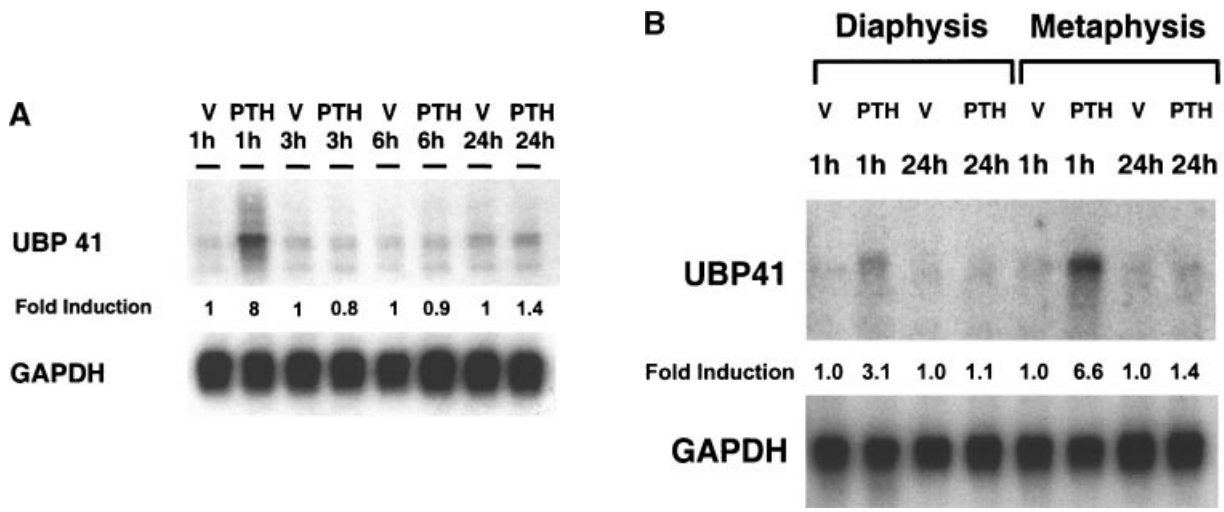


Fig. 2. Effect of hPTH (1–38) on UBP41 mRNA expression in rat femoral metaphysis and diaphysis. **A**: A representative autoradiograph showing the time course of hPTH (1–38) treatment on UBP41 mRNA expression in rat femoral metaphysis. **B**: Comparison of basal and PTH effects on UBP41 in metaphyseal and diaphyseal bone. RNA was isolated from the femoral metaphysis and diaphysis of young male rats (pooled, n = 4/group) at indicated times following a single PTH injection (8 μg/100 g, s.c.). Two micrograms of poly A^+ RNA were loaded per lane and analyzed for UBP41 expression by Northern blot hybridization. GAPDH was rehybridized as a control for RNA integrity and quantification. After normalization to GAPDH, the values are shown as fold induction over vehicle (V) treated controls (which is set as 1).

Jouishomme et al., 1992, 1994; Rixon et al., 1994]. In addition, because PTHrP can activate PTH receptor type 1 in bone to stimulate both the cAMP/PKA and PLC/PKC pathways [Juppner et al., 1988, 1991], we also examined the effects of hPTHrP (1–34), a potent analog of PTHrP. Rats injected with either vehicle or PTH analogs were sacrificed 1 h post-injection and UBP41 mRNA expression was analyzed in RNA isolated from femoral metaphyses. As demonstrated in Figure 3, only those analogs capable of significantly elevating intracellular cAMP levels [PTH (1–38), PTH (1–31), and PTHrP (1–34)] upregulated UBP41 mRNA expression. In contrast, other analogs of PTH that do not elevate cAMP levels [PTH (3–34) and PTH (7–34)] failed to significantly induce UBP41 mRNA expression.

To further elucidate UBP41 expression in bone, we examined whether UBP41 expression is affected by PGE_2, another osteotropic agent whose effects are partly dependent on the cAMP/PKA-signaling pathway. Animals, given either vehicle or a single dose of PGE_2 (6 mg/kg), were sacrificed at various time intervals (1, 6, or 24 h) after injection, and the femoral metaphysis was analyzed for UBP41 mRNA expression. As shown in Figure 4, UBP41 expression was rapidly and transiently induced by PGE_2 within 1 h, and returned to basal levels by 6 h.

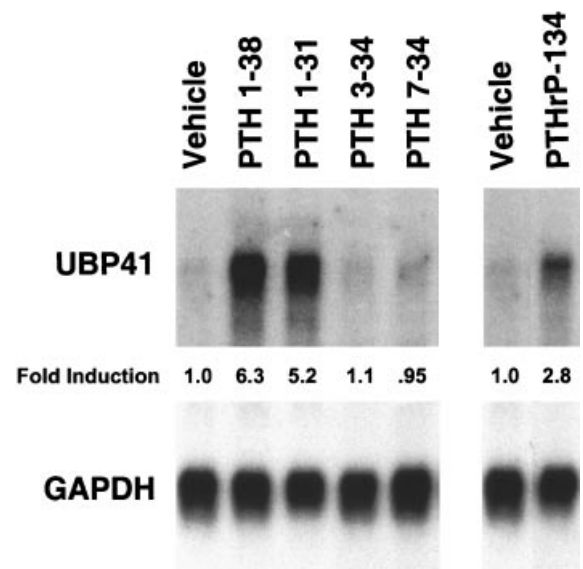


Fig. 3. Effects of PTH analogs and PTHrP on UBP41 mRNA expression in rat femoral metaphysis. Poly A^+ RNA, isolated from the distal femoral metaphysis of rats (pooled, n = 4/group) 1 h after injection (s.c.) with indicated PTH analogs (8 μg/100 g), PTHrP (8 μg/100 g), or vehicle equivalent, were analyzed for UBP41 expression by Northern blot hybridization. GAPDH was rehybridized as a control for RNA integrity and quantification. UBP41 mRNA levels normalized to GAPDH signals are expressed as fold induction over vehicle-treated control (which is set as 1).

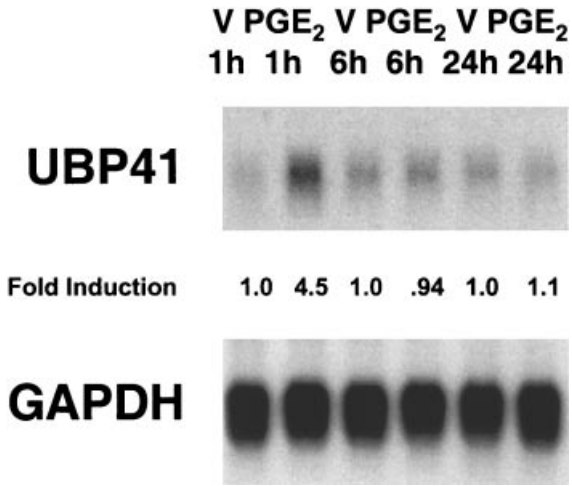


Fig. 4. Effect of PGE_2 on UBP41 mRNA expression in rat femoral metaphysis. Poly A^+ RNA isolated 1, 6, or 24 h after injection with PGE_2 (6 mg/kg) or vehicle equivalent was analyzed for UBP41 mRNA expression. Two micrograms per lane of poly A^+ RNA was used for Northern blot hybridization. GAPDH was rehybridized as a control for RNA integrity and quantification. UBP41 mRNA levels are normalized to GAPDH signals and are expressed as fold induction over vehicle-treated control (which is set as 1).

Effect of Continuous hPTH (1–38) Infusion on UBP41 mRNA Expression in Rat Femoral Metaphysis

As shown in the preceding figures (1–4) acute exposure to hPTH 1–38 induced a rapid and transient increase in UBP41. Because acute versus continuous exposure to PTH have different biological outcomes in bone (anabolic and catabolic, respectively), we next investigated the effect of continuous exposure by subcutaneous infusion of PTH on UBP41 expression. We examined the expression and potential role of UBP41 in the initiation of the catabolic state induced by continuous infusion of PTH (1–38) in parathyroidectomized (PX) rats. This is a well-established model of PTH-induced bone resorption and PX animals are used to reduce the confounding effects of endogenous PTH. The resorptive effects of continuous PTH in this animal model have recently been characterized by us and correlated with reciprocal expression of OPG and RANKL, and a concomitant decrease in the expression of bone formation related genes [Ma et al., 2001]. The expression studies for UBP41 were done under conditions that promoted optimal catabolic effects of PTH in PTX rats as we previously described [Ma et al., 2001].

Briefly, weanling rats were parathyroidectomized and maintained on a calcium-free diet. Rats were then infused with PTH (1–38) (20 μg/100 g/6 h) subcutaneously via Alzet minipump. UBP41 expression was examined in poly A^+ RNA isolated from metaphyseal bone at 1, 3, 6, and 24 h after treatment. As shown in Figure 5, continuous PTH infusion induced a rapid but sustained increase in UBP41 mRNA expression. UBP41 was induced as early as 1 h (3.6-fold) and the increase persisted for at least 24 h (5.6-fold). This is in contrast to the rapid but transient changes elicited by acute PTH exposure.

Expression and Regulation of UBP41 in Bone Cells In Vitro

To delineate the expression of UBP41 in various bone cell types, we examined UBP41 mRNA expression in poly A^+ RNA isolated from primary cultures of femoral metaphyseal and diaphyseal osteoblasts, rat osteoblast-like cells (UMR 106 and ROS 17/2.8 cells), mouse calveria-derived stromal/osteoblastic cells (BALC), and osteoclast-like cells (derived from M-CSF/RANKL/TGF-β1-treated bone marrow cells). We recently verified the authenticity of the osteoclast-like cells used in this study and they display the distinguishing characteristics of osteoclasts, such as the abilities to resorb bone and to express lineage-specific markers including TRAP and CTR [Sells Galvin et al., 1999]. As shown in Figure 6, we detected the expression of the 3.8–4 kb UBP41 mRNA transcript in the primary osteoblasts, UMR106 cells, ROS

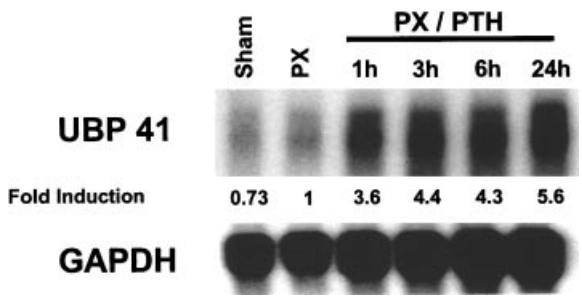


Fig. 5. Effect of continuous hPTH (1–38) infusion on UBP41 mRNA expression in rat femoral metaphysis. Weanling parathyroidectomized rats were fed a calcium-free diet and were infused with PTH (20 μg/100 g/6 h) via Alzet minipump. UBP41 mRNA was examined in 2 μg poly A^+ RNA isolated from metaphyseal bone at 1, 3, 6, and 24 h. GAPDH was rehybridized as a control for RNA integrity and quantification. UBP41 mRNA levels were normalized to GAPDH signals as described in preceding figures.

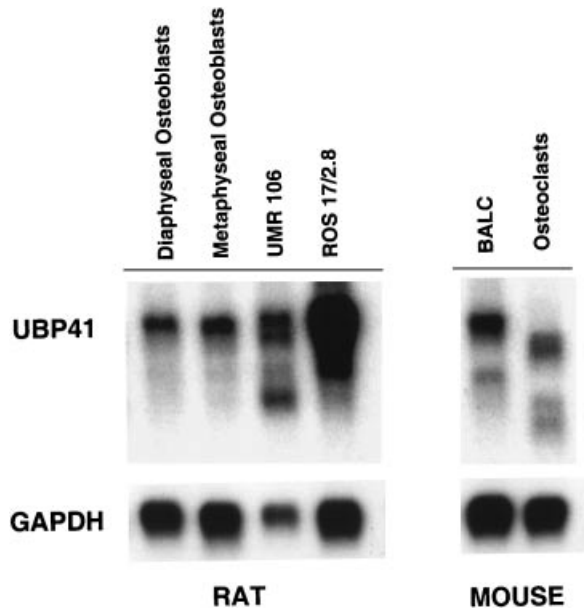
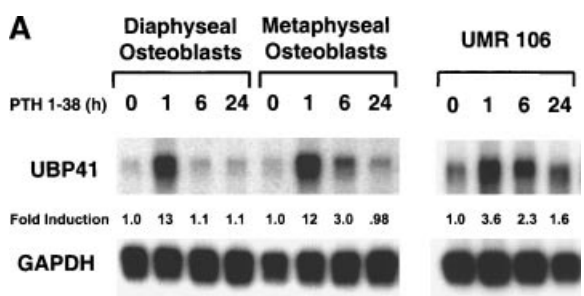


Fig. 6. Expression of UBP41 mRNA in bone cells. Two micrograms of poly A^+ RNA from various bone cells (primary cultures of osteoblasts derived from rat femoral diaphyseal and metaphyseal bone, UMR106 cells, ROS 17/2.8 cells, BALC stromal/osteoblastic cells and osteoclast-like cells derived from M-CSF/RANKL/TGF-β1-treated mouse bone marrow cells) were analyzed for UBP41 and GAPDH mRNA.

17/2.8 cells, and in the BALC cells. Additional transcripts of lower intensity were detected in UMR106 cells and BALC cells. The 3.8–4 kb transcript seen in bone and osteoblasts, was not detected in the osteoclast-like cells; instead, three lower molecular weight transcripts were detected. By comparison to other cells, UBP41 is expressed at very high levels in ROS 17/2.8 cells.

Because the effects of PTH on bone are mediated, in part, by direct actions on the osteoblast population, we examined the expression of UBP41 mRNA in primary osteoblast cultures (metaphyseal and diaphyseal osteoblasts), as well as in the PTH-responsive UMR106 cell line [Partridge et al., 1981, 1983; Forrest et al., 1985; Scott et al., 1992]. Cells were treated with PTH (1–38) (5×10^{-8} M) for various time intervals and UBP41 mRNA was evaluated. As shown in Figure 7A, both the primary osteoblast cultures and UMR106 cells expressed relatively low levels of UBP41 mRNA in the control state. Treatment with PTH dramatically increased UBP41 expression. In all three cultures, maximal increase in expression was evident at 1 h (3.6 to 13-fold). In the metaphyseal osteoblasts and UMR 106 cells, UBP41 mRNA remained elevated at 6 h (3.0 to 2.3-fold), but returned to control levels by 24 h.

Furthermore, we evaluated the involvement of the cAMP/PKA signaling pathway in PTH-induced UBP41 expression in UMR106 cells. We compared the effect of PTH (Fig. 7A) to forskolin (an activator of adenylate cyclase), dibutyryl cAMP (a membrane-permeable analog of cAMP), PGE_2 (whose effects on osteoblasts are partly dependent on the cAMP/PKA



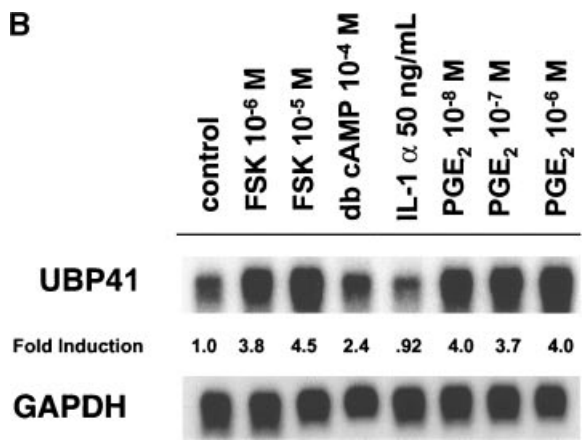


Fig. 7. Expression and regulation of UBP41 in bone cells in vitro. **A**: Time course of hPTH (1–38) effects on UBP41 mRNA expression in primary osteoblast cultures derived from the femoral metaphysis (metaphyseal osteoblasts) and diaphysis (diaphyseal obsteoblasts), as well as in UMR106 cells. Cells were treated with hPTH (1–38) (5×10^{-8} M) for 0, 1, 6, and 24 h. **B**: The effect of forskolin, dibutyryl cAMP, IL-1α, and PGE_2 on UBP41 mRNA expression in UMR106 cells. Cells were treated with indicated concentrations of either forskolin (FSK) or dibutyryl cAMP (db cAMP) or PGE_2 for 1 h. Poly A^+ RNA was isolated at the end of the treatments and UBP41 mRNA levels were determined by Northern analysis (2 μg/lane poly A^+ RNA). GAPDH was rehybridized as a control for RNA integrity and quantification. UBP41 mRNA levels normalized to GAPDH signals are expressed as fold induction over vehicle-treated control (which is set as 1).

signaling pathway), and IL-1α (which does not activate the cAMP/PKA signaling pathway in osteoblasts). As shown in Figure 7B, both forskolin (10^{-6} and 10^{-5} M) and dibutyryl cAMP (10^{-4} M) stimulated UBP41 mRNA 2.4 to 4.5-fold. Similarly, PGE_2 (10^{-8}, 10^{-7}, and 10^{-6} M) stimulated UBP41 mRNA 3.7 to 4.0-fold. In contrast, 50 ng/ml IL-1α did not stimulate UBP41 mRNA expression. Collectively, these results suggest that activation of the cAMP/PKA signaling pathway increases UBP41 expression in the osteoblast.

Tissue Expression of UBP41

Finally, we profiled the expression of UBP41 in non-osseous tissues by Northern blot analysis of poly A^+ RNA. As shown in Figure 8, UBP41 cDNA hybridized to mRNA transcripts in the heart, brain, liver, skeletal muscle, kidney, and testis. Under these conditions, no transcripts were detected in the spleen and lung. A 4 kb transcript (similar to that in bone) and a 3.6 kb transcript were detected in the heart and skeletal muscle. Only the 3.6 kb transcript was detected in the brain and kidney. In the liver, the 3.6 kb transcript and a smaller molecular weight transcript were detected. Quantitatively, significantly higher levels and numbers of transcripts were observed in the testis. In addition to the 4–4.2 kb transcript, three lower molecular weight transcripts were strongly expressed in the testis.

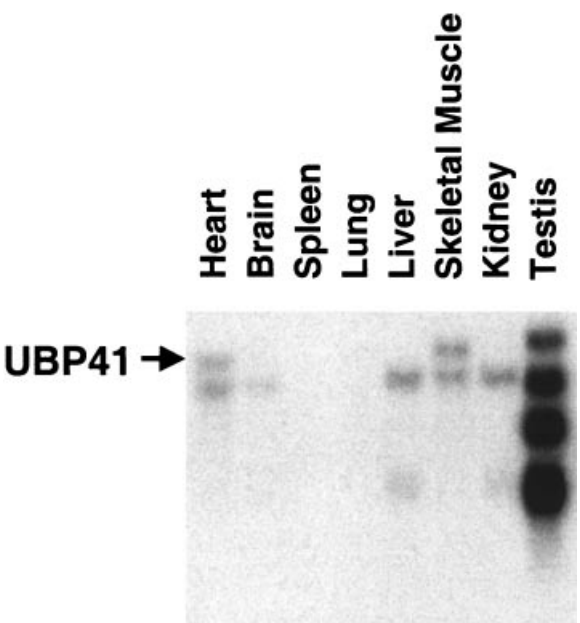


Fig. 8. Tissue distribution of UBP41 mRNA expression. Expression of UBP41 mRNA was examined using poly A^+ RNA from non-osseous tissues as indicated.

DISCUSSION

PTH has dual effects in bone depending on the mode of administration. PTH is anabolic when administered acutely or at low doses, and catabolic when administered continuously or at high doses [Hock et al., 1989c; Hock and Gera, 1992]. An anabolic regime of PTH increases the differentiation, function, and survival of osteoblasts while catabolic administration of PTH decreases type 1 collagen and extracellular matrix protein synthesis, and enhances protein turnover [Hock et al., 1994, 1995; Onyia et al., 1995; Schmidt et al., 1995; Jilka et al., 1999; Manolagas, 1999; Stanislaus et al., 2000b; Ma et al., 2001]. It has been speculated that PTH mediates its effects on bone metabolism, in part, by the induction of new genes/proteins that regulate intracellular proteolysis via the ubiquitin-mediated proteolytic pathway. However, the identity of these genes and the mechanisms by which they regulate protein metabolism in bone remain poorly defined. In the present study, we cloned and characterized the expression and regulation of an ubiquitin-specific protease, UBP41 in bone and bone cells. The in vivo and in vitro evidence demonstrate that UBP41 is robustly upregulated in bone and in osteoblastic cells after PTH treatment. PTH appears to mediate its stimulatory effects on UBP41 expression, in part, by the cAMP/PKA signal transduction pathway. These findings provide novel insight into the mechanistic basis for the metabolic effects of PTH and suggest that UBP41 may play regulatory roles in bone metabolism.

Our results show that in vivo acute exposure to a normal calcemic dose of PTH, induced UBP41 expression in a rapid and transient manner, characteristic of an early response gene. Similarly, UBP41 was rapidly and transiently induced by acute exposure to an anabolic dose of PGE_2. In contrast, continuous exposure to PTH, which induces a catabolic response, resulted in an early but prolonged activation of UBP41 up to 24 h, the latest time point examined. As we demonstrated previously in this resorption model, the sustained increase in UBP41 expression (3–24 h) corresponds to peak resorption and decreased expression of bone

formation genes/markers. Together, these results suggest that UBP41 may play a regulatory role in both anabolic and catabolic effects of PTH in bone. The rapid induction and short half-life of the UBP41 mRNA after acute PTH and PGE_2 exposure suggest that transient alterations in the levels of this protease may contribute to the initial events leading to anabolic actions of PTH and PGE_2 on bone metabolism. The prolonged activation of UBP41 associated with continuous PTH suggests that sustained expression of this protease may contribute to the cascade of events leading to bone turnover and destruction.

UBP41 was initially cloned and shown to encode a UBP, a large family of specific thiol proteases that cleave ubiquitin from ubiquitinated protein substrates [Baek et al., 1997]. UBPs have multiple functional roles through their regulation of ubiquitin-dependent processes within the cell [Wilkinson, 1997, 2000]. However, the functional role that has received most attention is the ubiquitin-mediated degradation. Several mechanisms are utilized by UBPs to cleave ubiquitin from ubiqutinated protein substrates [Wilkinson, 1997, 2000]. Some UBPs generate monomeric ubiquitin by cleaving either polymeric ubiquitin or ubiquitinated protein precursors, and thereby regulate the available cellular pools of free monomeric ubiquitin. Other UBPs (i.e., Doa-4), recycle ubiquitin from proteins partially degraded by the proteasome, and thereby accelerate proteasome-mediated degradation. Still other UBPs, such as fat facets protein (faf), remove ubiquitin from cellular target proteins and thus prevent their proteasome-mediated degradation [Huang et al., 1995]. Presently, the exact physiological role of UBP41 is unclear. Recent biochemical analysis of purified UBP41 with natural and engineered substrates suggest that UBP41 may regulate the ubiqutin-mediated degradation pathway in the recycling of ubiquitin by hydrolysis of branched polyubiquitin chains generated by the action of the proteasome on poly-ubiquitinated protein substrates. Alternatively, it may play a role in the production of free ubiquitin from linear polyubiqutin chains and certain ribosomal proteins from ubiquitin fusion proteins.

A regulatory role for the UB-proteasome system is clearly apparent in PTH control of bone metabolism and has been suggested in a few studies [Murray et al., 1998]. Many targets of PTH such as p27 Kip1, c-myc, c-fos, c-jun, RGS2, and OSF2 [Onyia et al., 1995; Liang et al., 1999; Miles et al., 2000b] have been shown to be regulated by the ubiquitin-proteasome system [Isaksson et al., 1996; Tintut et al., 1999; Nakayama et al., 2001]. These and many other important regulatory proteins in bone that are constitutively expressed are ubiquitinated and are natural substrates of the ubiquitin proteolytic pathways. The half-life of these proteins can be modulated via the UB-proteasome system to meet the cellular requirements necessary for PTH actions. Additionally, previous studies have demonstrated that the inhibitory effects of catabolic PTH on osteoblast proliferation in vitro require multicatalytic protease complex (MCP) or 26S proteasome activity [Murray et al., 1998]. In this study, PTH stimulated the accumulation of high molecular weight ubiquitinated protein substrates and significant levels of three major MCP endopeptidase activities (peptidylglutamyl-peptide bond hydrolase-, trypsin-, and chymotrypsin-like). PTH mediated its stimulatory effects on the osteoblastic UB-proteasome system, in part, by a PKA-mediated stimulation of MCP activities. Similarly in MC3T3-E1 cells, the catabolic effect of continuous activation of the cAMP pathway (measured by decreases in alkaline phosphatase, OSF2, bone sialoprotein, osteocalcin, and osteopontin) could be reversed by pretreatment with a proteasome inhibitor (perturbation of the ubiquitin dependent proteasome system) [Tintut et al., 1999]. These findings suggest that there is a central role for the ubiquitin-proteasome pathway in regulating the proper expression level/activity of specific regulatory factors controlling PTH effects in bone.

Evidence suggesting that UBPs can function in other alternative pathways that do not involve proteasome-mediated degradation has come from many independent studies showing that regulated ubiquitination can serve regulatory functions in the cell [Hochstrasser, 1996; Wilkinson, 1997, 1999; Chung and Baek, 1999; Hicke, 1999, 2001]. These studies suggest that reversible ubiquitination of proteins is similar or analogous to reversible phosphorylation of proteins and probably serves the same function, which is to modify the structure, activity, or localization of the target protein. For example, in yeast, ligand-induced ubiquitination of Ste2 receptor triggers receptor endocytosis and receptor targeting to vacuoles, not proteasomes

[Hicke and Riezman, 1996]. Also, the activation of IKBα kinase requires a rapid, inducible ubiquitination event, which is a prerequisite for specific phosphorylation of IKBα [Chen et al., 1996]. These ubiquitination events do not result in proteolysis and appear reversible, perhaps due to the action of a specific UBP. Similarly, specific UBPs have been shown to have growth regulatory properties. For example, continuous expression of DUB-1 in Ba/F3 cells (a murine pro-B-cell line) resulted in growth arrest in G1 phase [Zhu et al., 1996]. Two human proto-oncogenes (tre-z and unp) have been shown to code for active or dominant negative mutants of UBPs [Gupta et al., 1993, 1994; Papa and Hochstrasser, 1993]. The *Drosphila faf* gene regulates cell growth and differentiation during eye development [Huang et al., 1995]. Together, these studies demonstrate that other potential functional roles for UBP41 need to be explored. Future studies depleting or overexpressing UBP41 in animals will be required to elucidate the physiological role of UBP41.

Lastly, our results show a constitutive expression of UBP41 or an UBP41-like transcript in the tissues examined, including bone and bone cells (osteoblasts, stromal cells, and osteoclasts). This is consistent with an essential role for UBP41 in cellular function. Besides the primary transcript detected in bone, other transcripts were detected in heart, liver, skeletal muscle, testis, and UMR106 cells. These transcripts remain detectable even under very high stringency hybridization and washes (present study and data not shown), suggesting that they are alternate transcripts arising from the same gene or products of closely related genes. The specific expression and response of the 3.8–4 kb transcript to osteotropic agents PTH, PTHrp, and PGE_2 in bone and bone cells suggest a selective effect in bone metabolism.

In summary, our data suggest that a rapid burst of UPB41 is associated with PTH metabolic actions in vivo. We hypothesize that like other immediate early gene products, UBP41 plays a role in integrating the proximal signaling events of PTH with the downstream metabolic effects of PTH. Based on the different kinetics of gene expression between acute and continuous PTH treatment, we propose a role for UBP41 in both anabolic and catabolic effects of PTH in rat bone. The differential responsiveness of bone to these two regimes of PTH treatment may be explained in part by the differences in the magnitude and duration of signaling of UBP41.

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