Pyrophosphate Inhibits Mineralization of Osteoblast Cultures by Binding to Mineral, Up-regulating Osteopontin, and Inhibiting Alkaline Phosphatase Activity*

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Inorganic pyrophosphate (PPi), produced by cells inhibits mineralization by binding to crystals. Its ubiquitous presence is thought to prevent “soft” tissues from mineralizing, whereas its degradation to P1 in bones and teeth by tissue-nonspecific alkaline phosphatase (Tnap, Tnsalp, Alpl, Akp2) may facilitate crystal growth. Whereas the crystal binding properties of PPi are largely understood, less is known about its effects on osteoblast activity. We have used MC3T3-E1 osteoblast cultures to investigate the effect of PPi on osteoblast function and matrix mineralization. Mineralization in the cultures was dose-dependently inhibited by PPi. This inhibition could be reversed by Tnap, but not if PPi was bound to mineral. PPi also led to increased levels of osteopontin (Opn) induced via the Erk1/2 and p38 MAPK signaling pathways. Opn regulation by PPi was also insensitive to foscarnet (an inhibitor of phosphate uptake) and levamisole (an inhibitor of Tnap enzymatic activity), suggesting that increased Opn levels did not result from changes in phosphate. Exogenous OPN inhibited mineralization, but dephosphorylation by Tnap reversed this effect, suggesting that OPN inhibits mineralization via its negatively charged phosphate residues and that like P1, hydrolysis by Tnap reduces its mineral inhibiting potency. Using enzyme kinetic studies, we have shown that PPi inhibits Tnap-mediated P1 release from β-glycerophosphate (a commonly used source of organic phosphate for culture mineralization studies) through a mixed type of inhibition. In summary, PPi prevents mineralization in MC3T3-E1 osteoblast cultures by at least three different mechanisms that include direct binding to growing crystals, induction of Opn expression, and inhibition of Tnap activity.

Mineralization of bones, and tooth dentin and cementum, occurs in a collagen-rich extracellular matrix. Coincident with establishing an extracellular collagenous network in these tissues, osteoblasts, chondroblasts, odontoblasts, and cementoblasts all secrete additional noncollagenous matrix proteins that integrate with the collagen fibrils and provide additional functionality to the matrix (1). Although much of this functionality relates to cell adhesion and signaling, cell-free in vitro assays and in vivo studies using transgenic mice indicate that certain noncollagenous proteins (some tissue-specific) regulate the induction of mineralization and subsequently control hydroxyapatite crystal growth by binding to mineral surfaces (2).

In addition to the organic, mineral-binding noncollagenous proteins of bones and teeth, inorganic molecules in these tissues have also been proposed as molecular determinants of mineralization. Pyrophosphate (PPi) is a potent, mineral-binding small molecule inhibitor of crystal nucleation and growth (3), and recent studies using transgenic and naturally occurring mutant mice have identified the proteins/enzymes involved in producing and handling PPi (see references below).

PPi is a by-product of many intracellular metabolic reactions (4). PPi is also present in the extracellular matrix of most tissues and bodily fluids including plasma (5, 6), where it acts as a potent inhibitor of mineral nucleation and growth at micromolar concentrations (3, 7, 8). Although the mechanism by which PPi inhibits hydroxyapatite crystal growth is not entirely known, PPi is thought to adsorb specifically to crystal growth sites, thus preventing further apposition of mineral ions (7). Although some physicochemical studies have been conducted on the actions of PPi on crystal growth in cell-free systems, there is limited information available on putative direct effects of PPi on cells in general and, more specifically, on mineralizing cell culture models (9).

Extracellular matrix mineralization in bone is tightly linked to the P1/PPi ratio found in this tissue (10). Extracellular PPi deficiency leads to excess hydroxyapatite formation in the skeleton (11), whereas PPi elevation results in decreased skeletal mineralization (6, 12–14) and the formation of calcium pyrophosphate dihydrate crystals in joints (15, 16). The homeostatic regulation of local P1, levels by bone cells is thus an important part of their function related to mineralization. The major ectoenzyme responsible for generation of extracellular PPi is Enpp1 (ectonucleotide pyrophosphatase/phosphodiesterase 1;
also known as NPP1 and NPPS) (17); this enzyme is expressed at particularly high levels in bone, cartilage, and teeth (17–19). Intracellular PPi can also be exported into the extracellular compartment by the membrane transporter Ank (progressive ankylosis or ANKH), which is also highly expressed in bone, cartilage, and teeth (20–22). Deletion of the Ank gene in mice results in ectopic mineral formation in joint spaces and, eventually, complete joint ankylosis (23). Given the high expression levels of these two genes in mineralized tissues, removal of PPi is expected to be a prerequisite for physiologic mineralization, and thus regulation of the expression and activities of these two proteins is likely central to the control of mineralization. Tnap (tissue-nonspecific alkaline phosphatase; also known as Akp2), a well characterized marker of the osteoblast lineage (24), is capable of hydrolyzing PPi into Pi (25). In osteoblast cultures, Tnap is also responsible for the generation of Pi from phosphate esters such as β-glycerophosphate (βGP), which is commonly used as a source of organic phosphate for mineralization (26).

Another key inhibitor of mineralization found in bone is Opn (osteonectin), a highly phosphorylated glycoprotein with strong mineral binding properties (27). A link between the enzymes regulating extracellular PPi levels and nonenzymatic protein inhibitors of mineralization such as Opn has been demonstrated in Enpp1-deficient mice that show a decrease in both PPi and Opn levels and consequential hypermineralization particularly related to joints and ligaments (11). This phenotype is also observed in the naturally occurring mouse mutant ttw (tip-toe-walking mice; Ref. 28).

In view of recent findings linking PPI generators (Enpp1), transporters (Ank), and degraders (Tnap) with extracellular matrix protein inhibitors of mineralization (Opn), we have used the MC3T3-E1 murine preosteoblast cell line to examine whether there is a direct effect of PPi on Opn gene expression and the specific signaling pathways involved in this response. We report that PPi up-regulates Opn in osteoblasts via MAPK and the specific signaling pathways involved in this response.

**Pyrophosphate Inhibition of Mineralization**

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MC3T3-E1 murine calvarial osteoblasts (subclone 14) cells were a gift from Dr. R. T. Franceschi (University of Michigan, Ann Arbor, MI). The cultures were maintained in modified α-minimum essential medium (Invitrogen) supplemented with 1% fetal bovine serum (HyClone) and 1% penicillin-streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO2. All of the experiments were carried out at a seeding density of 50,000 cells/cm2. Cell differentiation and mineralization was initiated 24 h after plating by replacing the medium with α-minimum essential medium supplemented with 10% fetal bovine serum, 50 μg/ml ascorbic acid (Sigma), and 10 mM βGP (Sigma) or 5 mM sodium phosphate (Sigma). The medium was changed every 48 h. For inhibition experiments, sodium pyrophosphate tetrabasic, levasimole, kidney protein inhibitors of mineralization such as Opn has been demonstrated in the naturally occurring mouse mutant ttw (tip-toe-walking mice; Ref. 28).

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**Quantification of Mineralization**—Mineral was visualized by von Kossa staining using a 5% silver nitrate solution (Sigma) combined with exposure of the culture dishes to bright light. For quantification of insoluble calcium in the cell/matrix layer, the cultures were decalcified with 0.5% HCl, and calcium in the supernatant was determined colorimetrically using a calcium assay kit (Diagnostic Chemicals). Following decalcification, the cultures were solubilized with 0.1 NaOH and 0.1% SDS, and total protein content was measured with the BCA protein kit (Pierce). The calcium content of the cell/matrix layer was normalized to the protein content.

**Assay for Alkaline Phosphatase and Nucleotide Pyrophosphatase Phosphodiesterase Activity**—The cultures were washed three times with phosphate-buffered saline (PBS) and solubilized in 10 mM Tris, pH 7.4, 0.2% Igepal (Sigma), and 2 mM phenylmethylsulfonyl fluoride. After sonication and centrifugation, alkaline phosphatase activity in the supernatant was determined colorimetrically in a reaction mixture containing 50 mM Tris·HCl, pH 8.8, 10 mM MgCl2, and 20 mM p-nitrophenylphosphate. Calf intestinal alkaline phosphatase (Sigma) was used as a standard. One unit will hydrolyze 1 μmol of p-nitrophenylphosphate/min at 37 °C. Nucleotide pyrophosphatase phosphodiesterase activity was determined colorimetrically using p-nitrophenylthymidine monophosphate as a substrate (17).

**Enzyme Inhibition Kinetics**—All of the assays were performed in 50 mM Tris·HCl buffer, pH 8.8, with 10 mM MgCl2 at 37 °C and were repeated in triplicate. All of the enzymatic rates obtained are initial rates. Quantification of substrate reaction product (phosphate) was performed colorimetrically by the ammonium molybdate method. Inhibitor constants were obtained from averages of Dixon and s/i plots (29, 30).

**RNA Isolation and Quantitative Real Time PCR**—Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer’s protocol and quantified in a spectrophotometer by absorbance readings at 260 nm. RNA was treated with DNase (Invitrogen), and 1 μg was used for cDNA synthesis using the ThermoScript first strand cDNA synthesis reverse transcription-PCR System (Invitrogen) according to the manufacturer’s instructions. Primers (Invitrogen) for Ank, Enpp1, Tnap, and Gapdh were prepared as suggested by Foster et al. (31), and for Opn, as suggested by Ehara et al. (32). The sequences used are as follows: Ank, 5′-GAACTATCTGGCC-CAC-3′ and 5′-AGGCCGAGTAAAGCACA-3′; Enpp1, 5′-CGG-CACCGAGACTAAA-3′ and 5′-AGGAAATCATAGGTCGCG-3′; Tnap, 5′-GGGGAATGAGTAGTGGT-3′ and 5′-GGCGTGTATGGTTGGAG-3′; Gapdh, 5′-ACCACA-GTCCATGCCCAC-3′ and 5′-TCCACCACTCTTGTGCC-3′; and Opn, 5′-TCGCTAAGTACACAGCAGACA-3′ and 5′-CATGAGAAATTCGGAATTCCAC-3′.

The abbreviations used are: βGP, β-glycerophosphate; BSA, bovine serum albumin; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline.
Pyrophosphate Inhibition of Mineralization

PCR efficiency was optimized, and melting curve analysis of products were performed to ensure specificity. All of the primers were used at 0.5 μM except for those of Opn, which were used at 0.25 μM. PCR was carried out in an Applied Biosystems 7500 system using Power SYBR Green PCR Master Mix (Applied Biosystems). The sequential reaction conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Gene expression was quantified using the sequence detection software of the system (Applied Biosystems). Gapdh was used as the housekeeping gene for data normalization. Triplicate RNA samples from at least two separate experiments were used for these experiments.

Opn Enzyme-linked Immunosorbent Assay—Opn protein levels were measured using a mouse Opn assay kit (Assay Designs) according to the manufacturer’s instructions. Recombinant mouse Opn was used as a standard.

Western Blotting—The cell/matrix layer was harvested in buffer containing 10 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.5 mM EDTA (to release mineral-bound proteins) supplemented with protease inhibitor mixture (Sigma) containing 4-(2-aminophenyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin. Protein concentrations of cell lysates were determined by the BCA protein assay (Pierce), and 20 μg of total protein was separated by SDS-PAGE. The proteins were electrophoretically transferred to a nitrocellulose membrane (Pall Life Sciences). The membranes were blocked with 5% milk (from powder) in Tris-buffered saline/Tween 20 (TBS-T) and then probed with primary antibody in 5% bovine serum albumin (BSA) (Sigma) in TBS-T followed by the appropriate horseradish peroxidase-conjugated secondary antibody. The blots were visualized by chemiluminescence using ECL Plus (Amersham Biosciences). For densitometric analysis, the films were scanned and quantified with Quantity One software (Bio-Rad). Rabbit anti-Opn (LF-175) was kindly provided by Dr. Larry W. Fisher (National Institutes of Health, Bethesda, M). Anti-phospho-Erk1/2, phospho-p38, phospho-Jnk, pan-Erk1/2, pan-p38, pan-Jnk, and anti-rabbit horseradish peroxidase antibodies were purchased from Cell Signaling Technologies. Anti-Gapdh horseradish peroxidase was purchased from Abcam.

Immunofluorescence—The cells were cultured on 8-well chamber slides and then fixed in 3.7% formaldehyde. The cells were then first permeabilized with 0.25% Triton X-100 in PBS for 5 min or directly blocked with 2% BSA for 30 min. Actin cytoskeleton labeling was performed using phalloidin Alexa Fluor 564, and nuclei were observed using 4’,6-diamidino-2-phenylindole staining of DNA (Molecular Probes). Opn was immunolabeled using goat anti-mouse Opn from R & D Systems and chicken anti-goat Alexa Fluor 488 secondary antibody (Molecular Probes). The slides were mounted with Prolong Antifade (Molecular Probes).

Phosphorylated OPN and Dephosphorylation—Phosphorylated bovine milk OPN (Arla Foods, Denmark; prepared according to methods similar to those developed by E. S. Sorensen (33)) with 24 phosphorylations (of 28 potential sites) either was added directly to the cultures or was dephosphorylated at a concentration of 10 mg/ml in 50 mM Tris-HCl, pH 8.8, containing 10 mM MgCl₂ by passing through an immobilized-alkaline phosphatase column (Mobitech) according to the manufacturer’s protocol. Eluted protein was separated from the reaction mixture by precipitating with 12% trichloroacetic acid and washing with acetone. Dephosphorylation of OPN was confirmed using a phosphoprotein assay kit (Pierce).

Immunogold Labeling—Cells cultured in 35-mm dishes were fixed with 2% glutaraldehyde, dehydrated through a series of graded ethanol dilutions, and embedded in LR White acrylic resin (London Resin Company). Thin sections (80 nm) were then placed on Formvar-coated nickel grids. Post-embedding immunolabeling was performed on the grid-mounted sections as described previously (34) using goat anti-mouse Opn (R & D Systems) and protein A-collodial gold complex (15 nm; Dr. G. Posthuma, University of Utrecht, Utrecht, The Netherlands).

FIGURE 1. Effect of PPi on MC3T3-E1 osteoblast culture mineral deposition, enzyme activity, and gene expression. A, MC3T3-E1 cultures were treated with 50 μg/ml ascorbic acid and 10 mM βGP for 12 days to induce osteoblast differentiation and mineralization. Starting on day 6, the cultures were supplemented with the indicated dose of PPi, with, or without, TNAP (1 unit/ml) for the remainder of culture period. The medium was replaced every 48 h. On day 12, calcium content in decalcified extracts were measured colorimetrically by the Arsenazo method, and duplicate cultures were stained for mineral using 5% silver nitrate staining (von Kossa method). The extracts were normalized to cellular protein content, and the data are presented as the means ± S.E. B, PPi, bound to hydroxyapatite is TNAP hydrolysis-resistant. 1.8 mg of free PPi, or 1.8 mg of PP, adsorbed onto 2 mg of hydroxyapatite were incubated at 37 °C in a reaction mixture containing 50 mM Tris-HCl (pH 8.8), 10 mM MgCl₂ and 0.125 unit/ml TNAP, and P, release was measured colorimetrically by the ammonium molybdate complex method. Hydroxyapatite-bound PPi was predetermined using the EnzChek PPi, assay kit (Invitrogen). The data are presented as the means ± S.E., ***, p < 0.001, Student’s t-test. C, MC3T3-E1 cultures were treated as in A following which RNA was extracted and real-time RT-PCR analysis was performed to examine expression of the indicated genes (relative to Gapdh). D, MC3T3-E1 cultures were treated as in A following which Tnap and Enpp1 activities were measured using p-nitrophenylphosphate or p-nitrophenylthymidine monophosphate as substrates, respectively. The data are presented as the means ± S.E., * p < 0.05; **, p < 0.01; ***, p < 0.001, Student’s t-test relative to the 0 μM treatment control.
Pyrophosphate Inhibition of Mineralization

Previously published research has shown that the naturally occurring inorganic pyrophosphate (PPi) can inhibit mineralization of MC3T3-E1 osteoblast cultures (3). Because PPi has been shown to inhibit mineralization and contains one phosphate group per molecule, it is possible that PPi could be responsible for inhibition of the mineralization process. In this study, we examined the effect of PPi on mineralization and found that PPi dose-dependently inhibited mineralization of MC3T3-E1 osteoblast cultures (3).

RESULTS

Pyrophosphate Inhibits Mineralization of MC3T3-E1 Osteoblast Cell Cultures—To examine the role of PPi on osteoblast mineralization, we cultured MC3T3-E1 cells in PPi-supplemented medium, and mineral was visualized by von Kossa staining and quantified by a biochemical assay for calcium. As demonstrated in Fig. 1A, PPi inhibited mineralization in a dose-dependent manner with maximum inhibition occurring at a dose of 2.5 μM. Because PPi potently inhibits mineralization and because osteoblasts actively produce PPi, then enzymatic removal of this inhibitor from the extracellular matrix is likely a requirement for mineralization (18). Supplementation with PPi, inhibited cultures with exogenous TNAP (1 unit/ml) rescued the PPi-mediated abrogation of mineralization (Fig. 1A), suggesting that TNAP is a valid candidate for the degradation of PPi in the extracellular matrix. The addition of TNAP alone also increased mineralization as compared with the control, consistent with its ability to degrade endogenous PPi, and provide Pi for mineralization.

Because PPi has previously been shown to bind to growing crystals (3), we next examined whether mineral-bound PPi (versus free PPi) could also be degraded by TNAP. PPi adsorbed onto synthetic hydroxyapatite crystals were incubated with TNAP, and Pi release was assayed biochemically. As shown in Fig. 1B, free (unadsorbed) PPi was hydrolyzed to release Pi, whereas mineral-bound PPi was resistant to TNAP hydrolysis and yielded only negligible amounts of Pi. Together, these results indicate that PPi inhibits mineralization of MC3T3-E1 osteoblast cultures, and this inhibition can be reversed by TNAP, but not if PPi has bound to mineral.

Given that PPi/Pi ratios are regulated by the activities of Tnap, Enpp1, and Ank, we examined the effect of the above PPi treatments (Fig. 1A) on their gene expression and activity. Quantitative real time PCR showed that PPi caused a slight increase in Tnap, which was significant only at the 2.5 μM dose (Fig. 1C). Elevation of Tnap levels was anticipated because PPi would be expected to up-regulate the enzyme responsible for its degradation in an attempt to normalize its concentration. Furthermore, any PPi remaining unadsorbed to mineral could be hydrolyzed to Pi, which would then stimulate Tnap expression as has been shown previously (35).

The same 2.5 μM treatment of PPi, led to a decrease in Enpp1 expression (Fig. 1C), likely again reflecting an attempt by the cells to lower extracellular PPi. The addition of exogenous of TNAP resulted in increased Enpp1 expression, presumably representing an attempt by the cells to compensate for the increased PPi degradation. These data are consistent with the increased Enpp1 activity observed in Tnap-transfected osteoblasts (36).

Unexpectedly, we found that PPi dose-dependently increased Ank expression. That Ank, a gene whose encoded transporter protein outwardly pumps PPi out into the extracellular matrix, would be up-regulated in response to increased extracellular PPi, is surprising. Although the reason for this is not immediately obvious, we hypothesize that elevated Pi produced by TNAP hydrolysis of free, unadsorbed (to mineral) PPi could be responsible for increased Ank expression in cementoblasts (31). Furthermore, the structure and function of Ank is not entirely clear (20, 37), and Wang et al. (21) have demonstrated that blocking Ank expression led to decreases in both intra- and extracellular PPi levels, whereas Ank overexpression led to decreases in both intra- and extracellular PPi levels, suggesting a level of complexity in PPi handling and homeostasis that needs to be better clarified.

Increased Ank expression as a result of increased exogenous PPi,
Pyrophosphate Inhibition of Mineralization

could simply be an attempt by the cells to return extracellular PPi levels to normal by as yet an unknown mechanism.

The observed changes in Tnap and Enpp1 levels were reflected in corresponding activity assays, with PPi, causing a general increase in Tnap activity and suppression of nucleotide pyrophosphatase phosphodiesterase activity (includes Enpp1). The addition of exogenous TNAP appeared to normalize the elevated Tnap levels, whereas it greatly increased nucleotide pyrophosphatase phosphodiesterase activity (Fig. 1D).

Pyrophosphate Up-regulates Opn Expression—Recent data have shown that mineralizing cell culture systems (primarily osteoblasts and cementoblasts) are sensitive to local levels of Pi and PPi (22, 31, 35, 38). The role of anions such as PPi in skeletal and dental tissues appears not to be limited to their crystal binding properties. High doses of P (4–10 mM) have been shown to regulate levels of the crystal growth inhibitor protein Opn (39). To determine whether PPi could elicit a similar response, we examined levels of Opn by enzyme-linked immunosorbent assay and Western blotting in PPi-treated osteoblast cultures. 0.5 mM PPi treatment for 4 days led to a marked increase in total Opn levels (Fig. 2A). Most of the Opn was found in the cell/matrix layer, and Opn in the conditioned medium was also slightly (but not significantly) increased by the PPi treatment (Fig. 2A).

To determine whether the effect of PPi was reflected at the RNA level, we examined the PPi-treated cultures by real time PCR and observed that Opn mRNA was slightly, but significantly, up-regulated (Fig. 2B). Using Western blotting to further examine the up-regulation of Opn, we observed that PPi treatment led to a gradual cumulative increase in Opn levels over 4 days of PPi treatment (Fig. 2C).

We next examined whether this up-regulation of Opn was in fact directly attributable to PPi, and not to its hydrolysis end product, P, PPi treatment of MC3T3-E1 cultures in the presence of a specific Tnap inhibitor, levamisole, did not prevent induction of Opn, suggesting that PPi regulates Opn levels by a hydrolysis-independent mechanism (Fig. 2D). Furthermore, treatment of cultures with an equivalent dose of 0.5 mM P failed to increase Opn levels. This is consistent with previous work by Beck et al. (39) showing that Opn up-regulation by P1 in the MC3T3-E1 cell line requires a minimum dose of 4 mM and is maximal at 10 mM.

To further confirm that PPi and P regulate Opn by different mechanisms, we then treated MC3T3-E1 cultures with 0.5 mM PPi and with 10 mM P in the presence of a phosphate uptake inhibitor, foscarnet. 10 mM P was used here as a positive control for the phosphate transport-dependent (foscarnet-sensitive) system. Both Western blotting and immunofluorescence microscopy confirmed that whereas P-mediated up-regulation of Opn was sensitive to foscarnet, PPi-mediated induction of Opn was not (Fig. 3, A and B). Foscarnet failed to block Opn induction by PPi, and although this inhibitor caused a slight attenuation of the Opn signal in Ppi-treated cultures, this was negligible as compared with its effects on the P-mediated cultures where it completely abolished the strong Opn up-regulation by P. Localization of Opn to the extracellular matrix in the PPi-treated cultures is shown in Fig. 3C. Taken together, these results suggest that up-regulation of Opn by PPi is dependent on neither PPi, hydrolysis nor P uptake.

Pyrophosphate Regulates Opn via MAPK Signaling Pathways—Osteoblast-specific gene expression has previously been shown to be regulated by the MAPK pathways (40–42). To elucidate the mechanism by which PPi regulates Opn expression, we next examined the involvement of major cell signaling pathways using specific inhibitors. MC3T3-E1 cells were treated for 4 days with 0.5 mM PPi, in the presence of the selective MAPK inhibitors U0126 (Erk1/2 inhibitor), SB202190 (p38 inhibitor), or SP600125 (JNK inhibitor). Inhibition of Erk1/2 and p38 prevented induction of Opn by PPi (Fig. 4A), but Jnk inhibition had no significant effect. We then tested the ability of PPi to activate Erk1/2 and p38. Western blot analysis of Erk1/2 and p38 fol-
Pyrophosphate Inhibition of Mineralization

FIGURE 4. Role of MAPK signaling pathways in Opn regulation by PPi. A, MC3T3-E1 cells were pretreated with 30 μM U0126 (Erk1/2 inhibitor) or 30 μM SB202190 (p38 inhibitor) for 30 min and then treated with 0.5 mM PPi in the presence of the inhibitor for 96 h. The samples were then analyzed by Western blotting for Opn and the same blot reprobed for Gapdh. B, activation of MAPK signaling pathways by PPi. MC3T3-E1 cells were treated with 0.5 mM PPi for the indicated times, and the protein was extracted and analyzed by Western blotting using phospho-specific antibodies for the activated forms of Erk1/2 and p38. Densitometries are expressed as the means ± S.E. of the integrated densities obtained using Quantity One software adjusted to the total Erk1/2 or p38 bands. *, p < 0.05; **, p < 0.01, Student’s t test relative to the 0-min time point.

FIGURE 5. Effect of OPN phosphorylation status on MC3T3-E1 osteoblast mineral deposition. A, MC3T3-E1 osteoblast cultures were treated with 50 μg/ml ascorbic acid and 10 mM βGP for 12 days to induce cell differentiation and mineralization. Starting on day 6, the cultures were supplemented with the indicated dose of exogenous OPN or TNAP-dephosphorylated OPN (dephos. OPN) for the remainder of culture. The medium was replaced every 48 h. On day 12, the calcium content in decalcified extracts was measured colorimetrically by the Arsenazo method, and duplicate cultures were stained for mineral using 5% silver nitrate staining (von Kossa method). The extracts were normalized to cellular protein content. The data are presented as the means ± S.E. *** , p < 0.001, Student’s t test relative to the 0 M control treatment. B, transmission electron micrograph showing post-embedding, colloidal-gold immunolocalization of endogenous Opn in an 8-day MC3T3-E1 culture using anti-Opn followed by protein A-colloidal gold conjugate. Opn is localized to the margins of small mineralization foci dispersed among uncalcified collagen fibrils (Coll). LR White resin section counterstained with uranyl acetate and lead citrate. C, transmission electron micrograph of OPN binding to hydroxyapatite. Synthetic hydroxyapatite crystals were incubated with OPN followed by colloidal-gold immunolabeling showing localization of the protein at the surface of the hydroxyapatite crystals.
Opn Inhibits Mineralization via Its Phosphate Residues—Up-regulation of Opn by PPi is noteworthy in that although the former is a protein and the latter is an anionic small molecule, they nevertheless apparently share some remarkable similarities including mineral inhibition. As demonstrated in Fig. 1A, the inhibitory action of PPi is inactivated by the actions of TNAP. We thus reasoned that if the levels of one inhibitor are coupled to another, modulation of their activities might also be similarly coupled. To examine this, we added exogenous bovine OPN to MC3T3-E1 cultures that dose-dependently inhibited mineralization, with complete inhibition occurring at a dose of 1.2 μM (Fig. 5A). Dephosphorylation of the OPN with immobilized TNAP removed its ability to block mineralization at 1.2 μM (Fig. 5A). Another similarity between OPN and PPi is their high affinity for mineral. Using immunogold labeling, we demonstrated at the ultrastructural level that endogenous mouse Opn in mineralizing MC3T3-E1 cultures co-localized with mineral deposits (Fig. 5B). Furthermore, incubation of bovine OPN with synthetic hydroxyapatite crystals, where the protein was then visualized by immunogold labeling, likewise demonstrated that OPN can bind to hydroxyapatite (Fig. 5C).

Pyrophosphate Inhibits Phosphate Release from βGP and OPN—βGP is a monophosphoester commonly used as an organic source of phosphate in cell cultures (43, 44) whose
cleavage by TNAP yields free P_i for apatite crystal growth during mineralization (26). Mineralization of MC3T3-E1 cells can therefore be initiated with either βGP or P_i, with the main difference being that βGP-induced mineralization is Tnap-dependent. Although PP_i inhibited mineralization in both culture systems, the dose of PP_i required was differed. Whereas 5 μM PP_i was sufficient to inhibit βGP-induced mineralization, surprisingly, 50 μM PP_i was required to block P_i-induced mineralization (Fig. 6, A and B). This 10-fold difference in potency suggests that PP_i might be interfering with Tnap-mediated P_i release from βGP in addition to blocking mineral growth by binding to crystal surfaces. We thus monitored gene expression levels to determine whether cultures induced by both phosphate sources behaved in a similar manner when exposed to PP_i (Fig. 6C). Under both conditions, the effect of PP_i was more or less analogous, with PP_i causing a slight elevation of Tnap, Opn, and Ank. Enpp1 expression decreased at low doses of PP_i and increased at the higher doses. This unexpected increase in Enpp1, as well as the increase in Ank, might be attributable to the P_i released from the hydrolysis of PP_i, that is not bound to mineral. Regardless of the source of added phosphate for mineralization, there were no major differences in gene expression between the P_i- and βGP-treated cultures. The changes in Tnap and Enpp1 expression were reflected at the protein level in phosphatase and nucleotide pyrophosphatase phosphodiesterase enzyme activity assays (Fig. 6D). Although these assays reflect TNAP and Enpp1 protein levels, actual in vitro activity levels may differ because cell lysates were necessarily assayed here in the absence of culture medium, extracellular matrix, and endogenous inhibitors, which could affect activity in situ.

To test the hypothesis that PP_i might also be interfering with Tnap-mediated P_i release in the βGP-induced cultures, causing decreased mineralization of the cultures, we examined the direct effect of PP_i on the βGPase activity of Tnap in vitro. Cell lysates from mineralized MC3T3-E1 cultures contain high Tnap activity. Incubation of these extracts with βGP results in release of free P_i as measured colorimetrically (Fig. 7A). The addition of PP_i to the cell extract/βGP reaction mixture led to an inhibition of βGP hydrolysis and P_i release (Fig. 7A). To confirm that PP_i was having a direct effect on the enzymatic activity of Tnap, we performed the above experiment using purified TNAP in place of the cell lysate. As is shown in Fig. 7B, TNAP independently hydrolyzes both PP_i and βGP, but when the two substrates were combined, there was a reduction in P_i release from βGP (see also below, Fig. 8). These results therefore suggest that inhibition of MC3T3-E1 mineralization by PP_i also involves altered Tnap-mediated hydrolysis of βGP.

The ability of PP_i to inhibit Tnap activity may provide some insight into why Tnap present in fully differentiated osteoblasts does not enzymatically cleave the phosphates from Opn, which would in turn decrease binding of Opn to mineral. To test the hypothesis that phosphorylated Opn is at least partly protected by PP_i from the phosphatase actions of Tnap, we examined whether PP_i could inhibit TNAP hydrolysis of bovine OPN. As was demonstrated for βGP in Fig. 7 (A and B), hydrolysis of OPN phosphate by TNAP was significantly reduced in the presence of PP_i (Fig. 7C). To avoid the potential for substrate end product inhibition, all of the experiments were performed in...
Pyrophosphate Inhibition of Mineralization

A, Lineweaver-Burk plot. Reciprocal velocities (1/v) were plotted against 1/[βGP] at the following fixed concentrations of PPi: 0 mM (○), 0.5 mM (●), 1 mM (●), 2.5 mM (■), and 5 mM (▲). B, Dixon plot of 1/v against [PPi] at the following fixed concentrations of βGP: 0.1 mM (○), 0.25 mM (●), 0.5 mM (□), and 1 mM (▲). C, s/v against [PPi] at the following fixed concentration of βGP: 0.1 mM (○), 0.25 mM (●), 0.5 mM (□), and 1 mM (▲). D, summary of kinetic parameters obtained from data shown in A–C. K_m, K_s, and K_i are the dissociation constants.

The linear range of enzymatic activity as determined by our measuring of the enzyme kinetics (data not shown).

Kinetic Parameters of TNAP Inhibition by Pyrophosphate—To investigate the mode of TNAP inhibition by PPi, a Lineweaver-Burk plot of the initial velocity of βGP hydrolysis at various PPi concentrations was determined. Both the slope and the y-intercept increase with increasing inhibitor concentration, and thus the curves intersected to the left of the y axis and above the x axis indicate a mixed type of inhibition (Fig. 8A). The changes in both apparent K_m and V_max imply that PPi may bind to both free enzyme and to the enzyme-substrate complex.

To calculate the dissociation constant (K_i) of the PPi-TNAP complex, a Dixon plot of reciprocal velocities against PPi concentration was constructed (Fig. 8B), resulting in a K_i of 0.73 mM. The dissociation constant of the PPi-TNAP-βGP complex (K_i') was calculated from a plot of s/v against PPi concentration (Fig. 8C). The intersection of the curves results in a K_i' of 1.97 mM. Kinetic parameters obtained from the plots are summarized in Fig. 8D. In summary, a molecule of PPi may bind to either the TNAP active site (competitive inhibition) or to the TNAP-βGP complex at a secondary site that is only available after βGP binding (noncompetitive inhibition).

DISCUSSION

PPi, an anion consisting of two Pi molecules linked by an ester bond, is the simplest of the condensed phosphates. PPi inhibits hydroxyapatite crystal growth in cell-free systems in vitro (3, 7), and recent reports have documented PPi generation, transport, and activity in vivo, most notably for chondrocytes and the tissues of joints (10). Here, we have extended this understanding of the role of PPi in skeletal tissues, and more specifically its effects on biomineralization, by examining its influence on mineralizing MC3T3-E1 osteoblast cultures. We show that PPi directly affects osteoblast activity, and we present evidence that PPi inhibits mineralization of osteoblast cultures by at least three distinct mechanisms that include direct binding to mineral, up-regulation of Opn production, and inhibition of alkaline phosphatase activity.

Consistent with inhibition of crystal growth by PPi, in this study, we show that PPi inhibits mineralization of extracellular matrix in vitro produced by a well characterized osteoblast cell line, the MC3T3-E1 subclone M14 (45). We also show that this inhibition of mineralization by PPi is abrogated by the actions of tissue-nonspecific TNAP, which hydrolyzes PPi into its constituent Pi ions. The importance of PPi as a physiologic inhibitor of mineralization is evident from the skeletal and dental hypomineralization observed in patients with hypophosphatasia attributable to impaired TNAP activity. These patients, as well as Akp2<sup>-/-</sup> knock-out mice, demonstrate elevated plasma PPi levels in the absence of TNAP enzymatic activity (6, 13). Indeed, the bone and tooth hypomineralization of Akp2<sup>-/-</sup> mice is normalized in Akp2<sup>-/-</sup>;Enpp1<sup>-/-</sup> double-knock-out mice (36, 46), suggesting that homeostatic regulation of PPi levels by antagonistic Tnap enzyme activity modulates hydroxyapatite formation in these tissues. However, our results also indicate that there is rapid adsorption of PPi onto hydroxyapatite crystals and that once adsorbed, it is resistant to hydrolysis by TNAP (Fig. 1B). Furthermore, the dose of PPi required to inhibit βGP-induced mineralization is only 5 μM, and thus any hydrolysis would yield a negligible amount of Pi, relative to the 10 mM amount of βGP used here and commonly by others to provide a source of Pi for mineralization. The dose of PPi that we have used is in the physiologic range as reported for human plasma (3.5 μM average PPi) by Russell et al. (6).

Our results also demonstrate that PPi is a specific signal for the induction of Opn expression in osteoblasts. Previous studies have demonstrated that PPi deficiency in Enpp1<sup>-/-</sup> mice also results in Opn deficiency (11). In our study, we have identified the involvement of specific MAPK signaling pathways responsible for Opn regulation by PPi. Recently, others have shown that Pi itself can regulate osteoblast and cementoblast gene expression (31, 35, 38, 39). However, whereas Pi regulation of Opn is dependent on intracellular uptake via Na<sup>+</sup>/Pi co-transporters (39), most notably Pit-1 (47), we demonstrate here that PPi directly regulates Opn in a phosphate uptake-independent manner. Moreover, whereas previous studies by Beck et al. (39), have shown that Pi regulation of Opn is not dependent on the p38 MAPK pathway in osteoblasts (48), we report here that this pathway is activated by PPi to regulate Opn expression. It should be noted however, that although foscarnet is widely used as an inhibitor of Pi uptake, there are indeed other mechanisms for Pi transport that may be not be foscarnet-sensitive. Nevertheless, because the inhibitor levamisole prevents Tnap-mediated Pi hydrolysis to Pi, and this treatment had no effect on the induction of Opn, then these data, plus the failure of an equivalent dose of Pi to induce Opn, collectively indicate that Pi regulates Opn by a mechanism distinct from that of Pi.

The addition of exogenous PPi resulted in an increase in Opn mRNA, which led to a gradual cumulative increase in Opn protein levels over 4 days of treatment, with 4 days representing roughly one-third of the 12 days of culture typically used to assay for full MC3T3-E1 osteoblast differentiation and mineralization of the extracellular matrix. Indeed, induction of Tnap
activity during differentiation by the addition of ascorbic acid and $\beta$GP typically takes up to 6 days in control cultures. Compared with this 6-day induction time for Tnap, Opn induction at 4 days is consistent with this same time frame. Furthermore this might explain why a relatively high dose of PPi (0.5 mM) might be needed to induce Opn over 4 days compared with the low dose (5 $\mu$M) required to inhibit mineralization after 12 days. The 0.5 mM concentration of PPi, required to induce Opn is far less than the $P_i$ concentration required (4–10 mM) to have a similar induction (39).

The mechanism by which extracellular PPi leads to activation of intracellular signaling pathways remains unknown. Based on its size and charge, PPi, likely cannot passively cross the cell membrane. In prokaryotes, chloroplasts, and mitochondria, an ATP-ADP translocator has been reported to import PPi, (49–51), but there is no known importer or extracellular receptor for PPi in mammalian cells (10). The possibility that PPi acts through a plasma membrane receptor is not unreasonable. Other reported examples of small molecule signaling through osteoblast receptors include the ion-sensing Ca$^{2+}$-sensing receptor (52), the nucleotide P2-purinoreceptor family (53), and PPi analogues (bisphosphonates) acting to open connexin 43 hemi-channels (54), all of which in turn activate intracellular signaling pathways. Work is ongoing in our laboratory to clarify upstream effectors of the signaling pathways involved in PPi action as well as to identify transcription factors and genes regulated by PPi.

Based on the known inhibitory actions of Opn on hydroxyapatite crystal growth in cell-free systems (55–57), in cell cultures models (this study Fig. 5A, and Wada et al. (58)), and in vivo in mineralized tissues (59), the up-regulation of Opn by PPi, would likely act to further inhibit and thus regulate mineralization. Recent evidence for this in vivo is demonstrated by the elevated Opn levels in $Akp2^{-/-}$ mice that have elevated PPi, in the absence of Akp2 enzymatic activity and by the fact that in $Akp2^{-/-};Opn^{-/-}$ double-knock-out mice, there is partial rescue of the bone hypomineralization phenotype otherwise observed in the $Akp2^{-/-}$ knock-out alone (60). We also propose here, as an alternative (or simultaneous) activity for this elevated Opn when PPi, levels are high, that increased Opn might prevent calcium pyrophosphate crystal deposition in fluids and tissues as is commonly seen in joints in pseudogout (61). Studies are currently in progress to assess the ability of Opn to inhibit calcium pyrophosphate crystal growth and to stabilize mineral ions and putative amorphous precursor phases in solution.

Phosphorylation of Opn appears to be ordered in triplet clusters that favor its interaction with calcium in the hydroxyapatite crystal lattice (62, 63). Opn binds directly to hydroxyapatite crystal surfaces both in vivo (64) and in vitro (34), and dephosphorylation of Opn by Tnap prevents much of its mineral binding and crystal growth inhibiting activity (56, 65, 66) (Fig. 5A). Our high resolution, immunogold localization of Opn at crystal growth sites in the extracellular matrix of MC3T3-E1 osteoblast cultures is consistent with previous data demonstrating a similar, electron microscopic localization of Opn in primary rat osteoblast cultures (34). Dose-dependent inhibition of calcification in vitro by the addition of exogenous Opn has been shown previously for vascular smooth muscle cell cultures (58).

Our study in osteoblast cultures confirms this finding and further demonstrates that Tnap modulation of Opn phosphorylation levels is important for inhibition of mineralization. Using a gelatin gel crystal growth system, Gericke et al. (67) likewise demonstrated the importance of phosphorylation status of Opn in inhibiting hydroxyapatite formation, and similar phosphorylation influences were observed for inhibition of hydroxyapatite and also calcium oxalate crystal growth by peptides of Opn (55, 57).

Although differentially phosphorylated forms of Opn have been observed in rat bone cell cultures (68), the amount of Opn dephosphorylation resulting from Tnap activity in vivo remains unknown. Indirect in vivo data supporting a link between Tnap activity and Opn and its phosphorylation status is evidenced by the altered distribution of Opn in bone from $Akp2^{-/-}$ mice (69), although direct measurement of phosphorylation level of Opn in this case has not been determined.

Another finding in our study is that the inhibition of mineralization by PPi, that is abrogated by the actions of Tnap can be reduced when another Tnap substrate is present. In vitro, this second substrate is $\beta$GP, but in vivo, other substrates would be present including mineral-inhibiting phosphoproteins such as Opn. Mineralization of osteoblast cultures can be initiated in vitro with a variety of phosphate esters with $\beta$GP being the organic phosphate source most commonly used (26, 70–72). Although high doses of $P_i$ have also been demonstrated to inhibit Tnap activity (73), our kinetic experiments clearly demonstrate that PPi may function as a mixed type of inhibition system. It is possible that with substrate ($\beta$GP, Opn) already occupying the active site in Tnap, PPi, might bind to a second site on the enzyme, causing conformational change that inhibits its activity. This possibility has previously been suggested for the Tnap activity associated with matrix vesicle-mediated mineralization (72) and is also supported by recent data demonstrating that PPi induces conformational changes in Escherichia coli alkaline phosphatase leading to inhibition of its enzymatic activity (74). Our data showing that PPi acts as an inhibitor of TNAP when another substrate such as $\beta$GP (in vitro) or Opn (or other phosphoproteins, in vivo) is present also provides a hypothesis to explain why osteoblast Tnap does not inactivate the mineral-inhibiting potential of endogenous Opn in the extracellular matrix (Fig. 7). Recent evidence (75) that PPi, also inhibits PHEX activity (an enzyme whose inactivating mutations cause X-linked hypophosphatasia in humans) implicates multiple additional factors affecting the $P_i$/PPi balance regulating mineralization, and more work in this area is required to elucidate their respective contributions.

In summary, the in vitro data presented here for $P_i$, Tnap, and Opn, together with our recent in vivo data in transgenic mice overexpressing Tnap in collagen extracellular matrices (18), suggest the following partial scenario for induction and regulation of extracellular matrix mineralization. Mineralization initially involves a collagen matrix and enzymatic removal of $P_i$. The enzymatic hydrolysis of PPi, by Tnap also produces additional $P_i$ potentially available for hydroxyapatite mineral deposition. Once hydroxyapatite crystal growth is initiated by an as yet unknown mechanism, the enzymatically (Tnap) maintained $P_i$/PPi balance regulates further mineralization in part by

**Pyrophosphate Inhibition of Mineralization**
inhibiting crystal growth via multiple mechanisms that include direct binding to crystals, up-regulation of mineral-inhibiting Osn and inhibition of Tnap enzymatic activity. Thus, mineralization is not only inhibited by enzymatic inactivation by Tnap of these two molecular determinants of mineralization, one a protein and the other a small anion inhibitor, but is further enhanced by the fact that PPI also up-regulates Osn. Both inhibitors are found at high levels in bone, both bind to mineral via negatively charged phosphate residues, and both are inactivated by Tnap. Inhibition of Tnap by PPI, (in the presence of other Tnap substrates, e.g. Osn and potentially other matrix phosphoproteins) also maintains the phosphorylated form of Osn to further regulate mineral growth. Given that skeletal and dental mineralization occurs to at least some extent in transgenic mice deficient in these and other mineral-regulating proteins, it is likely that other molecular determinants also are important in regulating crystal nucleation and growth within the extracellular matrix.

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REFERENCES

1. McKee, M. D., and Sodek, J. (2000) in The Osteoporosis Primer (Henderson, J. E., and Golzmann, D., eds) pp. 46–63, Cambridge University Press, Cambridge, UK
2. Boskey, A. L., Young, M. F., Kilts, T., and Verdelis, K. (2005) Cells Tissues Organs 181, 144–153
3. Fleisch, H., Russell, R. G., and Straumann, F. (1966) Nature 212, 901–903
4. Kornberg, A. (1962) in Horizons in Biochemistry (Henderson, J. E., and Golzmann, D., eds) pp. 251–264, Academic Press Inc., New York
5. Fleisch, H., and Bisaz, S. (1962) Am. J. Physiol. 203, 461–475
6. Russell, R. G., Bisaz, S., Donath, A., Morgan, D. B., and Fleisch, H. (1971) J. Clin. Invest. 50, 970–980
7. Moreno, E. C., Aoba, T., and Margolis, H. C. (1987) Compend. (Suppl. 8) S256–S266
8. Meyer, J. L., and Nancollas, G. H. (1973) Calcif. Tissue Res. 13, 295–303
9. Tenenbaum, H. C., Torontali, M., and Sukhu, B. (1992) J. Clin. Investig. 90, 1093–1104
10. Terkeltaub, R. A. (2001) Am. J. Physiol. 281, C1–C11
11. Johnson, K. A., Hesse, L., Vaingankar, S., Wennberg, C., Mauro, S., Nakano, S., Godle, J. W., Sano, K., Millan, J. L., and Terkeltaub, R. (2000) Ann. J. Physiol. 279, R135–R177
12. Villiotte, P. J., Riefsnyder, P. C., Bloomstone, J. A., and Howland, J. L. (2005) J. Clin. Investig. 115, 249–255
13. Wang, D., Christensen, K., Chawla, K., Xiao, G., Krebsbach, P. H., and Hunter, G. K. (2004) J. Biol. Chem. 279, 95421–95429
14. Beck, J. G. R., Jr., Zerler, B., and Moran, E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8352–8357
15. Chen, C., Koh, A. J., Datta, N. S., Zhang, J., Keller, E. T., Xiao, G., Franceschi, R. T., D'Silva, N. J., and McCauley, L. K. (2004) J. Biol. Chem. 279, 29121–29129
16. Xiao, G., Gopalakrishnan, R., Jiang, D., Reith, E., Benson, M. D., and Franceschi, R. T. (2002) J. Bone Miner. Res. 17, 101–110
17. Xiao, G., Jiang, D., Thomas, P., Benson, M. D., Juan, K., Karsenty, G., and Franceschi, R. T. (2000) J. Biol. Chem. 275, 4453–4459
18. Ercot-Charrier, B., Glorieux, F. H., van der Rest, M., and Pereira, G. (1983) J. Cell Biol. 96, 639–643
19. Bellows, C. G., Aubin, J. E., Heersche, J. N., and Antosz, M. E. (1986) Calcif. Tissue Int. 38, 143–154
20. Wang, D., Christensen, K., Chawla, K., Xiao, G., Krebsbach, P. H., and Franceschi, R. T. (1999) J. Bone Miner. Res. 14, 893–903
21. Hesse, L., Johnson, K. A., Anderson, H. C., Narisawa, S., Sali, A., Godle, J. W., Terkeltaub, R., and Millan, J. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9445–9449
22. Li, X., Yang, H. Y., and Giachelli, C. M. (2006) Mol. Cell Biochem. 282, 101–108
23. Zhao, G., Gopalakrishnan, R., Jiang, D., Reith, E., Benson, M. D., and Franceschi, R. T. (2000) J. Bone Miner. Res. 17, 101–110
24. Hui, M., and Tenenbaum, H. C. (1998) Compend. 21, 15–29
25. Beck, J. G. R., Jr., Zerler, B., and Moran, E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8352–8357
26. Plotkin, L. I., Manolagas, S. C., and Bellido, T. (2002) J. Bone Miner. Res. 17, 249–255
27. Beck, G. R., Jr., Zerler, B., and Moran, E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8352–8357
28. Chen, C., Koh, A. J., Datta, N. S., Zhang, J., Keller, E. T., Xiao, G., Franceschi, R. T., D’Silva, N. J., and McCauley, L. K. (2004) J. Biol. Chem. 279, 29121–29129
29. Xiao, G., Gopalakrishnan, R., Jiang, D., Reith, E., Benson, M. D., and Franceschi, R. T. (2000) J. Biol. Chem. 275, 4453–4459
30. Villiotte, P. J., Riefsnyder, P. C., Bloomstone, J. A., and Howland, J. L. (1991) FEMS Microbiol. Lett. 62, 293–296
31. Mansurova, S. E. (1989) Biochim. Biophys. Acta 977, 237–247
32. Lunn, J. E., and Douce, R. (1993) Biochim. J. 290, 375–379
33. Mocuta, V. M., and Risjord, D. C. (2004) Cell Calcium 35, 249–255
34. Hofer, D., Bojanic, L. M., Ullrich, W., Doty, S. B., Butler, W. T., and Stress Analysis.
57. Hoyer, J. R., Asplin, J. R., and Otvos, L. (2001) *Kidney Int.* **60**, 77–82
58. Wada, T., McKee, M. D., Steitz, S., and Giachelli, C. M. (1999) *Circ. Res.* **84**, 166–178
59. Boskey, A. L., Spevak, L., Paschalis, E., Doty, S. B., and McKee, M. D. (2002) *Calcif. Tissue Int.* **71**, 145–154
60. Harmey, D., Johnson, K. A., Zelken, J., Camacho, N. P., Hoylaerts, M. F., Noda, M., Terkeltaub, R., and Millan, J. L. (2006) *J. Bone Miner. Res.* **9**, 1377–1386
61. Ryan, L. M., and Rosenthal, A. K. (2003) *Curr. Opin. Rheumatol.* **15**, 311–314
62. Sorensen, E. S., Hojrup, P., and Petersen, T. E. (1995) *Protein Sci.* **4**, 2040–2049
63. Christensen, B., Nielsen, M. S., Haselmann, K. F., Petersen, T. E., and Sorensen, E. S. (2005) *Biochem. J.* **390**, 285–292
64. McKee, M. D., and Nanci, A. (1995) *Ann. N. Y. Acad. Sci.* **760**, 177–189
65. Jono, S., Peinado, C., and Giachelli, C. M. (2000) *J. Biol. Chem.* **275**, 20197–20203
66. Hunter, G. K., Kyle, C. L., and Goldberg, H. A. (1994) *Biochem. J.* **300**, 723–728
67. Gericke, A., Qin, C., Spevak, L., Fujimoto, Y., Butler, W. T., Sorensen, E. S., and Boskey, A. L. (2005) *Calcif. Tissue Int.* **77**, 45–54
68. Kasugai, S., Zhang, Q., Overall, C. M., Wrana, J. L., Butler, W. T., and Sodek, J. (1991) *Bone Miner.* **13**, 235–250
69. Tesch, W., Vandenbos, T., Roschgr, P., Fratzl-Zelman, N., Klaushofer, K., Beertsen, W., and Fratzl, P. (2003) *J. Bone Miner. Res.* **18**, 117–125
70. Chung, C. H., Golub, E. E., Forbes, E., Tokuoka, T., and Shapiro, I. M. (1992) *Calcif. Tissue Int.* **51**, 305–311
71. Kawazoe, Y., Shiba, T., Nakamura, R., Mizuno, A., Tsutsumi, K., Uematsu, T., Yamaoka, M., Shindoh, M., and Kohgo, T. (2004) *J Dent Res.* **83**, 613–618
72. Garimella, R., Bi, X., Anderson, H. C., and Camacho, N. P. (2006) *Bone* **38**, 811–817
73. Coburn, S. P., Mahuren, J. D., Jain, M., Zabovic, Y., and Wortsman, J. (1998) *J. Clin. Endocrinol. Metab.* **83**, 3951–3957
74. Kananovich, S. Z., and Mazhul, V. M. (2003) *J. Appl. Spectrosc.* **70**, 765
75. Boileau, G., Tenenhouse, H. S., Desgroseillers, L., and Crine, P. (2001) *Biochem. J.* **355**, 707–713