Osteoblast tissue-nonspecific alkaline phosphatase antagonizes and regulates PC-1

K. A. JOHNSON,1 L. HESSLE,2,3 S. VAINGANKAR,1 C. WENNBERG,2,3 S. MAURO,2 S. NARISAWA,2 J. W. GODING,4 K. SANO,5 J. L. MILLAN,2,3 AND R. TERKELTAUB1
1Veterans Affairs Medical Center/University of California San Diego, La Jolla 92161; 2Burnham Institute, La Jolla, California 92037; 4Monash Medical School, Prahran, Australia 3181; 3Medical Biosciences, Medical Genetics, Umea University, Umea S-90185, Sweden; and 5Kobe University, Kobe 650-0017, Japan
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Osteoblast tissue-nonspecific alkaline phosphatase antagonizes and regulates PC-1. Am J Physiol Regulatory Integrative Comp Physiol 279: R1365–R1377, 2000.—Tissue-nonspecific alkaline phosphatase (TNAP) is essential for bone matrix mineralization, but the central mechanism for TNAP action remains undefined. We observed that ATP-dependent 45Ca precipitation was decreased in calvarial osteoblast matrix vesicle (MV) fractions from TNAP−/−mice, a model of infantile hypophosphatasia. Because TNAP hydrolyzes the mineralization inhibitor inorganic pyrophosphate (PPi), we assessed phosphodiesterase nucleotide pyrophosphatase and phosphatase (PDNP3) activity, which hydrolyzes ATP to generate PPi. Plasma cell membrane glycoprotein-1 (PC-1), but not the isozyme B10 (also called PDNP3) colocalized with TNAP in osteoblast MV fractions and pericellular matrix. PC-1 but not B10 increased MV fraction PPi and inhibited 46Ca precipitation by MVs. TNAP directly antagonized inhibition by PC-1 of MV-mediated 45Ca precipitation. Furthermore, the PPi content of MV fractions was greater in cultured TNAP−/−Than TNAP+/+ calvarial osteoblasts. Paradoxically, transfection with wild-type TNAP significantly increased osteoblast MV fraction NTTPPPH. Specific activity of NTTPPPH was also twofold greater in MV fractions of osteoblasts from TNAP+/+ mice relative to TNAP−/−mice. Thus TNAP attenuates PC-1/NTTPPPH-induced PPi generation that would otherwise inhibit MV-mediated mineralization. TNAP also paradoxically regulates PC-1 expression and NTTPPPH activity in osteoblasts.

inorganic pyrophosphate; hypophosphatasia; PDNP3; B10; transglutaminase

OSTEOBLASTS INITIATE MINERALIZATION of the pericellular matrix by promoting formation of hydroxyapatite crystals in the sheltered interior of extracellular membrane-limited vesicles (3, 4, 9, 10, 23). These structures, called matrix vesicles (MVs), are shed from specialized areas of the osteoblast plasma membrane (2, 3). MVs contain factors that regulate the composition of the extracellular matrix (3, 9, 10, 23). By an unknown mechanism, MVs are markedly enriched in tissue-nonspecific alkaline phosphatase (TNAP) relative to both whole cells and the plasma membrane (3).

Physiological bone matrix mineralization is hypothesized to be dependent on the availability of Pi released from a variety of substrates by certain MV ectoenzymes (3, 23). For example, ATP is hypothesized to drive the initiation of calcification by MVs in vivo, and a specific bone and cartilage ATPase appears to be responsible for the ATP-dependent calcium and Pi-depositing activity of bone and cartilage-derived MVs in vitro (23).

TNAP is the only tissue-nonrestricted isozyme of a family of four homologous human alkaline phosphatase (AP) genes (EC. 3.1.3.1) (31). Expressed as an ectoenzyme transported to the osteoblast plasma membrane and anchored via a phosphatidylidyinositol glyco moiety, TNAP has been demonstrated to play an essential physiological role during osteoblastic bone matrix mineralization (31, 44). Specifically, defective bone mineralization (osteomalacia) occurs in TNAP deficiency (hypophosphatasia) (44). The severity of hypophosphatasia is variable and modulated by the nature of the TNAP mutation (18, 21, 31, 40, 44, 46).

Skeletal TNAP can catalyze Pi release from ATP (23), and TNAP catalyzes several transphosphorylation reactions (44). Although TNAP does not appear to dephosphorylate membrane proteins (17), TNAP has been hypothesized to modulate bridging of MVs to matrix collagen (44). TNAP has been demonstrated to bind calcium (14). Moreover, TNAP degrades at least three phosphocompounds [phosphoethanolamine, pyridoxal 5’-phosphate, and inorganic pyrophosphate (PPi)] that accumulate endogenously in hypophosphatasia (45).

The central function(s) of TNAP in conditioning mineralization has not been completely defined (31, 44). Importantly, aberrant localization of TNAP can occur, including defective transport of TNAP to the plasma membrane associated with hypophosphatasia (16, 18). This has suggested that TNAP might act at the level of

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plasma membrane-derived structures such as MVs. In subjects with perinatal hypophosphatasia, the most severe form of the disease, MVs were present in approximately normal numbers and distribution, and these MVs contained internal hydroxyapatite crystals (4). However, propagation of hydroxyapatite crystals outside of isolated MVs was impaired in perinatal hypophosphatasia, but by an undefined mechanism (4).

The ability of TNAP to hydrolyze PPi to P_i (45) has been hypothesized to be central to the ability of TNAP to promote osteoblastic mineralization (4, 44). A major action of P_i is to suppress both the deposition and propagation of hydroxyapatite crystals in vitro (27, 30). Thus critically timed removal or exclusion of P_i at sites of mineralization appears to be necessary for active crystal deposition to proceed (27, 30).

P_i is generated by multiple biochemical reactions (38), including hydrolysis of the phosphodiester I bond in purine and pyrimidine nucleoside triphosphates (nucleoside triphosphate pyrophosphohydrolase activity) by ectoenzymes of the phosphodiesterase I nucleotide pyrophosphatase (PDNP/NTPPPP) family (EC 3.6.1.8, EC 3.1.4.1) (19). Significantly, cultured fibroblasts from subjects with infantile hypophosphatasia retain an apparently normal capacity to generate extracellular P_i through NTPPPP activity (13). It also should be noted that osteoblasts have particularly high NTPPPP-specific activity (12, 26, 33, 34).

Plasma cell membrane glycoprotein-1 (PC-1) is a NTPPPP isozyme expressed by cultured osteoblastic cells (19, 26, 41). Furthermore, similar to the case for TNAP and other ectoenzymes, PC-1 expression and the extent of PC-1 distribution to MVs are regulated by certain growth factors and calcitropic hormones, including transforming growth factor-β, basic fibroblast growth factor, and 1,25-dihydroxyvitamin D_3 (7, 26, 33, 34, 41). Osteoblast-derived MV PC-1 appears to function directly to increase MV fraction P_i content and to restrain mineralization by isolated MVs in vitro (26). In this regard, a two- to fourfold increase in osteoblast PC-1 expression decreases, by >80%, the amount of hydroxyapatite deposited in the pericellular matrix of osteoblasts in vitro (26). Moreover, the effects of PC-1 on mineralization have been demonstrated to be physiologically significant in ttw/ttw mice, which are homozygous for a naturally occurring PC-1 truncation mutation and develop osteoblast-mediated and chondrocyte-mediated hyperostosis and articular cartilage calcification in early life (32).

PC-1 does not account for all the NTPPPP activity of osteoblastic cells (41). Moreover, several tissues that actively synthesize a collagenous extracellular matrix, including articular chondrocytes and hepatocytes, express not only PC-1 but also the closely related isozyme B10 (also called PDNP3) (19, 27).

In this study, we investigated and compared the potential interactions, in osteoblastic cells and MV fractions derived from these cells, among TNAP, PC-1, and B10/PDNP3 (principally referred to as B10 in the text). To do so, we employed the clonal immortalized murine calvarial osteoblast line MC3T3-E1 (abbreviated here as MC3T3) (26) and also studied primary calvarial osteoblasts from TNAP-knockout (ko or —/—) mice (31), which serve as a model of infantile hypophosphatasia.

**MATERIALS AND METHODS**

**Reagents**

Unless otherwise indicated, all chemical reagents were from Sigma-Aldrich (St. Louis, MO).

**Mouse Genotyping and Isolation and Culture of Primary Calvarial Osteoblasts**

To indirectly determine the TNAP genotype of pups from our established breeding colony, we measured AP activity of mouse sera by colorimetric assay by using p-nitrophenyl phosphate (pNPP) as substrate (31). Southern blot of tail tissue (31) was later used to confirm the genotype of each mouse.

Primary cultures of osteoblasts were isolated from calvariae of 1- to 4-day-old pups that were hybrids of C57Bl/6 × 129/SvJ mouse strains with wild-type, heterozygote, and homozygote TNAP-null genotypes. Isolation was performed by a slightly modified version of the time sequential collagenase technique described by Boonekamp et al. (8). In brief, calvariae of the same genotype were pooled and three 10-min incubations in 4 mM EDTA, 137 mM NaCl, 2.7 mM KCl, 3 mM Na_2HPO_4, pH 7.2, were performed, followed by seven 10-min digestions in EDTA-free buffer, containing 180 U/ml collagenase type II (Worthington Biochemical, Lakewood, NJ). All isolations were performed at 37°C in a shaking water bath. The last five collagenase digestions, containing an enriched cell population of osteoblastic phenotype, were pooled and seeded at 4 × 10^6 cells/cm^2 in MEM collagenase type II (Worthington Biochemical, Lakewood, NJ). All isolations were performed at 37°C in a shaking water bath. The last five collagenase digestions, containing an enriched cell population of osteoblastic phenotype, were pooled and seeded at 4 × 10^6 cells/cm^2 in MEM-α (GIBCO-BRL, Grand Island, NY), containing 10% heat-inactivated FCS, penicillin (50 U/ml) and streptomycin (0.5 mg/ml).

**Culture of Osteoblastic Cells Under Mineralizing Conditions**

To study mineralization, we used conditions under which we observed that osteoblasts normally formed von Kossa stain-positive and alizarin red-positive nodules at 7–10 days in culture. In brief, primary calvarial osteoblasts were cultured in complete MEM-α media as described above, supplemented with β-glycerophosphate (10 mM) every third day and l-ascorbic acid (50 μg/ml) daily. MC3T3 cells (26) were maintained in the same medium and for each experiment were plated at 80% confluence (1 × 10^6 cells in a 10-cm tissue-culture plastic dish in 5 ml) and received β-glycerophosphate and l-ascorbic acid as for primary osteoblasts above.

**Immunofluorescence/Confocal Microscopy**

For immunofluorescent staining/confocal microscopy to localize NTPPPP isozymes and AP, MC3T3 cells were cultured for up to 7 days as described above. Cells were initially seeded on 18-mm^2 coverslips coated with poly d-lysine at a density of 3 × 10^5 cells/coverslip. At the indicated time points, cells were rinsed with PBS, fixed in 4% paraformaldehyde in PBS for 30 min at 22°C, and then washed three times. Where indicated, cells were permeabilized with 0.1% Triton X-100 in blocking buffer for 10 min, and cells were again treated with blocking buffer for 45 min.

To detect AP activity, fixed cells were stained with a 1:1 solution of 0.2 mg/ml naphthol AS-MX and 1.2 mg/ml fast red
TR salt dissolved in 0.2 M Tris-HCl for 30 min in the dark at 25°C. This was followed by blocking (in PBS with 2% goat serum and 0.02% thimerosal) for 1 h. Where indicated, cells were permeablized by using 0.1% Triton X-100 in the blocking buffer for 15 min, followed by continued incubation in blocking buffer for another 45 min.

To detect PC-1 and B10, the murine anti-PC-1 alloantibody IR518 (42), or a rabbit antibody to the COOH terminus (amino acids 580–875) of rat B10 (5), was diluted in blocking buffer and added to the cells for 18 h at 4°C, washed three times in PBS, and incubated with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) or goat anti-rabbit FITC (Sigma) at 1:400 in blocking buffer for 1 h at 22°C. Coverslips were mounted with SlowFade media (Molecular Probes, Eugene, OR) and examined with a Leica DMR epifluorescence microscope equipped with epifluorescence channels to detect AP staining.

**Western Blotting, Densitometry, and Immunoprecipitation**

For Western blotting, all samples were treated with lysis buffer (1% Triton X-100 in 0.2 M Tris base with 1.6 mM MgCl₂, pH 8.1), and protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Protein (0.03 mg) from each sample was separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose.

NTPPPH-specific, polyclonal antibodies to PC-1 (R1769) and B10 (26, 27) and rabbit antibody to tubulin (Sigma) served as primary antibodies in Western blotting, performed as described (26, 27). Washed nitrocellulose membranes were incubated with horseradish peroxidase-conjugated secondary antibody in blocking buffer for 1 h and washed again, and immunoreactive products were detected by using the enhanced chemiluminesence system (Amershams, Arlington Heights, IL). Where indicated, semiquantitative analyses of Western blots were performed, using a previously described densitometry protocol (26).

To specifically immunoprecipitate TNAp from osteoblast cell lysates, we used a previously described method (41). We used a rabbit polyclonal antibody against rat TNAp that cross-reacts with mouse TNAp (22) (a gift from Prof. Yuki Ikehara, Fukuoka, Japan).

**Transfections of PC-1, B10 and TNAp**

For transfection studies, the cDNA expression constructs for wild-type human PC-1 and human B10 in pcDNA3.1 were as previously described (27). A 2.4-kb Hind III/Bgl II fragment from the human TNAp cDNA (ATCC no. 59635) was used as the template to generate the enzyme-deficient mutant by site-directed mutagenesis, as described by Tomic et al. (43), using the primer 5’-CCAGGGTTGTGGAGCTGAC-CCTTGAGGATGCAGGCAGCCGTC 3’ to introduce the R54C substitution. The wild-type and mutant TNAp cDNAs were subcloned into pcDNA 3.1 expression vector (Hind III/ Spe I) downstream from SV40 early promoter. Transient transfections were performed by using 5 μg of plasmid DNA and Lipofectamine Plus (Life Technologies, Gaithersburg, MD), as previously described, and this reproducibly achieved 40–45% transfection efficiency in osteoblastic cells in this study (27).

**MV Fraction Isolation and ⁴⁵Ca Precipitation Assay: Electron Microscopy of MV Fractions**

For MV mineralization assays, conditioned media from cultured cells were collected at the time points indicated and initially centrifuged at 20,000 g for 20 min at 4°C to pellet cellular debris, as previously described (26). This was followed by centrifugation at 100,000 g for 1 h to isolate the MV fraction, which was resuspended in Hanks’ balanced salt solution.

MV fractions (0.04 mg protein in 0.025 ml) were added in triplicate to 0.5 ml of “calcifying medium” [(in mM) 2.2CaCl₂ (1 μCi/ml ⁴⁵Ca), 1.6 KH₂PO₄, 1 MgCl₂, 85 NaCl, 15 KCl, 10 NaHCO₃, 50 mM N Tris, and, where indicated, 1 ATP disodium salt, and/or 0.1% Triton X-100, pH 7.6] and vortexed and incubated at 37°C for 24 h (26). Samples were then centrifuged at 14,000 g for 10 min at 4°C. The pellet was washed twice with cold calcifying medium without ATP. The ⁴⁵Ca in the mineral phase was solubilized in HCl and counted in 5 ml scintillation fluid.

The MV fraction of MC3T3 cells at 10 days in culture was assessed by conventional EM, using previously described methods (11). For specimen processing, we carried out double fixation (using 2% glutaraldehyde in cacodylate buffer, followed by 1% OsO₄ in H₂O), which was followed by dehydration in 2% uranyl acetate in 70% ethanol, and then in 100% ethanol, which was then followed by infiltration using acetone/nitrite/Epon (11).

**RT-PCR Analysis**

For RT-PCR, total RNA was isolated by using 0.5 ml TriZol (Life Technologies, Gaithersburg, MD)/60-mm plate, with RNA extracted in chloroform and then precipitated in isopropanol overnight at −20°C. Six hundred nanograms of total RNA was reverse transcribed by using 5 mM MgCl₂, 1× PCR buffer [200 mM Tris·HCl (pH 8.4), 500 mM KCl], 1.25 mM of each dNTP, 2.5 μM Oligo d(T) primer, 0.25 U/ml RNase Inhibitor (Sigma), 2.5 U/ml MuLV RT in a volume of 20 μl at 42°C for 40 min, 99°C for 5 min, and 4°C for 5 min. One tenth of this reaction was used for a single round of RT-PCR, as previously described in detail (26, 27). Rodent B10 primers were sense 5’-TTAGCCACGGAGGACCCATTAAAG 3’ and antisense 5’-AGCCTTTGTAATCGTGACGACGAGCTC 3’ (5), which amplified a 378-bp product at the 5’ end of mouse B10 cDNA that hybridized to rat B10 cDNA in Southern blotting. Primers for mouse PC-1 and the ribosomal housekeeping gene L30 were as previously described and characterized (26, 41).

**PP₆, Enzyme, and Cellular DNA Assays**

PP₆ was determined by differential adsorption on activated charcoal of UDP-β-[6-³H]glucose (Amershams) from the reaction product 6-phospho-[6-³H]glucosamine (Amershams) from the reaction product 6-phospho-[6-³H]glucosamine, as previously described (27). PP₆ was equalized for the DNA concentration in each well, determined chromogenically after precipitation in perchlorate (27). We determined specific activity of NTTPPPH and AP by previously described assays (27). Units of NTTPPPH and AP were designated as micromoles of substrate hydrolyzed per hour (μmol protein in each sample).

To measure transglutaminase activity, we added 30 μg of protein in 0.08 ml of 5 mM Tris·HCl (pH 7.5), 0.25 mM sucrose, 0.2 mM MgSO₄, 2 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, and 0.4% Triton X-100 to an equal volume of 300 mM Tris·HCl, pH 7.5, 10 mM CaCl₂, 20 mM DTT, 10 mM/g N,N’-dimethylcasein and 10 μCi/ml [³H]putrescine (15) for 30 min at 37°C. The reaction was stopped by adding cold 10% TCA and 0.5% tannic acid. The precipitate was washed with 10% TCA and 0.25% tannic acid. The pellet was resuspended in 0.1 ml 1 M NaOH. Samples were then treated with 2% H₂O₂ for 4 h at 22°C, followed by 4 h of treatment at 40°C, and then counted for 5 min in 5 ml scintillation fluid.
Transglutaminase activity was designated as nanomoles of putrescine incorporated into casein (per mg protein in each sample).

**Statistical Analysis**

Where indicated, error bars represent SD. Statistical analysis was performed by using the Student's $t$-test (paired 2-sample testing for means), applied on Microsoft Excel 5.0 for the Macintosh computer.

**RESULTS**

**Impaired ATP-Dependent Calcium Precipitation by Detergent-Treated Osteoblast-Derived MV Fractions From TNAP-Deficient Mice**

Extravesicular ATP-dependent extension of mineral deposition is impaired in MVs from patients with perinatal hypophosphatasia (4). We prepared MV fractions from osteoblastic cells as described in MATERIALS AND METHODS and visualized MVs in such fractions that had characteristic morphology and size and the capacity to develop electron-dense internal deposits consistent with crystals (Fig. 1). We first assessed the ability of murine heterozygous and homozygous TNAP-deficient osteoblast MV fractions to precipitate calcium. In doing so, aliquots of the MV fractions were treated, where indicated, with the detergent Triton X-100 (0.1%), which is capable of enhancing ATP-initiated MV mineralization via MV membrane perturbation (under conditions where such treatment does not modulate MV enzyme activities) (24).

In the absence of detergent treatment, TNAP-deficient osteoblasts and control osteoblasts released MV
fractions that were comparably able to precipitate calcium in an ATP-dependent manner (Fig. 2). However, treatment of the MV fractions with Triton X-100 unmasked a significant defect in the ATP-dependent calcium-precipitating ability of TNAP−/− osteoblast-derived MV fractions relative to TNAP+/+ MV fractions (Fig. 2). The defect directly correlated with the extent of TNAP deficiency, because it was greater in MV fractions derived from TNAP−/− mice than from TNAP+/− mice (Fig. 2). The defect was exposed by using ATP as a substrate and was active at the level of the MV fractions. Because NTPPPH activity hydrolyzes ATP and is associated with MVs, we assessed NTPPPH expression in osteoblasts and osteoblast-derived MV fractions.

**Differential Localization of PC-1 Relative to B10 in Osteoblasts and Osteoblast-Derived MV Fractions**

Both PC-1 and B10 mRNA expression were consistently detected by RT-PCR in cultured primary calvarial osteoblasts isolated from TNAP+/+ mice and from TNAP +/+ and TNAP−/− mice (Fig. 3). In addition, PC-1 and B10 mRNA expression were both detected in MC3T3 cells, which were grown under the same mineralizing conditions used for primary calvarial osteoblasts (Fig. 3).

PC-1 protein expression also was readily detected in cell lysates and in the MV fractions of primary calvarial osteoblasts of TNAP+/+ mice, and of TNAP+/− and TNAP−/− mice (Fig. 4A). However, PC-1 was more readily detected in equal amounts of protein from MV fractions of the osteoblasts of the osteoblasts of TNAP+/+ mice than TNAP−/− mice (Fig. 4A). PC-1 and B10 both were detected in cell lysates of primary calvarial osteoblasts and MC3T3 cells (Fig. 4). In contrast, in MV fractions from calvarial osteoblasts of all mice tested, B10 was below the limits of detection in Western blotting (Fig. 4A), and B10 could not be detected in MV fractions of MC3T3 cells (Fig. 4B). Semiquantitative densitometric analyses of these Western blot findings suggested a greater than eightfold increase in MV fraction PC-1 in MV fractions from TNAP+/+ compared with TNAP−/− mice, and levels of cell lysate PC-1 were more than 50% greater in TNAP+/− cells than in TNAP−/− cells, under conditions where tubulin was not greater in cells of TNAP+/+ mice compared with TNAP−/− mice (not shown).

Because we detected PC-1 but not B10 expression in MV fractions of primary calvarial osteoblasts and MC3T3 cells, we assessed whether direct upregulation of B10 expression would be associated with localization of B10 in MV fractions. To do so, we transfected MC3T3 cells with human PC-1 and B10 cDNAs, which are more than 80% identical to their respective rodent homologs (16). We cultured the MC3T3 cells under mineralizing conditions as used for primary calvarial osteoblasts.

![Fig. 3. Plasma cell membrane glycoprotein-1 (PC-1) and B10 mRNA are expressed in MC3T3 cells and primary calvarial osteoblasts in culture. Total RNA isolation and RT-PCR analysis were performed to determine whether PC-1 and B10 mRNA were expressed in cultured MC3T3 cells and primary calvarial osteoblasts from TNAP wild-type, heterozygotic, and homozygous knockout mice (designated as in Fig. 2). Cells were cultured for the number of days indicated and as described in MATERIALS AND METHODS. Assay of the ribosomal "housekeeping gene" L30 served as the control for RNA loading.](http://ajpregu.physiology.org/doi/abs/10.1152/ajpregu.00802.2017)

![Fig. 4. Preferential localization of PC-1 relative to B10 in MV fractions of primary calvarial osteoblasts (A) and MC3T3 cells (B). A: primary calvarial osteoblasts from TNAP +/+ , +/− and −/− mice were cultured, and MV fractions isolated as described above. Then, 30 µg of protein from cell lysates (at days 6 and 10 in culture) and MV fractions (at day 10) were analyzed by SDS-PAGE and Western blotting as described in MATERIALS AND METHODS. Detection of immunoreactive products, performed as described in MATERIALS AND METHODS, is illustrated for PC-1 and B10 (each ~130 kDa) and for tubulin (~70 kDa) as the housekeeping gene control. B: MC3T3 cells were cultured with or without transfection with empty plasmid, or PC-1 or B10 in pcDNA3.1, as indicated, using the procedure described in MATERIALS AND METHODS. Cell lysates and MV fractions (30 µg of protein) were studied by Western blotting after isolation at day 7 in culture (all lanes indicated were taken from the same gel).](http://ajpregu.physiology.org/doi/abs/10.1152/ajpregu.00802.2017)
Transfection of PC-1 increased immunoreactive PC-1 in MC3T3 cells (Fig. 4B) and elevated both cell-associated and MV fraction NTPPPH activity in MC3T3 cells (Fig. 5). PC-1 also significantly elevated MV fraction PPi, but not extracellular PPi, suggesting enrichment of PC-1 in the MV fractions (Fig. 5C). In contrast, transfection of B10 elevated cell-associated NTPPPH activity but did not augment MV fraction NTPPPH activity in MC3T3 cells (Fig. 5A). B10 transfection also did not elevate MV fraction PPi (Fig. 5C). Transfected MC3T3 cells demonstrated an increase in PC-1 or B10 protein (Fig. 4B), but only PC-1 was detectable by Western blotting in MV fractions derived from the cells transfected with either NTPPPH (Fig. 4B).

Because PC-1 but not B10 appeared to localize in isolated osteoblast-derived MV fractions, we assessed and compared the distribution of PC-1 and B10, relative to TNAP, in mineralizing MC3T3 cells, using confocal microscopy (Fig. 6). Before matrix mineralization, at 48 h in culture, TNAP staining was readily detected on the surface of the subconfluent MC3T3 cells. Surface PC-1 and B10 immunostaining were weak at this point in time (Fig. 6A). Permeabilization of MC3T3 cells revealed that B10 staining was predominantly intracellular at 48 h (Fig. 6A).

At 7 days in culture, when mineralization of the matrix by confluent MC3T3 cells was detectable, PC-1 and TNAP staining colocalized in broad areas of the pericellular matrix (Fig. 6B). Osteoblast surface and pericellular matrix B10 staining remained weak at this time (Fig. 6C). Moreover, permeabilization of the MC3T3 cells revealed that B10 staining remained predominantly intracellular in MC3T3 cells in a distribution distinct from that shared by PC-1 and TNAP (Fig. 6D).

**Functional Relationship Between TNAP and PC-1 in MV Fractions**

Our results revealed that TNAP and PC-1 were co-expressed and colocalized in mineralizing osteoblastic cells and their MV fractions and pericellular matrix. Thus we concluded this study by directly assessing the relationship between TNAP and PC-1 functions in MV fractions. To do so, we first transfected MC3T3 cells...
Fig. 6. Confocal microscopy: PC-1 but not B10 colocalizes with TNAP on the surface and in the pericellular matrix of mineralizing MC3T3 cells. MC3T3 cells were cultured with ascorbate and β-glycerophosphate as described above and stained for AP activity by using naphthol AS-MX and fast red TR salt and immune stained for PC-1 and B10 as described in MATERIALS AND METHODS. Where indicated, cells were permeabilized with 0.1% Triton X-100, as described in MATERIALS AND METHODS, in blocking buffer for 10 min, and then again treated with blocking buffer for 45 min. Cells were studied by using a Zeiss Axiovert 100M laser scanning microscope using the FITC channel to detect PC-1 or B10 staining, and the Texas red channel to detect AP staining. Results are shown before onset of mineralization (at 48 h in culture) (A), and during mineralization by confluent cells (at 7 days in culture) (B-D). In D, all cells were permeabilized. Colocalization of PC-1 or B10 with TNAP was detected by yellow or orange staining. LM, light microscopy control.
with cDNAs encoding wild-type TNAP and the R54C catalytically inactive mutant of TNAP.

Transfection of wild-type TNAP significantly elevated cell-associated and MV fraction-associated AP activity in MC3T3 cells and decreased the PPi associated with MV fractions derived from MC3T3 cells transfected with PC-1 (Fig. 5, B and C). Paradoxically, transfection of wild-type TNAP was associated with a significant increase in cell-associated NTPPPH, and an even greater increase in MV fraction-associated NTPPPH activity in MC3T3 cells (Fig. 5A) but no significant change in activity of transglutaminase activity, which served as a control enzyme for these experiments. We established that this result was not attributable to intrinsic NTPPPH activity of TNAP. Specifically, immunoprecipitation of TNAP from osteoblastic cell lysates isolated >85% of the TNAP activity but was not associated with any communoprecipitation of NTPPPH activity (starting mean cell lysate NTPPPH of 1.7 U and AP of 4.3 U; beads after immunoprecipitation with antibody to TNAP had mean of 0.09 U NTPPPH and 4.1 U alkaline phosphatase; beads after immunoprecipitation with antibody to PC-1 had mean of 1.3 U NTPPPH and 0.11 U AP, n = 5).

Because TNAP appeared to regulate the NTPPPH activity of osteoblastic MC3T3 cells, we assessed whether this effect was dependent on TNAP enzymatic activity. Wild-type TNAP, but not the enzyme-inactive mutant of TNAP, induced an increase in both MV fraction NTPPPH and AP activity (Fig. 7, A and B). In contrast to wild-type TNAP, the mutant TNAP failed to decrease MV fraction PPi (Fig. 7C).

The results presented in Fig. 4A had indicated that immunoreactive PC-1 decreased progressively over time in culture in TNAP−/− osteoblasts relative to primary osteoblasts from TNAP+/− and TNAP+/+ mice. Thus we also assessed the relationship among TNAP deficiency and MV fraction PPi-generating NTPPPH activity and PPi concentration in primary osteoblasts. Cell-associated NTPPPH but not transglutaminase activity progressively decreased over time in culture in osteoblasts from TNAP−/− mice, relative to cells from TNAP+/− and TNAP+/+ mice, and was significantly less in TNAP−/− cells than in TNAP+/+ cells at 14 days (Fig. 8). Moreover, NTPPPH activity but not transglutaminase activity was significantly lower in MV fractions derived from days 10–13 from TNAP−/− mice compared with MV fractions from osteoblasts of TNAP+/− and TNAP+/+ mice, with the values for MV fraction NTPPPH activity being intermediate for TNAP+/− osteoblasts (Fig. 9). Despite the presence of the lowest MV fraction NTPPPH specific activity, it was in the TNAP−/− state that the highest MV fraction-associated concentration of the mineralization inhibitor PPi was observed (Fig. 9).

Last, we directly assessed the effects of TNAP, PC-1, and B10 on MV fraction-mediated mineralization (Fig. 10). Transfection of PC-1, but not B10, significantly inhibited ATP-dependent calcium precipitation by MV fractions (detergent-treated and untreated) isolated from the transfected MC3T3 cells (Fig. 10). Wild-type but not mutant TNAP significantly antagonized the inhibitory effect of PC-1 on calcium precipitation by MV fractions derived from the MC3T3 cells (Fig. 10).
Thus TNAP functioned to directly antagonize the inhibitory effect of PC-1 on the mineralizing activity of osteoblast-derived MV fractions.

DISCUSSION

A definition of the function of TNAP is pivotal to understanding how the skeleton mineralizes. We previously demonstrated that PC-1 suppressed osteoblast MV-mediated mineralization under conditions where sodium phosphate was provided as the major phosphate source for hydroxyapatite formation (26). The present study employed β-glycerophosphate, which, to provide free phosphate, requires hydrolysis by inorganic phosphatase activity such as that exerted by TNAP. PC-1 markedly inhibited ATP-dependent calcium precipitation by MV fractions under these conditions in this study. We also demonstrated that TNAP and PC-1 were concomitantly expressed and TNAP and PC-1 colocalized in both cultured osteoblasts that formed mineralized nodules and the MV fractions derived from the mineralizing cells.

TNAP functioned to directly antagonize the inhibitory effects of PC-1 on ATP-dependent precipitation of calcium by MV fractions. In this regard, a forced increase in TNAP localization in MV fractions diminished basal MV fraction-associated PPi and prevented PC-1 from augmenting MV fraction PPi. Our results suggest that TNAP promotes MV-mediated mineralization, in large part, by hydrolyzing the MV-associated mineralization inhibitor PPi generated by PC-1 through catabolism of exogenous ATP.

A selective impairment of extravesicular propagation of hydroxyapatite by ATP-treated MVs has been demonstrated in subjects with perinatal hypophosphatasia (4). This suggested the possibility of defective metabolism of PPi at the exterior face of MVs. In previous studies the majority of both NTPPPH activity (38) and TNAP activity (3, 25) have been found to be active on the external face of MVs. We did not directly assess surfaces where the enzyme activities were localized in the MVs obtained in the present study. The observation that detergent-induced perturbation of MV fractions was required to reveal a defect in ATP-dependent MV calcium precipitation associated with TNAP deficiency could be consistent with external-face orientation of TNAP enzyme activity and/or release of a mineralization inhibitor such as PPi from MVs.

Significantly, a mineralizing defect correlated directly with the extent of TNAP deficiency measured in osteoblast-derived MV fractions. In contrast, the total concentration of MV fraction-associated PPi was less clearly related to the mineralizing defect observed in TNAP+/− and TNAP−/− osteoblasts. The finding that MV fractions from TNAP+/− and TNAP−/− osteoblasts both demonstrated a mineralizing defect may help explain in vitro differences in mineralization between osteoblasts from TNAP+/− and TNAP+/+ mice (43a). More specifically, when TNAP+/− and TNAP+/+ mice at day 2–3 after birth are compared, no morphological differences could be detected in the bones in vivo. However, initiation of mineralization in vitro was delayed in the TNAP+/− osteoblast cultures. In this regard, in vitro cultures evaluated at different time points (days 4, 6, 8) revealed a delayed increase in TNAP activity in the TNAP+/− cultures compared with TNAP+/+ cultures, accompanied by a later onset of mineralization, as demonstrated by von Kossa staining and measurement of deposited calcium (43a).

We speculate that the foci in which PPi might be concentrated at MVs may be more critical in regulating mineralization than the total PPi concentration associated with MVs. Alternatively, our results suggest that PPi is not likely to be the sole potent inhibitor of MV
mineralization in normal or TNAP-deficient states. For example, the ability to precipitate calcium dramatically declined for MV fractions of normal primary calvarial osteoblasts recovered at days 10–13 in culture, when much of the mineralization of nodules had already been completed. In contrast, the concentration of PPi associated with MV fractions changed little over 13 days in culture of the normal calvarial osteoblasts. The relative stability of PPi in MV fractions produced at various time points may reflect parallel changes in MV AP and NTPPPH activity observed in normal osteoblasts. Significantly, TNAP expression directly promoted an increase in MV fraction NTPPPH specific activity in MC3T3 cells in this study. Moreover, TNAP-expressing primary calvarial osteoblasts ultimately produced MV fractions with significantly more NTP-PPH activity than MV fractions produced by TNAP-deficient osteoblasts. Our results suggested that TNAP, by an enzyme activity-dependent mechanism, acts to control mineralization in part by modulating the content of its own inhibitor in osteoblasts and osteoblast-derived MVs. It will be of interest to determine what other constituents of MVs that regulate mineralization are modulated by TNAP activity in osteoblasts.

In this study, we demonstrated that PC-1 preferentially distributed to osteoblast MV fractions, compared with the closely related NTPPPH B10. MV fraction-associated NTPPPH and PPi were increased by a direct elevation in expression of PC-1 but not of B10. The ability of PC-1 to suppress MV-mediated mineralization was not shared by B10. We observed that B10 remained in a predominantly intracellular distribution distinct from that of PC-1 and TNAP in mineralizing
osteoblastic cells. These findings help explain how osteoblast-mediated hyperossification occurs in PC-1-deficient mice, despite the fact that mouse osteoblasts also contain abundant B10 and despite the physiological expression (as a component of BMP-2-mediated chondroosseous differentiation) of a distinct PDNP/NTPPPH isozyme called autotaxin, which is a predominantly secreted species (6).

The genes for PC-1 and B10 are located in close proximity on chromosome 6q21–23, presumably reflecting an antecedent gene duplication event (19). Each gene encodes a class II (intracellular NH2 terminus) transmembrane glycoprotein of 120–130 kDa that shares a highly homologous extracellular domain containing two somatomedin B-like regions and a conserved catalytic site (19). However, B10 has a unique extracellular RGD cell adhesion motif, and the cytosolic tails of PC-1 and B10 share no significant homology (19). Moreover, differential localization and function of PC-1 have been observed in other tissues. For example, PC-1 translocates to the basolateral surface and B10 to the apical surface in a polarized cell type (hepatocytes), an effect attributable to differences in the cytosolic tail (39). Furthermore, PC-1 but not B10 preferentially localizes to the plasma membrane in human articular chondrocytes and only PC-1 causes an increase in extracellular PPi in these cells (32).

In conclusion, TNAP promotes mineralization, in part, by removing the profound inhibitory effect of PC-1-generated PPi on ATP-dependent MV-mediated mineralization. It will be of interest to determine whether TNAP does so by not only hydrolyzing PPi but also by dephosphorylating ATP that would otherwise be used by PC-1 to generate PPi. This study established that PC-1 preferentially distributes to osteoblast MV fractions compared with another NTPPPH isozyme, B10 (also known as PDNP3). It will be of interest to determine whether the signals responsible for PDNP/NTPPPH-selective basolateral membrane localization of PC-1 in polarized epithelia and PC-1 distribution to MVs are the same or different. Results of such studies would help elucidate how selective concentration of specific ectoenzymes occurs in MVs. We also conclude that TNAP upregulates the amount of NTPPPH expressed by osteoblasts, which influences the amount of NTPPPH activity distributing to osteoblast MVs. Once their bone-making duties in the basic multicellular unit of bone are completed, the majority of osteoblasts must become less active in bone formation (29). We speculate that the control of PC-1 expression by TNAP may be one of the physiological means by which normal osteoblasts regulate their bone-making activity.

**Perspectives**

The association of osteomalacia with TNAP deficiency (hypophosphatasia) has illustrated the essential function of TNAP in osteoblastic bone matrix mineralization. TNAP acts on multiple substrates and has several potential physiologically significant functions in mineralization. However, the ability of TNAP to hydrolyze PPi to Pi has been hypothesized to be central to the ability of TNAP to promote osteoblastic mineralization. This study indicated that TNAP has at least one native antagonist, the NTPPPH PC-1, which hydrolyzes ATP to generate PPi, an inhibitor of osteoblast
MV-mediated mineralization. The results of this study indicated that PC-1 (but not another NTPPPH isozyme, B10/PDNP3) acts as a mineralization inhibitor at the level of MVs. PC-1 has the potential to inhibit osteoblastic mineralization not only by generating PP_i associated with MVs but also by hydrolyzing ATP that may be utilized, in part, by MV TNAp to mineralize. Our results suggested that TNAp acts to promote mineralization, in part, by removing the profound inhibitory effect of PC-1-generated PP_i on ATP-dependent MV-mediated mineralization. Whether the effects of TNAp are mediated not only by PP_i hydrolysis but also by TNAp-induced dephosphorylation of the PC-1 substrate ATP will be of interest for further investigation.

One surprising finding of this study was that TNAp modulated the expression of its own antagonist, PC-1, in osteoblasts, which was associated with changes in the distribution of osteoblastic MV fractions of NTPPPH activity. Finally, in view of the association of deficient PC-1 expression with hyperossification in vivo, the results of this study suggest the testable hypothesis that a marked attenuation of PC-1 expression in vivo may have the potential to augment bone matrix mineralization in the setting of TNAp deficiency.

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