

Site-specific localization of two distinct phosphatases along the osteoblast plasma membrane: tissue non-specific alkaline phosphatase and plasma membrane calcium ATPase

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Abstract

In osteoblasts, alkaline phosphatase has been reported to be restricted to the basolateral domains. In recent studies, we have demonstrated phosphatase activities different from those of tissue non-specific alkaline phosphatase (TNSALP) along the osteoidal aspect of osteoblast membrane at alkaline and neutral pH on undecalcified freshly frozen sections of rat bones. In the present study, we sought to further characterize and define the nature of membrane-associated phosphatases along the osteoidal aspect of osteoblasts. Histochemical properties of the enzymes and their localization *in vivo* were examined in long bones of normal Wistar rats and TNSALP null mutant mice and their wild type littermates. Molecular profiles of the enzymes in the osteoblast extracts were also examined. The enzymatic activity of the phosphatase along the osteoidal surface of osteoblasts proved to be activated by both Mg^{2+} and Ca^{2+} . Unlike TNSALP, the activity was inhibited by vanadate but resistant to levamisole, implicating a similarity between this enzyme and plasma membrane Ca^{2+} transport ATPase (PMCA). Immunohistochemistry showed that PMCA immunoreactions were restricted to the osteoidal domain of the plasma membrane. Native-PAGE analysis of osteoblast extracts suggested the presence of two phosphatases corresponding, respectively, to TNSALP and PMCA. Western blot analysis after SDS-PAGE of osteoblast extracts confirmed the existence of PMCA (140 kDa) and TNSALP (80 kDa). Gel-chemical analysis of the osteoblast extract from TNSALP null mutant mice depicted phosphatase activity, which was resistant to levamisole.

These data suggest the presence of a phosphatase different from TNSALP, most plausibly PMCA, on the osteoidal surface of osteoblasts.
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Introduction

Alkaline phosphatase (ALPase) [EC 3.1.3.1] is a member of the phosphoric monoester hydrolases [EC

3.1.3]. Human ALPase is classified as four isoenzymes (intestinal/tissue-nonspecific/germ cell/placental) [1]. Tissue-nonspecific alkaline phosphatase (TNSALP) has three isoforms: bone, kidney, and liver types, attributed to the variety of post-transcriptional *N*- and *O*-glycosylation [2]. These enzymes have in common that their pH optimum in the test tube is in the alkaline range. Natural substrate(s) and actual working pH for these enzymes have not sufficiently been clarified. In bone tissue,

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TNSALP is localized mainly at the plasma membrane of osteoblasts [3,4] and is bound via a glycosylphosphatidylinositol (GPI) anchor [5–7]. This enzyme has been used as a marker for phenotyping osteoblastic cells in vitro [8].

Hypophosphatasia is a clinically heterogeneous heritable disorder characterized by defective bone mineralization, which is caused by an impaired TNSALP activity due to mutation of the TNSALP gene [9,10]. Data from studies on hypophosphatasia patients and TNSALP mutant mice showing symptoms of hypomineralization suggest that TNSALP is one of the factors involved in bone mineralization [9–18].

Two types of adenosine triphosphatase (ATPase) [EC 3.6.1.3], Na, K-ATPase and plasma membrane Ca^{2+} transport ATPase (PMCA) have been reported to be located on the osteoblast plasma membrane [19–22]. The natural substrate of these ATPases, ATP, is also hydrolyzed by TNSALP in vitro [23] and is therefore supposed to be one of the candidates of the natural substrate of TNSALP. Despite an abundance of data on TNSALP and ATPases in osteoblasts, the physiological roles and kinetics of these phosphatases on the osteoblast plasma membrane have yet to be determined [19–22,24–32].

In initial mineralization, it is well known that matrix vesicles (MV) present TNSALP and ATPase activities on the outer surface of their membrane and contribute to induction of mineral deposition [33–35]. Though the presence or absence of MV in the osteoid undergoing appositional mineralization had long been uncertain, Bab et al. [36] demonstrated by biochemical methods the existence of MV in the osteoid as well as the presence of both ATPase and TNSALP in these MV fractions. Co-existence of TNSALP and ATPase on the osteoblast-derived MV is therefore feasible. Interestingly, however, in osteoblasts, enzyme reactions of TNSALP have been reported to be associated with the basolateral aspects of plasma membrane, facing away from the bone matrix, when examined in sections of routinely fixed and decalcified specimens [3,4]. Immunoreactivity of TNSALP was also shown to be restricted to these sites [37,38]. On the other hand, Morris et al. [39,40] reported the presence of TNSALP along the entire surface of the osteoblast membrane. Furthermore, Stains et al. [22] recently reported a polarized localization of PMCA on the cultured osteoblasts and claimed an association of PMCA on the basolateral domains of these cells. In our previous studies, however, we used undecalcified bone sections and could successfully demonstrate phosphatase activities along the osteoidal surface of osteoblasts at both alkaline and near neutral pH, and suggested the presence of enzyme(s) different from TNSALP [37]. Therefore, it appears necessary to further examine the precise spatial localization and enzymatic properties of these phosphatases in the osteoblasts to elucidate their roles in the mineralization of osteoid matrix.

Materials and methods

Tissue preparation

Protocols for animal experiments had been approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University and the local institutional review board of the Academic Medical Centre in Amsterdam, The Netherlands. All experiments were properly carried out under the Guideline for Animal Experimentation at Tokyo Medical and Dental University.

Whole body sectioning of freshly frozen animals

One- or 2-week-old Wistar rats (Charles River Japan, Inc, Kanagawa, Japan) were anesthetized by ether inhalation followed by an intraperitoneal injection of 8% chloral hydrate solution (400 mg/kg B.W.), and subjected to whole body freeze sectioning according to Kawamoto's procedures [41,42]. In brief, the deeply anesthetized animals were frozen by quenching in cold hexane kept at -94°C in the cooling bath of a freeze dryer (Neocool Bath, Yamato Kagaku, Tokyo, Japan). The frozen animals were rapidly embedded in 5% carboxymethylcellulose (CMC) gel and further frozen in cold hexane. The frozen CMC blocks of animals were set in a cryomicrotome (CM 3500, Leica Microsystems, Wetzlar, Germany) kept at -20°C and cut with a disposable type carbide tungsten steel blade (Leica Microsystems, Nußloch, Germany). When the appropriate plane of CMC-embedded frozen animal was exposed, an adhesive tape made of polyvinylidene chloride film (Asahikasei Kogyo, Tokyo, Japan) was glued onto the cut surface. In this way 5- μm -thick frozen sections supported by a polyvinylidene chloride film were collected one by one. The whole body frozen sections were freeze-dried in the cold chamber of the cryomicrotome and stored at -80°C until use. Anesthetized 8- to 12-day-old TNSALP null mutant mice and their wild type littermates (Jackson Laboratory, Animal Resources, Bar Harbor, ME, USA) were decapitated and immediately frozen in liquid nitrogen. The frozen bodies were processed for whole body sectioning in the same manner.

Paraffin or cryosectioning of chemically fixed specimens

A group of Wistar rats (1–5 week old) were similarly anesthetized and perfused via the left cardiac ventricle with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). Upper and lower jaws and hind limbs of perfused animals were excised and further immersed in the fixative for 1 day at 4°C . They were processed either for paraffin sectioning or for cryosectioning with or without prior EDTA decalcification. Before cryosectioning, specimens were immersed overnight in 30% sucrose/PBS solution for cryoprotection. A support film cryosectioning method [41,42], as already described for whole body sectioning, was applied for the undecalcified specimens.

Semi-thin sectioning of freshly frozen, freeze-substituted specimens

Three-week-old Wistar rats were anesthetized (as described above) and killed by phlebotomy. Maxillae, mandible, tibiae, and femora were immediately excised, cut into small pieces, and quenched in liquid propane cooled with liquid nitrogen for rapid freezing. The frozen pieces were freeze-substituted with absolute ethanol at -80°C for 4 days, gradually brought to 4°C , and embedded in Technovit 7100 (Heraeus Kulzer GmbH and Co. KG., Werrheim, Germany) at 0°C . Two-micron-thick sections were cut by glass knives on a Histoknife (Diatome, Bienne, Switzerland) attached to the ultramicrotome (Leica ULTRA CUT, Leica Aktiengesellschaft, Vienna, Austria) and were adhered to the glass slide without heating.

Enzyme histochemistry of phosphatases

To detect phosphatase reactions, the Technovit sections of freshly frozen, freeze-substituted specimens were incubated either by the Azo-dye method or the lead salt method. The Azo-dye method was performed basically according to the Burstone's method [43]. Briefly, the sections were incubated in the medium comprising 1.5 mM Naphthol AS-MX phosphate as substrate and 0.5 mM Fast Red Violet LB salt as capture agent, in 0.1 M Tris-HCl buffer (pH 9.2), containing 3 mM MgSO_4 at 37°C for 60 min. For the lead salt method, sections were incubated in the medium containing 3 mM ATP as substrate, 8% glucose, 3 mM MgSO_4 , and 3 mM PbNO_3 in 0.1 M Tris-maleate buffer (pH 9.2). After completion of the incubation for 60 min at 37°C , the sections were thoroughly rinsed in distilled water and treated with 1% ammonium sulfide solution to visualize sites of lead precipitation.

Specificity tests of enzyme reactions

To test metal ion dependency of the phosphatase, some Technovit sections of freshly frozen, freeze-substituted specimens adhering to the glass side were immersed in 5% EDTA for 1 day at 4°C to eliminate metal ions. Demineralized sections were then reactivated by immersion in 0.1 M Tris-maleate buffer (pH 7.3) supplemented either with 50 mM MgSO_4 or 50 mM CaCl_2 , for 3 days at 4°C [44], followed by an incubation in Azo-dye medium containing 3 mM MgSO_4 or 3 mM CaCl_2 . The influence of phosphatase inhibitors was also tested on undecalcified freshly frozen, freeze-substituted Technovit sections by adding levamisole (5 mM), a specific inhibitor of TNSALP [45] or vanadate (0.2 mM), a potent inhibitor of $\text{Mg}^{2+}/\text{Ca}^{2+}$ -ATPase [46,47] to the incubation medium by the lead salt method, using ATP as substrate.

Preparation of osteoblast extracts

Anesthetized 5-week-old Wistar rats were decapitated and their limbs immediately excised. Subsequently, the

periosteum of femora, tibiae, humeri, and radii was carefully removed, leaving the osteoblast layer remaining on the bone surface [48]. The osteoblasts were then collected by scraping the bone surface by a scalpel under the dissection microscope at a magnification of $\times 10$. Osteoblasts were similarly collected from skeletal tissue of 8- to 12-day-old TNSALP wild type or null mutant mice, which had been kept frozen at -80°C following decapitation. Collected osteoblasts were then transferred to 0.1 M Tris-HCl buffer (pH 7.3) supplemented with or without 1% Triton X-100. Subsequently, the samples in the Triton-free buffer were briefly homogenized using an ultrasonic homogenizer (Sonic and Materials, Inc., Newtown, CT, USA). After $10,000 \times g$ centrifugation at 4°C for 30 min, the supernatant was obtained as the osteoblast extract.

Electrophoresis of osteoblast extract

Native-polyacrylamide gel disc electrophoresis

The osteoblast extract prepared after a brief ultrasonic homogenization in Triton-free buffer was diluted with 60% sucrose solution (3.5 μg protein per tube) and applied on a 7.5% polyacrylamide gel (pH 8.9). Electrophoresis was carried out at a constant current of 2.0 mA per gel tube in Tris-glycine buffer (pH 8.3) in a cold chamber [49,50]. Subsequently, the gels were removed from the glass tubes and incubated in medium for the detection of phosphatase activity either by the Azo-dye or the lead-salt method containing ATP as substrate at pH 9.2 for 60 min at 37°C . To determine the effect of inhibitors, either 0.5 mM levamisole or 0.35 mM vanadate was added to the incubation media.

SDS-PAGE

To determine the molecular mass of target proteins, the osteoblast extract prepared in Triton-supplemented buffer was dissolved in 0.32 M Tris containing 10% glycerol 2% SDS and 2 β -mercaptoethanol (7.0 μg protein per well), boiled for 5 min, and run on a 12% SDS-polyacrylamide gel at 200 V.

Western blot analysis

After electrophoresis (native PAGE or SDS-PAGE), proteins were transferred to nitrocellulose or PVDF membrane using a semi-dry transfer system. The membrane was incubated in blocking reagent (Hoffmann-La Roche Inc., Nutley, NJ, USA) and primary/secondary antibody treatment was performed. The primary antibodies used were rabbit anti-human TNSALP (1:500) [51] and mouse anti-human PMCA (1:1000) (Affinity Biologicals Inc., Golden, Colorado, USA). HRP-conjugated goat anti-rabbit IgG (WAKO, Tokyo, Japan) (1:1000) and AP-conjugated goat anti-mouse IgG (KPL, Inc., Gaithersburg, MD, USA) (1:5000) were employed as secondary antibodies, respectively. Reactive protein bands were visualized by treating

the membranes with 2% 3' 3-diaminobenzidine (DAB) or NBT/BCIP reagent.

Immunohistochemistry

Paraffin sections or film-supported undecalcified frozen sections were immersed in a solution of 0.3% H₂O₂ in absolute methanol to inhibit endogenous peroxidase activity. Following a blocking treatment with a mixture of normal swine (10%) and goat (5%) sera, rabbit anti-human TNSALP antibody (1:100) and mouse anti-human PMCA antibody (1:2000) were applied as primary antibodies for 12 h. A biotin-conjugated swine anti-rabbit antibody (1:200) or HISTOFINE simple stain mouse MAX-PO (NICHIREI Corporation, Tokyo, Japan) was used as the secondary antibody and incubated for 1 h. The site of immunoreaction was visualized by treating sections with a 2% DAB solution supplemented with 0.002% H₂O₂. Normal rabbit or mouse immunoglobulins were used as negative control of primary antibodies. For TNSALP immunostaining, HRP conjugated streptavidin treatment (Dako Cytomation, Glostrup, Denmark) was performed for 30 min before DAB treatment.

For PMCA immunostaining on TNSALP null mutant or wild type mice, immunocomplex of primary antibody (4000 times diluted mouse anti-human PMCA) and secondary antibody (HISTOFINE biotin conjugated rabbit anti-mouse Igs (NICHIREI Corporation)) supplemented with 5% normal mouse IgG was prepared before antibody incubation. The sections treated by 0.3% H₂O₂ in absolute methanol and 10% normal rabbit serum were immersed in the immunocomplex solution overnight, after which HRP conjugated streptavidin treatment was performed for 30 min, and the site of immunoreaction was visualized by 2% DAB treatment.

Results

Metal ion dependency

In sections of conventional aldehyde fixed and decalcified specimens, phosphatase reactions in osteoblasts were undetectable without reactivation with metal ions, such as Mg²⁺ or Ca²⁺. In reactivated sections, distinct phosphatase reactions appeared along the plasma membrane of osteoblasts but only along the basolateral aspects (Fig. 1A). On the freshly frozen and freeze-substituted Technovit sections, on the other hand, an intense phosphatase reaction emerged along the entire perimeter of osteoblasts including the osteoidal surface (Fig. 1B). In whole body cryo-sections of freshly frozen rats, osteoblasts in all types of bones displayed identical localization of phosphatase reactions (data not shown).

To characterize the phosphatase(s) responsible for the intense reactions along the osteoidal aspect of osteoblast plasma membrane, the dependency for metal ions (Mg²⁺ and Ca²⁺) was tested on Technovit sections of the freshly

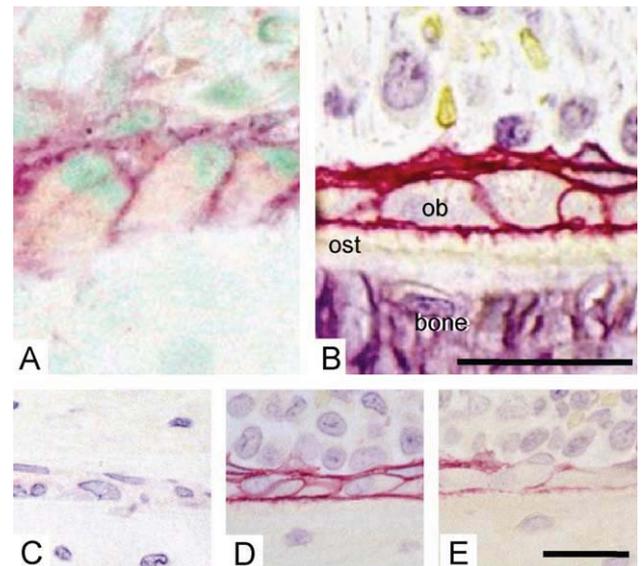


Fig. 1. Phosphatase reactions on the osteoblast layer and its metal ion dependency by the Azo-dye method. (A) Frozen section of a chemically fixed and EDTA-decalcified specimen demonstrates reactions only along the basolateral aspects of osteoblasts after Mg²⁺ reactivation. (B) Technovit section of a freshly frozen, freeze-substituted specimen shows intense reactions along the whole perimeter of osteoblasts (ob). (C) A total loss of reactions in Technovit section of a freshly frozen, freeze-substituted specimen after EDTA-chelation before incubation. (D, E) Recovery of enzyme reactions in EDTA-chelated sections after reactivation with Mg²⁺ (D) or Ca²⁺ (E) ions. Osteoid: ost. Scale bars = 25 μm.

frozen, freeze-substituted specimens. The phosphatase reactions along the perimeter of osteoblasts (Fig. 1B) were totally abolished by EDTA chelation before histochemical incubation according to the Azo-dye method (Fig. 1C). Unlike conventional aldehyde fixed and decalcified sections, the phosphatase reaction on the freshly frozen sections was restored along the entire perimeter of the osteoblasts by pre-treatment of chelated sections with Mg²⁺ (Fig. 1D) and less efficiently with Ca²⁺ (Fig. 1E).

Inhibition experiment

When the lead salt method was applied on freshly frozen, freeze-substituted Technovit sections, for histochemical localization of phosphatase activities, intense reactions were also detected along the entire perimeter of the osteoblast plasma membrane, using ATP as substrate (Fig. 2A). The reaction was abolished from the basolateral domains of the osteoblast plasma membrane in the presence of levamisole, while the osteoidal aspect of the same cells retained the reaction (Fig. 2B). Inversely, the phosphatase reaction on the osteoidal aspect was inhibited by vanadate, whereas the activity remained on the basolateral membrane (Fig. 2C).

Immunolocalization of TNSALP and PMCA

TNSALP immunoreaction along the osteoblast plasma membrane was restricted to the basolateral domains and was

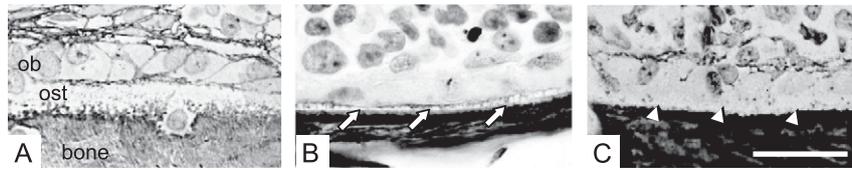


Fig. 2. Phosphatase reactions on Technovit sections of undecalcified freshly frozen and freeze-substituted specimens using ATP as substrate. (A) Control without inhibitor. (B) Levamisole (5 mM) abolishes the reaction on the basolateral domains but causes no effect on the phosphatase along the osteoidal surface (arrows). (C) Vanadate (0.2 mM) inhibits phosphatase activity along the osteoidal surface (arrowheads) but much less on the basolateral aspects of osteoblasts. osteoblast, ob; osteoid, ost. Scale bar = 25 μ m.

lacking or negligible along the osteoidal surface (Figs. 3A, E). With anti-PMCA antibody, on the other hand, the immunoreaction was observed only along the osteoidal surface of osteoblasts and negligible along the basolateral aspects (Figs. 3C, F). No reaction products were depicted when the primary antibodies were replaced with normal immunoglobulin (Figs. 3B, D).

Gel electrophoresis and Western blot analysis of osteoblast extract

To further characterize and distinguish the phosphatase reactions on the osteoblast plasma membrane, the osteoblast extracts from long bones of normal rats (Fig. 4B) were subjected to micro-disc Native-PAGE. When the micro-disc gel thus prepared was incubated for phosphatase reactions, two intensely reactive bands were detected (Fig. 4A). Similar bands were also confirmed when the gel was incubated by the Azo-dye method (data not shown). The upper band was inhibited by levamisole dose dependently,

but resistant to vanadate, whereas the lower band was resistant to levamisole but inhibited by vanadate. Western blot analysis of micro-disc gel revealed single bands immunopositive either for PMCA or TNSALP. Since the molecular weight of these bands cannot be determined by this system, we then examined these bands after SDS-PAGE. As the result, we depicted single bands positive either for PMCA or TNSALP antibodies and confirmed the molecular mass of these bands to be 140 and 80 kDa, respectively (Fig. 5).

Analysis of osteoblast extract of TNSALP mutant mice

To examine the phosphatase reaction on the osteoblast plasma membrane of TNSALP null mutant mice, a protocol similar to that for normal rats was employed and data were compared with those of wild type animals.

Native-PAGE gel analysis of the osteoblast extract of TNSALP wild type mice revealed two distinct bands each showing phosphatase activities, using ATP as substrate (Fig. 6A, +/+). As predicted, the osteoblast extracts from TNSALP null mutant mice only displayed a single reactive band (Fig. 6A, -/-), which was resistant to levamisole (data not shown). As shown in Figs. 6B and C, only one of

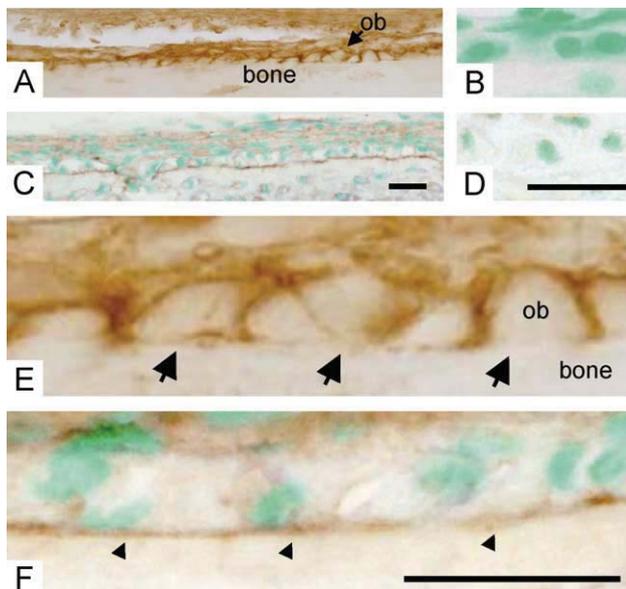


Fig. 3. Immunolocalization of TNSALP (A, E) and PMCA (C, F) in osteoblasts of rat bone. (A, E) TNSALP is located along basolateral domains of osteoblasts and is lacking or negligible on osteoidal aspect (arrows in E). Decalcified paraffin section. Osteoblast: ob. (C, F): PMCA is detected mainly on osteoidal aspect (arrowheads in F). Undecalcified frozen section. (B, D): negative control of TNSALP (B) and PMCA (D). Scale bars = 25 μ m.

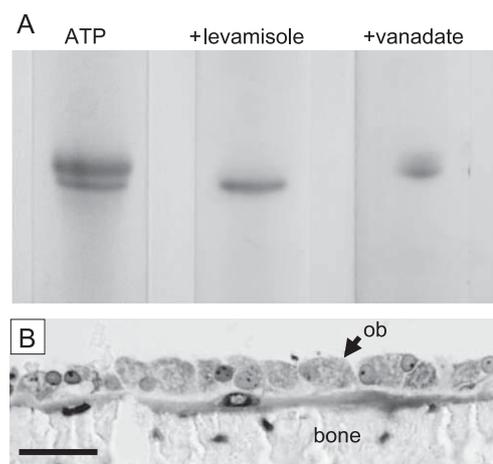


Fig. 4. Gel analysis of osteoblast extracts of normal rat. (A) Native PAGE gel incubated for phosphatase activity using ATP as substrate by the lead salt method with or without inhibitors (0.5 mM levamisole or 0.35 mM vanadate). (B) Photomicrograph of the osteoblast layer (ob) remaining on bone after removal of periosteum. These osteoblasts were scraped off and collected to obtain osteoblast extracts for Gel analysis. Technovit section of undecalcified rat femur. Scale bar = 25 μ m.

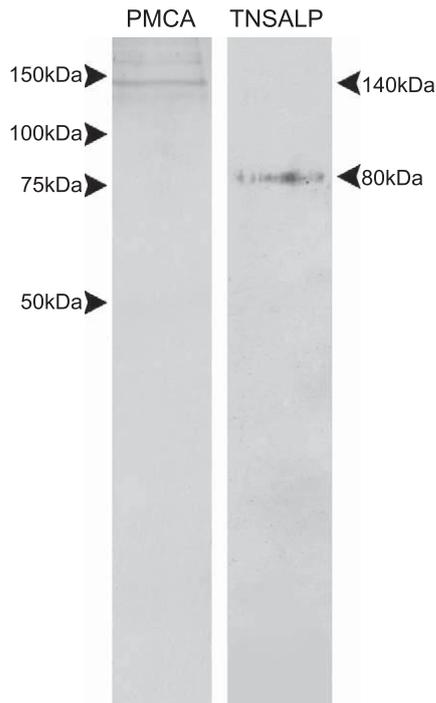


Fig. 5. Western blot analysis of osteoblast extract from normal rats for PMCA and TNSALP following SDS-PAGE. The molecular mass of PMCA and TNSALP positive bands are near 140 and 80 kDa, respectively.

the two phosphatase reactive bands in TNSALP wild type lane was immunopositive either for TNSALP or PMCA, whereas TNSALP null mutant lane was lacking a positive band for TNSALP and showed a single immunopositive band for PMCA after Western blotting.

Immunostaining of PMCA on the whole body fresh frozen sections of TNSALP null mutant and wild type mice demonstrated the immunoreactivity for this protein associated with the osteoidal domain of osteoblasts (Fig. 7).

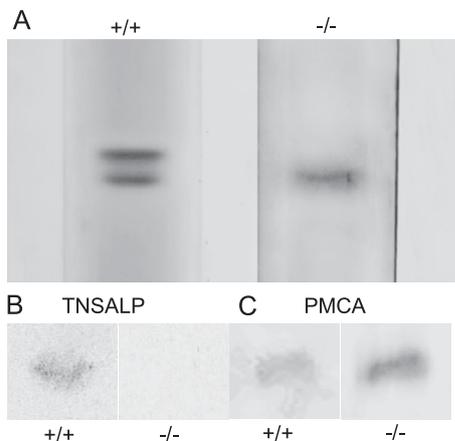


Fig. 6. (A) Native PAGE gel of the osteoblast extracts of TNSALP wild type (+/+) and null mutant (-/-) mice incubated for phosphatase activities using ATP as substrate. (B and C) Immunodetection of TNSALP (B) and PMCA (C) on the TNSALP +/+ and -/- lane. TNSALP -/- lane shows a band immunopositive for PMCA but not for TNSALP.

Discussion

In the present study, we could successfully obtain histochemical and immunohistochemical evidence showing the presence of two distinct phosphatases, plasma membrane Ca^{2+} transport ATPase (PMCA) and tissue non-specific alkaline phosphatase (TNSALP), located on the osteoidal and basolateral domains of the plasma membrane of osteoblasts, respectively.

Phosphatase along the osteoidal surface

In our previous study, we reported the presence of intense phosphatase activity along the osteoidal aspect of the plasma membrane of osteoblasts, and suggested that this enzyme is different from TNSALP, which is known to be located along the basolateral domains of the same cells [37]. The scarcity of information regarding the phosphatase activity associated with the osteoidal domain of the osteoblast plasma membrane is attributable at least in part to the difficulty to demonstrate histochemically the enzyme in routinely processed specimens, most notably chemically fixed and EDTA-decalcified specimens. Our data from the metal ion dependency test (Fig. 1) indicate that the phosphatase reactions along the osteoidal aspect are reactivated and become detectable by supplementation of both Mg^{2+} and Ca^{2+} in the chelated (EDTA treated) specimens if the specimens had not been treated with chemical fixatives. It is now clear that a combined treatment of the specimens with chemical (aldehyde) fixation and EDTA-chelation irreversibly abolish the phosphatase reactions on the osteoidal domain of the osteoblasts, but not from basolateral domains. A reactivation treatment of such specimens with divalent cations before histochemical staining [44] is not efficient for the enzyme located along the osteoidal surface for unknown reasons.

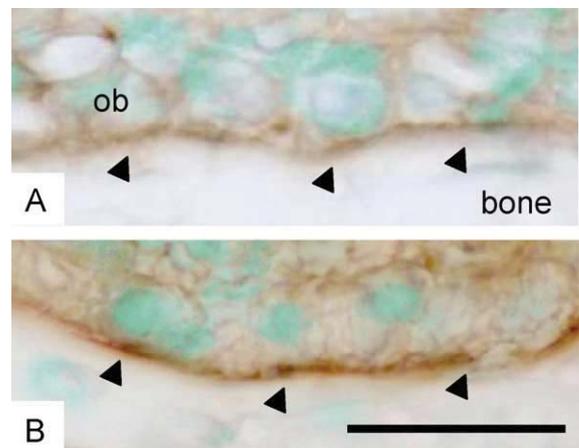


Fig. 7. Immunolocalization of PMCA in the osteoblast layer of TNSALP null mutant (A) and wild type (B) mice showing significant reactions associated with the osteoidal aspect of osteoblasts (arrowheads). Undecalcified frozen sections of bone. Osteoblast: ob. Scale bar = 25 μm .

It is well known that PMCA is resistant to levamisole [52] but sensitive to vanadate [46,47], whereas TNSALP is sensitive to levamisole [45] but is reported to be less sensitive to vanadate than PMCA [53]. In our histochemical observations (Fig. 2), the phosphatase activity located on the osteoidal surface was shown to be inhibited by vanadate but resistant to levamisole in an inverse manner to the phosphatase (TNSALP) associated with the basolateral domains of osteoblasts. Taking substrate specificity, metal ion dependency, and sensitivity to inhibitors together, PMCA appears to be the most plausible candidate for the phosphatase associated with the osteoidal aspect of the osteoblast plasma membrane. In fact, our Western blot analysis of Native-PAGE of osteoblast extracts confirmed the presence of PMCA in the osteoblast layer (Fig. 4). Immunoreactivity for PMCA did indeed reveal the presence of the enzyme on the osteoidal domain of osteoblasts (Figs. 3,7).

A careful comparison between the histochemical data and the two phosphatase-reactive protein bands which activity could be inhibited either by vanadate or levamisole on the Native-PAGE gel (Fig. 4) has clearly shown that the levamisole-sensitive but vanadate-resistant band on the gel corresponds to TNSALP and the other band to PMCA. The molecular weights of the PMCA-positive band (140 kDa) and the TNSALP-positive band (80 kDa) as shown by the SDS-PAGE of the osteoblast extract correspond to those reported previously [2,51,54,55]. The lower concentration of inhibitor needed to abolish phosphatase activity on the gel relative to that in histochemical sections may be due to a difference in the state and/or microenvironment of the enzyme between the in situ and laboratory conditions.

A polarized protein localization of PMCA in osteoblasts had already been reported in previous in vitro [22] studies. Interestingly, however, the distribution patterns of PMCA demonstrated were opposite to those found in our current experiment. This discrepancy may be attributed at least in part to possible alteration in the polarization of functional domains of the osteoblastic cells in cell culture system. Data in a previous cytochemical study of Ca^{2+} -ATPase in

osteoblasts [56] also appear to be different from our observations. This contradiction may be due at least in part to the fact that the authors of the latter study employed en bloc incubation for the lead salt method, which might not have allowed enough penetration of the substrate and/or the capture agent in the incubation medium to the site of enzyme reaction (osteoidal surface).

TNSALP mutant mice

In this study, we could also confirm the presence of a phosphatase different from TNSALP in the osteoblast extract of TNSALP null mutant mice. The gel electrophoretic profiles of the osteoblast extract by Native-PAGE treated for the expression of phosphatase activities, and the data from the Western blot analysis, all indicate characteristics of an enzyme different from TNSALP. Since the phosphatase detected in the osteoblast extract of TNSALP mutant mice is capable of hydrolyzing ATP and is resistant to levamisole, this enzyme is most likely PMCA. In fact, PMCA immunoreactivity was in close association with the osteoidal surface of the osteoblasts in TNSALP null mutant mice (Fig. 7A).

Role of PMCA

Under physiological conditions, osteoblast TNSALP hydrolyzes inorganic pyrophosphate (PPi) [57], a natural inhibitor of crystallization, and promotes mineralization of bone matrix [10]. It is noteworthy that, in TNSALP null mutant mice, bone mineralization proceeds to some extent despite severe hypophosphatasia [11–16], which accompanies elevation of PPi [57]. Nevertheless, a slow but steady progression of mineralization of osteoid under these conditions may implicate the existence of enzymes other than TNSALP that might at least partially hydrolyze and lower the local level of PPi, and promote mineralization of osteoid matrix. Though data are not available yet as to whether PMCA can hydrolyze PPi, the enzyme associated

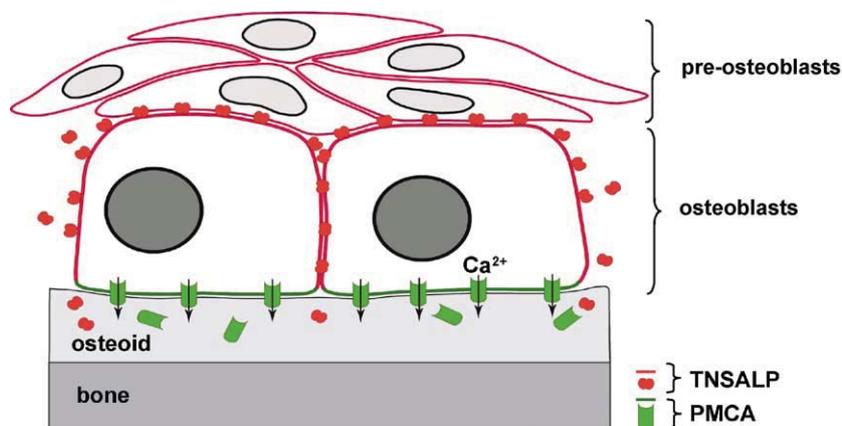


Fig. 8. Summary diagram of localization of TNSALP (red) and PMCA (green) on the osteoblast plasma membrane and its possible diffusion into the intercellular spaces and also into the osteoidal matrix. Arrows in PMCA positive osteoidal plasma membrane indicate the direction of putative Ca^{2+} -extrusion pump.

with the osteoidal aspect of osteoblast plasma membrane and/or that released into osteoid matrix may contribute to the regulation of PPI concentration. Under severe hypophosphatemic conditions like in TNSALP null mutant animal, this system may perhaps remain functional.

Aside from the possible role as a regulator of pyrophosphate concentration, the known physiological role of PMCA is to maintain intracellular Ca^{2+} concentration by regulating intra/extra-cellular Ca^{2+} homeostasis [54,55,58–60]. PMCA is an outwardly directed Ca^{2+} pump that extrudes intracellular Ca^{2+} fuelled by hydrolysis of ATP [54,55,58–60]. As illustrated in the summary diagram of plausible localization patterns of TNSALP and PMCA based on our current observations (Fig. 8), the exclusive, polarized localization of PMCA on the osteoidal aspect of osteoblast plasma membrane may also imply a role of this enzyme as a potential calcium pump that provides Ca^{2+} ions toward the osteoid. This calcium pump may act synergistically with the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which is also reported to be present on the osteoidal aspects of osteoblasts [22,61].

In case of the periodontal ligament, 10% of TNSALP is associated with its extracellular matrix and 90% of the enzyme is firmly bound to cell membranes [62]. Thus, partial release of PMCA from the osteoidal aspect of osteoblast membrane into the osteoid matrix may also be possible. Rather broad band-like immunoreactivity for PMCA along the osteoidal surface (Figs. 3,7) support this hypothesis. Liberated PMCA in the osteoid matrix may function as a regulator of local pyrophosphate concentrations, whereas the membrane-bound PMCA may serve as a Ca^{2+} -exclusion pump. Takano et al. [63] provided cytochemical evidence for such a liberation of PMCA from the plasma membrane of presecretory ameloblasts into the osteoid-like predentin matrix at the early stage of tooth formation.

Conclusion

In conclusion, the phosphatase associated with the osteoidal aspect of the osteoblast plasma membrane is different from TNSALP, which is restricted to the basolateral domains of osteoblasts. Current histochemical and immunochemical data indicate that PMCA is the most likely candidate enzyme for the phosphatase located on the osteoidal aspects of the osteoblast plasma membrane though possible co-localization of minor activities of phosphatase(s) such as inorganic pyrophosphatase should not be disregarded.

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