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Functional Involvement of PHOSPHO1 in Matrix Vesicle-Mediated Skeletal Mineralization

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Conflicts of Interest

All authors have no conflicts of interest.

ABSTRACT: PHOSPHO1 is a phosphatase highly expressed in bone. We studied its functional involvement in mineralization through the use of novel small molecule inhibitors. PHOSPHO1 expression was present within matrix vesicles and inhibition of enzyme action caused a decrease in the ability of matrix vesicles to calcify.

Introduction: The novel phosphatase, PHOSPHO1, belongs to the haloacid dehalogenase superfamily of hydrolases and is capable of cleaving phosphoethanolamine (PEA) and phosphocholine to generate inorganic phosphate. Our aims in this study were to examine the expression of PHOSPHO1 in murine mineralizing cells and matrix vesicles (MV) and to screen a series of small-molecule PHOSPHO1-specific inhibitors for their ability to pharmacologically inhibit the first step of MV-mediated mineralization.

Materials and Methods: q-PCR and immunohistochemistry were used to investigate the expression and localization profiles of PHOSPHO1. Inhibitors of PHOSPHO1's PEA hydrolase activity were discovered using high throughput screening of commercially available chemical libraries. To asses the efficacy of these inhibitors to inhibit MV mineralization, MVs were isolated from TNAP deficient (*Akp*2 $^{-/-}$) osteoblasts and induced to calcify in their presence.

Results: q-PCR revealed a 120-fold higher level of PHOSPHO1 expression in bone compared to a range of soft tissues. The enzyme was immunolocalized to the early hypertrophic chondrocytes of the growth plate and to osteoblasts of trabecular surfaces and infilling primary osteons of cortical bone. Isolated MVs also contained PHOSPHO1. PEA hydrolase activity was observed in sonicated MVs from *Akp*2 $^{-/-}$ osteoblasts but not intact MVs. Inhibitors to PHOSPHO1 were identified and

characterized. Lansoprazole and SCH202676 inhibited the mineralization of MVs from *Akp*2 $^{-/-}$ osteoblasts by 56.8 % and 70.7%, respectively.

Conclusions: The results show that PHOSPHO1 localization is restricted to mineralizing regions of bone and growth plate and that the enzyme present within MVs is in an active state, inhibition of which decreases the capacity of MVs to mineralize. These data further support our hypothesis that PHOSPHO1 plays a role in the initiation of matrix mineralization.

Keywords: PHOSPHO1; mineralization; osteoblasts, growth plate; alkaline phosphatase.

Introduction

During the process of endochondral bone formation, chondrocytes and osteoblasts are believed to mineralize their extracellular matrix by promoting the initial formation of crystalline hydroxyapatite (HA) in the sheltered interior of membrane-limited matrix vesicles (MVs).[1] This is followed by the modulation of matrix composition to further promote propagation of apatite outside of the MVs. [2,3] Regulation of this biphasic two-step mineralization process depends on a regulated balance of a number of factors such as Ca^{2+} and inorganic phosphate (P_i) concentrations, the presence of matrix proteins and of adequate mineralization inhibitors including inorganic pyrophosphate (PP_i), matrix gla protein and osteopontin.[4-8] Three osteoblast molecules have been identified as affecting the controlled deposition of bone mineral by regulating the extracellular levels of PP_i, and in turn of osteopontin, i.e., tissue-nonspecific alkaline phosphatase (TNAP); NPP1 (a nucleotide pyrophosphatase/phosphodiesterase isozyme) and the ANK gene product.[9-17]

In bone, TNAP is confined to the cell surface of osteoblasts and chondrocytes, including the membranes of their shed MVs.[18,19] It has been proposed that the role of TNAP in bone matrix is to generate P_i for HA crystallization.[20-22] However, TNAP has also been hypothesized to hydrolyze the mineralization inhibitor PP_i [4] to facilitate mineral precipitation and growth.[23-25] A variety of loss-of-function mutations in the human TNAP gene (*ALPL*) lead to hypophosphatasia, an inborn error-of-metabolism characterized by rickets and osteomalacia.[26] Mice with null mutations in the orthologous *Akp2* gene phenocopy infantile hypophosphatasia[27,28] including elevations in the known substrates of TNAP, i.e., pyridoxal-5'-phosphate and PP_i. Electron microscopy revealed that TNAP-deficient MVs, both in patients with hypophosphatasia as well as in *Akp2*$^{-/-}$ mice, contain apatite crystals, but that

extravesicular crystal propagation is retarded.[29,30] This crystal growth retardation, referred to as the second step of MV-mediated calcification, most likely results from the accumulated levels of PP_i in the extracellular matrix as a consequence of the lack of TNAP's pyrophosphatase function and the concomitant pyrophosphate-induced increase in osteoblast production of osteopontin, another potent inhibitor of calcification.[17] But, why are *Akp2*$^{-/-}$ mice born with a mineralized skeleton and still contain HA crystals inside their MVs? . TNAP is known to sit on the outer surface of the MV membrane and although it has not been adequately resolved that there is no TNAP inside MVs, it is likely that another enzyme is responsible for either cleaving PP_i or elevating the intravesicular concentration of P_i so as to achieve a P_i/PP_i ratio conducive for crystallization.

We have previously proposed that PHOSPHO1, a novel phosphatase, plays the important role of increasing the P_i/PP_i ratio inside MVs and thus control the first step of HA crystal deposition inside MVs. [31] MVs have long been recognised to contain high levels of Pi.[32] Since its identification in the chicken[33], PHOSPHO1 orthologues have also been identified in a number of other species including humans, mice and zebrafish.[34,35] However, to date no information exists on the expression of PHOSPHO1 in the mammalian skeleton. PHOSPHO1, a member of the haloacid dehalogenase superfamily, is localised to mineralizing surfaces in both bone and cartilage of the chicken where its expression precedes the deposition of mineral suggesting that it is involved in the initial events of mineral formation.[31,36] It is a soluble cytosolic enzyme that has specificity for phosphoethanolamine (PEA) and phosphocholine (PCho)[37,38] but not for PPi and a number of other potential substrates.[37] PEA and PCho are the two most abundant phosphomonoesters in cartilage.[39] In addition, the proportions of membrane phospholipids containing these

groups decrease in MVs during mineralization, whilst 1,2-diacyl glycerol accumulates, indicative of phospholipase C activity.[40] This gives rise to the possibility of a novel mechanism whereby plasma membrane bound phosphate may be released through the action of PHOSPHO1 and phospholipase C to contribute to the P_i concentration inside the MV.

The critical first step of mineralization mediates the deposition of the initial crystals of HA. We hypothesize that a TNAP-independent step involves PHOSPHO1 functioning to increase the local concentration of P_i inside the MVs. Thus, we would envisage that an inhibition of PHOSPHO1 activity would result in decreased mineralization within MVs. Therefore, to test this hypothesis experimentally we have now examined the expression of PHOSPHO1 in murine mineralizing cells and MVs, screened for and characterized a series of small-molecule PHOSPHO1-specific inhibitors and used these compounds to pharmacologically inhibit the first step of MV-mediated mineralization.

Materials and Methods

Chemical libraries

The LOPAC^{1280} (Sigma, St Louis, USA) and Spectrum (Microsource Discovery, Connecticut, USA) libraries were used as a source of potential small-molecule PHOSPHO1 inhibitors. The LOPAC library consists of pharmacologically active compounds covering most of the major target classes i.e. G protein coupled receptors and kinases whereas the Spectrum library contains known bioactives, natural products and their derivatives. The complete libraries (3280 compounds) were screened. The use of these two libraries allowed the evaluation of hundreds of marketed drugs and biochemical standards. Each compound within these collections

was dissolved in 10% DMSO at approximate concentrations of 100 μM and tested at a final concentration of approximately 10 μM.

Recombinant PHOSPHO1

A cDNA corresponding to Met19-Cys267 of human PHOSPHO1 was amplified and cloned into the pBAD TOPO TA vector (Invitrogen) as previously described.[37] Briefly, the construct was designed to express PHOSPHO1 fused to a V5 epitope and 6 His-tag at the C-terminus. A clone containing the PHOSPHO1 fragment in the correct orientation was identified by restriction digestion of plasmid minipreparations. *E. coli* were grown in Lauria-Bertani broth (10 litres, 37^0C) and recombinant protein expression was induced by treatment with 0.1% (w/v) L-arabonose for 4 h.

Inhibitor Screening

The semi-automated screening utilized a Beckman Coulter dual bridge Biomek FX liquid handler, consisting of a 96 tip head bridge for full plate pipetting. The reactions were measured in 96-well plates containing 25 μl 20 mM MES-NaOH, pH 6.7, 0.01% (w/v) BSA, 0.0125% (v/v) Tween 20, 2mM $MgCl_2$, 62.5 μM PEA, 10 μM test compound and 500 ng (15.5 pmoles) of purified recombinant PHOSPHO1. Substrate addition was used to initiate the reaction thus allowing for an enzyme/compound pre-incubation. Reactions were allowed to proceed for 60 min at room temperature then stopped by the addition of 50 μl BIOMOL green reagent (Biomol International, L. P., Plymouth Meeting, PA). The absorbance of each well was measured at 620 nm and the inhibitory effect of each compound calculated as a percentage in relation to controls containing 1% (v/v) DMSO, since each compound was dissolved in 10% DMSO giving 1% in the final reaction. Each compound that exhibited an inhibition of 40% or more using the automated system was reconfirmed

manually, in duplicate, to eliminate the possibility of false positives. The assay used included negative controls (i.e. no inhibition, contained both enzyme and PEA) and positive control (i.e. 100% inhibition, contained only PEA)

Characterisation of Inhibitors

The IC50 for each inhibitor was determined using the phosphatase assay detailed above. The inhibitor concentration was varied between 100 and 0.3 μM with each individual reaction repeated in triplicate. The data of absorbance vs. inhibitor concentration was plotted using SigmaPlot and four parameter logistic curve fitted. The IC50 was calculated using the equation; $y = min + (max\text{-}min/1+ 10^{(logEC50-x)Hillslope})$. Effect of inhibitors on recombinant mammalian expressed TNAP protein[41] was assessed under optimal conditions; 1M diethanolamine buffer pH 9.6, 1mM $MgCl_2$, 20 μM $ZnCl_2$, 20 μM inhibitor, 0.5mM p-nitrophenolphosphate (*p*NPP). This assay was repeated using recombinant PHOSPHO1 under the optimal conditions described below. Absorbance's were measured at 405 nm.

The continuous phosphatase assay to determine kinetic parameters of the reactions involved monitoring the dephosphorylation of *p*NPP which causes an absorbance change at 405 nm. The reactions were measured in 96-well plates containing 20 mM MES-NaOH, pH 6.7, 0.01% (w/v) BSA, 0.0125% (v/v) Tween 20, 2mM $MgCl_2$, and 1.5 μM purified recombinant PHOSPHO1 at room temperature. *p*NPP and inhibitor concentrations were varied accordingly. Absorbance's were measured continuously at 405 nm using a VICTOR HTS plate-reader.[37]

Immunolocalization of PHOSPHO1 within long bones

Ten-day old male mice were euthenized by cervical dislocation and tibiae were fixed in 4% paraformaldeyde in PBS for 24 h before decalcification in 0.5M EDTA (pH 8.0) for a further 24 h at 4^0C. The fixed tissue was dehydrated and paraffin

embedded using standard techniques. Paraffin sections (6 μm) were dewaxed in xylene and rehydrated through a graded series of alcohol solutions and antigen retrieval was achieved by heating in sodium citrate for 90 minutes at 70 °C followed by extensive washing in PBS. Endogenous peroxidases were blocked by incubating the sections with 3% hydrogen peroxide (in methanol), followed by 3 washes in PBS. Unspecific protein binding was blocked by normal goat serum (1:5) diluted in PBS for 30 min. Rabbit antisera to mouse PHOSPHO1 (a generous gift from Professor Ikramuddin Aukhil, University of Florida, USA) was diluted 1:200 in PBS and incubated with the tissue section at 4°C overnight. Control sections received a similar dilution of normal rabbit serum. Following this the sections were washed in PBS, and incubated with a 1:100 dilution of goat anti rabbit IgG - peroxidase (DAKO, Cambridgeshire, UK) for 60 min. DAB substrate reagent (0.06% DAB, 0.1% H_2O_2 in PBS) was incubated for 8 min, rinsed in PBS and counterstained with Mayer's hematoxylin (Sigma) for 5 min. The sections were finally dehydrated and mounted in DePeX.

Isolation of MVs from Chick Growth Plate Cartilage

All animal studies and protocols were approved by the Institutional Animal Users Committees of both Roslin Institute and Burnham Institute for Medical Research. Under sterile conditions, growth plate cartilage from 3-week-old male broiler chickens was collected and diced. MVs were released by incubating the tissue at 37 °C with 0.45% collagenase (Worthington, type II) in 50mM Tris-HCl pH 7.6, 120 mM NaCl and 10 mM KCl for 3 h with constant agitation. The digested tissue was passed through a 40 μM sieve to remove undigested material. MVs were harvested from the digest by differential centrifugation as previously described.[42]

Briefly, the digest was centrifuged for 30 min at 1500 g to collect chondrocytes, then at 30, 000 g to remove sub – cellular debris and finally at 250, 000g to pellet MVs.

Isolation of MVs from primary cultured calvarial osteoblasts

Mouse calvarial cells were isolated from 3-day-old mice through sequential collagenase digestion, as previously described.[11,16] Calvarial cells from *Akp2*$^{-/-}$, *Akp2*$^{+/-}$ and wild-type mice were pooled separately and plated at a density of 20,000/cm^2 in alpha-MEM (Gibco, Paisley, UK) containing 10% FBS, 50 μg/ml ascorbate for a period of 21 days. The cell monolayer was washed with 50mM Tris-HCl pH 7.6, 120 mM NaCl and 10 mM KCl, and then incubated with 0.45% collagenase (Worthington, type II) in 50 mM Tris-HCl pH 7.6, 120 mM NaCl and 10 mM KCl at 37ºC for 120 min at 37ºC, with constant agitation. This cell suspension was subjected to differential centrifugation as described above to isolate both cells and MVs**.**

MV phosphatase activity and mineralization ability

Phosphatase activity within chick and mouse MVs was determined using the standard discontinuous colorimetric assay. [43] In brief, reactions were measured in 96-well plates containing 200 μl of 25% (w/v) glycerol, 20 mM TBS, pH 7.2, 25 μg/ml BSA, 2.5 mM PEA, 2 mM $MgCl_2$ and 12 μg MV protein.[37] PHOSPHO1 inhibitors at a final concentration of 1mM were used where appropriate. The mouse MVs were either left intact or ruptured by sonication to release the cytosolic contents. TNAP activity was determined using the Thermo-line ALP reagent (Melbourne, Australia). Total TNAP activity was expressed as nmoles pNPP hydrolysed/ min/mg protein. The in-vitro calcification ability of MV was determined by their ability to form calcium phosphate in vitro.[44] In brief, samples of MV protein (15μg of chick MVs and 20μg of TNAP null osteoblast derived MVs) were incubated in calcification

buffer in the presence of 0 to 3 mM phosphoester substrate for 5.5 h at 37°C. The reaction was terminated by centrifugation at 8800g for 30 minutes to pellet both MVs and any calcium phosphate mineral formed during incubation. The pellet was then solubilised with 0.6N HCl for 24 h and used directly in for calcium quantification using the *O*-cresolpthalein complexone method (CPC Kit, Thermotrace).

Western Blotting of PHOSPHO1 within MVs

Murine MVs were analysed for the presence of PHOSPHO1 by immunoblotting. MV preparations were lysed in PBS containing 1.6 mg/ml of Complete® protease inhibitor cocktail (Roche, Lewes, UK). Samples corresponding to 10 μg total protein were incubated at 70˚C for 10 minutes in LDS sample buffer before loading. Samples were ran on a 10% Bis-Tris NuPAGE gel and electroblotted to nitrocellulose, which were incubated in blocking solution (5% non-fat milk in Tris buffered saline with 0.1% Tween 20). The membranes were then probed with a 1:750 dilution of rabbit-anti-PHOSPHO1 antisera in blocking solution and washed three times with PBS. Blots were then incubated with goat anti rabbit IgG-peroxidase (DAKO) diluted 1:2,000 in blocking solution. The immune complexes were then visualized by enhanced chemiluminescence

Real Time (Quantitative) Polymerase Chain reaction (qPCR) of PHOSPHO1 within murine tissues

Tissues from several 10 day-old male mice were pooled and RNA was isolated by phenol/chloroform extraction and used directly in a quantitative RT-PCR reaction. The Brilliant® SYBR® Green QRT-PCR Master Mix Kit (Stratagene) method was utilised to allow quantification by fluorescence during the PCR reaction. Briefly 25μl SYBR green mastermix was added to 10ng RNA along with 0.2μM forward and reverse primers (forward: GACAATGAGCGGGTGTTTTC reverse:

GGGGATGGTCTCGTAGACAG). The RT-PCR reaction was cycled in a Perkin-Elmer Applied Biosystems Prism 7700 sequence detector as follows: 50°C for 30 minutes (RT step), 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds 57°C for 30 seconds and 72°C for 1 minute. Each tissue sample tested was tested in triplicate and compared to 18S RNA (classic II primers, Ambion) external control which allowed normalisation of results. A dilution series of both gene of interest and external control were carried out and subjected to an identical PCR to allow estimation of PCR efficiency. Relative differences in expression were calculated using the $2^{-\triangle CT}$ method.

Semi Quantitative PCR of osteoblast specific genes

Murine osteoblasts (*Akp2*$^{-/-}$ and wild-type) were cultured in alpha-MEM containing 10% FBS, 50 μg/ml ascorbate and ß-glycerophosphate (10mmol/L) for up to 14-days. Total RNAs were extracted by phenol/chloroform extraction and treated with DNase I (Ambion) according to the manufacturer's instructions. The RT-PCR reaction was carried out using the SUPERSCRIPT - First Strand synthesis system for RT-PCR with Oligo dT (Roche). Primer pairs for β-Actin RNA were used as a control. The reaction mixture contained 5μg RNA, 500ng Oligo dT, 2μl 10 x RT buffer, 2mM $MgCl_2$, 10mM DTT, 0.5mM DNTP's, 200ul Superscript enzyme. The reaction was then cycled as follows; 25°C for 10 min, 42°C for 50 min and 70°C for 15 min. The cDNA was used directly in a PCR reaction containing; 0.2mM dNTP mix (Promega), 5μl 10x PCR Buffer (Roche), 5 units Taq polymerase (Roche), 0.5μM of the forward and reverse primers and 1μl cDNA. This was then cycled in at: 94°C for 5 minutes, thirty cycles of 94°C for 30 seconds 57°C for 30 seconds and 72°C for 1 minute and finally one step on 72°C for 10 minutes. The primers sets were designed to span at least one intron so that any amplification from contaminating genomic DNA would be identified. The primers were PHOSPHO1 (forward:

GACAATGAGCGGGTGTTTTC reverse: GGGGATGGTCTCGTAGACAG) TNAP (forward: ACTACCACTCGGGTGAACCA reverse: TGAGATCCAGGCCATCTAGC) ß-actin (forward: TCCATCATGAAGTGTGACGT reverse: ACGATGGAGGGGCCGGACTC) Each reaction was analyzed on 1.5% agarose gels run in the presence of ethidium bromide (250 μg/l).

Statistical analysis

Analysis of variance (ANOVA) was performed to determine the significance of a given result. General Linear Model analysis incorporating pair-wise comparisons using Tukeys test was used to compare groups within the ANOVA models. All data are expressed as the mean +/- SD. Statistical analysis was performed using Minitab 14. Statistical significance was accepted at $P<0.05$.

Results

PHOSPHO1 Expression in Whole Tissues

To investigate the levels of PHOSPHO1 gene expression in different mammalian tissues, we used qPCR to obtain relative expression values. Bone had the highest expression levels while the least amount of PHOSPHO1 transcript was found in the liver (Fig. 1). The difference in expression between these two tissue types was ~120 fold. Furthermore, low transcript levels were detectable in all tissues examined; heart (5.67), bone marrow (3.57), adipose tissue (3.38), brain (1.96) and gut (1.20) (numbers in brackets represent the fold difference when compared to PHOSPHO1 expression in liver, which was arbitrarily set as 1 for comparison).

Immunolocalization of PHOSPHO1 to skeletal cells and Identification of PHOSPHO1 in MVs

The high expression level of PHOSPHO1 in bone observed by qPCR was confirmed and extended by the immunolocalization of PHOSPHO1 to primary regions of ossification in both growth plate cartilage and trabecular and cortical bone. PHOSPHO1 was restricted to the hypertrophic zone of the growth plate (Fig 2a). No staining was observed in the proliferating chondrocytes and in comparison to the pre-hypertrophic chondrocytes the terminally differentiated cells displayed little staining. PHOSPHO1 immunoreactivity was also observed within the chondrocytes of the developing secondary ossification centre (Fig 2b). PHOSPHO1 was also present on the surface of the trabecular bone of the metaphysis as well as within the osteoblasts lining the bone forming surfaces of the primary osteons within the periosteal region of cortical bone (Figs. 2c and d). All control sections were negative. Confirmation that PHOSPHO1 is a strong candidate as a modulator of matrix mineralization was obtained by the detection of PHOSPHO1 in MVs isolated from mouse calvarial osteoblasts (Fig. 2e). A single band of 29 KDa was detected, which agrees with the expected protein size for one of the PHOSPHO1 splice variants (Roberts et al. unpublished observation).

Identification of Active PHOSPHO1 in MVs

To determine if PHOSPHO1 present in murine MVs was active, we tested for hydrolase activity within intact and sonicated MVs isolated from cultures of *Akp2*$^{-/-}$, *Akp2*$^{+/-}$ and wild-type osteoblasts. This strategy was adopted to eliminate the possibility that TNAP hydrolysis of PEA may mask PHOSPHO1 activity. Cell phenotype was confirmed using ALP histochemistry and mineralization capability by von Kossa staining (data not shown). TNAP activity of intact MVs purified from these cultures mirrored that of TNAP histochemistry (Fig.3a). MV preparations were used directly in the standard discontinuous colorimetric assay. It was found that the

wild-type and *Akp2*$^{-/-}$ intact MVs had a hydrolase activity of 3.3 ± 0.4 and 0.07 ± 0.4 nmol.min^{-1}mg^{-1} MV protein, respectively. TNAP heterozygous MVs had an activity of 1.68 ± 0.5 nmol.min^{-1}mg^{-1} MV protein (Fig. 3b). Sonication of the MV preparation, to assess PHOSPHO1 and TNAP combined activity, caused an increase of approximately 1 nmol.min^{-1}mg^{-} in all cases ($P<0.05$), indicting that the enzyme responsible for this increased hydrolysis is cytosolic.

Is PHOSPHO1 Expression influenced by the presence of TNAP?

To analyse whether PHOSPHO1 expression was altered by TNAP expression, RNA was extracted from cells cultured for up to 14 days under mineralizing conditions. The TNAP genotype was confirmed by PCR and PHOSPHO1 expression was not altered by the background TNAP status of the cells nor by continuous culture for up to 14-days (Fig. 4).

Identification and Characterization of PHOSPHO1 Inhibitors

In order to be able to conduct studies to examine the effects of PHOSPHO1 inhibition on MV-mediated calcification, we first needed to identify and characterize small molecule inhibitors specific for PHOSPHO1. Consequently we screened two well known chemical libraries, i.e., the LOPAC and the Spectrum libraries and identified seventeen compounds capable of inhibiting recombinant PHOSPHO1 activity with IC_{50} values of 10 μM or less. From these seventeen compounds, three were selected based on certain criteria, i.e. solubility, reconfirmed inhibitory potential of 80% or more and also the absence of any reactive groups such as thiols etc. This led to the identification of SCH 202676; Lansoprazole and Ebselen as compounds for further investigation (structures shown in Fig. 5a).

The calculated IC_{50} values for each of these compounds when tested against PHOSPHO1-mediated hydrolysis of PEA were 1.97 ± 0.01 μM (SCH 202676), 4.71 ±

0.1 μM (Lansoprazole) and 2.81 ± 0.04 μM (Ebseleln) (Fig. 5b).. The PHOSPHO1 inhibition displayed by each of these compounds was also investigated by analysing data of initial reaction velocity in the presence of varying concentrations of inhibitor and pNPP. From analysis of the resultant Line-Weaver Burke plots all four inhibitors displayed lines which intercept after the y axis, close to the x axis which is a hallmark of non-competitive inhibition (data not shown). A Michaeles-Menten curve was constructed under saturating quantities of pNPP which also indicated that the inhibitors are of a non-competitive nature (data not shown) The inhibitor constant K_i, for the inhibitor/enzyme complex, was also calculated for each of these reactions with SCH 202676, Lansoprazole and Ebseleln displaying K_i values of 1.08 ± 0.83 , 71.28 ± 9.21and 31.00 ± 0.74 μM, respectively.

Effect of PHOSPHO1 Inhibitors on MV Phosphatase Activity

With these novel inhibitors at hand, we used chick MV protein directly as a source of wild-type PHOSPHO1 in a discontinuous colorimetric assay to assess the effect of inhibiting PHOSPHO1 activity on P_i generation. Each inhibitor was used at a concentration of 1 mM. The uninhibited MV protein exhibits a PEA hydrolase activity of 87.1 ± 1.8 nmol min^{-1} mg^{-1} whereas in the presence of Lansoprazole and SCH202676 this activity was reduced to 62.9 ± 1.1 nmol min^{-1} mg^{-1} and 73.0 ± 2.7 nmol min^{-1} mg^{-1} respectively (Fig. 6a). In comparison to the uninhibited reactions these activities relate to a reduction in activity of approximately 28% for Lansoprazole ($P<0.001$) and 16% for SCH202676 ($P<0.001$). Neither Lansoprazole nor SCH202676 interfered with the ability of TNAP to catalyse the hydrolysis of pNPP under optimal conditions, but did exhibit inhibition of PHOSPHO1 mediated hydrolysis of pNPP similar to that seen when using PEA as a substrate (Fig. 6b).

Effect of PHOSPHO1 Inhibitors on MV Calcification

Using the *in vitro* calcification assay we found that PEA supports the calcification of chick MVs, purified from growth plate cartilage, comparable to that shown by the phosphoester ß-glycerophosphate, 1.0 ± 0.01 and 1.3 ± 0.04 units of precipitated calcium (Fig. 7a). Chick MVs preparations were subsequently incubated with 1mM of Lansoprazole or SCH202676 which resulted in a slight decrease in calcifying potential in the presence of PEA (approximately 10% with each inhibitor ($P<0.001$), (Fig. 7b).

To increase the sensitivity of this assay the inhibitor actions were examined on MVs extracted from cultured *Akp2*$^{-/-}$ osteoblasts. This strategy eliminated the possibility of TNAP hydrolysing the phosphoester and potentially masking the effects of the PHOSPHO1 inhibitors. Using this strategy, a much more pronounced inhibitory effect was seen with Lansoprazole and SCH202676. In comparison to uninhibited reactions, Lansoprazole exhibited a 56.8 % inhibition of calcification ($P<0.01$) whereas SCH202676 inhibited calcification by 70.7% ($P<0.001$) (Fig 7c).

Discussion

The mechanisms and proteins regulating matrix mineralization are not yet fully understood, and we are still unclear if under physiological conditions mineralization is due to the presence of promoters of mineralization (active process) or a lack of mineralization inhibitors (passive process).[45] Notwithstanding the presence of an optimum balance of both inhibitors and promoters, the mineralization process is clearly dependent on the attainment of sufficiently high concentrations of P_i for the formation of *de novo* calcium phosphate crystals. Generation of P_i for mineralization has long been attributed to the actions of TNAP[6,20] but it is now clear that the production of Pi for matrix calcification is not entirely attributable to TNAP activity.

In newborn *Akp2* knockout mice, bone development and mineralization appear normal even though hypomineralization and other abnormalities do subsequently appear.[11,27,28] Although mechanisms involving *in utero* protection cannot be ruled out the postnatal mineralization defects in *Akp2*$^{-/-}$ mice are likely to be due to a build up of PP_i, a known substrate of TNAP[21] and a potent inhibitor of HA crystal formation.[4] Indeed, hypomineralization is greatly reduced in [*Akp2*$^{-/-}$; *Enpp1*$^{-/-}$] double-knockout mice where extracellular PP_i (ePP_i) concentrations return to normal after ablating NPP1 enzyme activity, i.e., the enzyme that produces PP_i at the surface of osteoblasts and MVs.[10,11] Hypomineralization in these double knockout mice is corrected in a site-specific manner in the calvaria and vertebrae while residual hypomineralization remains in metatarsals and long bones.[6] Mechanistically, the aforementioned build-up of ePP_i in *Akp2*-/- mice in turn leads to upregulated osteopontin expression by *Akp2*-/- osteoblasts[17] and it has recently been shown that it is the combined accumulation of ePP_i and OPN that causes osteomalacia in *Akp2*-/- mice.[17]

The location of TNAP to the outer surface of MV membrane and the demonstrated role of this enzyme as a pyrophosphatase *in vivo* suggests that other molecules or mechanisms are responsible for increasing intravesicular P_i levels so as to achieve a P_i/PP_i ratio conducive for crystallization. The sodium-dependent phosphate type III transporter Pit-1 (GLvr-1)[46-48] and other phosphatases such as, pyrophosphatase, AMPase and ATPase, known to be present in MVs[6,49,50] may be involved. In this present study we now provide the first functional evidence that PHOSPHO1 is a MV phosphatase involved in skeletal mineralization.

The immunolocalization of PHOSPHO1 in the mouse is consistent with its presumed role in matrix mineralization. PHOSPHO1 was localized in chondrocytes

in the early hypertrophic zone of the growth plate and to bone forming surfaces in both long bones (trabecular and cortical) and the site of the developing secondary ossification centre. This distribution, together with the absence of strong PHOSPHO1 immunoreactivity in the hypertrophic chondrocytes situated deep in the mineralized zone, suggests that PHOSPHO1 has a pivotal role in the initial stages of the mineralization process and is required for the *de novo* formation of the inorganic phase but not for the continued crystal growth of hydroxyapatite. This concurs with studies of the developing chick skeleton, where the expression of PHOSPHO1 in the mid-shaft of long bones preceded alizarin red staining, indicative of calcium deposition.[31] This localisation of PHOSPHO1 to bone forming surfaces mirrors that of TNAP [51,52], and perhaps indicates synergy between the two enzymes to achieve HA crystal growth. Although likely working in concert, no evidence was obtained to suggest that PHOSPHO1 expression was influenced by the presence/absence of TNAP possibly indicating that PHOSPHO1 is functioning independently from TNAP.

The matrix staining of PHOSPHO1 on bone forming surfaces is likely to reflect the presence of the enzyme in osteoblast derived MVs, which are deposited within newly formed osteoid. [53] Indeed the presence of PHOSPHO1 in MVs derived from calvarial osteoblasts was confirmed by immunoblotting as was the ability of TNAP null MV lysates to catalyse the hydrolysis of PEA. This indicates that the enzyme responsible for this degradation is cytosolic and, as according to the KEGG database[54] only TNAP and PHOSPHO1 have the ability to cleave PEA, it is likely that this cytosolic enzyme is in fact PHOSPHO1.

The high expression of PHOSPHO1 in bone noted by qPCR is consistent with the immunolocalization of PHOSPHO1, however, its low basal expression in a number of soft tissues may indicate other putative roles for this enzyme. Phosphocholine is an

important regulatory compound for the metabolism of phosphatidylcholine, the major phospholipid in mammalian tissues.[55] In heart muscle it has been documented that the PCho pool is much lower than that in the liver or HeLa Cells [56], however the enzyme responsible for this hydrolysis remains unknown.[57] It is interesting to speculate that this step may be controlled by the PCho activity of PHOSPHO1[37,38] thereby explaining the low expression in the heart and implicating PHOSPHO1 in a possible novel pathway which may be mediated by one of the alternate transcript from the PHOSPHO1 gene.

From the high throughput screening of compounds from the two chemical libraries LOPAC and SPECTRUM, 17 inhibitors of PHOSPHO1 mediated hydrolysis of PEA were observed. Many of these inhibitors, however, had undesirable properties, such as cisplatin that conceivably intercalates between the disulphide bridge in PHOSPHO1. In addition, compounds such as the dimeric disulphide containing compound thiram would be susceptible to nucleophlic attack and reduction. Other compounds deemed unsuitable included those which possess thiol groups. The functional group of the amino acid cysteine is indeed a thiol, therefore it is likely that compounds such as mercaptobenzothiazole, which was identified during this screen, would cause an inhibition through chemical modification of the PHOSPHO1 structure. This compound structure screening led to the identification of the three inhibitors; SCH 202676, Lansoprazole and Ebseleln deemed suitable for further characterization.

Lansoprazole and SCH 202676 decreased the amount of liberated P_i using isolated chick MV by 28% and 16% respectively. Ebselen has no effect on PEA hydrolase activity of MVs thus it is likely that this inhibitor is not effective against the wild-type enzyme. As this is a non-competitive inhibitor and binds at a site distinct

from the active site, it is conceivable that Ebselen is binding recombinant PHOSPHO1 at the C terminal tagged region thus inhibiting the recombinant but not the wild-type enzyme. SCH 202676 is a thiadiazole compound that acts upon and inhibits signalling through G protein-coupled receptors.[58] It is thought that this compound is an allosteric modulator of G protein-coupled receptors and may recognise an intracellular regulatory domain of the protein, however it's exact mode of inhibition remains unknown.[59] In contrast, Lansoprazole is an extremely well-characterised compound. This drug belongs to a class of compounds known as the 2-(2-pyridylmethylsulfinyl)-1*H*-benzimidazoles and is an inhibitor of H+ and K+ (H+/K+)-ATPase of stomach parietal cells. Under acidic conditions, Lansoprazole is converted into an acid activated cationic sulphenamide form (AG2000) which acts as a proton pump inhibitor[60], blocking the final step of acid production.

The effect of Lansoprazole and SCH 202676 on P_i release by chick MVs was greater than their inhibitory effect on MV calcification which was only ~10% with each inhibitor. This imbalance is possibly due to TNAP activity of the MVs preparations masking the effects of the PHOSPHO1 inhibitors by promoting PP_i hydrolysis and/or maintaining P_i levels at about the minimum threshold for mineralization to occur normally. Alternatively, preformed mineral crystals found within MVs may be acting as local nucleators allowing mineral propagation, even in the presence of PHOSPHO1 inhibitors. To avoid interference from TNAP in the assay system, we used MVs isolated from calvaria of TNAP null mice. In this case a more profound inhibition was observed; SCH 202676 and Lansoprazole caused a 71 and 57% decrease in calcification, respectively. This indicates that PHOSPHO1 which is sequestered within the lumen of the MV has the ability to hydrolyse PEA to increase the intravesicular concentrations of P_i to allow mineralization to occur. This is likely

to occur in synergy with phosphate[46-48] and calcium[61-63] transporters to facilitate the production of the initial crystals for hydroxyapatite deposition. SCH202676 is an allosteric antagonist of dopamine receptors and Lansoprazole is a proton pump inhibitor, and both are already being used in the treatment of patients, however to the best of our knowledge their use has not been reported to be associated with rickets or osteomalacia.

In conclusion the *Phospho1* gene is highly expressed in bone tissue and the PHOSPHO1 protein localization is restricted to sites of skeletal mineralization in the mouse. Further, PHOSPHO1 protein is cytosolic and active within murine osteoblast-derived MVs retaining the ability to hydrolyse the PEA, as previously demonstrated with the recombinant enzyme.[37,38] In addition, the PHOSPHO1 inhibitors SCH 202676 and Lansoprazole have the ability to modulate the *in vitro* mineralization of MVs. These data further strengthens the hypothesis that PHOSPHO1 has a role in bone mineralization, likely to be linked to the glycerolipid metabolism pathways involving the degradation of phosphatidylethanolamine and phosphatidylcholine and the production of P_i for MV mediated mineralization.

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Figure Legends

Fig. 1. qPCR of PHOSPHO1 in murine tissues. Data shows relative expression of PHOSPHO1 with reference to that of the liver which is set at 1 due to the lowest expression level of this tissue. RNA was isolated from mouse tissues by phenol chloroform extraction and used directly in the qPCR reaction utilising the DNA intercalating dye, SYBR green. Results are mean ± SD (n = 3).

Fig. 2. Expression of PHOSPHO1 in mouse tibial sections and MV. (A). Strong specific localization within the growth plate was limited to the early hypertrophic chondrocytes (arrows). Chondrocytes of the proliferating zone were negative (*). Trabecular bone surfaces within the metaphyseal region of the tibia were also positive (arrow-heads) (Scale Bar = 100μm) **(B).** Positive PHOSPHO1 staining in the developing secondary ossification center of the tibia (arrows) (Scale bar = 100μm**) (C).** Higher magnification of positively stained osteoblasts lining the bone forming surfaces of primary osteons in cortical bone (arrows) (Bar = 50μm). **(D).** Trabecular bone surfaces within the metaphysis containing positively stained osteoblasts (arrows) (Scale Bar = 50μm). Control sections are displayed in the lower panel of each pair (B-D). **(E).** Immunoblot showing the localisation of PHOSPHO1 to murine MVs isolated from calvarial osteoblasts. Immunoreactivity is compared to that of recombinant PHOSPHO1. The recombinant protein is of greater mass due to the presence of a C-terminal tagged region.

Fig. 3. Hydrolase activity of MVs from Wt, *Akp2*$^{+/-}$ and *Akp2*$^{-/-}$ osteoblasts. (**A**) pNPP and (**B**) PEA phosphatase activity of MV preparations. Data is presented as the mean ± SD of nine replicates from each genotype. PEA hydrolase activity was significantly higher ($p<0.05$) in sonicated vs. intact MVs.

Fig. 4. Analysis of TNAP and PHOSPHO1 expression in Wt and *Akp2*$^{-/-}$ osteoblasts Comparison of TNAP and PHOSPHO1 expression between Wt and *Akp2*$^{-/-}$ osteoblasts over a 14 day culture period.

Fig. 5. Identification of PHOSPHO1 inhibitors. (A). Structures of three compounds found to inhibit recombinant PHOSPHO1 by over 80%. **(B).** IC_{50} determination of each PHOSPHO1 inhibitor. Recombinant human PHOSPHO1 was incubated with various concentrations of SCH 202676, Lansoprazole or Ebseleln at room temperature for 60 min and phosphate released during the reaction was measured. Results are mean ± SD (n = 3).

Fig. 6. PEA hydrolase potential of chick MVs in the presence of PHOSPHO1 inhibitors. (A) Chick MVs were isolated from tibial growth plate cartilage and used directly to measure phosphate released, as an indicator of PEA hydrolase activity, in the presence of Ebselen (EBS), Lansoprazole (LAN) or SCH 202676 (SCH). **(B)** Effect of inhibitors on PHOSPHO1 (open bars) and TNAP (shaded bars) pNPP hydrolase activity under optimal conditions. Results are mean ± SD (n = 3, *** = $p<0.001$ when inhibited reaction compared to uninhibited reaction).

Fig. 7. Potential for MVs to calcify in vitro in the presence of PEA and PHOSPHO1 Inhibitors. (A). The ability of chick MVs to induce calcification in the presence of 3mM PEA and 3mM ß-glycerophosphate (RU, relative units). **(B).** Calcification of chick MVs in the presence of 1mM Lansoprazole (LAN) or SCH 202676 (SCH) **(C).** Calcification of murine TNAP null MVs in the presence of 1mM Lansoprazole (LAN) or SCH 202676 (SCH). Results are mean ± SD (n = 3, *** = $p<0.001$, ** = $p<0.01$ when inhibited reaction compared to uninhibited reactions).