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Phosphate Depletion Enhances Tissue-Nonspecific Alkaline Phosphatase Gene Expression in a Cultured Mouse Marrow Stromal Cell Line ST2

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Alkaline phosphatases (ALP) are highly ubiquitous enzymes present in the majority of animals from bacteria to higher vertebrate. Although their wide distribution in nature has suggested that these enzymes should perform important biological functions, their detailed roles or natural substrates remain unknown. In *Escherichia coli,* the extracellular phosphate (Pi) limitation induces the ALP gene, indicating the role of extracellular Pi in ALP gene regulation. However, little is known about the similar mechanisms in mammalian cells. This study was designed to examine the effect of low Pi medium on the ALP activity and its expression in the mouse stromal cell line ST2. The enzymatic property was classified into tissue-nonspecific ALP (TNSALP). After treatment by Pi starvation for 3 days, there was a 2-fold increase in the specific activity of TNSALP. RT-PCR analysis revealed that the mRNA of the TNSALP gene was highly stimulated. These results indicated that the effect of Pi depletion on ALP activity was regulated at the TNSALP transcriptional level, suggesting that the possible role of the Pi sensing system for biological functions of ALP might have been conserved in evolution. Our findings also made it possible to discuss the physiological roles of ALP *in vivo.* © 1999 Academic Press

In most animals except homonidae, alkaline phosphatase (ALP; orthophosphoric monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) is classified into two types; tissue-nonspecific (liver/bone/kidney; TNSALP) and intestinal types (1). In humans, there are at least four types of genetically different isozymes, namely, tissue-nonspecific, intestinal, placental and placental-like types (2–4).

cDNAs for several types of ALP have been isolated and characterized. A comparison of these genes strongly supports the hypothesis that multiple species of ALP genes are the products of a series of duplications of a single ancestral gene (2). The structure in the catalytically important region is well conserved in the ALPs of humans, animals and *E. coli* (2).

The expression of ALP in *E. coli* is regulated by extracellular phosphate (Pi). The ALP of *E. coli* is encoded by a pho A gene and its synthesis is induced when the cells are starved of Pi (5, 6). The function of the pho A gene protein may be regulated by the global control of the cellular metabolism during signaling the activation of "the phosphate regulon system" (5). Elevated bone-type ALP in hypophosphatemic rickets raises the possibility of similar regulation of ALP in osteoblasts. In cultured osteogenic cells, the ALP expression is reported to be modulated by the growth conditions and various factors including bone morphogenetic proteins (BMPs), glucocorticoid, vitamin D, parathyroid hormone, etc. (7–12). However, no detailed investigation has yet been done on the regulation of the ALP gene expression by Pi starvation in mammalian cells. In the present study, we attempted to examine the direct effects of Pi depletion of the ALP gene expression in a cultured stromal cell line ST2 using specific primers for the reverse transcription-polymerase chain reaction (RT-PCR) analysis.

MATERIALS AND METHODS

Cell culture. The mouse stromal cell line ST2 (Riken Cell Bank, Tsukuba, Japan) was cultured in MEM-α medium (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (Gibco BRL) and antibiotics (100 U/ml of Penicillin-G and 100 μg/ml of Streptomycin sulfate) at 37°C in a humidified atmosphere of air plus 5% CO_2. In order to determine the effect of phosphate concentration in the medium, phosphate-free Earl's solution containing Eagle's amino

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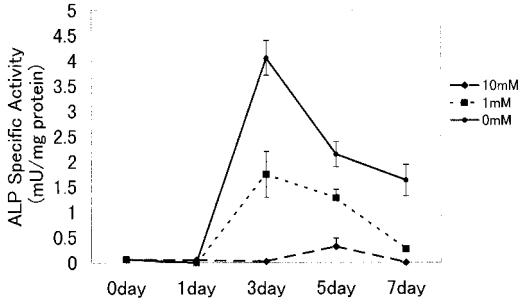

FIG. 1. Time course of specific ALP activity of ST2 cells cultured in three different concentrations of phosphate in the medium. Each value represents mean ± S.E. from triplicate experiments.

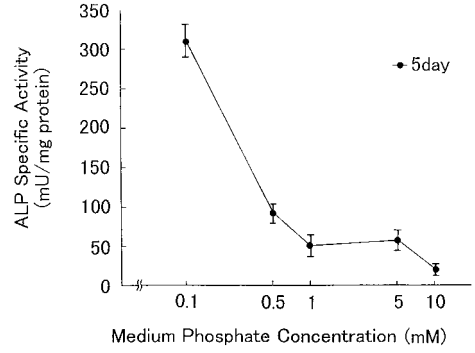

FIG. 2. Effect of phosphate concentration on specific ALP activity. ST2 cells were cultured in various concentrations of phosphate in the medium for 3 days. Each value represents mean ± S.E. from triplicate experiments.

acid and vitamin mixture (Nissui Co., Japan) was supplemented with various concentrations of phosphate (NaH_2PO_4) and adjusted to the same pH as that of D-MEM.

ST2 cells were spread on 24-cell plates at a density of approximately 1×10^4 cells/cm^2 and incubated for 3-4 days to 80% confluency. Cell washing was performed 3 times with phosphate-free Earl's solution. Earl's solution containing the desired concentrations of phosphate was added, and the cells were cultured from 0 to 7 days.

Enzyme assay. ALP activity was determined using 10 mM *p*-nitrophenylphosphate as the substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer (pH 10.0) containing 5 mM $MgCl_2$ and incubated at 37°C. To analyze the properties of ALP, an inhibitory assay using levamisole (Lev) and a thermostability assay were performed as previously described (13). The enzyme activity was determined by the rate of hydrolysis of *p*-nitrophenyl phosphate and expressed in units (U = mol *p*-nitrophenol formed/min). Protein concentrations were determined using the bicinchoninic (BCA) protein assay reagent (Pierce, Rockford, IL). Triplicate experiments were carried out in this study.

Statistics. Data are presented as mean values ± S.E. Quantitative data were analyzed by Student's *t*-test.

Preparation of RNA and polymerase chain reaction (PCR). The total RNA from about 2.0×10^5 cells was extracted using the acid guanidinium thiocyanate-phenol-chloroform method (14). As a template for PCR, single strand cDNA was prepared from 1 μg using the Superscript preamplification system (Gibco BRL). Two pairs of PCR primers: a sense primer E1A and an antisense primer E2 for 1A-type ALP (15, 16), and a sense primer E1B and an antisense primer E2 for 1B-type ALP (17), were used under the previously described PCR conditions (18). The amplified sample (10 μl) was analyzed using 5.25% polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was stained with ethidium bromide solution. Stained gels were observed with UV light. The density of the photograph was determined with a densitometer (AE-6920M, ATTO Co., Tokyo, Japan).

RESULTS

Effects of Phosphate on ALP Activity in ST2 Cells

Figure 1 shows the time course of the ALP activity in ST2 cells cultured in media of 3 different Pi concentrations (0, 1, 10 mM). The ALP activity was measured at day 0, 1, 3, 5 and 7 for all cell samples. The specific activity of the cells cultured in the medium with the Pi treatment (0 mM and 1 mM) gradually increased until the 3rd day and then decreased. The enzyme activity in the lowest Pi medium (0 mM) showed the highest value at day 3. When expressed as specific activity, about a 231% enhancement by Pi starvation (0 mM) was obtained compared with the value in 1 mM Pi medium at day 3. Therefore, the effect of the Pi concentration on the ALP activity was investigated in detail on day 3 of the culture of the ST2 cells. As shown in Fig. 2, the specific activity of the cultured cells became higher as the Pi concentration in the medium decreased.

Properties of ALP in ST2 Cells

The results of the inhibition and thermal inactivation experiments are shown in Fig. 3. The experiments were done on day 3 of the Pi treatment (1 mM and 0 mM). The inhibition and thermal inactivation experiments showed that Lev was a strong inhibitor of ALP

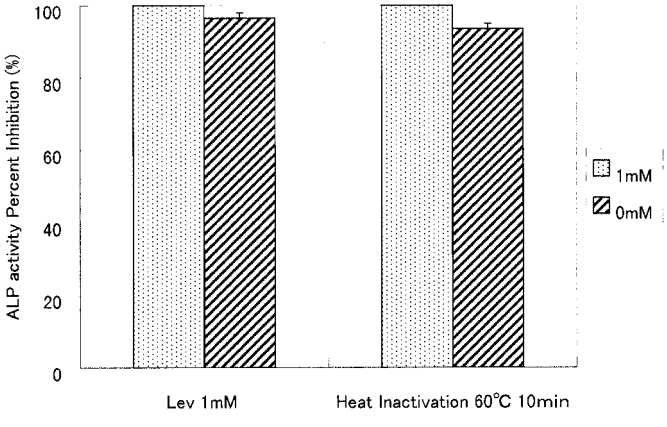

FIG. 3. Comparison of ALP properties of the ST2 cells. Activity was assayed by the rate of hydrolysis of *p*-nitrophenyl phosphate. Inhibited activities by various treatments were expressed as a percentage of the untreated control. Effects of inhibitors were determined in the presence of 5 mM $MgCl_2$ in the assay mixture. Lev, levamisole (1 mM) and heat treatment at 60°C for 10 min. Each value represents mean ± S.E. from triplicate experiments.

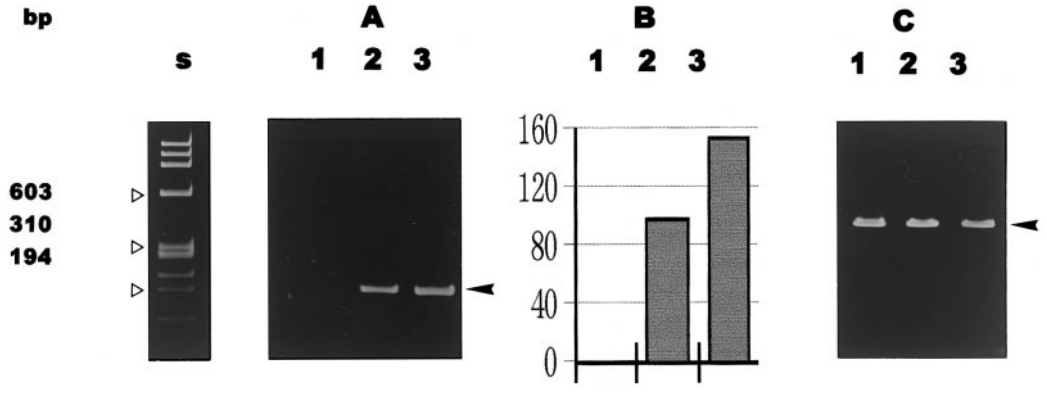

FIG. 4. Detection of mRNAs for 1A-type TNSALP (A) and (C) at day 3. RT-PCR products were analyzed in 5.25% polyacrylamide gel electrophoresis. (A) E1A and E2 primers detected 1A-type TNSALP mRNA expression. (B) The diagram shows the relative density of the products of 1A-type (A). (C) GAPDH primers detected GAPDH mRNA. S: molecular-size standard, *Hae*III digest of phai-X (New England Bio-lab). Lane 1, Pi (10 mM) treatment; lane 2, Pi (1 mM) treatment; lane 3, Pi (0 mM) treatment.

in ST2 cells and that the enzyme was 100% inhibited by heat treatment (60, 10 min). Moreover, ALP in the ST2 cells enhanced by Pi starvation of ST2 cells was effectively inhibited by Lev, and the enzyme was heat-labile. These inhibition pattern is typical of mammalian TNSALP isozyme.

Effects of Phosphate on ALP Expression in ST2 Cells

RNAs were extracted from ST2 cells, and RT-PCR analysis using specific primers was performed. As shown in Fig. 4, polyacrylamide gel electrophoresis (PAGE) analysis of the PCR products of ST2 cells at day 3 for the 1A-type transcripts in ST2 cells in 1 mM Pi medium revealed a band at 180 bp (Fig. 4A, lane 3). However, the band at 180 bp was not detected in the cells of the Pi-rich medium (10 mM) (Fig. 4A, lane 1). More than 150% enhancement by Pi starvation (0 mM) was obtained compared with the value in 1 mM Pi medium (Fig. 4B). 1B-type TNSALP mRNA at 194 bp was not detected in the ST2 cells even after Pi starvation treatment (data not shown). All bands for the mouse glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA showed essentially the same intensity (452 bp) (Fig. 4C).

DISCUSSION

In the case of *E. coli,* it is well-known that ALP is induced when the cells are starved of Pi; oppositely, a high concentration of Pi reduces ALP synthesis. Newly synthesized ALP under a low Pi condition is secreted into the periplasmic space to accelerate Pi transport into the cell. The Pi level in the environment modulates the "phosphate regulon system" in *E coli;* in other words, a promoter of the ALP gene is operated by Pi depletion (5). In mammalian cells, there is a short report indicating that Pi deprivation can stimulate the ALP synthesis in the rat osteoblastic cell line ROS 17/2.8 (19). In this study, we clarified that low Pi conditions regulated TNSALP activity in the mouse stromal cell line ST2 and targeted the transcription of ALP mRNA. Therefore, the "phosphate regulon system" modulated by environmental Pi may be a conserved system even in mammalian cells.

Multiple regulation mechanisms of TNSALP gene expression respond to environmental signals. The expression of the mouse TNSALP gene is regulated by the presence of two leader exons that are controlled by distinct promoters, resulting in the synthesis of two alternatively spliced mRNAs (1A and 1B types) (11). The exon 1A-derived transcript is expressed in the kidney, placenta and osteoblasts such as MC3T3-E1 cells (20). In the 5′-flanking region of the mouse TNSALP 1A exon, several consensus sequences were reported to be associated with the osteoblast differentiation including a cbfa1 site (21) and nine e-boxes (22). In this study, the ALP activity in the ST2 cells was increased by Pi depletion, and the expression of the bone-type TNSALP mRNA (1A) was enhanced by the same condition. Based on our findings, we could conclude that the extracellular Pi concentration is one of the important environmental signals regulating the TNSALP 1A gene expression.

The possible role of the extracellular Pi even for human TNSALP regulation could exist. The structural organization of the human gene is strikingly similar to the rat and mice TNSALP genes (16). The presence of two leader exons (bone-type and liver-type) and their promoters were described in the rat and human TNSALP gene. A series of our studies on the TNSALP expression in human cells have shown that the regulatory expression of the type specific mRNAs significantly resembles that of the mice counterparts (23–26). These findings show that the regulatory systems of the

TNSALP promoter have been highly conserved among mammalian cells.

The physiological roles of ALP have not been well understood, but informative findings should be provided by the human and mouse studies such as the rare genetic disease hypophosphatasia and mice lacking the TNSALP function (27–32). These findings provide ideas on the physiological roles of ALP; ALP is possibly supplying inorganic phosphate for Pi compensation by hydrolyzing the physiological substrate of the phosphate compounds. In clinical cases, high ALP activity in serum is commonly found under low Pi conditions such as familial hypophosphatemia as well as vitamin D-deficient rickets (33). Although a possible explanation for these symptoms must be based on several factors such as hormones, our findings from an *in vitro* study here might propose an *in vivo* compensatory mechanism for Pi starvation through the ALP induction by a low Pi level. Our proposal is based on these findings for the conserved mammalian TNSALP promoter and the physiological roles of ALP mentioned above.

In this paper, we present valuable data concerning ALP regulation by Pi levels in mammalian cells *in vitro*, and propose the physiological roles of mammalian ALP from the point of view of the Pi metabolism, which might be involved in the phylogenetically conserved "phosphate regulon system." Our findings raise questions mainly divided into two categories. The first question concerns the relationships between the Pi depletion and the other osteoblastic phenotypes, since ALP is also well known as an early marker of osteoblastic phenotypes. Our observations of the other phenotypes such as osteocalcin in this system were too complicated to understand these relationships. Since these osteoblastic markers were expressed after ALP induction, which could compensate for the Pi depletion, we could not analyze the direct effects of the Pi depletion on the other phenotypes. The other question is related to the cell type specificity of this phenomenon. Our pilot study indicated that the ALP seemed to be more clearly induced in premature osteoblastic cells than in mature osteoblastic cells, suggesting that the "phosphate regulon system" might target the premature osteoblasts *in vivo*. Even though ALPs are expressed in other tissues such as embryonic stem cells, kidney, liver, intestine, and placenta, the clinical symptoms strongly suggest that the major targeted cell of the Pi depletion might be the premature osteoblast. Further studies on the promoter region of the TNSALP gene and on the presence of the Pi sensing system in mammalian cells could provide the mechanism for the induction of ALP by Pi depletion and the full understanding of the physiological functions of the ALPs.

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