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Extracellular Inorganic Phosphate Regulates Gibbon Ape Leukemia Virus Receptor 2  
/Phosphate Transporter mRNA Expression In Rat Bone Marrow Stromal Cells

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## ABSTRACT

In mammalian cells, several observations indicate not only that phosphate transport probably regulates local inorganic phosphate (Pi) concentration, but also that Pi affects normal cellular metabolism, which in turn regulates apoptosis and the process of mineralization. To elucidate how extracellular Pi regulates cellular functions of pre-osteoblastic cells, we investigated the expression of type III sodium-dependent Pi transporters in rat bone marrow stromal cells and ROB-C26 pre-osteoblastic cells. The mRNA expression level of gibbon ape leukemia virus receptor (Glv-2) was increased by the addition of Pi in rat bone marrow stromal cells, but not in ROB-C26 cells or in normal rat kidney (NRK) cells. In contrast, the level of Glv-1 mRNA was not altered by the addition of extracellular Pi in these cells. The induction of Glv-2 mRNA by Pi was inhibited in the presence of cycloheximide. Moreover, mitogen-activated protein kinase (MEK) /extracellular-signal-regulated kinase (ERK) pathway inhibitors; U0126 and PD98059 inhibited inducible Glv-2 mRNA expression, but p38 mitogen-activated protein kinase inhibitor SB203580 did not inhibit the induction of Glv-2 mRNA expression, suggesting that extracellular Pi regulates *de novo* protein synthesis and

MEK/ERK activity in rat bone marrow stromal cells and through these, induction of Glvr-2 mRNA. Although Pi also induced osteopontin mRNA expression in rat bone marrow stromal cells but not in ROB-C26 cells and NRK cells, changes in cell viability with the addition of Pi were similar in both cell types. These data indicate that extracellular Pi regulates Glvr-2 mRNA expression, provide insights into possible mechanisms whereby Pi may regulate protein phosphorylation, and suggest a potential role for the Pi transporter in rat bone marrow stromal cells.

## INTRODUCTION

Inorganic phosphate (Pi) has a fundamental role in the biosynthesis of cellular components, including nucleic acids, proteins, lipids, and sugars (Torriani-Gorini et al., 1994). Phosphate and calcium are the major constituents of the mineral phase of cartilage, bone, and dentin, which suggests that regulatory mechanisms for the sensing and uptake of both ions might be present in the cells that are responsible for mineralizing these tissues (Wang et al., 2001). Montessuit *et al.* (1991, 1995) first identified and characterized a phosphate transport mechanism in matrix vesicles isolated from the growth plates of cartilage, and this phosphate transporter was sodium-dependent, sensitive to hormone stimulation and to phosphate deprivation. Recent researchers have reported that sodium-dependent Pi transporters are expressed in osteoblasts and chondrocytes, and are regulated by several factors such as insulin-like growth factor (IGF-1) (Campbell et al., 1992; Palmer et al., 1997), fluoride (Burgener et al., 1995), parathyroid hormone (PTH) (Caverzasio et al., 1996), platelet-derived growth factor (PDGF) (Zhen et al., 1997), protein kinase C (Jobbagy et al., 1999), basic fibroblast growth factor (bFGF) (Suzuki et al., 2000) and extracellular Pi (Kavanaugh et al., 1994;

Montessuit et al., 1995). Therefore, they have a role in regulating Pi handling in bone forming cells (Palmer et al., 1999; Wang et al., 2001).

#### Gibbon ape leukemia virus receptor (Glv<sub>r</sub>) –1 and Glv<sub>r</sub>-2, type III

Na-dependent Pi transporters, were originally identified as receptors for gibbon ape leukemia virus (O'Hara et al., 1990) and amphotropic murine retrovirus, respectively (Miller et al. and Zeijl et al., 1994). These two proteins are approximately 60% identical in amino acid sequence (Zeijl et al., 1994) and closely resemble each other in function and characterization (Kavanaugh and Kabat, 1996), but they represent no significant sequence homology with the type I and type II Na-dependent Pi transporters (Werner et al., 1991; Magagnin et al., 1993) that are expressed in kidney cells. Nielsen *et al* (2001) showed that Glv<sub>r</sub>-1 mRNA expression was increased during osteoblastic differentiation in MC3T3-E1 cells, indicating a relationship between Glv<sub>r</sub>-1 mRNA expression and osteoblastic differentiation. High levels of expression have also been demonstrated in the resting and proliferative zones of murine cartilage growth plates, but a lack of Glv<sub>r</sub>-1 mRNAs detected in fully differentiated hypertrophic chondrocytes indicates that Glv<sub>r</sub>-1 is selectively expressed in a subset of hypertrophic chondrocytes during endochondral

bone formation (Palmer et al., 1999).

Pi can directly regulate expression of certain mRNAs in yeast (Ogawa et al., 2000) and in cultured mammalian cells (Kido et al., 1999). In *Saccharomyces cerevisiae*, the PHO regulatory pathway (the inorganic phosphate metabolic system in yeast) is involved in the uptake of Pi (Ogawa et al., 2000). When extracellular Pi concentrations are low, several genes are transcriptionally induced by this pathway, which induces the Pho4 transcriptional activator. However, the roles of Pi in the regulation of mRNA expression in mammalian cells have not been clearly established.

In this study we show that addition of Pi enhances Glvr-2 but not Glvr-1 mRNA expression in rat bone marrow stromal cells. In contrast, in both ROB-C26 cells (a calvaria-derived potential osteoblast precursor cell line; Yamaguchi et al., 1991) and normal rat kidney (NRK) cells (Nemir et al., 1989), the addition of Pi has no effect on the induction of Glvr-2 mRNA expression. Also, we show that the induction of Glvr-2 mRNA results from activation of the mitogen activated protein kinase (MAPK) pathway by the addition of Pi. Furthermore, in rat bone marrow stromal cells, osteopontin mRNA levels appear to be positively regulated by the addition of Pi to the culture medium.

## **MATERIALS AND METHODS**

### **Cell cultures**

Rat bone marrow stromal cells were harvested from 6-week old wistar rat femurs using the method described by Maniopoulos *et al* (1988). Cells were plated at a density of  $1 \times 10^6$  cells per/cm<sup>2</sup> in alpha modified minimum essential medium ( $\alpha$ -MEM; Invitrogen, Carlsbad, CA) containing 200 mg/l of kanamycin monosulfate (Sigma, St. Louis, MO) with 15% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) in 100-mm culture dishes (Corning, New York, NY) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. All experiments using rat bone marrow stromal cells were performed only on the first passages. ROB-C26 cells (Yamaguchi *et al.*, 1991) were kindly provided by Dr. A. Yamaguchi (Nagasaki University, Nagasaki, Japan). NRK cells (Nemir *et al.*, 1989) were purchased from RIKEN Cell Bank (Tsukuba, Japan). ROB-C26 and NRK cells were cultured in  $\alpha$ -MEM containing 10% FBS at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

## **Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from the cells using the Rneasy Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Qiagen) to remove any contaminating genomic DNA. Complementary DNA (cDNA) was synthesized with omniscrypt reverse transcriptase (Qiagen) using (dT)<sub>15</sub> primer (1 μM). Subsequent amplification for the detection of rat Glvr-1 and Glvr-2 cDNAs was performed for 30 cycles under the following conditions: 94°C for 25 sec, 58°C for 30 sec, and 72°C for 1 min. The detection of rat osteopontin cDNA was performed for 30 cycles under the following conditions: 94°C for 25 sec, 55°C for 30 sec, and 72°C for 1 min and of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed for 25 cycles under the following conditions: 94°C for 25 sec, 53°C for 30 sec, and 72°C for 1 min. The primer sequences used for PCR amplification were designed based on cDNA sequences of rat Glvr-1 (Tatsumi et al., 1998) (Genbank AB000489, 2352-2757 nt), Glvr-2 (Miller et al., 1994) (Genbank NM\_017223, 975-1311 nt), osteopontin (Singh et al., 1992) (Genbank NM\_012881, 638-1177 nt) and GAPDH (Tso et al., 1985) (Genbank NM\_017008, 34-333 nt). The primer sequences used (each at a final concentration of 1

pM) were 5'-TCC CTT TTC TCC TGG GCT AT-3' and 5'-CTT AGC AAC ACG TCC ACG AA-3' for Glvr-1, generating a 406 bp fragment; 5'-GTC CGT GGA TGA AGA GGA AA-3' and 5'-GTC ATT CCT CAT GTG GCC TT-3' for Glvr-2, generating a 337 bp fragment; 5'-ATC AAG GTC ATC CCA GTT GC-3' and 5'-TCA GGG CCC AAA ACA CTA TC-3' for osteopontin, generating a 540 bp fragment; 5'-GTG AAG GTC GGT GTC AAC G-3' and 5'-GGT GAA GAC GCC AGT AGA CTC-3' for GAPDH, generating a 300 bp fragment. All these primers were synthesized by Hokkaido System Science (Sapporo, Japan). Amplified products were visualized by agarose gel electrophoresis after staining with ethidium bromide. PCR experiments were performed using samples from at least three different cell preparations and results were confirmed by triplicate PCR experiments from the same cell samples.

### **Quantitative real time RT-PCR**

Quantification of rat Glvr-1, Glvr-2, osteopontin and GAPDH mRNAs was performed using a Smart Cycler<sup>®</sup> apparatus (Cepheid, Sunnyvale, CA). One µg of each RNA sample was subjected to reverse transcription using the omniscrypt reverse

transcriptase and RNase-free DNase Set (Qiagen) in a total volume of 20  $\mu$ l. Then, two  $\mu$ l of the reaction mixture were incubated with the double-stranded DNA dye SYBR<sup>®</sup> Green I (Takara biochemicals, Shiga, Japan) in a total volume of 25  $\mu$ l. The primers used for detection were as described above. All reactions were run in a Smart Cycler<sup>®</sup> PCR machine with a hot start pre-incubation step of 30 sec at 95°C, following by cycles of 5 sec at 95°C, 15 sec at 60°C, and 15 sec at 72°C. We performed these experiments using samples from at least three different cell preparations and quantification of mRNA was confirmed using the same cell sample at least in triplicate. The amount of template was quantified using the second derivative maximum method as included in the Smart Cycler<sup>®</sup> software package. The second-derivative method was used to calculate the threshold cycle. Melting curve analysis showed a single sharp peak for all samples.

### **Preparation of cDNA templates for riboprobe**

Total RNA was isolated from rat bone marrow stromal cells using Isogen (Nippongene, Tokyo, Japan), and the RNA was reverse-transcribed using reverse transcriptase (TaKaRa Biomedicals, Shiga, Japan) and oligo (dT)<sub>15</sub> primers according to

the manufacturer's instructions. Subsequently, cDNAs of rat osteopontin and rat GAPDH were amplified with a PCR profile of 95°C for 30 sec, 60°C for 30 sec and 72°C for 90 sec for 40 cycles followed by heating at 72°C for 7 min. Primer sequences from 5' to 3' for PCR were 5'-ATG AGA CTG GCA GTG GTT-3' and 5'-GCT TTC ATT GGA GTT GCT-3' for osteopontin, generating a 243 base pair (bp) fragment (Oldberg et al., 1986); and 5'-CCA TGG AGA AGG CTG GGG-3' and 5'-CAA AGT TGT CAT GGA TGA CC-3' for GAPDH, generating a 194 bp fragment (Yaoita et al., 2000). For subsequent Northern hybridization studies, amplified PCR products were subcloned into the pGEM<sup>®</sup>-T Vector (Promega, Madison, WI) using the pGEM<sup>®</sup>-T Vector System II and the sequences of the subcloned cDNAs were checked by a DNA sequencer (Prism 310, Applied Biosystems, Foster city, CA). The digoxigenin (DIG) labeled RNA probes for Northern blot hybridization analysis were prepared with a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturers' instructions.

### **Northern blot analysis**

Total RNA (15 µg per lane) was electrophoresed in 1.0% agarose gels in 7.4%

formaldehyde and transferred to a nylon membrane (Zeta-Probe<sup>®</sup> Membranes; Bio-Rad, Hercules, CA) using a pressure blotter (Stratagene, La Jolla, CA). The membranes were UV cross-linked, and then prehybridized at 69°C for 2 h in buffer (50% formamide, 5 X saline-sodium citrate buffer [SSC (pH7.0): 3 M NaCl, 0.3 M trisodium citrate, 50 mM NaPO<sub>4</sub> (pH 7.0), 2% blocking buffer, 0.1% laurylsarcosine, 7% sodium dodecyl sulfate (SDS), and 100 µg/ml of salmon sperm DNA]. Hybridization was performed at 69°C for 15 h with DIG-11-dUTP labeled RNA probe in the same buffer. After hybridization, the membranes were washed in 2 X SSC containing 0.1% SDS for 3 times 15 min at room temperature, followed by 0.1 x SSC containing 0.1% SDS for 3 times 15 min at 75°C. Signals were detected by chemiluminescence using a detection kit with disodium 3-(4-methoxyspiro {1, 2-dioxetane-3, 2'-(5'-chloro) tricyclo [3.3.1.1<sup>3,7</sup>] decan}-4-yl) phenyl phosphate (CSPD) as substrate (Roche Diagnostics, Mannheim, Germany).

### **MTT cell viability assay**

This assay is based on the ability of viable mitochondria to convert MTT (3-(4,5-dimethyl-thiazoyl-2-yl) 2, 5-diphenyltetrazolium bromide), a water-soluble

tetrazolium salt, into a water-insoluble formazan precipitate. This conversion is catalyzed by cellular mitochondrial dehydrogenase. Because the rate of this reaction is proportional to the number of surviving cells, the MTT assay is widely used to quantify viable cells. To observe the effect of Pi on cell viability, subconfluent proliferating cells in 24-well plates were exposed to 20 mM inorganic phosphate before culture. Cells were washed and then incubated with 100  $\mu$ l of MTT at 37°C for 3 h. The reagent was removed, and 100  $\mu$ l of isopropanol/HCl (400  $\mu$ l of HCl plus 100 ml of isopropanol) was added to each well. The solution was mixed and the absorbance of each well was determined at 570 nm using a micro plate reader (Tosoh, Shinnanyou, Japan). All MTT assays were repeated at least three times in triplicate.

#### **Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay**

To detect cells undergoing apoptosis, a colorimetric TUNEL system (Promega, Madison, WI) was used. After subconfluent proliferating cells were exposed to 20 mM inorganic phosphate for 24 h, the cells were washed with 10 mM PBS and fixed with 4%

paraformaldehyde in 0.1 M sodium phosphate buffer. Cells were then washed with 10 mM PBS and permeabilized with Triton X-100 for 5 min. After a rinse with 10 mM PBS, each slide was equilibrated and then incubated with terminal deoxynucleotidyl transferase at 37 °C for 60 min. The reaction was stopped with 2 × SSC, and the cells were examined under a microscope.

## **Reagents**

PD98059 (2'-Amino-3'-methoxyflavone), DMSO (Dimethyl sulphoxide), phosphonoformic acid (PFA) and MTT were purchased from Sigma (St. Louis, MO). U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]butadiene) and SB203580 [4-(4'-fluorophenyl)-2-(4'-methyl-sulfinylphenyl)-5-(4'pyridyl)imidazole] were purchased from Promega (Madison, WI).

## **Statistical analysis**

All experiments were repeated three to six times. Values were calculated as the means ± standard deviation (SD). Some data were subjected to two-way analysis of

variance (ANOVA), as indicated in the results, and two-way Student's *t*-test was used for analysis of the difference between the groups tested. A *P* value of less than 0.05 was accepted as significant.

## **RESULTS**

### **Inorganic phosphate (Pi) regulates type III sodium-dependent phosphate transporter mRNAs in rat bone marrow stromal cells**

We first examined the effects of 24 h exposure to extracellular Pi concentrations from 0 to 30 mM on the expression of Glvr-1 and -2, type III sodium-dependent phosphate transporters, in rat bone marrow stromal cells. RT-PCR analysis of total RNA isolated from these cells revealed the presence of transcripts of Glvr-1 and -2. Glvr-2 mRNA expression was dramatically and dose-dependently increased by the addition of 5-20 mM Pi, while Glvr-1 mRNA expression did not change (Fig. 1). We were unable to detect either NaPi-2a or NaPi-2b mRNA expression in these cells by RT-PCR or quantitative real-time RT-PCR analysis (data not shown). We next examined Glvr-1 and -2, sodium-dependent Pi transporter mRNAs, in the rat undifferentiated mesenchymal cell line ROB-C26 cells and in NRK cells. These two cell types expressed Glvr-1 and Glvr-2 mRNAs, but there was no change in either with the addition of Pi (Fig. 2).

## **Analysis of time dependent changes in type III sodium-dependent phosphate transporter mRNAs in response to the addition of Pi**

Time dependent changes in Glvr-2 mRNA expression in response to the addition of Pi revealed that expression was up regulated after 6 h then remained constant up to 72 h in rat bone marrow stromal cells (Fig. 3A). To further confirm the specific induction of expression of these transcripts by Pi, we performed quantitative real-time RT-PCR analysis. The Glvr-2 mRNA level was significantly increased to about 2-fold within 6 h and 3-fold within 12 h compared to no Pi controls, after which levels were maintained up to 72 h, while Glvr-1 mRNA levels remained constant after the addition of Pi, indicating a selective activation of Glvr-2 mRNA expression in response to Pi (Fig. 3B).

## **Involvement of protein synthesis, the MAPK signaling pathway and phosphate transport in induction of Glvr-2 mRNA expression by Pi in rat bone marrow stromal cells**

To examine the potential role of protein synthesis in the induction of Glvr-2

mRNA expression by Pi, we used the inhibitor of protein synthesis, cycloheximide to block *de novo* synthesis of protein. With the addition of cycloheximide, up-regulation of the Glvr-2 mRNA level was reduced. In contrast there was no change in the Glvr-1 mRNA level during a 24 h culture period in the presence of cycloheximide (Fig. 4A). The MAPK signaling pathway was previously suggested to mediate the expression of Glvr-1 by growth factors in osteoblast-like cells. To further address the mechanism and involvement of MAPK by which Pi induces Glvr-2 mRNA expression in rat bone marrow stromal cells, we used three MAPK inhibitors; PD98059 to block mitogen-activated protein kinase (MEK), U0126 to block activation of ERK1/2 and p38 MAPK inhibitor and SB203580 to block p38 MAPK activity. PD98059 reduced the response of Glvr-2 mRNA induction in these cells (Fig. 4A). Similar results were obtained with U0126 producing a reduction in inducible mRNA levels. However, SB203580 increased steady-state Glvr-2 mRNA levels, and there was no statistically significant induction of Glvr-2 mRNA levels in the presence of Pi (Fig. 4A). In order to examine the involvement of sodium-dependent phosphate transport on the induction response of Glvr-2 mRNA, we used PFA, a pyrophosphate analog of the specific phosphate transport

inhibitor; to block phosphate transport in these cells. PFA did not reduce the induction of Glvr-2 mRNA observed with addition of Pi (Fig. 4B).

### **Regulation of osteopontin mRNA expression by the addition of Pi in rat bone marrow stromal cells, ROB-C26 cells and NRK cells**

To elucidate whether Pi exerts effects on the mRNA expression of osteopontin in rat bone marrow stromal cells, the cells were cultured with various concentrations of inorganic phosphate for 72 h. Treatment with 25 mM inorganic phosphate dramatically up-regulated osteopontin mRNA levels, with a further increase at 30 mM inorganic phosphate (Fig. 5A). At various time points ranging from 1-72 h, the temporal expression level of osteopontin mRNA was examined by Northern blot analysis. Fig. 5B shows that there was no marked increase following the addition of inorganic phosphate in these cells up to 24 h. However, at time periods beyond 48 h, there was an up-regulation in osteopontin mRNA expression (Fig. 5B). As internal control, mRNA levels of the housekeeping gene GAPDH are not subject to regulation by treatment with inorganic phosphate in culture. To further confirm the specific induction of expression of the

osteopontin transcripts by Pi, we performed quantitative real time RT-PCR analysis. The osteopontin mRNA levels were significantly higher with the addition of Pi in rat bone marrow stromal cells at 72 h, averaging  $7.5 \pm 2.1$ -fold (Fig. 6). To explore the possibility that Pi induces osteopontin mRNA expression in other cells, ROB-C26 cells and NRK cells, which express osteopontin mRNA, were examined for osteopontin mRNA expression after treatment with Pi. As shown in Fig. 6, osteopontin mRNA levels were not regulated by the addition of 20 mM inorganic phosphate for 72 h in culture in ROB-C26 cells or NRK cells.

### **Effect of Pi on cell viability and apoptosis in rat bone marrow stromal cells,**

#### **ROB-C26 cells and NRK cells**

Since it has been shown that Pi reduces cell viability, we assessed the effect of Pi on viable cell numbers and apoptosis. In the presence of 20 mM medium Pi concentration for 72 h in culture, the cell viability of rat bone marrow stromal cells is about 86.5% compared with untreated cells (Fig. 7A). In either ROB-C26 cells or NRK cells, the cell viability was also decreased by treatment with 20 mM inorganic phosphate,

to 70% and 55%, respectively (Fig. 7A). In the presence of 30 mM medium Pi concentration for 72 h in culture, viable cells are reduced to almost 50% in rat bone marrow stromal cells, ROB-C26 cells and NRK cells (Fig. 7A). Moreover, in the presence of 20 mM medium concentration of Pi for 24 h, DNA fragmentation was detected by TUNEL assay in rat bone marrow stromal cells (Fig. 7B). In contrast, no DNA fragmentation was observed in these cells without Pi treatment (Fig. 7B).

## DISCUSSION

In the current study, we show that the addition of extracellular Pi regulates mRNA expression of *Glv-2*, one of the type III Na-dependent Pi transporters, and of osteopontin, an extracellular matrix protein, in rat primary bone marrow stromal cells, but not in ROB-C26 cells or NRK cells.

Phosphate is an essential molecule for all organisms and regulatory mechanisms have evolved for the acquisition, storage, and release of phosphate. In the yeast *Saccharomyces cerevisiae*, the PHO regulatory pathway regulates expression of ‘PHO’ genes, which are critically involved in the scavenging and specific uptake of Pi from extracellular sources (Oshima, 1997; Ogawa et al., 2000). The PHO regulatory system consists of at least five regulatory proteins, the Pho-81 CDK inhibitor, the Pho80-Pho85 cyclin-cyclin dependent protein kinase complex, and Pho2 and Pho4 transcription factors, which have been shown to contribute to Pi acquisition and phosphate metabolism in yeast. Also, in mammalian cells it is known that extracellular Pi regulates not only Pi uptake but also cellular functions. For example, high extracellular Pi increases IGF-1 mRNA within 12 h in MC3T3-E1 cells (Kanatani et al., 2002). High

Pi concentration induces parathyroid hormone (PTH) mRNA and secretion of PTH in parathyroid tissue *in vivo* and *in vitro* while parathyroid vitamin D receptor mRNA levels are not modified (Kilav et al., 1995; Hernandez et al., 1996; Almaden et al., 1998). In our study, the difference in dose and time responsiveness in the induction of Glvr-2 and osteopontin mRNA expression with the addition of Pi to the culture medium of rat bone marrow stromal cells, suggests diverse inductive mechanisms by which extracellular Pi may regulate expression of these genes in mammalian cells. Since there is no significant difference in the reduction of cell viability with Pi treatment between the three cell lines observed, the authors conclude that the increases in Glvr-2 and osteopontin mRNA levels can not be associated only with their changes in viability in response to Pi.

Induction of Glvr-2 and osteopontin mRNA by Pi is observed only in rat bone marrow cells, not in ROB-C26 cells or NRK cells. This shows that changes in gene expression in response to Pi are cell specific. Here, we demonstrate that the MEK/ERK pathway but not the p38 MAPK pathway is involved in the induction of Glvr-2 mRNA expression by Pi in rat bone marrow stromal cells, suggesting that extracellular Pi regulates intracellular kinase activities in these cells. In this study, we could not

determine what cellular mechanisms or molecules induce the MEK/ERK pathway with the addition of Pi. Similar molecules to the PHO pathway in yeast may mediate the cellular Pi-response in certain mammalian cells. Genistein could not block Pi-induced Glvr-2 mRNA expression in rat bone marrow cells (data not shown), indicating that protein tyrosine kinases that are inhibited by genistein do not mediate Glvr-2 mRNA induction by Pi in such cells. Furthermore, addition of PFA, a sodium-dependent phosphate transport inhibitor, did not block the induction of Glvr-2 mRNA expression by Pi, suggesting that phosphate transporter(s) could not mediate the effects of Pi on Glvr-2 mRNA expression in these cells.

Although the stromal cells of bone marrow originally support hematopoiesis of blood cells such as erythrocytes, platelets, monocytes, granulocytes, and lymphocytes *in vivo*, mesenchymal stem cells are also found among these cells and they are able to differentiate into several cell types, not only mesenchymal cells such as osteoblasts, chondrocytes, and adipocytes (Prockop, 1997; Pittenger et al., 1999) but also neural cells (Azizi et al., 1998) and endothelial cells (Reyes et al., 2002), *in vivo* and/or *in vitro*. On the other hand, ROB-C26 cells are a clonal established cell line isolated from rat calvaria

and are known as a potential mesenchymal precursor cell line that is also capable of differentiating into osteoblasts, muscle cells and adipocytes (Yamaguchi et al., 1991).

Therefore, extracellular Pi concentration contributes to the regulation of certain cellular functions associated with Glvr-2 mRNA induction in bone marrow stromal cells. In this study, mRNA levels of other matrix proteins such as collagen  $\alpha$ 1(I), bone sialoprotein and osteocalcin were not significantly changed by the addition of Pi to rat bone marrow stromal cells (data not shown), suggesting a specific role in the induction of osteopontin mRNA by extracellular Pi in bone marrow stromal cells but not calvaria- or kidney-derived cells. Osteopontin is expressed in a variety of cells of connective tissue lineages. Although expression of this gene has been demonstrated in multiple tissues including bone marrow stromal cells, its function is not well understood (Denhardt et al., 2001). This protein is secreted into the mineralizing extracellular matrix by osteoblasts during bone development and it is believed to facilitate the attachment of cells to the extracellular matrix. Several other functions have been suggested for osteopontin; its protein levels are up-regulated during cell injury and are associated with the pathology of many tissue diseases. In our experiments, apoptotic cell death was detected in rat bone

marrow stromal cells following treatment with Pi. The induction of osteopontin in direct response to increased phosphate levels suggests it may be associated with this cellular damage in bone marrow stromal cells.

Pi is essential for the formation and mineralization of calcified tissues such as bones and teeth. Pi enters cells through a sodium-dependent phosphate transport system present in the plasma membrane. In cultured osteogenic cells and matrix vesicles in mineralized tissues, several observations suggest that Pi transport plays an important role in the initiation of extracellular matrix calcification. Although the mRNA of type III Pi transporters (Glv-1 and -2) are expressed in rat bone marrow stromal cells, type I and II Pi transporter mRNAs were not detected in these cells by RT-PCR analysis (data not shown). Therefore, Glv-1 and -2 may mediate Pi transport in bone marrow stromal cells. Induction of Glv-2 mRNA by the addition of Pi indicates a possibility that Glv-2 may regulate Pi transport in these cells in cases of high extracellular Pi concentration. Several reports have described that expression of Glv-1 and -2 mRNA is increased by phosphate deprivation. Kavanaugh *et al.* (1994) reported that the mRNA levels of Glv-1 and -2 increase in rat fibroblastic 208F cells in response to Pi removal from the culture medium.

Furthermore, in response to Pi removal in culture, the Glvr-2 mRNA level increases in human osteosarcoma-derived HOS and 143B cells but not in HeLa and U937 cells (Chien *et al.*, 1997), suggesting that the response of Glvr-2 mRNA induction to Pi depletion is dependent upon a cell-type specific phenomenon. Our results provide a new insight into the regulation of the phosphate transporter by Pi; not only Pi depletion but also Pi addition up-regulates Glvr-2 mRNA expression in certain mammalian cells.

Extracellular phosphate ions also cause apoptosis and this role may be linked to chondrocyte maturation and mineralization of the extracellular matrix. In our study, apoptotic cell death was detected with the addition of Pi to the culture medium of rat bone marrow stromal cells. Meleti *et al.* (2000) showed that Pi induced osteoblast apoptosis and this effect was blocked by treating the cells with phosphonoformic acid, an inhibitor of the plasma-membrane Na-Pi transporter. Jono *et al.* (2000) showed dose-dependent increases in mineral deposition from phosphate-containing media in human aortic smooth muscle cell cultures, and these effects were mediated by Glvr-1, the specific transporter through which Pi enters the cells, suggesting that the phosphate transporter may directly stimulate cells to undergo phenotypic changes that predispose to calcification and

offering a novel explanation of the phenomenon of vascular calcification under hyperphosphate conditions. Recently, Wang *et al.* (2001) demonstrated that regulation of the expression and activity of Glvr-1 by extracellular phosphate was restricted to an early stage of differentiation of CFK2 cells, as evidenced by expression of type II collagen, proteoglycan, and Ihh. The above investigations and our results suggest that phosphate and the phosphate transporter may regulate differentiation and cellular functions in bone marrow stromal cells.

In conclusion, the results of this study suggest that the phosphate transporter Glvr-2 is up-regulated by extracellular Pi and has putative physiological significance for osteoblastic differentiation in rat bone marrow stromal cells. The physiological role of this regulatory process may be important in the control of matrix calcification of cells derived from bone marrow stroma.

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## FIGURE LEGENDS

Fig. 1 Dose dependent changes in mRNA expression of type III phosphate transporters in rat bone marrow stromal cells *in vitro* with the addition of Pi.

Rat bone marrow stromal cells were plated at  $6 \times 10^5$  cells/cm<sup>2</sup> in 100 mm culture dishes.

After cells reached 70-80% confluence, the medium was changed and 0-30 mM

Tris-phosphate was added, after which cells were cultured for a further 24 h. Total

cellular RNA was isolated, and RT-PCR analysis was performed as described in Materials

and Methods. PCR was carried out with primers for rat Glvr-1, -2 and GAPDH cDNA.

The data shown represent one of three independent experiments with similar results.

Lane 1: 0 mM Pi, lane 2: 5 mM Pi, lane 3: 10 mM Pi, lane 4: 20 mM Pi, lane5: no reverse transcriptase control.

Fig. 2 Expression of type III phosphate transporters mRNA with the addition of Pi in rat bone marrow stromal cells, ROB-C26 cells and NRK cells.

Rat bone marrow stromal cells, ROB-C26 cells, and NRK cells were plated at  $6 \times 10^5$

cells/cm<sup>2</sup> in 100 mm culture dishes. After cells reached 70-80% confluence, the medium

was changed and 20 mM Tris-phosphate (+) or vehicle (-) was added. Cells were then cultured for a further 24 h, after which total cellular RNA was isolated, and RT-PCR analysis was performed as described in Materials and Methods. The data shown represent one of three independent experiments with similar results.

Fig. 3 Time dependent changes in mRNA expression of type III phosphate transporters in rat bone marrow stromal cells in vitro with the addition of Pi.

(A) Rat bone marrow stromal cells were plated at  $6 \times 10^5$  cells/cm<sup>2</sup> in 100 mm culture dishes. After cells reached 70-80% confluence, the medium was changed and 20 mM Tris-phosphate was added, after which the cells were cultured for a further 0-72 h. Total cellular RNA was isolated, and RT-PCR analysis was performed as described in Materials and Methods. (B) Real-time PCR was carried out with primers for rat Glvr-1, -2 and GAPDH cDNA. Expression levels were normalized to the level of GAPDH mRNA, and are shown as fold change compared to the GAPDH mRNA expression level. The data shown represent one of three independent experiments with similar results.

Fig. 4 Effects of cycloheximide, MAPK inhibitors and a sodium-dependent phosphate transport inhibitor on Pi- induced Glvr-2 mRNA expression in rat bone marrow stromal cells.

(A) After cells reached 70-80% confluence, rat bone marrow stromal cells were cultured for 24 h with 20 mM Tris-phosphate in the presence or absence of 10 µg/ml cycloheximide (CHX), 20 µM PD98059, 0.5 µM U0126, or 1 µM SB203580 in α-MEM supplemented with 15% FBS. (B) After cells reached 70-80% confluence, rat bone marrow stromal cells were cultured for 12 h with 20 mM Tris-phosphate in the presence or absence of 2 mM phosphonoformic acid (PFA) in α-MEM supplemented with 15% FBS. Then, total cellular RNA was isolated, and RT-PCR analysis was performed as described in Materials and Methods. These data were evaluated from one of three independent experiments with similar results.

Fig. 5 Induction of osteopontin mRNA expression by Pi in rat bone marrow stromal cells.

Cells were plated at  $5 \times 10^5$  cells/cm<sup>2</sup> in 60 mm cell culture dishes and cultured in

$\alpha$ -MEM supplemented with 15% FBS. After the cells reached 70-80% confluence, they were incubated with Tris-phosphate at the concentration indicated for 72 h (A), or with 30 mM Tris-phosphate for the period indicated. (B). Total cellular RNA was isolated, and Northern blot analysis was performed (15  $\mu$ g of total cellular RNA per lane) using a DIG-11-labeled osteopontin RNA probe as described in Materials and Methods. Equal loading in all lanes was confirmed by hybridization with DIG-11-labeled GAPDH as a probe. Arrows indicate the positions of 28 S and 18 S ribosomal RNA.

Fig. 6 Quantification of osteopontin mRNA expression levels in rat bone marrow stromal cells, ROB-C26 cells and NRK cells with the addition of Pi.

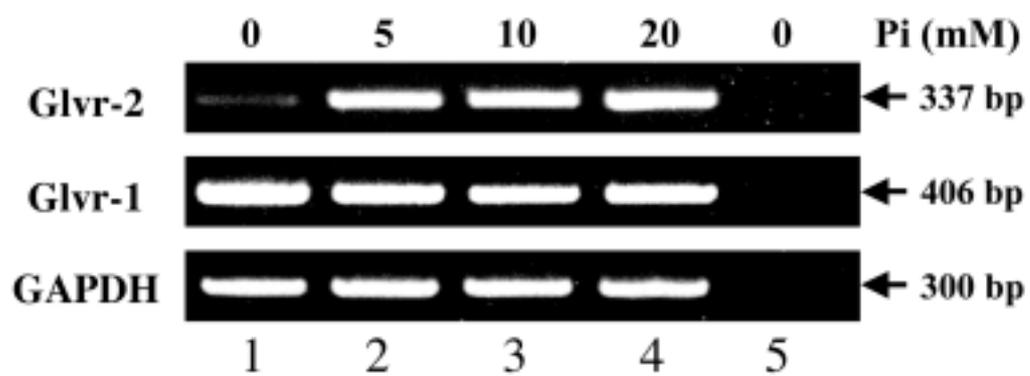
After cells reached 70-80% confluence, the medium was changed and 30 mM Tris-phosphate (+) or vehicle (-) was added, after which cells were cultured for a further 72 h. Total cellular RNA was extracted and reverse transcribed (2  $\mu$ g from each sample), and then real-time PCR was performed using primers for osteopontin cDNA. Expression levels were normalized to the level of GAPDH mRNA, and are shown as fold change compared to rat bone marrow stromal cells in the absence of Tris-phosphate. The data

shown represent one of three independent experiments with similar results and are shown as mean  $\pm$  S.D. \*P < 0.01 compared with vehicle.

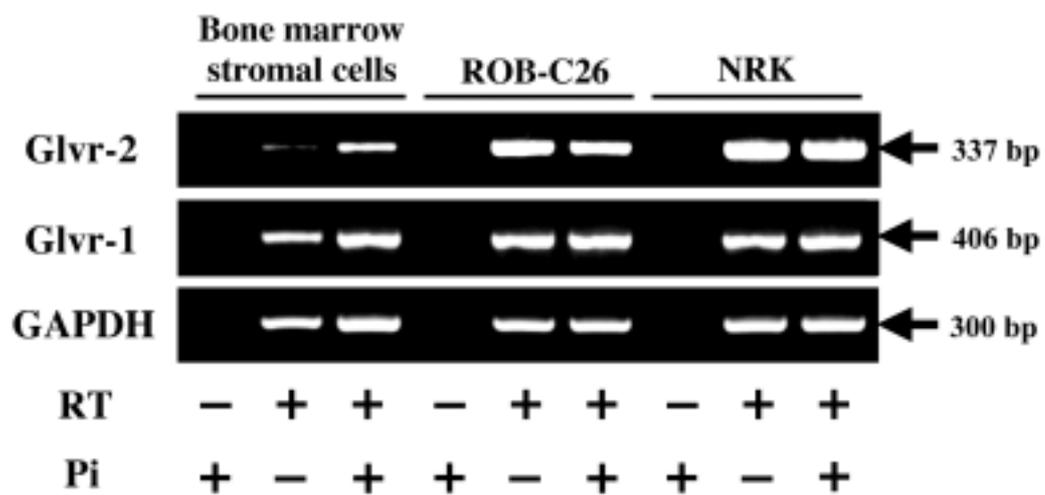
Fig. 7 Cell viability and apoptosis of rat bone marrow stromal cells, ROB-C26 cells, and NRK cells in culture with the addition of Pi.

Rat bone marrow stromal cells, ROB-C26 cells, and NRK cells were plated at  $5 \times 10^5$  cells/cm<sup>2</sup> in 35 mm culture dishes. (A) After cells reached 70-80% confluence, the medium was changed and 0-30 mM Tris-phosphate (+) or vehicle (-) was added. Cells were then cultured for a further 72 h. Cell viabilities were measured by the MTT method as described in Materials and Methods. \*P < 0.05 compared with vehicle. (B) After cells reached 70-80% confluence, the medium was changed and 20 mM Tris-phosphate (right) or vehicle (left) was added. Cells were then cultured for a further 24 h. The TUNEL assay was performed as described in Materials and Methods.

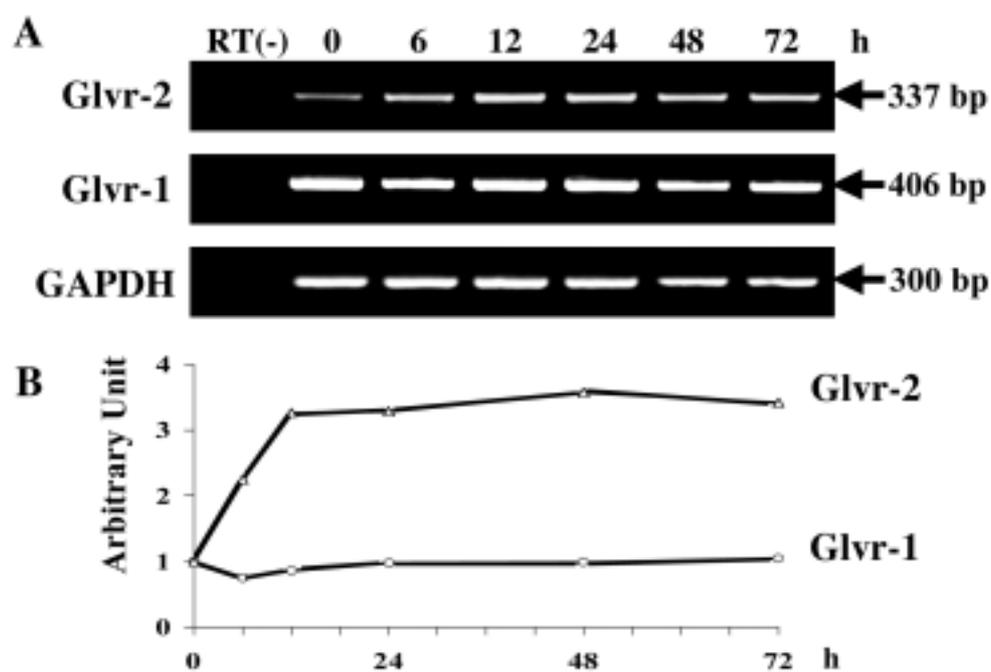
**Fig. 1**



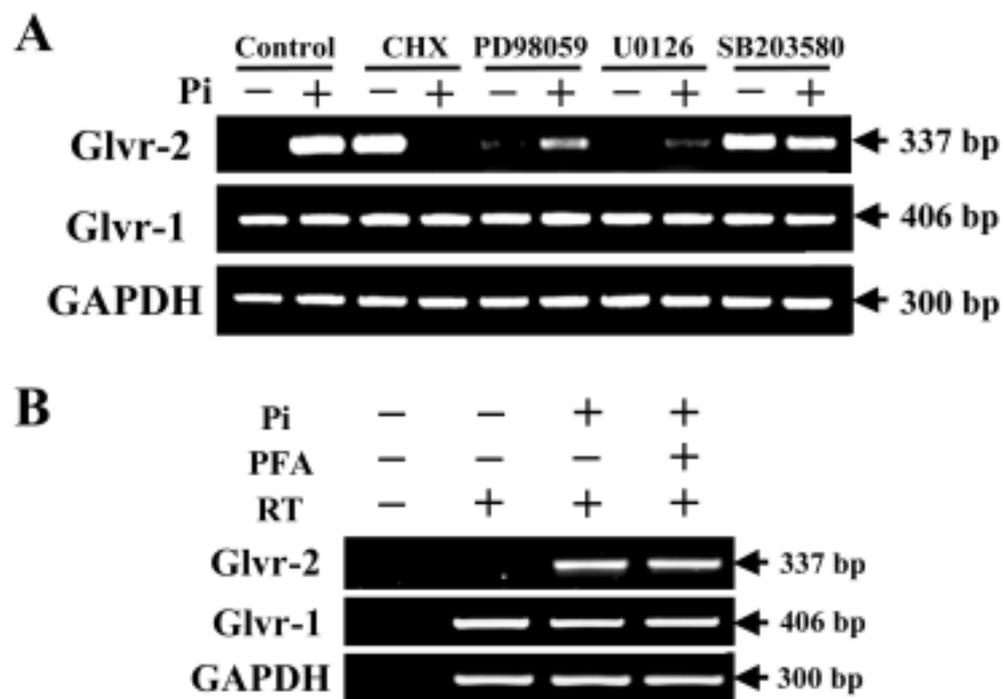
**Fig. 2**



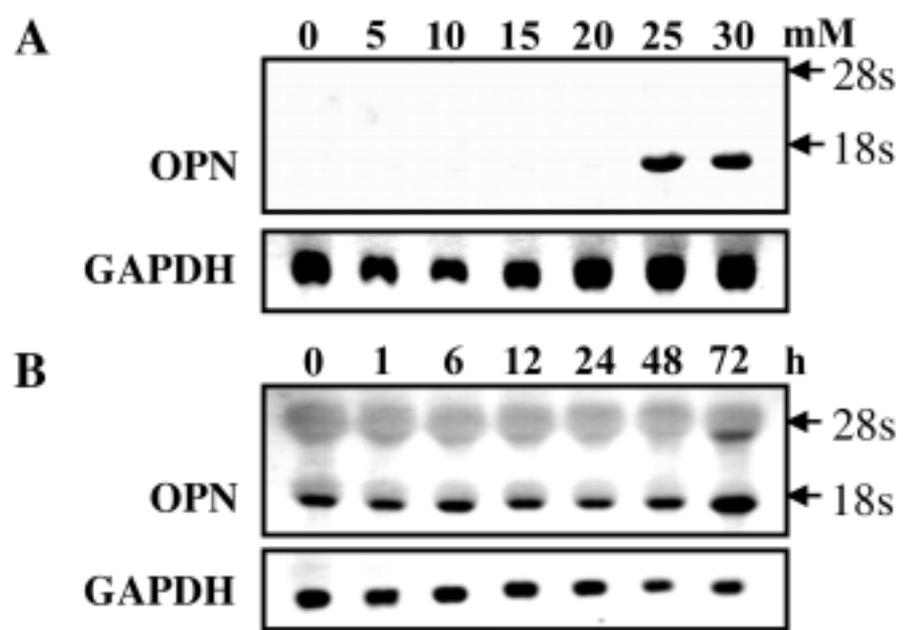
**Fig. 3**



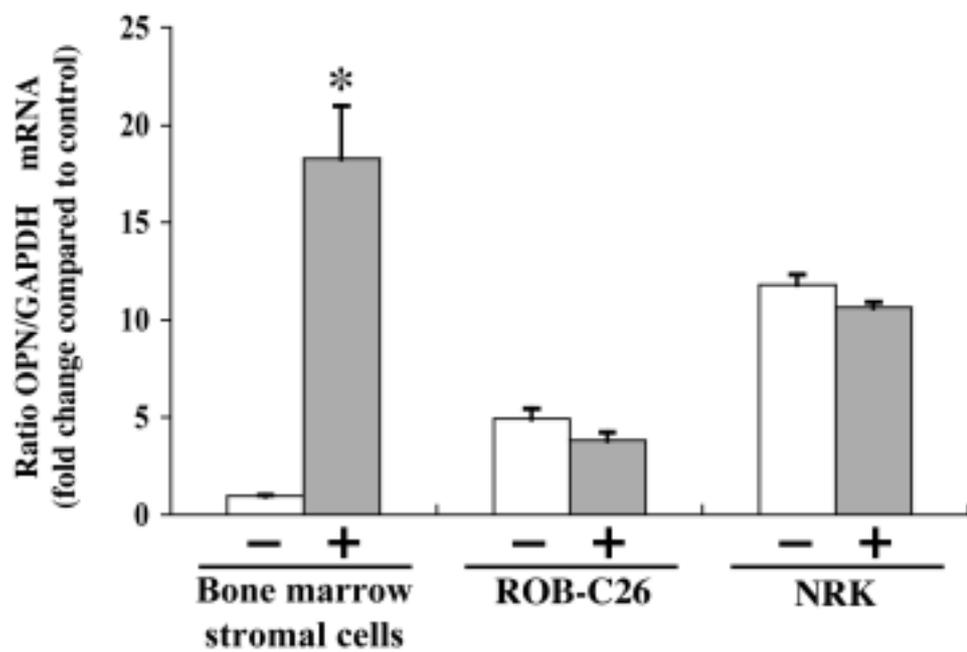
**Fig. 4**



**Fig. 5**



**Fig. 6**



**Fig. 7**

