Osteopontin Regulation by Inorganic Phosphate Is ERK1/2-, Protein Kinase C-, and Proteasome-dependent*

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The generation of inorganic phosphate by alkaline phosphatase during osteoblast differentiation represents an important signaling event, although the molecular and cellular consequences are currently undefined. We have previously described osteopontin as a gene regulated by an increase in inorganic phosphate not only in osteoblasts but also in other cell types. We describe here the identification of specific signaling pathways required for the stimulation of osteopontin expression by inorganic phosphate. We have determined that phosphate selectively activates the extracellular signal-regulated kinase (ERK1/2) signaling pathway but does not activate the other mitogen-activated protein kinase signaling proteins, p38, or the c-Jun N-terminal kinase. In addition, our results suggest that cellular exposure to 10 mM inorganic phosphate causes a biphasic ERK1/2 activation. The second ERK1/2 activation is required for osteopontin regulation, whereas the first is not sufficient. Analysis of common protein kinase families has revealed that phosphate-induced osteopontin expression specifically uses a protein kinase C-dependent signaling pathway. In addition, our results suggest that protein kinase C and ERK1/2 are not part of the same pathway but constitute two distinct pathways. Finally, we have determined that the proteasomal activity is required not only for phosphate-induced expression of osteopontin but also for the induction of osteopontin in response to 12-O-tetradecanoylphorbol 13-acetate and okadaic acid. The data presented here define for the first time the ability of increased inorganic phosphate to stimulate specific signaling pathways resulting in functionally significant changes in gene expression and identify three important signaling pathways in the regulation of osteopontin.

The process of osteoblast differentiation requires the integration and coordination of multiple intra- and extracellular signals. Previous analysis of a MC3T3-E1 murine preosteoblast cell line repressed for alkaline phosphatase activity identified inorganic phosphate generated in the medium through the interaction of alkaline phosphatase and β-glycerol phosphate as a potentially important signaling molecule in osteoblast differentiation (1). The importance of inorganic phosphate in

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We describe here a systematic evaluation of many of the major signaling pathways with the goal of identifying required signaling molecules in the up-regulation of osteopontin by inorganic phosphate. Analysis of the mitogen activated protein kinases (MAPK) ERK1/2, p38, and c-Jun N-terminal kinase (JNK) reveals that ERK1/2 is specifically required for osteopontin expression in response to elevated phosphates. In addition, we demonstrate that phosphate causes a biphasic phosphorylation of ERK1/2. Analysis of protein kinase signaling molecules reveals that protein kinase c (PKC) is also required in the phosphate-signaling pathway. Finally, we determine that proteasomal activity is required for increased osteopontin expression, not only in response to elevated phosphate but also in response to other regulators of osteopontin, including the tumor promoters okadaic acid and 12-O-tetradecanoylphorbol-13-acetate (TPA). The requirement of proteasomal activity for osteopontin expression represents a novel and potentially globally important pathway in the regulation of this gene and also identifies a possible target in the modulation of osteopontin in various disease states. These data not only provide insights into the regulation of osteopontin but also identify key signaling cascades initiated by an increase in intracellular inorganic phosphate.

**EXPERIMENTAL PROCEDURES**

**Materials**—Penicillin and streptomycin were purchased from Invitrogen. Sodium phosphate mono and di-basic, phosphononic acid (foscarnet), and anisomycin were obtained from Sigma Chemical Co., sodium sulfate from ICN Biochemicals Inc. (Aurora, OH). Radiochemicals were obtained from PerkinElmer Life Sciences. Inhibitors U0124, U0126, SB202190, SB203580, H-89, calphostin C, bisindolylmaleimide I (GP109203X), cytochalasin D, proteasome inhibitor I, lactacystin, MG-132, wortmannin, LY294002, N\(^2\)-monomethyl-L-arginine, N-acetyl-\(\text{L}\)-cysteine, KN-62, and okadaic acid were purchased from CalBiochem. Inhibitors SP600125, KT 5823, and ML-9 were purchased from Tocris (Ellisville, MO). TPA, from Alexis (San Diego, CA), was kindly provided by Nancy Colburn (National Cancer Institute, Frederick, MD).

**Cell Culture**—Low-passage MC3T3-E1 cells were a gift from Brad Zeller (Locas Discovery, Blue Bell, PA). Cells were maintained in α-minimal-essential medium, (Irvin Scientific, Santa Ana, CA) plus 10% fetal bovine serum (Invitrogen) as described previously (1). Inorganic phosphate was used in the form of NaPO\(_4\), pH 7.4, and for all experiments described, sodium sulfate was used a control. When inhibitors were added, an equivalent amount of MeSO\(_4\) was used as a vehicle control.

**Northern Blots**—Total cell RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. 10 µg of RNA was loaded in each lane and separated by electrophoresis through a 1% formaldehyde-agarose gel. The RNA was transferred to a Hybond-N membrane (Amersham Biosciences) according to the manufacturer's protocol. Membranes were probed for osteopontin and probes were visualized by chemiluminescence development, Western blotting detection system (Amersham Biosciences).

**Antibodies**—The OPN-specific antibody LF-123 was provided by Larry Fisher (27) (see Fig. 2A) and a second OPN-specific antibody was purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA) (see Figs. 2B and 7C). The anti-phospho-ERK1/2, phospho-JNK, phospho-p38, and JNK antibodies were purchased from Cell Signaling Technologies Inc. (Beverly, MA), the antibody to p38 from Santa Cruz Biotechnologies, and the antibody to ERK1/2 from Promega (Madison, WI).

**RESULTS**

**Phosphate Induction of Osteopontin Requires ERK1/2**—The ability of inorganic phosphate to regulate expression of osteopontin represents a novel and potentially important signaling mechanism, not only in osteoblasts but also in other cell types in which osteopontin is expressed. We hypothesized that the up-regulation of osteopontin mRNA expression that occurs in response to elevated inorganic phosphate requires specific signaling pathways. Previous findings established that phosphate must enter the cell through a sodium-dependent transporter to induce osteopontin expression (5). Because many stimuli use pathways that include one or more members of the MAPK family, we first examined ERK1/2, JNK, and p38 for possible roles in phosphate regulation of osteopontin. MC3T3-E1 cells were pretreated with the indicated inhibitor for 30 min then followed by 24 h of treatment with either 10 mM sulfate (control) or phosphate, and the samples were analyzed by Northern blotting. Pretreatment with the ERK1/2-specific MEK1/2 inhibitor U0126 (30 µM) effectively blocked up-regulation of osteopontin in response to inorganic phosphate (Fig. 1A). The inactive form of the compound, U0124 (30 µM), had no effect on phosphate-induced osteopontin expression. Treatment with two specific p38 inhibitors, SB202190 and SB203580 (29), at 30 µM had little effect on the up-regulation of osteopontin by phosphate. A selective inhibitor of JNK 1/2/3 at doses up to 10 µM (30), the highest dose the cells would tolerate for 24 h, did not inhibit the phosphate up-regulation of osteopontin (Fig. 1A).

To determine whether the inhibition of osteopontin by U0126 is dose-dependent, MC3T3-E1 cells were treated as described above and Northern analysis was performed. The data indicate that pretreatment with U0126 at 10 µM resulted in a decrease of approximately 50% in osteopontin expression in response to phosphate (Fig. 1B), whereas 30 µM resulted in complete inhibition. A concentration of 3 µM resulted in no inhibition (data not shown). The inactive form of the compound, U0124, was again used as a control and showed no inhibition. These results reveal that the up-regulation of osteopontin in response to elevated inorganic phosphate is dose-dependently inhibited by the MEK1/2 inhibitor U0126 and suggest that activation of the ERK1/2 MAP kinase signaling pathway is required.

**Phosphate Causes a Biphasic Activation of ERK1/2**—The ability of the MEK1/2 inhibitor to block up-regulation of osteopontin in response to inorganic phosphate suggests that elevated inorganic phosphate results in the phosphorylation and hence activation of ERK1/2. To test this hypothesis,
MC3T3-E1 cells were treated with 10 mM phosphate for the indicated times (Fig. 2) and harvested for Western analysis. Equal amounts of protein were separated by 10% SDS-PAGE. Use of a phospho-specific antibody demonstrated that treatment with phosphate resulted in a biphasic activation of ERK1/2 (Fig. 2A). The activation was initiated within the first 15 min and returned to basal levels by 2 h. A second, more sustained period of activation occurred starting at 8 to 12 h and continued at least through 32 h. An antibody to non-phosphorylated forms of ERK1/2 was used to demonstrate equal protein loading. Evaluation of the time course for osteopontin protein levels revealed a detectable increase in osteopontin protein that follows the second increase in ERK1/2, starting at ~12 h. These results reveal that increased levels of inorganic phosphate lead to phosphorylation and activation of the ERK1/2 signaling proteins and that this pathway is required for phosphate-induced osteopontin RNA and protein (Figs. 1 and 2A).

We have demonstrated previously that the concentration of inorganic phosphate required to up-regulate osteopontin expression was inversely proportional to the amount of time the cells were exposed to elevated phosphate (6). The results discussed thus far describe the response to 10 mM phosphate. To determine whether the inverse relationship between time and dose holds true for ERK1/2 activation, a time-course experiment was performed in response to treatment with 4 mM inorganic phosphate (Fig. 2B). The results suggest that 4 mM inorganic phosphate requires ~2 days for the first ERK1/2 phosphorylation, with the second peak occurring at ~4 days.

The up-regulation of osteopontin again follows the second ERK1/2 phosphorylation and is detectable at 5 days. These results agree with the inverse dosetime relationship identified by Northern analysis (6) and suggest that the same events that occur after treatment with 10 mM inorganic phosphate for 24 h also occur in response to lower concentrations of phosphate at longer time points. 

**Phosphate Stimulation of ERK1/2 Is Specific**—Our initial analysis using inhibitors of the traditional MAPK signaling proteins suggested that neither p38 nor JNK was required in the regulation of osteopontin by phosphate. It is possible that these signaling proteins were activated by phosphate but not required for osteopontin expression. To determine whether these signaling proteins were activated by phosphate, or whether phosphate specifically activated ERK1/2, MC3T3-E1 cells were treated with phosphate for the indicated times (Fig. 3). One sample was additionally treated with 2.5 μg/ml anisomycin, a known activator of both p38 and JNK, for 30 min as a control. Western analysis using phospho-specific antibodies to p38 and JNK revealed no detectable phosphorylation of either p38 or JNK in response to phosphate at any time point analyzed (Fig. 3A). However, these proteins were phosphorylated in response to anisomycin, as determined by the positive response to phospho-specific antibodies. Antibodies to the non-phosphorylated forms of p38 and JNK demonstrated that there are ample amounts of these proteins in MC3T3-E1 cells. These results suggest that although the MAP kinases p38 and JNK are abundantly expressed and capable of activation in these cells, inorganic phosphate specifically stimulates ERK1/2 phosphorylation.

To further test the specificity of the MEK1/2 inhibitor, MC3T3-E1 cells were pretreated with 30 μM U0126 for 30 min.
followed by treatment with either 10 mM inorganic phosphate or 2.5 μg/ml anisomycin for 30 min. The resulting protein samples were analyzed by Western blotting (Fig. 3B). Treatment with 10 mM phosphate resulted in an increase in the phosphorylation of ERK1/2 that was completely inhibited by pretreatment with U0126. Elevated inorganic phosphate did not cause detectable phosphorylation of either p38 or JNK with the antibodies used; however, treatment with anisomycin resulted in phosphorylation of both p38 and JNK. Pretreatment with U0126 did not block phosphorylation of either protein in response to anisomycin, suggesting specificity of this compound for the ERK1/2 signaling pathway.

To determine whether one or both peaks of ERK1/2 phosphorylation (activation) are required for osteopontin expression, we again used Northern analysis. MC3T3-E1 cells were treated with 10 mM phosphate followed by treatment with U0126 at the indicated times (Fig. 4A) after phosphate treatment and all samples were harvested at 24 h. Surprisingly, addition of U0126 at 4 and 8 h after phosphate treatment, well after the first peak of ERK1/2 phosphorylation (1h), resulted in essentially complete inhibition of osteopontin expression, whereas addition of U0126 at 16 h after phosphate treatment resulted in no inhibition of osteopontin expression. Addition of U0126 at 12 h caused a partial inhibition of osteopontin (data not shown). The ability of U0126 to inhibit osteopontin expression after the first ERK1/2 activation peak has occurred suggests that the first peak alone is not sufficient to regulate osteopontin expression and that the second peak of ERK1/2 activation at 8 to 12 h after phosphate treatment is required for induction of osteopontin expression. This does not rule out the requirement of the first peak but does suggest that it is insufficient.

We confirmed these results using a different approach. MC3T3-E1 cells were treated with 10 mM inorganic phosphate for the indicated times “pulsed” (Fig. 4), followed by replacement of the phosphate-containing medium with normal growth medium. All samples were harvested at 24 h from the initial phosphate treatment and used for Northern analysis. The results (Fig. 4B) demonstrate that neither the pulse at 3 h nor the pulse at 6 h shows an increase in osteopontin expression, whereas the pulses at both the 9 and 12 h show a clear increase in expression. As a control, the medium was changed on a corresponding 12 h sample without phosphate treatment (−) and showed no increase in osteopontin, confirming that the change in medium itself was not the cause of increased osteopontin expression. The timing of osteopontin regulation in

**Fig. 3. Specificity of phosphate stimulation of ERK1/2.** A, MC3T3-E1 cells were treated with either 10 mM inorganic phosphate (for the indicated times) or 2.5 μg/ml anisomycin (Ani) for 30 min. The resulting samples were separated by electrophoresis on a 10% polyacrylamide gel, and duplicate membranes were immunoblotted with phosphorylation specific antibodies (p-ERK1/2, p-p38, and p-JNK) or the corresponding non-phosphorylation specific antibodies (ERK1/2, p38, and JNK) as indicated. B, MC3T3-E1 cells were pretreated with U0126 (30 μM), followed by treatment with either 10 mM inorganic phosphate or 2.5 μg/ml anisomycin (Ani) for 30 min. The resulting samples were analyzed by Western and immunoblotted as in A.

**Fig. 4. Regulation of osteopontin by phosphate: timing of ERK1/2 activation.** A, MC3T3-E1 cells were treated with 10 mM inorganic phosphate followed by treatment with U0126 (30 μM), at the indicated times. All samples were harvested 24 h after phosphate treatment for Northern analysis. The resulting membrane was probed for osteopontin (OPN) and the autoradiograph is shown. Ethidium bromide staining of the 18S and 28S ribosomal RNA is shown as a loading control. B, MC3T3-E1 cells were pretreated with U0126 (30 μM), followed by treatment with either 10 mM inorganic phosphate or 2.5 μg/ml anisomycin (Ani) for 30 min. The resulting samples were analyzed by Western and immunoblotted as in A.
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Figure 5. Requirement of protein kinase C for phosphate-induced osteopontin expression. MC3T3-E1 cells were pretreated with the PKC inhibitors calphostin C (CalC) and GF109203X (GFX), the PKA inhibitor KT-5823 (KT), or the PKA inhibitor H-89 at the indicated concentrations for 30 min followed by treatment with 10 mM phosphate for 24 h. The resulting Northern blot was probed for OPN. Ethidium Bromide staining of the 18S and 28S ribosomal RNA is shown as a loading control.

Figure 6. ERK1/2 and PKC represent distinct signaling pathways. A, MC3T3-E1 cells were pretreated for 30 min with the indicated inhibitor: GF109203X (5 μM, GFX), calphostin C (1 μM, CalC), or U0126 (30 μM), followed by treatment with 10 mM inorganic phosphate for 30 min. The resulting cell lysates were separated by electrophoresis on a 10% polyacrylamide gel, and the membrane was immunoblotted with the indicated antibodies. B, MC3T3-E1 cells were pretreated with either 300 μM foscarnet (Fos), 500 mM calphostin C (CalC), or 5 μM GF109203X (GFX) followed by treatment with 10 mM inorganic phosphate for 20 h. The resulting cell lysates were separated by electrophoresis on a 10% polyacrylamide gel, and the membrane was immunoblotted with the indicated antibodies.

these two experiments corresponds to the timing of the second ERK1/2 activation in Fig. 2A and supports the idea that at least 8 to 12 h of exposure to 10 mM phosphate is required for increased osteopontin expression. Taken together, these results strongly suggest that the first ERK1/2 peak demonstrated in Fig. 2A is insufficient to cause the increase in osteopontin expression and that the second ERK1/2 activation peak is required. It should be pointed out that this does not rule out the requirement of the first peak but does establish that it is insufficient.

Phosphate-induced OPN Expression Is PKC-dependent—The availability of numerous and relatively specific PKC inhibitors allows for the determination of the requirement of this family in signal transduction events. Although these inhibitors can reveal the requirement of the family as a whole, their use becomes somewhat more controversial in determining the exact family member required. We used two different relatively broad-spectrum PKC inhibitors, calphostin C (31–33) and GF109203X (34, 35), to evaluate the requirement of the PKC family in phosphate-induced osteopontin expression. MC3T3-E1 cells were pretreated with the indicated concentration of inhibitor (Fig. 5) for 30 min, followed by treatment with 10 mM phosphate for 24 h. The resulting samples were analyzed by Northern blotting. Data indicate that up-regulation of osteopontin in response to phosphate is dose-dependently inhibited calphostin C starting at 100 nM and resulting in complete inhibition at 500 nM (Fig. 5). Although this inhibitor is thought to be specific for the PKC family, it is possible that it also inhibits other signaling molecules. To address this, we used another PKC inhibitor, GF109203X. This compound also dose-dependently (1 to 5 μM) inhibited the increase in osteopontin expression in response to elevated phosphate (Fig. 5). Finally, because some PKC inhibitors are also known to inhibit protein kinase A (PKA) and protein kinase G, usually at higher doses, we sought to determine whether specific inhibitors of these kinases would block the increase in osteopontin expression in response to phosphate. Neither the PKA inhibitor H-89 (30 μM) nor the protein kinase G inhibitor KT-5823 (5 μM) had any inhibitory effect on osteopontin expression (Fig. 5). Taken together, these results provide evidence for the requirement of at least one member of the PKC family in the regulation of osteopontin in response to phosphate. Further studies will be required to ascertain the actual PKC isoform(s) involved.

PKC and ERK1/2 Represent Required and Distinct Pathways—Signaling pathways have been elucidated in which the activation of PKC is upstream of the activation of ERK1/2. To test whether this is the case in response to stimulation with inorganic phosphate, MC3T3-E1 cells were pretreated with the indicated inhibitor, followed by treatment with 10 mM phosphate for the indicated times and the cells were harvested for Western analysis. Total cell lysate was analyzed for ERK1/2 phosphorylation at either 30 min (Fig. 6A) or 20 h (Fig. 6B) after exposure to phosphate. Evaluation of the 30-min time point revealed that neither 1 μM calphostin C nor 5 μM GF109203X, concentrations known to block osteopontin expression, inhibited phosphorylation of ERK1/2 in response to treatment with inorganic phosphate. The ERK1/2 inhibitor U0126 (30 μM) and the phosphate transport inhibitor foscarnet (300 μM) (data not shown) blocked the phosphorylation of ERK1/2. Analysis of the 20-hour time point also revealed that neither of the PKC inhibitors (GF109203X (5 μM) and calphostin C (500 nM)) inhibited phosphorylation of ERK1/2 in response to phosphate, whereas the phosphate transport inhibitor foscarnet (300 μM) blocked the phosphorylation. In fact, the PKC inhibitors actually seem to enhance the phosphorylation of ERK1/2. These data suggest that PKC activation is not required upstream of ERK1/2 activation. In addition, the inability of the PKC inhibitors to block phosphorylation of ERK1/2 suggests that the PKC inhibitors are not inhibiting osteopontin expression by blocking entry of phosphate into the cells. Considering that it is unlikely that PKC activation occurs downstream of ERK1/2, these results imply that PKC and ERK1/2 represent distinct and required pathways in the regulation of osteopontin expression in response to elevated phosphate.

Phosphate Stimulation of Osteopontin Requires the Activity of the Proteasome—The proteasome is a multiprotein complex that is the major non-lysosomal mechanism for cellular protein degradation. To determine whether proteasomal activity is required for increased osteopontin expression in response to increased phosphate, Northern analysis was again used. Many proteasome inhibitors will also inhibit calpains and cathepsins depending on the concentration used. We therefore used multiple proteasome inhibitors with different properties. MC3T3-E1 cells were pretreated with the indicated inhibitor.
(Fig. 7) for 30 min followed by treatment with 10 mM inorganic phosphate for 24 h. The resulting Northern blot was probed for osteopontin levels (Fig. 7A). Ethidium bromide staining of the 18 S and 28 S RNA is shown as a loading control. Data shown are representative of multiple experiments. The peptide aldehyde MG-132 dose-dependently inhibited the up-regulation of osteopontin expression in response to phosphate at relatively low doses (100–500 nM). Another peptide aldehyde, benzylxycarbonyl-Ile-Glu-(O-tart-butyl)-Ala-leucinal also dose-dependently inhibited osteopontin expression in response to phosphate at doses up to 1 μM (data not shown). Lactacystin, a Streptomyces sp. metabolite considered the most specific proteasome inhibitor available (36), completely inhibited phosphate-induced osteopontin expression at concentrations between 1 and 10 μM (Fig. 7B). Taken together, these data indicate that proteasomal activity is required for phosphate-induced osteopontin expression.

To determine whether the requirement of proteasomal activity is a required aspect of phosphate signaling in general or a unique property of osteopontin regulation, we sought to determine whether another known phosphate responsive gene, HMGA2 (6), was inhibited. Analysis of HMGA2 expression on the same blots revealed that up-regulation of HMGA2 in response to phosphate is not inhibited by pretreatment with either proteasome inhibitor (Fig. 7, A and B). This suggests that the requirement of proteasomal activity for phosphate-induced osteopontin expression is probably an aspect of osteopontin regulation as opposed to a general phenomenon of phosphate signaling.

To determine whether the inhibition of osteopontin expression by proteasome inhibitors is also reflected at the protein level, Western analysis was performed. The resulting membrane was immunoblotted with an osteopontin-specific antibody, which revealed that treatment with 10 mM inorganic phosphate for 24 h caused an increase in osteopontin protein and that this increase is completely blocked by treatment with the proteasome inhibitor MG-132 (1 μM) (Fig. 7C). Taken together with the Northern analysis, the data strongly support the requirement of proteasomal activity in the up-regulation of osteopontin in response to inorganic phosphate.

Proteasomal Activity Is Required for OPN Stimulation in Response to Varied Stimuli—The requirement for proteasomal activity in the regulation of osteopontin represents a novel and potentially important requirement in understanding osteopontin expression. We were interested in determining whether the proteasome is necessary for osteopontin expression in response to other stimuli. Both the tumor promoter TPA and the protein phosphatase 2A inhibitor okadaic acid are known to stimulate osteopontin expression (37–39). To test the hypothesis that proteasomal activity is required for osteopontin expression to diverse stimuli, MC3T3-E1 cells were pretreated with 1 μM MG-132 for 30 min followed by treatment with the indicated stimulus. Northern analysis revealed that treatment with either TPA (10 ng/ml) or okadaic acid (100 nM) resulted in induction of osteopontin RNA; this induction is completely blocked in the presence of the proteasome inhibitor (Fig. 7D). Similar results were obtained by Western analysis in response to stimulation with either TPA or okadaic acid (data not shown). These observations suggest that proteasome activity is an important aspect of osteopontin expression in response to a variety of stimuli and may be required for induced osteopontin expression in general.

Specificity of the Phosphate Signaling Pathways Not Required—To demonstrate the specificity of phosphate signaling, we have compiled a table of compounds that were evaluated but demonstrated no inhibition of osteopontin expression in response to 10 mM inorganic phosphate. Table I represents a summary of experiments in which MC3T3-E1 cells were pretreated with the listed compound for 30 min followed by treatment with 10 mM phosphate for 24 h. All samples were analyzed by Northern blotting and represent multiple experiments. In general, the concentrations listed represent the maximum dose the cells would tolerate for 24 h, although multiple doses were evaluated, or the highest dose that has previously been shown to be specific. Two different phosphatidylinositol 3-kinase inhibitors, wortmannin and LY294002, showed no ability to inhibit osteopontin expression. Other kinase inhibitors, including the calcium/calmodulin kinase inhibitor KN-62, the myosin light chain kinase inhibitor ML-9, and the focal adhesion kinase inhibitor cytchalasin D showed no effect. Inorganic phosphate does not seem to increase osteopontin expression by generating reactive oxygen species, because N-acetyl-l-cysteine does not inhibit up-regulation. The nitric-oxide synthase inhibitor Nω-monomethyl-l-arginine at 5 mM also had no effect on osteopontin expression, suggesting that the generation of nitric oxide is not a signaling mechanism. As demonstrated in Figs. 1, 3, and 5, the inhibitors of p38 (SB202190 and SB203580), JNK (SP600125), protein kinase A (H-89), and protein kinase G (KT 5823) showed no effect on phosphate-induced osteopontin expression. Taken together, these results demonstrate the surprising specificity of...
signaling in the up-regulation of osteopontin expression in response to elevated phosphate.

**DISCUSSION**

We previously defined the influx of inorganic phosphate as a stimulus capable of ultimately regulating the transcription of the matrix-associated protein osteopontin not only in osteoblasts but also in other cell types. The potential importance of inorganic phosphate as a novel signaling molecule in osteoblast development and the links between osteopontin regulation and multiple disease states prompted us to begin to define the pathways by which phosphate regulates osteopontin expression. The results establish that elevated inorganic phosphate levels, generated during osteoblast differentiation, initiate very specific signaling pathways upon entering the cell and point to three important signaling events in the regulation of osteopontin: ERK1/2, PKC, and proteasomal activity. The inability of inhibitors of many other kinases, such as PKA, protein kinase G, p38, JNK, and p38, to block the up-regulation of osteopontin suggests a high degree of specificity in the phosphate-induced signaling cascade.

The MAP kinase family of proteins has been suggested to play an important role in osteoblast differentiation (40), although the specific functions of these proteins in the differentiation process are only beginning to be understood. We report here a novel signaling mechanism for the activation of ERK1/2 in osteoblasts. As osteoblasts differentiate the levels of the membrane-bound enzyme, alkaline phosphatase rise; given a source of organic phosphate, the interaction will create an increase of inorganic phosphate in the extracellular environment (3). Data presented here suggest that elevated levels of inorganic phosphate cause a biphasic activation of ERK1/2. Experiments analyzing the requirement of the individual activation peaks determined that the up-regulation of osteopontin requires the second activation of ERK1/2 and that the first is not sufficient; however, this does not rule out a requirement for the first peak. The requirement of ERK1/2 signaling in osteopontin regulation has been previously demonstrated in osteoblasts in response to oscillatory fluid flow (41) and in response to the addition of medium from PC-3 and MCF-7 tumor cells (42). Induced expression of osteopontin also requires ERK1/2 in cell types other than the osteoblast lineage, including the induction by TPA in HL-60 cells and mouse epidermal JB6 cells (43, 44), the response to injury of rat arterial smooth muscle cells (45), and the response to angiotensin II in cardiac microvascular endothelial cells (46). This growing list of cell types and stimuli suggests that ERK1/2 activation may be a required event for osteopontin regulation regardless of the environment. Although the downstream targets of ERK1/2 in the regulation of osteopontin have yet to be elucidated, a number of transcription factors have been demonstrated to regulate osteopontin including the AP-1 family of proteins (39, 47), and these proteins are also known to be downstream targets of ERK1/2 activation (48).

The results presented here also identify the requirement of the PKC family of serine/threonine kinases in the up-regulation of osteopontin in response to elevated inorganic phosphate. Currently, 11 PKC isozymes have been identified and are classified by structure and regulation into three groups (49). The conventional PKCs (α, splice variants βI and βII, and γ) are activated by calcium and diacylglycerol; the novel PKCs (δ, ε, η, θ, and μ) are activated by diacylglycerol; and the activator(s) for the atypical PKCs (ζ and η) remains unknown. Osteoblasts are known to express PKC isozymes α, β, δ, ε, η, θ, ζ, and η, although cell line differences seem to occur even among osteoblasts. The requirement of the PKC family in the regulation of osteopontin has been previously reported in MC3T3-E1 cells in response to the addition of the medium from PC-3 and MCF-7 cells (42), and in other cell types, including TPA in HL-60 cells and JB6 cells (43, 44, 51) and in response to glucose in rat aortic smooth muscle cells (52). The TPA study used various selective PKC inhibitors to determine that the novel PKC ε is probably the required PKC isozyme (44). The ability of both calphostin C and GF102903X, relatively specific inhibitors of PKC, and the failure of the PKA and protein kinase G inhibitors to block the increase in osteopontin expression suggest that one or more members of PKC family of isozymes are required. The GF102903X compound is believed to possess some degree of specificity for PKC family members. At low doses (10−20 μM range), it is believed to inhibit the conventional PKCs; only at higher doses is this compound believed to block the novel and atypical PKCs (53). The inability of the 1 μM dose to block the expression of osteopontin in response to phosphate suggests that the PKC isozyme required is not from the conventional PKC group. The exact behavior of the many available PKC inhibitors toward individual isozymes is currently the focus of much research but remains unclear, making it difficult to determine the isozyme required for osteopontin elevation.

It is not uncommon that PKC activation may be required upstream of ERK1/2 in various signaling pathways (54). We have determined that in response to inorganic phosphate, PKC is not required upstream of ERK1/2; therefore, these two important signaling molecules are likely to constitute two individual and required pathways. Given that both ERK1/2 and PKC have been demonstrated to be required for osteopontin

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**TABLE I**

| Target                  | Compound              | Concentration |
|-------------------------|-----------------------|---------------|
| Phosphatidylinositol 3-kinase | Wortmannin            | 30 μM         |
| Phosphatidylinositol 3-kinase | LY294002              | 30 μM         |
| Myosin light chain kinase | ML 9                  | 10 μM         |
| Ca2+/Calmodulin kinase  | KN-62                 | 20 μM         |
| Focal adhesion kinase   | Cytochalasin D        | 500 nM        |
| Nitric oxide increase   | Nα-monomethyl-l-arginine | 5 mM  |
| Free radicals           | N-acetyl-l-cysteine    | 5 mM          |
| p38                     | SB203580              | 30 μM         |
| p38                     | SB202190              | 30 μM         |
| JNK                     | SP600125              | 10 μM         |
| Protein kinase A        | H-89                  | 30 μM         |
| Protein kinase G        | KT 5823               | 5 μM          |
expression in response to varied stimuli, and that the PKCs are a family of isozymes likely to be differentially responsive to various stimuli, one may speculate that ERK1/2 activation is a common requirement for induced osteopontin expression, whereas the PKC isoform required may confer stimulus and cell-type specificity. It is also not surprising that at least two signal pathways would be required. A number of stimuli present during osteoblast differentiation are capable of activating ERK1/2, but the up-regulation of osteopontin occurs only during a discrete time. In addition, ERK1/2 signaling is often associated with proliferative signals, but osteopontin expression and elevated phosphate levels do not occur until the cells have exited the cell cycle. This not only emphasizes the need for multiple signaling pathways but also suggests a role for ERK1/2 signaling in addition to growth stimulatory pathways in osteoblasts.

Whereas both PKC and ERK1/2 signaling molecules have been previously implicated in the regulation of osteopontin in response to other stimuli, to our knowledge, the data presented here are the first identification of the proteasome as an important regulator of osteopontin expression. The proteasome is not only required for expression of osteopontin in response to inorganic phosphate but also in response to at least two other known inducers of osteopontin, TPA and okadaic acid. Proteasomal activity has been demonstrated to regulate gene expression by at least two mechanisms: the degradation of cytoplasmic inhibitors, such as IκB, resulting in the activation of transcription factors or the select degradation of various transcription factors (55–57). Little is known about the role of the proteasome in osteoblast differentiation, although it has been demonstrated to be essential for osteoblast proliferation (58). Our data suggest that the proteasome may be an important component in the regulation of osteopontin, not only in osteoblasts in response to elevated phosphate but in the global regulation of osteopontin expression in response to a host of stimuli. Furthermore, given the correlation between elevated osteopontin RNA levels and the metastatic phenotype of many cancer cells (25, 59) the data presented here may provide insight into evaluating proteasome inhibitors as cancer therapeutics (60, 61). The ability of the proteasome inhibitor to block induction of osteopontin in response to varied stimuli that use different signaling pathways suggests that the target of the proteasome function may act at or near the promoter. The identification of the target of the proteasome will be important in understanding and potentially targeting the regulation of osteopontin expression.

In summary, the data presented here establish for the first time that entry of inorganic phosphate into osteoblasts is capable of initiating specific signal transduction pathways, including the activation of ERK1/2. In addition, we have used this model to determine specific signaling pathways, including ERK1/2 and PKC, in the regulation of the matrix-associated protein osteopontin, and a proteasome requirement in osteopontin regulation emerges for the first time. The ability of proteasome inhibitors to block the up-regulation of osteopontin in response to multiple stimuli suggests the proteasome may be a global regulator of osteopontin. These findings should prove valuable in understanding the effect of elevated inorganic phosphate, not only on the process of osteoblast differentiation but also in other cell types and disease states. Targeting ERK1/2, PKC, or proteasome activity presents intriguing possibilities for intervening in bone metabolism or metastasis via regulation of osteopontin.

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