MAPK Pathways Activate and Phosphorylate the Osteoblast-specific Transcription Factor, Cbfa1*

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(Received for publication, August 6, 1999, and in revised form, November 5, 1999)

The bone-specific transcription factor, Cbfa1, regulates expression of the osteocalcin (OCN) gene and is essential for bone formation. However, little is known about the mechanisms regulating Cbfa1 activity. This work examines the role of the MAPK pathway in regulating Cbfa1-dependent transcription. Stimulation of MAPK by transfecting a constitutively active form of MEK1, MEK(SP), into MC3T3-E1 preosteoblast cells increased endogenous OCN mRNA, while a dominant negative mutant, MEK(DN), was inhibitory. MEK(SP) also stimulated activity of a 147-base pair minimal OCN promoter, and this stimulation required an intact copy of OSE2, the DNA binding site for Cbfa1. Effects of MEK(SP) were specific to Cbfa1-positive osteoblast-like cells. A purified His-tagged Cbfa1 fusion protein was directly phosphorylated by activated recombinant MAPK in vitro. Furthermore, 32P metabolic labeling studies demonstrated that MEK(SP) clearly enhanced phosphorylation of Cbfa1 in intact cells, while MEK(DN) decreased phosphorylation. The specific MEK1/MEK2 inhibitor, PD98059, inhibited extracellular matrix-dependent up-regulation of the OCN promoter, indicating that the MAPK pathway and, presumably, Cbfa1 phosphorylation are also required for responsiveness of osteoblasts to extracellular matrix signals. This study is the first demonstration that Cbfa1 is controlled by MAPKs and suggests that this pathway has an important role in the control of osteoblast-specific gene expression.

Differentiation of the osteoblast, the cell responsible for the production of mineralized bone tissue, requires secretion of a type I collagen-containing extracellular matrix (ECM) by an osteoblast precursor. Production of this matrix is followed by induction of a set of genes associated with terminally differentiated cells, including osteocalcin (OCN), bone sialoprotein, alkaline phosphatase, and the parathyroid hormone/parathyroid hormone-related protein receptor, and ultimately, mineralization of the matrix (1–5). Until recently, however, little was known about the transcriptional events regulating matrix-induced gene expression.

In 1995, Ducy and Karsenty (6) characterized osteoblast-specific element 2 (OSE2), a cis-acting sequence in the promoter of the murine OCN gene 2 (mOG2) that is required for its expression in osteoblastic cells (7). The factor that binds this element, initially termed Osf2, was subsequently identified as Cbfa1, a member of the Cbf/Runt family of transcription factors, which share a DNA binding motif that is homologous to the Drosophila protein, Runt (8). Cbfa1, which is also known as PEBP2αA1 and AML3, is essential for the differentiation of osteoblasts from mesenchymal precursors, since homozygous Cbfa1−/− mice show a complete lack of functional osteoblasts (9, 10). Moreover, this factor is required for bone matrix synthesis by differentiated osteoblasts (11), indicating that it regulates osteoblast gene expression at multiple levels.

We recently showed that Cbfa1 at least in part mediates the response of preosteoblasts to matrix signals (12, 13). Specifically, ECM production by murine MC3T3-E1 preosteoblast cells dramatically increases OSE2-dependent transcription of the OCN promoter and in vitro binding of Cbfa1 to the OSE2 sequence. This stimulation requires an αβ1 integrin-collagen interaction, thus linking for the first time matrix production with osteoblast-specific transcription. Interestingly, this increase in transcriptional activity is not accompanied by a significant change in Cbfa1 mRNA or protein, suggesting that Cbfa1 is activated either by a posttranslational modification or through an accessory factor.

The mitogen-activated protein kinase (MAPK) pathway provides a plausible link between cell surface integrin activation by ECM and subsequent stimulation of Cbfa1-dependent transcription. Consistent with this model, Takeuchi and co-workers showed that the binding of type I collagen to the αβ1 integrin of MC3T3-E1 preosteoblasts activates MAPK and that this activation is necessary for ECM-dependent stimulation of the bone marker, alkaline phosphatase (14). The present study was undertaken to further explore the involvement of the MAPK pathway in osteoblast differentiation and function by directly testing the effects of an exogenously added MAPK pathway intermediate on Cbfa1-dependent transcription. As will be shown, transfection of MC3T3-E1 preosteoblast cells with a constitutively active MAPK kinase stimulates expression of both endogenous OCN mRNA and an mOG2 promoter-driven reporter construct. MAPK-dependent transcription of this promoter requires both Cbfa1 and its DNA binding site, OSE2. Furthermore, transcriptional activation is accompa-

* This work was supported by National Institutes of Health (NIH) Grants DE 11723 and DE12211 (to R. T. F.), Training Program in Organogenesis Postdoctoral Fellowship T32-HD007505 (to G. X.), NIH Grant GM51586 (to K. G.), NIH Grant AR45548 and March of Dimes Foundation Basic Research Award IFY92-0871 (to G. K.), and Michigan Multipurpose Arthritis Center Grant AR20557. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: ECM, extracellular matrix; Cbfa1, core binding factor 1; OCN, osteocalcin; OSE2, osteoblast-specific element 2; mOG2, mouse osteocalcin gene 2; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis.
nied by phosphorylation of Cbf1a, thus providing evidence that a MAPK-dependent phosphorylation cascade regulates Cbf1a activity.

**EXPERIMENTAL PROCEDURES**

**Reagents—**α-[32P]dCTP (3000 Ci/nmol) and [33P]orthophosphate were purchased from Amersham Pharmacia Biotech. Anti-mouse Cbf1a serum was prepared in rabbit using a synthetic peptide as described previously (13). All other chemicals were of analytic grade.

**Cell Lines—**Subclone 4 and subclone 42 MC3T3-E1 cells (MC-4 cells and MC-42 cells), two previously described strongly differentiating subclonal lines (12, 15), were maintained in ascorbic acid-free minimal essential medium (Life Technologies, Inc.), 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin/streptomycin. F9 teratocarcinoma (from the American Type Culture Collection) and ROS 17/2.8 osteosarcoma cells (16), a gift from Dr. Laurie McCauley (University of Michigan School of Dentistry) were grown in minimal essential medium, the fetal bovine serum, 1% penicillin/streptomycin. COS-7 cells (from the American type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), 10% FBS, 1% penicillin/streptomycin.

**DNA Constructs and Production of Recombinant Cbf1a Peptides—** All luciferase reporter plasmids were constructed by cloning mOG2 promoterless luciferase expression vector as described previously (6). The Cbf1a expression plasmids, pCMV5Cbf1a, pCMV5Cbf1a(M1–108), and pCMV5Cbf1a(AS25–528), containing cDNAs encoding either wild type Cbf1a or deletions thereof under cytoskeletal promoter control, were also described previously (17). The Cbf15β-gal expression plasmid, used as a negative control for Cbf1a overexpression, was constructed by ligating a full-length bacterial β-galactosidase cDNA from plasmid pGAL10 into HindIII/BamHI sites of the pCMV5 vector. Expression plasmids pCMV5MEK(SP) and pCMV5MEK(DN), encoding, respectively, a constitutively active and dominant negative mutant of MAPK were constructed by cloning mOG2 promoterless expression plasmid that encodes a constitutively active form of MAPK1 (also known as MAPK) into the mammalian expression vector pDsRed (Clontech, CA) (18, 19). The expression plasmids of mouse Cbf1 fusion protein were constructed in a similar manner. Mouse Cbf1 coding region was taken from the originally reported Ofs2/Cbf1a cDNA (8) using a primer designed in the 3′-end of mouse full-length Cbf1a cDNA. The cDNA sequence included a HindIII and EcoRI site for expression in HEK293. The resulting construct was named Cbf1a-ΔCbf1a and was used to transfect MC3T3-E1 cells.

**Preparation of nuclear extracts—** COS-7 cells were transfected and cultured for 30 h in DMEM containing 10% FBS and then transfected to and incubated for 12 h in DMEM containing 0.1% FBS. Labeling was conducted for 5 h in phosphate-free DMEM containing 0.1% FBS (dialyzed against phosphate-free DMEM) and 200 μCi of [33P]orthophosphate (Amersham Pharmacia Biotech) per ml. Preparation of nuclear extracts was described previously (12).

**Metabolic Labeling and Preparation of Nuclear Extracts—** COS-7 cells were transfected and cultured for 30 h in DMEM containing 10% FBS and then transfected to and incubated for 12 h in DMEM containing 0.1% FBS. Labeling was conducted for 5 h in phosphate-free DMEM containing 0.1% FBS (dialyzed against phosphate-free DMEM) and 200 μCi of [33P]orthophosphate (Amersham Pharmacia Biotech) per ml. Preparation of nuclear extracts was described previously (12).

**Immunoprecipitation of Cbf1a—** Nuclear extracts were preclaved twice with 50 μl of protein A-agarose beads (Life Technologies, Inc.) for 30 min followed by pelleting of beads. 5 μl of rabbit polyclonal anti-Cbf1 antibody was added and incubated for 2 h at 4 °C with gentle rocking. The immune complexes were collected and washed with 200 μl of PBS, centrifuged, and the supernatants were collected. Precipitates were suspended in one time in washing buffer (20 mM Tris-HCl, pH 8.3, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 50 mM NaCl, 2 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride), and the immunoprecipitated complexes were suspended in SDS sample buffer and analyzed by SDS-PAGE and autoradiography. Incorporation was measured using a Packard A2024 InstantImager.

**RESULTS**

**MAPK Stimulates Cbf1a-dependent OCN Gene Transcription—** Previous studies showed that the MAPK signaling pathway is involved in the ECM-stimulated induction of alkaline phosphatase, an osteoblast-related enzyme (14). Specifically, forced expression of a MAPK-specific phosphatase inhibited matrix-induced alkaline phosphatase activity in MC3T3-E1 cells, while introduction of a constitutively active MAPK kinase enhanced this activity. To determine whether the MAPK pathway can stimulate the transcriptional activity of an osteoblast-related gene, we performed a similar set of experiments using expression of both the endogenous OCN gene and an mOG2 promoter-driven luciferase reporter as assays.

**Western Blots—** Whole cell extracts were prepared by harvesting cells in 1× SDS-PAGE loading buffer (2% SDS, 2× sample buffer, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 10 mM Tris-HCl, 0.002% bromphenol blue). Proteins were separated on SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell), and probed with a specific rabbit anti-mouse Cbf1a antisera as described previously (13). Immunoreactivity was determined using the ECL chemiluminescence reaction (Amersham Pharmacia Biotech).

**RNA Extraction and Northern Blot Analysis—** Total RNA was isolated from cell layers as described by Chomczynski and Sacchi (20). Aliquots of total RNA were fractionated on 1.0% agarose-formaldehyde gels and blotted onto nitrocellulose paper as described by Thomas (21). The mouse OCN cDNA was from Dr. John Wozney (Genetics Institute, Boston, MA) (22). The cDNA insert was excised from plasmid DNA with the appropriate restriction enzymes and purified by agarose gel electrophoresis before labeling with α-[32P]dCTP using a random primer kit (Roche Molecular Biochemicals). Hybridizations were performed as described previously using a Belco Autoblot hybridization oven (4) and quantitatively scanned using a Packard A2024 InstantImager. All values were normalized for RNA loading by probing blots with cDNA to 18 S rRNA (23).

**In Vitro Phosphorylation of His-tagged Mouse Cbf1a Fusion Protein—** Phosphorylation of purified His-tagged fusion proteins was conducted in 25 mM Tris HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, 40 μM ATP, 20 μCi of [γ-33P]ATP, 0.5 mM EGTA, in the presence of 0.5 μl (300 units) of recombinant activated MAPK (Calbiochem). Samples were incubated at 25 °C for 20 min in a final volume of 25 μl. Boiling with 20 μl of 2× SDS gel loading buffer terminated the reaction. Aliquots were then analyzed by SDS-PAGE.

**Statistical Analysis—** All transfection data are reported as means ± S.D. based on triplicate independent cell cultures from a representative experiment. All experiments were repeated at least twice, and qualitatively identical results were obtained.
dominant negative MEK1 mutant suppressed basal levels of OCN mRNA in cells grown in 10% serum (Fig. 1, left two lanes). As shown in Fig. 2, MEK(SP) and MEK(DN) had similar effects on the expression of a luciferase reporter plasmid driven by a 147-base pair mOG2 promoter (p147-luc), which has been shown to have osteoblast-specific, matrix-induced activity (6, 13); i.e. MEK(SP) stimulated promoter activity, while MEK(DN) was inhibitory. However, when a 2-base pair mutation that renders the OSE2 sequence nonfunctional as a Cbfa1 binding site was introduced into this promoter fragment, both MEK(SP) stimulation and MEK(DN) suppression were abolished.

Two additional studies were carried out to confirm the involvement of OSE2 and its binding partner, Cbfa1, in the MEK response. In the first, the ability of MEK(SP) to stimulate transcription of OSE2/34-luc, a reporter plasmid containing six copies of OSE2 upstream of a minimal 34-base pair mOG2 promoter, was examined in Cbfa1-positive (MC-4 and ROS17/2.8 cells) and Cbfa1-negative cell lines (Fig. 3). A Western blot confirmed that only the two osteoblast-like cell lines expressed immunoreactive Cbfa1 protein (Fig. 3A). As shown in Fig. 3, B–E, MEK(SP) stimulated the low basal activity of OSE2/34-luc only in Cbfa1-positive cells; MEK(SP) stimulated luciferase activity 5-fold in MC-4 and 2.5-fold in ROS17/2.8 cells, while MEK(DN) had no effect. As shown in Fig. 3B, OSE1/34-luc, a reporter plasmid containing six copies of OSE1, which is the other known osteoblast-specific enhancer in the mOG2 promoter (6), was only slightly stimulated by MEK(SP). Furthermore, this stimulation was not dependent on the presence of an intact OSE1 site because it was not abolished by mutation (compare results with OSE1/34-luc versus OSE1mt/34-luc plasmids). Therefore, of the two known osteoblast-specific elements in the mOG2 promoter, only OSE2 is specifically stimulated by MEK, and this stimulation is only seen in cells that contain Cbfa1.

The second study examined effects of MEK(SP) and MEK(DN) on cells transfected with OSE2/34-luc and a Cbfa1 expression plasmid (Fig. 4). In both MC-4 and ROS17/2.8 cell lines, transfection with Cbfa1 increased basal promoter activity, and this stimulation was enhanced an additional 6–11-fold by MEK(SP), but not by MEK(DN) (A and B). Furthermore, as expected, Cbfa1 and MEK(SP) responsiveness in MC-4 cells was totally lost with mutation of the OSE2 sequence in the reporter plasmid (OSE2mt/34-luc reporter plasmid; Fig. 4A). Transfection of the two nonosteoblastic cell lines (COS-7 or F9 cells) with the Cbfa1 expression plasmid increased the very low basal OSE2/34-luc activity approximately 5–10 fold, and this activity was stimulated an additional 8–18-fold by the MEK(SP) expression plasmid (Fig. 4, C and D). The MEK(DN) plasmid had no consistent effect on Cbfa1-dependent activity in F9 or COS-7 cells. Thus, both COS-7 and F9 cells acquired the ability to be stimulated by MEK(SP) when they were transfected with the Cbfa1 plasmid. Taken together, these results indicate that MAPK stimulation of OCN transcription requires binding of Cbfa1 to OSE2.

MAPK Stimulates the Phosphorylation of Cbfa1—We next addressed whether MEK could also stimulate the phosphorylation of Cbfa1 in vitro and in intact cells. First, the ability of MAPK to phosphorylate a recombinant His-tagged Cbfa1 fusion protein was examined in vitro. As shown in Fig. 5A, incubation of fusion protein purified by nickel affinity chromatography with active MAPK, but not heat-inactivated MAPK, in the presence of [γ-32P]ATP, yielded a labeled band of the expected size on SDS-polyacrylamide gels. In contrast, an N-terminal Cbfa1 fusion protein, missing residues 44–528, was not labeled although it contains several MAPK consensus phosphorylation sites. We next performed 32P metabolic labeling studies to establish whether intact cells phosphorylate Cbfa1 and to assess the relationship between this phosphorylation and MAPK-stimulated gene expression. COS-7 cells were transfected as in Fig. 4D with plasmids expressing β-galactosidase or Cbfa1 alone or Cbfa1 with MEK(SP) or MEK(DN). Cells were then labeled with [32P]orthophosphate, and nuclear extracts were prepared for Western blots or immunoprecipitation and autoradiography. As shown in Fig. 5B, only samples transfected with the Cbfa1 expression plasmid gave a positive signal on Western blots. This analysis verified that amounts of Cbfa1 protein were similar in all three conditions. Replicate samples were immunoprecipitated with anti-Cbfa1 antibody, and 32P was visualized by autoradiography (Fig. 5C) or quantified with an Instant Imager (Packard Instruments, Meriden, CT) (Fig. 5D). Only samples transfected with the Cbfa1 vector contained immunoprecipitated 32P. Furthermore, cells cotransfected with MEK(DN) showed reduced incorporation, while those treated with MEK(SP) showed a 2.1-fold increase in 32P incorporation. Thus, Cbfa1 can be phosphorylated in
intact cells, and this phosphorylation is increased by exogenous MEK(SP) but not by MEK(DN).

The PST Domain of Cbfa1 Is Necessary for MAPK Activation—As a first step in the identification of the residues necessary for MEK responsiveness, we conducted cotransfection experiments with two previously described Cbfa1 deletion mutants, Cbfa1(SP) and Cbfa1(DN).

**FIG. 3.** MEK(SP) stimulates OSE2-driven transcription only in Cbfa1-positive cells. A, Cbfa1 Western blot analysis. Whole cell extracts from osteoblast-like cells (MC-4, ROS17/2.8) and nonbone cells (COS-7, F9) were probed with a specific anti-Cbfa1 antibody as described under "Experimental Procedures." B–E, OSE2-driven transcription. MC-4, ROS17/2.8, F9, or COS-7 cells were transfected as in Fig. 2 except that the reporter plasmids used were OSE2/34-luc, OSE1/34-luc, or OSE1mt/34-luc as indicated. Expression plasmids were the same as in Fig. 2.

**FIG. 4.** Transfection with a Cbfa1 expression plasmid enhances MEK responsiveness in osteoblast-like cells and confers MEK responsiveness to nonbone cell lines. MC-4, ROS17/2.8, F9, or COS-7 cells were transfected with OSE2/34-luc or OSE2mt/34-luc reporter plasmids and the indicated expression plasmid. Cbfa1, pCMV5Cbfa1 expression plasmid. Other abbreviations are as in Figs. 2 and 3.
mutants (17). Both deletions were in areas of the Cbfa1 sequence containing potential MAPK phosphorylation sites. These studies showed that COS-7 cells cotransfected with expression vectors encoding OSE2/34-luc, MEK(SP), and a Cbfa1 construct lacking the entire proline-serine-threonine (PST) domain (258–528) exhibited none of the reporter stimulation observed in cells transfected with wild type Cbfa1 (Fig. 6). In contrast, deletion of the N-terminal 108 amino acids of Cbfa1, while greatly reducing the basal transactivation ability of the protein, did not effect the magnitude of MEK-induced stimulation. This result is consistent with the in vitro phosphorylation study shown in Fig. 5A, which failed to detect 32P labeling of a 44-amino acid N-terminal Cbfa1 fragment. Taken together, these results suggest that the MAPK signaling pathway is able to regulate bone-specific OCN transcription through phosphorylation of residues in the PST domain of Cbfa1.

ECM-dependent Induction of the mOG2 Promoter Requires MAPK Signaling—The studies described above exclusively used transfected MEK1 expression vectors as a means of activating MAPK. The experiment shown in Fig. 7 examined whether MAPK signaling is necessary for activation of the OCN promoter by ECM synthesis. This study used MC-42 cells, a highly differentiating MC3T3-E1 subclone containing stably integrated copies of a 1.3-kb mOG2-luc reporter plasmid. Previous worked showed that ECM-dependent induction of luciferase activity in these cells parallels induction of the endogenous OCN mRNA (12). Cells were grown for 10 days in control or ascorbic acid-containing medium (to induce ECM synthesis) and then treated for 6 h with increasing concentrations of the specific MEK1/MEK2 inhibitor, PD98059 (24). This compound selectively inhibited ECM-dependent promoter activity by approximately 70%. Effects of PD98059 were specific in that this compound did not alter the luciferase activity of control cultures; nor did it reduce either cell DNA or protein (result not shown). The concentration of PD98059 required for maximal inhibition of ECM-dependent promoter activity (100 μM) is in the range reported by others as being necessary to block both MEK1- and MEK2-dependent phosphorylation of MAPK (25).

DISCUSSION

Studies with Cbfa−/− animals and transgenic experiments (7, 9–11) conclusively established the essential role of Cbfa1 in osteoblast formation and regulation of bone ECM synthesis. However, the molecular mechanism of Cbfa1 action is unknown. This study provides the first evidence that the MAPK pathway is important for Cbfa1-dependent transcription. We show that activation of MAPK signaling by transfection of osteoblast cells with a constitutively active form of MEK1 induces transcription of the osteoblast-specific OCN gene and that this activation is accomplished through modulation of Cbfa1 transcriptional activity coincident with phosphorylation of Cbfa1. Furthermore, specific inhibition of the MAPK pathway was shown to selectively and rapidly block ECM-depend-
absence of ascorbic acid for 10 days and then treated for 6 h with the specific MEK1/MEK2 inhibitor, PD98059. Cells were harvested and assayed for luciferase activity, DNA, and protein. Results were normalized to total cell protein.

Activation of osteoblast-specific gene expression requires a complex and poorly understood dialogue between bone cells and their ECM. This laboratory and others showed that osteoblasts must secrete an ECM before they will express osteoblast-related genes such as OCN in vitro (1–5, 12) and in vivo (26, 27). An important component of this response is the interaction of osteoblasts with type I collagen via α2β1 and α1β1 integrins (13, 14, 28). Because the ECM-dependent increase in OCN gene transcription requires an intact promoter binding site for Cbfa1 (i.e. OSE2) and is accompanied by increased binding of Cbfa1 to OSE2 DNA in the absence of any change in Cbfa1 protein levels, we hypothesized that Cbfa1 must undergo some type of activation process to become transcriptionally active (13).

Heterodimerization of Cbfa proteins with a common β subunit represents one potential mechanism for activating this class of transcription factors (29). For example, binding of Cbfa2 to Cbfb stimulates DNA binding and transcriptional activity and is essential for the in vivo function of Cbfa2 (AML1) in hematopoiesis (30, 31). Although we have not examined whether matrix signals affect the ability of Cbfa1 to heterodimerize with other nuclear factors, heterodimer formation with Cbfb is unlikely due to the presence of a unique QA domain in the N-terminal region of Cbfa1, which blocks in vitro heterodimerization with the β subunit (17). Furthermore, mice heterozygous for the Cbfb deletion do not exhibit skeletal abnormalities, which would be expected if Cbfa1 function required Cbfb (30, 31). The possibility that Cbfa1 can heterodimerize with other nuclear proteins has not yet been examined.

Covalent modification is another potential route for Cbfa1 regulation. Because previous studies pointed to involvement of the MAPK pathway in the stimulation of osteoblast-specific gene expression by ECM (14), we examined the possibility that Cbfa1 activity could be regulated by MAPK-dependent phosphorylation. Our demonstration that transfection of osteoblasts with a constitutively active MEK1 leads to the phosphorylation and activation of Cbfa1 may point to a general mechanism for regulating gene expression in osteoblasts. The MAPK pathway is a major point of convergence for a variety of intracellular signals initiated by ECM-integrin interaction (32), growth/differentiation factor binding to receptor tyrosine kinases (33), and mechanical stimulation (34). In addition, there is considerable cross-talk between this pathway and other signaling events such as those mediated by protein kinase C (35) and BMPs (36). The canonical model for MAPK activation after either receptor tyrosine kinase ligandig or integrin occupancy involves dimerization and tyrosine phosphorylation of the receptor tyrosine kinase or, in the case of integrins, phosphorylation of the focal adhesion kinase, pp125FAK. In either case, phosphorylation is followed by recruitment of an adapter protein-guanine nucleotide exchange protein complex (GRB2-mSOS), leading to GTP exchange and activation of Ras. Ras binds and activates the serine/threonine kinase, Raf, which phosphorylates and activates the dual specificity kinase, MEK. MEK subsequently phosphorylates and activates MAPK (also known as extracellular signal-regulated kinase). Two extracellular signal-regulated kinases (1 and 2) can subsequently stimulate gene expression by phosphorylating specific transcription factors (reviewed in Ref. 37).

We are clearly at the very early stages of investigating what is likely to be a complex set of related pathways for regulating Cbfa1 action. Our work highlights several directions for future study. First, although a recombinant MAPK was able to stimulate the in vitro phosphorylation of Cbfa1, we do not yet know whether MAPK can directly phosphorylate Cbfa1 in vivo. It is equally plausible that effects of MEK(SP) transfection on Cbfa1 are indirect and mediated by other signal transduction pathways that are directly activated by MAPK. Similarly, inhibitor studies showing a requirement for MEK1 and MEK2 in the activation of the OCN promoter by ECM synthesis do not address the possibility of there being additional steps between MAPK and Cbfa1. Interestingly, AML1, which plays a major role in hematopoiesis and cell transformation in leukemias, is phosphorylated and activated by extracellular signal-regulated kinase 1 (38). Although the two phosphorylated serine residues in the PST domain of AML1 are conserved in Cbfa1, we found no changes in MEK responsiveness after these sites were mutated, indicating that other regions of the PST domain must be required for MAPK activation. We are currently examining the Cbfa1 sequence in greater detail to identify sites in the 260-amino acid PST domain necessary for phosphorylation and biological activity and to determine if these sites are direct substrates for MAPK.

A second area for future work deals with the identification of the ECM-associated signals regulating Cbfa1 and elucidation of the overall pathway for transcriptional activation. As noted above, a large number of signals can stimulate the MAPK cascade, but only a select number of these factors can affect osteoblast differentiation. It is likely that MAPK stimulation must be presented together with other appropriate stimuli for Cbfa1 activation to take place. For example, a recent study by Suzawa and co-workers showed that the ECM of MC3T3-E1 cells contains BMP2 and BMP4 and that these factors, together with type I collagen, are necessary for the induction of alkaline phosphatase activity observed when MC3T3-E1 cells are plated on ECM isolated from previously differentiated cells (39). Furthermore, recent evidence suggests that cells in chronic contact with their ECM, as is the case during osteoblast differentiation, may use a distinct mechanism to activate the MAPK cascade that involves protein kinase C and cytoskeleton-associated factors (35). Future work will be required to resolve the degree to which other matrix molecules and non-MAPK signal transduction pathways are involved in the response of osteoblasts to their ECM.

REFERENCES
1. Gerstenfeld, L. C., Chipman, S. D., Glowacki, J., and Lian, J. B. (1987) Dev. Biol. 122, 49–60
2. Orren, T. A., Arnow, M., Shalhoub, V., Barone, L. M., Wilmng, L., Tassani, M. S., Kennedy, M. B., Peckwise, S., Lian, J. B., and Stein, G. S. (1990) J. Cell. Physiol. 143, 420–430
3. Franceschi, R. T., and Iyer, B. S. (1992) J Bone Miner. Res. 7, 235–246
4. Franceschi, R. T., Iyer, B. S., and Cai, Y. (1994) J. Bone Miner. Res. 9, 843–854
5. Franceschi, R. T. (1999) Crit. Rev. Oral Biol. Med. 10, 40–57
6. Ducy, P., and Karsenty, G. (1995) Mol. Cell. Biol. 15, 1858–1869
7. Frenod, J. L., Xiao, G., Fuchs, S., Franceschi, R. T., Karsenty, G., and Ducy, P. (1998) J. Biol. Chem. 273, 30509–30516

G. Xiao and R. T. Franceschi, unpublished results.
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8. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997) Cell 89, 747–754
9. Komori, T., Yagi, H., Nenura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) Cell 89, 755–764
10. Otto, F., Thurin, A. P., Crompton, T., Denzel, A., Gilmore, K. C., Rosewell, I. R., Stamp, G. W., Beddington, R. S., Mundlos, S., Olsen, B. R., Selby, P. B., and Owen, M. J. (1997) Cell 89, 765–771
11. Ducy, P., Starbuck, M., Priemel, M., Shen, J., Pinero, G., Geoffroy, V., Amling, M., and Karsenty, G. (1999) Genes Dev. 13, 1025–1036
12. Xiao, G., Cui, Y., Ducy, P., Karsenty, G., and Franceschi, R. T. (1997) Mol. Endocrinol. 11, 1103–1113
13. Xiao, G., Wang, D., Benson, M. D., Karsenty, G., and Franceschi, R. T. (1998) J. Biol. Chem. 273, 32988–32994
14. Takeuchi, Y., Suzawa, M., Kikuchi, T., Nishida, E., Fujita, T., and Matsumoto, T. (1997) J. Biol. Chem. 272, 29309–29316
15. Wang, D., Christensen, K., Chawla, K., Xiao, G., Krebsbach, P. H., and Franceschi, R. T. (1999) J. Bone Miner. Res. 14, 993–903
16. Majeska, R. J., Rodan, S. B., and Rodan, G. A. (1980) Endocrinology 107, 1494–1503
17. Thirunavukkarasu, K., Mahajian, M., McLaren, K. W., Stifani, S., and Karsenty, G. (1998) Mol. Cell. Biol. 18, 4197–4208
18. Wu, X., Noh, S. J., Zhou, G., Dixon, J. E., and Guan, K. L. (1996) J. Biol. Chem. 271, 3265–3271
19. Sugimoto, T., Stewart, S., Han, M., and Guan, K. L. (1998) EMBO J. 17, 1717–1727
20. Chomezynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
21. Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5201–5205
22. Celeste, A. J., Rosen, V., Buecker, J. L., Kriz, R., Wang, E. A., and Wozney, J. M. (1986) EMBO J. 5, 1885–1890.
23. Renkawitz, R., Gerbi, S. A., and Glazter, K. H. (1979) Mol. Gen. Genet. 173, 1–13
24. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 27489–27494
25. Hodge, C., Liao, J., Stofega, M., Guan, K., Carter-Su, C., and Schwartz, J. (1998) J. Biol. Chem. 273, 31327–31336
26. Takigawa, A., Araki, M., Nakagawa, S., Banno, A., Aoki, M., and Matsumoto, S. (1995) Jpn. J. Pharmacol. 68, 255–261
27. Mahmoudian, F., Gosiewska, A., and Peterkofsky, B. (1996) Arch. Biochem. Biophys. 336, 86–96
28. Jikko, A., Harris, S. E., Chen, D., Mendrick, D. L., and Damsky, C. H. (1999) J. Bone Miner. Res. 14, 1057–1063
29. Ogawa, E., Inuzuka, M., Maruyama, M., Satake, M., Naito-Fujimoto, M., Ita, Y., and Shipesada, K. (1993) Virology 194, 314–331
30. Wang, Q., Stacy, T., Miller, J. D., Lewis, A. F., Gu, T. L., Huang, X., Bushweller, J. H., Borges, J. C., Alt, F. W., Ryan, G., Liu, P. F., Wynshaw-Boris, A., Binder, M., Marin-Padilla, M., Sharpe, A. H., and Speck, N. A. (1996) Cell 87, 697–708
31. Sasaka, K., Yokoh, M., Bronson, R. T., Tominaga, K., Matsunashi, T., Deguchi, K., Tani, Y., Kishimoto, T., and Komori, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12359–12363
32. Chen, Q., Knche, M. S., Lin, T. H., Burridge, K., and Juliano, R. L. (1994) J. Biol. Chem. 269, 26602–26605
33. Cobb, M. H., Boulton, T. G., and Robbins, D. J. (1991) Cell Regul. 2, 965–978
34. Schmidt, C., Pommerenke, H., Durr, F., Nebe, B., and Rychny, J. (1998) J. Biol. Chem. 273, 5081–5085
35. Howe, A. K., and Juliano, R. L. (1998) J. Biol. Chem. 273, 27268–27274
36. Ghosh-Choudhury, N., Celeste, A., Harris, S. E., and Ghosh-Choudhury, G. (1998) Bone 23, F012
37. Robinson, M. J., and Cobb, M. H. (1997) Curr. Opin. Cell Biol. 9, 180–186
38. Tanaka, T., Kurokawa, M., Ueki, K., Tanaka, K., Imai, Y., Mitani, K., Okazaki, K., Sagata, N., Yazaki, Y., Shihata, Y., Kadewaki, T., and Hirai, H. (1996) Mol. Cell. Biol. 16, 3967–3979
39. Suzawa, M., Takeuchi, Y., Fukushima, S., Kato, S., Ueno, N., Miyazono, K., Matsumoto, T., and Fujita, T. (1999) Endocrinology 140, 2125–2133