Fibroblast Growth Factor 2 Induction of the Osteocalcin Gene Requires MAPK Activity and Phosphorylation of the Osteoblast Transcription Factor, Cbfa1/Runx2*

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Fibroblast growth factor 2 (FGF-2) is an important regulator of bone formation and osteoblast activity. However, its mechanism of action on bone cells is largely unknown. A major route for FGF signaling is through the mitogen-activated protein kinase (MAPK) pathway. We showed recently that this pathway is important for activation and phosphorylation of Cbfa1/Runx2, an osteoblast-related transcription factor. Xiao, G., Jiang, D., Thomas, P., Benson, M. D., Guan, K., Karsenty, G., and Franceschi, R. T. (2000) J. Biol. Chem. 275, 4453–4459. The present study examined the mechanism of FGF-2 regulation of the mouse osteocalcin gene in MC3T3-E1 preosteoblastic cells. FGF-2 stimulated osteocalcin mRNA and promoter activity in a dose- and time-dependent manner in MC3T3-E1 preosteoblastic cells. Similar results were obtained in mouse bone marrow stromal cells. This stimulation required Runx2 and its DNA binding site in the osteocalcin promoter. FGF-2 also dramatically increased phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) followed by phosphorylation of Runx2. Furthermore, a specific ERK1/2 phosphorylation inhibitor, U0126, completely blocked both FGF-2-stimulated Runx2 phosphorylation and osteocalcin promoter activity, indicating that this regulation requires the MAPK pathway. Deletion studies showed that the C-terminal PST domain of Runx2 is required for the FGF-2 response. This study is the first demonstration that Runx2 is phosphorylated and activated by FGF-2 via the MAPK pathway and suggests that FGF-2 plays an important role in regulation of Runx2 function and bone formation.

Fibroblast growth factor 2 (FGF-2) is an important regulator of bone growth and development that can stimulate bone formation and mineralization (1–4). This factor can also regulate intracellular signaling cascades. FGFRs have intrinsic protein-tyrosine kinase activity and are capable of autophosphorylation (19, 20). Although all the different splice variants of the four FGFRs can be activated by FGF1 (acidic fibroblast growth factor), most other FGFRs have narrower specificity for different FGF ligands. In particular, FGF-2 activates the splice variant FGFR2-IIIc but not FGFR2-IIIb (21). FGF-2 binding to FRG induces receptor dimerization and subsequent transphosphorylation that initiates intracellular signaling cascades. FGFRs signal through both the MEK/ERK branch of the mitogen-activated protein kinase (MAPK) pathway and protein kinase C (PKC) in a number of cell types including osteoblasts (22–25). Core binding factor A1 (Cbfa1, also known as Runx2) is an osteoblast-related transcription factor that is essential for bone formation (26). This factor functions by binding to specific store bone mass in the ovariectomized rat (2, 4, 5), a well established model for postmenopausal bone loss. Consistent with FGF-2 having a role in bone formation, overexpression of FGF-2 in transgenic mice causes premature mineralization, achondroplasia, and shortening of long bones (6), whereas disruption of the FGF-2 gene leads to decreased bone mass and bone formation (7). All FGFRs signal through a group of high affinity transmembrane receptors (FGFR1 to FGFR4). Activating mutations in FGFRs have been linked to a number of human autosomal dominant skeletal disorders including craniosynostosis, thus providing further evidence that FGF signaling is important for skeletogenesis. For example, mutations in the FGFR1 gene are associated with Pfeiffer syndrome, which is characterized by the premature fusion of several calvarial sutures and hand and foot anomalies including syndactyly and shortened digits (8). Similarly, activating mutations in FGFR2 give rise to Apert syndrome, which is also associated with craniosynostosis (9).

Studies with cultured osteoblast precursors also indicate that FGFRs can act as local regulators of bone formation. Osteoblasts synthesize FGF-2 and store it in a bioactive form in the extracellular matrix (10–12). FGF family members control osteoblast gene expression in a biphasic fashion, dependent upon the stage of osteoblast maturation (2, 3, 11, 13–15). FGF-2 stimulates osteoblast proliferation and the production of osteocalcin (OCN), an osteoblast-specific protein, in immature preosteoblastic cells (13–16). In contrast, phenotypically more mature osteoblast cells do not respond to FGF-2 in this way. For example, FGF-2 suppresses differentiation markers in ROS17/2.8 osteosarcoma cells (11, 17). Similarly, FGF-2 reduces bone sialoprotein (BSP) expression in differentiated osteoblasts from calvarial explants while it induces BSP expression in other cells in close proximity to the site of application (18). Taken together, these studies indicate that FGF-2 is an important regulator of bone formation.

All FGFRs have intrinsic protein-tyrosine kinase activity and are capable of autophosphorylation (19, 20). Although all the different splice variants of the four FGFRs can be activated by FGF1 (acidic fibroblast growth factor), most other FGFRs have narrower specificity for different FGF ligands. In particular, FGF-2 activates the splice variant FGFR2-IIIc but not FGFR2-IIIb (21). FGF-2 binding to FGFR induces receptor dimerization and subsequent transphosphorylation that initiates intracellular signaling cascades. FGFRs signal through both the MEK/ERK branch of the mitogen-activated protein kinase (MAPK) pathway and protein kinase C (PKC) in a number of cell types including osteoblasts (22–25).

Core binding factor A1 (Cbfa1, also known as Runx2) is an osteoblast-related transcription factor that is essential for bone formation (26). This factor functions by binding to specific
enhancer sequences in target genes where it activates transcription through a mechanism that is currently not well understood. We showed recently that Runx2 is phosphorylated and activated by the MEK/ERK branch of the MAPK pathway (27). Specifically, stimulation of MAPK by transfecting a constitutively active form of MEK1, MEK(SP), into MC3T3-E1 preosteoblastic cells increased endogenous OCN mRNA, whereas a dominant negative mutant, MEK(DN), was inhibitory. MEK(SP) also stimulated activity of the OCN promoter, and this stimulation required an intact copy of the Runx2 DNA binding site, OSE2 (osteoblast-specific element 2). Significantly, transfection of cells with MEK(SP) also increased Runx2 phosphorylation. In addition, Runx2 was phosphorylated by activated recombinant MAPK in vitro. Finally, the specific MEK1/2 inhibitor, U0126, inhibited ECM-dependent up-regulation of OCN and BSP mRNAs and OCN promoter activity (28), indicating that the MAPK pathway and presumably Runx2 phosphorylation are also required for responsiveness of osteoblasts to ECM signals.

These studies led us to speculate that MAPK-dependent phosphorylation of Runx2 may also be required for FGF2 induction of the OCN gene. To test this hypothesis, FGF-2 actions were examined in murine MC3T3-E1 preosteoblast cells and primary bone marrow stromal cells (BMSCs). As will be shown, this growth factor stimulates OCN gene expression and promoter activity in a dose- and time-dependent manner. Stimulation requires Runx2 and its DNA binding site, OSE2. Furthermore, FGF-2 stimulates Runx2 phosphorylation through a pathway requiring MAP kinase activity.

**EXPERIMENTAL PROCEDURES**

Reagents—Tissue culture medium and fetal bovine serum were obtained from HyClone (Logan, UT). Other reagents were obtained from the following sources: U0126 from Promega (Madison, WI), U0124 from Calbiochem (La Jolla, CA), p44/42 MAP kinase antibody or phospho-p44/42 MAP kinase (Thr-202/Tyr-204) antibody from Cell Signaling (Beverly, MA), Rabbit anti-mouse Runx2 antibody from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), horse-radish peroxidase-conjugated goat anti-rabbit IgG from In vitrogen, and FGF-2 from Upstate Biotechnology (Lake Placid, NY). All other chemicals were of analytical grade.

Cell Cultures—Two previously described MC3T3-E1 subclonal cell lines with high osteoblast differentiation potential were used in this study (28). Both subclones (MC-4 and MC-42 cells) express osteoblast phenotypic marker genes and mineralize only after growth in ascorbic acid-containing medium. MC3T3-E1 subclone 42 (MC-42) cells have the same phenotype as MC-4 cells and also contain stably integrated copies of a 1.3-kb mouse osteocalcin gene 2 (mOG2) promoter driving a firefly luciferase reporter gene. Luciferase expression closely follows levels of endogenous OCN mRNA (29). Both cell lines were maintained in ascorbic acid-free H9251 MEM (Invitrogen), 10% FBS, 1% penicillin/streptomycin and were not used beyond passage 15. F9 teratocarcinoma cells (from the American Type Culture Collection, Manassas, VA) were grown in MEM, 10% FBS, 1% penicillin/streptomycin C3H10T1/2 fibroblasts, and COS-7 cells (from the American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium, 10% FBS, 1% penicillin/streptomycin.

Mouse Bone Marrow Stromal Cell Cultures (BMSCs)—Isolation of mouse BMSCs was described previously (30). Briefly, 6-week-old male C57BL/6 mice were sacrificed by cervical dislocation. Tibiae and femurs were isolated and the epiphyses were cut. Marrow was flushed with Dulbecco's modified Eagle's medium containing 20% FBS, 1% penicillin/streptomycin, and 10⁻⁸ M dexamethasone into a 80-mm dish, and the
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Cell suspension was aspirated up and down with a 20-gauge needle in order to break clumps of marrow. The cell suspension (marrow from two mice/flask) was then cultured in a T75 flask in the same media. After 10 days, cells reach confluence and are ready for experiments.

**DNA Constructs**—All luciferase reporter plasmids were constructed by cloning the mOG2 promoter insert and multimeric oligonucleotides into the p4luc promoterless luciferase expression vector as described previously (31). The Cbfα1 expression plasmids pCMV5Cbfα1 and pCMV5Cbfα1 (Δ258–528), containing cDNAs encoding either wild type Cbfα1 or deletion thereof under CMV promoter control, were also described previously (32).

**Transfection**—Cell lines were plated on 35-mm dishes at a density of 5 × 10^5 cells/cm^2. After 24 h, cells were transfected with LipofectAMINE (Invitrogen) according to manufacturer's instructions. Each transfection contained 0.5 μg of the indicated plasmid plus 0.05 μg of pRL-SV40, containing a cDNA for Renilla reformis luciferase to control for transfection efficiency. Cells were harvested and assayed using the dual luciferase assay kit (Promega) on a Monolight 2010 luminometer (Pharmingen).

**Western Blot Analysis**—Whole cell extracts were prepared by rinsing cell layers in phosphate-buffered saline containing 100 μl of Sigma protease inhibitor mixture per 10^7 cells. Cells were then harvested in 1× SDS-PAGE loading buffer (2% SDS, 2.5 m urea, 10 mM dithiothreitol, 10% glycerol, 10 mM Tris HCl, 0.002% bromphenol blue, 1.0 mM phenylmethylsulfonyl fluoride). Whole cell extracts were subjected to SDS-PAGE on 10% gels. Separated proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Primary rabbit antibody (p44/p42 MAP kinase antibody, phospho-p44/p42 MAP kinase (Thr-202/Tyr-204), or Runx2 antibody) was used at a dilution of 1:1,000. Second antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) was used at a dilution of 1:10,000. Blocking and reaction with antibodies were conducted as described previously (33). Immunoreactivity was determined using the ECL chemiluminescence reaction (Amersham Biosciences).

**RNA Analysis**—RNA was isolated using TriZOL reagent according to the manufacturer's protocol (Invitrogen). Poly(A)-containing RNA was purified by oligo(dT)-cellulose chromatography (34). Aliquots were fractioned on 1.0% agarose-formaldehyde gels and blotted onto nitrocellulose paper as described by Thomas (35). Mouse cDNA probes used for hybridization were obtained from the following sources: GCN (36) from Dr. John Wozney (Genetics Institute, Boston, MA) and mouse α-actin from the American Type Culture Collection. All cDNA inserts were 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 Bellco Autoblot hybridization oven (37) and scanned quantitatively using a Packard A2024 InstantImager. All values were normalized for RNA loading by probing blots with cDNA to 18 S rRNA (38).

**Metabolic Labeling and Immunoprecipitation of Runx2**—MC-4 cells were cultured for 30 h in ascorbic acid-free α-MEM containing 10% FBS and then transferred to and incubated for 12 h in ascorbic acid-free α-MEM containing 0.1% FBS. Labeling was conducted for 9 h in phosphate-free ascorbic acid-free α-MEM containing 0.1% FBS (dialyzed against phosphate-free ascorbic acid-free α-MEM) and 200 μCi of [32P]orthophosphate (Phosphorus-32; Amersham Biosciences) per ml. FGF-2 was added into the cultures for the last 6 h. Cells were then harvested in cold 1× phosphate-buffered saline containing 100 μl of Sigma protease inhibitor mixture per 10^7 cells. The cell pellet was resuspended in 1× lysis buffer containing 100 μl of protease inhibitor mixture per 10^7 cells (20 mM HEPES, pH 7.6, 350 mM NaCl, 50 mM KCl, 1 mM dithiothreitol, 0.25% Nonidet P-40, 5 mM NaF, 1 mM EDTA, 5 mM MgCl2, 0.25 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1 mM sodium orthovanadate) for 20 min on ice. Whole cell extracts were precleared twice with 50 μl of protein A/G-agarose beads (Stratagene, La Jolla, CA) for 30 min followed by pelleting of beads. 5 μl of rabbit polyclonal anti-Runnx2 antibody was added and incubated for 2 h at 4 °C with gentle rocking. The immune complexes were collected upon addition of 30 μl of protein A/G-agarose beads, and incubation for 1 h at 4 °C was followed by centrifugation. Precipitates were washed five times with 1× lysis buffer (20 mM HEPES, pH 7.6, 50 mM KCl, 1 mM dithiothreitol, 0.25% Nonidet P-40, 5 mM NaF, 1 mM EDTA, 5 mM MgCl2, 0.25 mM phenylmethylsulfonyl fluoride), and the immunoprecipitated complexes were suspended in SDS sample buffer and analyzed by SDS-PAGE and autoradiography. [32P] incorporation was measured using a Packard A2024 InstantImager.

**Statistical Analysis**—All experiments were repeated a minimum of two times, and qualitatively identical results were obtained. Statistical analyses were performed using Instat 3.0 (GraphPAD Software, Inc., San Diego, CA). Unless indicated otherwise, each value reported is the mean and standard deviation of triplicate independent samples.

**RESULTS**

**FGF-2 Stimulates Osteocalcin Gene Expression and Promoter Activity**—Actions of FGF-2 on osteoblast gene expression were examined using the endogenous OCN gene and a 1.3-kb mOG2 promoter luciferase reporter gene. As shown in Fig. 1, A and B, treatment of MC-4 cells with FGF-2 (25 ng/ml) for 12 h resulted in more than a 5-fold increase in OCN mRNA expression. As shown in Fig. 1, C and D, FGF-2 also stimulated OCN gene expression in mouse primary BMSCs. To study the effect of FGF-2 on mOG2 promoter activity, MC-4 cells that stably integrated copies of a 1.3-kb mOG2 promoter luciferase construct were treated with the indicated concentration of FGF-2 for 12 h. Luciferase activity was normalized to total protein. B, time course. Cells were treated for the indicated times with 25 ng/ml FGF-2.
The 1.3-kb mOG2 promoter contains a number of enhancer sequences including two copies of the specific Runx2 binding site, OSE2. The 3′-OSE2 is known to be essential for osteoblast-specific expression of OCN in vivo (39). To determine whether OSE2 and Runx2 are both necessary for FGF-2 stimulation, MC-4 cells that contain high levels of Runx2 (27) were transfected with 6OSE2/34-luc, which is a reporter plasmid containing six copies of OSE2 upstream of a minimal 34-bp mOG2 promoter. After transfection, cells were treated with 25 ng/ml FGF-2 for 12 h in 0.1% FBS in MEM before harvesting. 

Fig. 3 shows that FGF-2 dramatically stimulated OSE2-dependent luciferase activity. The dose response and time course for stimulation were similar to that seen with the 1.3-kb promoter in Fig. 2 (results not shown). In contrast, introduction of a 2-bp mutation that renders the OSE2 sequence nonfunctional as a Runx2 binding site completely abolished the FGF-2 response. As shown in Fig. 3, B–D, FGF-2 did not stimulate 6OSE2/34-luc in COS-7 cells, F-9 cells, and 10T1/2 cells, which do not contain detectable levels of Runx2 protein. However, transfection of these cells with a Runx2 expression vector rendered them responsive to FGF-2 treatment. 

As a first step in the identification of regions in Runx2 necessary for FGF-2 responsiveness, we found that COS-7 cells cotransfected with 6OSE2-luc reporter plasmid and renilla luciferase normalization plasmid and cultured in 10% FBS medium for 24 h. Cells were then switched to 0.1% FBS in the absence or presence of 25 ng/ml FGF-2 for 12 h. Firefly luciferase activity was normalized to renilla luciferase activity. B–D, transfection with a Runx2 expression plasmid confers FGF-2 responsiveness to non-bone cell lines. F9, C3H10T1/2, or COS-7 cells were transfected with 6OSE2-luc reporter plasmid and renilla luciferase normalization plasmid in the absence or presence of pCMV5Runx2 expression plasmid (Runx2). Cells were then treated as in A. Also shown in D is the lack of FGF-2 responsiveness in cells transfected with a pCMV5 expression plasmid encoding a Runx2 deletion mutant lacking the entire C-terminal PST domain (ΔPST).
orthophosphate in the absence or presence of increasing FGF-2 (2.5, 12.5, and 25 ng/ml) with or without U0126 (FGF2/H9262); 25 ng/ml FGF-2 plus 10 μM U0124 (a negative control for U0126) (FGF2/U0124); 10 μM U0126 alone (U0126), 10 μM U0124 alone (U0124).

To determine whether FGF-2 can stimulate Runx2 phosphorylation, we used U0126, a specific inhibitor of MAPK signaling. This compound binds MEK1 and MEK2 regardless of activation state to noncompetitively inhibit phosphorylation of ERK1/2. We also used the inactive analogue, U0124, as a negative control for U0126 (40). As shown in Fig. 4, U0126 at a concentration of 10 μM completely blocked FGF-2-stimulated promoter activity. However, the same concentration of U0124 was without effect. Also, no cell toxicity was observed under the conditions of this experiment in that inhibitor treatment did not affect cell morphology or number. In conclusion, FGF-2 stimulated Runx2-dependent transcription, and this stimulation required the MEK/ERK branch of the MAPK pathway.

FGF-2 Stimulation of the Phosphorylation of Runx2—The results described previously indicate that FGF-2 has an important role in the regulation of Runx2 activity and OCN gene expression. To further explore the molecular mechanism of this regulation, we first tested the effects of FGF-2 on ERK1/2 phosphorylation. MC-4 cells were treated with or without 25 ng/ml FGF-2 for different times (from 0.2 to 12 h) in 0.1% FBS α-MEM. Whole cell extracts (25 μg/lane) were loaded for Western blot analysis using antibodies against phosphorylated/active ERK1/2, total ERK1/2, and Runx2, respectively. As shown in Fig. 5A, FGF-2 dramatically stimulated ERK1/2 phosphorylation. This stimulation was detected after 10 min and lasted for at least 12 h. In contrast, FGF-2 did not change total ERK1/2 protein levels. Similar results were obtained in MC-42 cells (data not shown). Also, Runx2 protein levels as measured by Western blot were not influenced by FGF-2 treatment. Runx2 mRNA levels as measured by Northern blot analysis were also not affected by FGF-2 treatment (result not shown).

To determine whether FGF-2 can stimulate Runx2 phosphorylation, 32P metabolic labeling was carried out in MC-4 cells as described previously (27). Briefly, cells were labeled with [32P]orthophosphate in the absence or presence of increasing concentration of FGF-2 (2.5, 12.5, and 25 ng/ml) with or without U0126 (10 μM) for 6 h. The whole-cell extracts were then used for immunoprecipitation using antibody against mouse Runx2. As shown in Fig. 5, B (top) and C, FGF-2 dramatically stimulated Runx2 phosphorylation. Furthermore, this stimulation was completely abolished by U0126 indicating that MAPK activity was required for the phosphorylation response. Again, Western blot analysis (Fig. 5B, lower panel) showed that Runx2 protein levels were not changed by FGF-2 treatment. The time course of FGF2 stimulation of Runx2 phosphorylation is shown in Fig. 5D. This response was considerably slower than that observed for ERK1/2 phosphorylation, peaking 4–6 h after FGF-2 addition. However, Runx2 phosphorylation preceded the induction of OCN promoter activity that peaked after 6 h (Fig. 2B).

Taken together, these results show that FGF-2 phosphorylates and activates the bone-specific transcription factor, Runx2, via activation of the MAPK pathway.

DISCUSSION

Although in vivo studies have clearly established an essential role for FGF-2 in bone formation (see Introduction), the mechanism of FGF-2 action in osteoblasts is not well understood and appears to be complex, involving multiple signaling pathways and factors. The most detailed studies have focused on regulation of osteocalcin, human interstitial collagenase (matrix metalloproteinase I, MMP1), and bone sialoprotein (16, 41, 42).

Boudreaux and Towler (16) showed that FGF-2 could stimulate OCN expression and promoter activity in MC3T3-E1 cells. Furthermore, this stimulation was synergistically enhanced by forskolin or by 8-bromocyclic AMP, implying a role for protein kinase A in this response. An AP-1-like site in the OCN promoter (5'-GCACCTA) was shown to be necessary for FGF-2/forskolin stimulation. This FGF response element (FRE) shows enhanced binding to nuclear proteins after FGF-2 treatment. However, the protein-DNA complex does not contain members of the ATF, Fos, or Jun families of transcription factors. Furthermore, this FRE was not sufficient for the FGF-2/forskolin response because three tandem copies of the sequence could not reconstitute the response when placed upstream of the Rous sarcoma virus minimal promoter. Interestingly, this FRE is immediately 5' to the Runx2 binding site (OSE2) studied in the present work, although no evidence was provided for the involvement of OSE2 in the FGF-2 response (see below).

The same group identified another FRE in the human matrix metalloproteinase I promoter. This FRE contains a core AP-1 binding site and an adjacent 5'-Ets site, which are both required for FGF-2 responsiveness. FGF-2 up-regulated nuclear factors capable of interacting with the AP-1 site including Fra1 and c-Jun and undefined factors interacting with the Ets site. Surprisingly, FGF-2 induction of the collagenase promoter could not be inhibited by a variety of pharmacological inhibitors including those blocking protein kinase A and MEK/ERK pathways.

Finally, Shimizu-Sasaki and co-workers (42) identified a distinct FRE in the BSP promoter containing the core AP-1-like sequence, GGTGAGAA. However, this induction was not blocked with protein kinase A inhibitors. Instead, inhibition was achieved by blocking tyrosine kinases including Src as well as the MEK/ERK branch of the MAPK pathway. No evidence was obtained for the involvement of AP-1 factors in this response. In further examining the mechanism of FGF-2 regulation of the OCN gene, we discovered a novel control mechanism involving MEK/ERK-mediated phosphorylation of Runx2. Our studies demonstrate: 1) FGF-2 stimulates OCN gene expression in both MC-4 and BMSC cultures through a mechanism involving...
Runx2 and its cognate DNA binding site, OSE2; 2) FGF-2 stimulates ERK1/2 and Runx2 phosphorylation; 3) The MEK/ERK MAP kinase pathway is required for this FGF-2 response. This discovery clearly adds a new dimension to our understanding of FGF-2 actions on bone and provides a physiologically relevant example of how growth factor-mediated phospho-
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REFERENCES


