Stimulus-selective Inhibition of Rat Osteocalcin Promoter Induction and Protein-DNA Interactions by the Homeodomain Repressor Msx2*  

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Osteocalcin (OC) is a matrix calcium-binding protein expressed in osteoblasts and odontoblasts undergoing mineralization. OC expression is up-regulated in part by signals initiated by basic fibroblast growth factor (FGF2), cyclic AMP or forskolin (FSK), and calcitriol via defined elements and DNA-protein interactions in the OC promoter. We identified the OC gene as a target for transcriptional suppression by Msx2, a homeodomain transcription factor that controls ossification in the developing skull. In this study, we examine the role of Msx2 expression on OC promoter activation (luciferase reporter) by FGF2/FSK and calcitriol in MC3T3-E1 osteoblasts. Expression of Msx2 decreases basal activity of the 1-kilobase (−1050 to +32) rat OC promoter by 80%; however, the promoter is still inducible 3-fold by calcitriol. By contrast, OC promoter induction by FGF2/FSK is completely abrogated by Msx2. Because intrinsic Msx2 DNA binding activity is not required for the Msx2 suppressor function, we assessed whether Msx2 represses OC activation by regulating DNA-protein interactions at the FGF2 response element (OCFRE) and compared these interactions with those occurring at the calcitriol response element (VDRE). Treatment of MC3T3-E1 cells with FGF2/FSK or calcitriol up-regulates specific DNA-protein interactions at the OCFRE or VDRE, respectively, as detected by gel shift assay. Preincubation of crude nuclear extracts with recombinant glutathione S-transferase (GST)-Msx2 dose-dependently inhibits OCFRE DNA binding activity, whereas GST has no effect. Msx2 itself does not bind the OCFRE. Residues 132–148 required for Msx2 core suppressor function in transfection assays are also required to inhibit OCFRE DNA binding activity. By contrast, GST-Msx2 has no effect on calcitriol-regulated DNA-protein interactions at the VDRE. Using gel shift as an assay, the OCFRE DNA-binding protein factor A1 is a global transcriptional regulator of osteoblast terminal differentiation (7–9). Recently, OC gene expression has also been detected in tissues undergoing heterotopic mineralization, such as calcified atherosclerotic plaques (2). The physiologic functions of OC are only beginning to be elucidated. In vitro, OC inhibits mineralization by binding calcium ions and preventing hydroxyapatite mineral nucleation (3). Consistent with this observation, mice that possess targeted disruption of the OC genes-1 and -2 develop a postnatal osteopetrotic syndrome characterized by enhanced mineral deposition by bone-forming osteoblasts (4). An endocrine role for OC may also exist because OC circulates at nanomolar concentrations in plasma and can recruit and activate osteoclasts and cells of the monocytic series (1, 5).

OC expression increases as osteoblasts and odontoblasts mature at the onset of tissue mineralization (1, 6). Recently, a member of the core-binding factor family, core-binding factor A1, has been shown to regulate both OC expression and the onset of osteoblast mineralization, thus suggesting that core-binding factor A1 is a global transcriptional regulator of osteoblast terminal differentiation (7–9). However, OC gene expression is also under the control of exogenous hormonal stimuli. For example, calcitriol up-regulates rat OC expression and promoter activity via protein-DNA interactions occurring at the vitamin D response element (VDRE; Ref. 10). This occurs through binding of calcitriol to the vitamin D receptor (VDR), which binds as a heterodimer with retinoid X receptor to its bipartite DNA cognate in the OC promoter (11, 12). The VDR has been shown to activate transcription via contacts with TFIIA, a component of the basal transcription machinery (13). Recently, we demonstrated that OC expression in immature MC3T3-E1 cavitary osteoblasts is synergistically up-regulated by the combination of basic fibroblast growth factor (FGF2) and cAMP or forskolin (FSK; Ref. 14). Again, a bipartite element in the proximal rat OC promoter was shown to mediate this response, and we demonstrated that FGF2/FSK up-regulates

The abbreviations used are: OC, osteocalcin; VDRE, vitamin D (calcitriol) response element; VDR, vitamin D (calcitriol) receptor; TFIIA, transcription factor IIB; FGF2, basic fibroblast growth factor; FSK, forskolin; kb, kilobase(s); OCFRE, osteocalcin fibroblast growth factor response element; GST, glutathione S-transferase; OCFREB, osteocalcin fibroblast growth factor response element-binding protein; CMV, cytomegalovirus; LUC, luciferase; FPLC, fast protein liquid chromatography.
specific DNA-protein interactions at this element (14). We and others have demonstrated that the homeodomain protein Msx2 suppresses OC expression in calvarial osteoblasts (15–18). Using OC promoter suppression as an assay, we have shown that the core suppressor domain of Msx2, residues 55–208, is sufficient for Msx2 function (19). This region overlaps the DNA binding homeodomain. However, intrinsic Msx2 DNA binding activity is not required for suppressor function, and basal OC promoter suppression is mediated at least in part via protein-protein interactions between Msx2 and TFIIF (19).

We wished to examine whether Msx2 alters hormonal regulation of the OC promoter in osteoblasts. The inductive effects of FGF2/FSK and calcitriol were examined in both the presence and absence of Msx2. A 1-kb fragment (−1050 to +32) of the rat OC promoter coupled to a luciferase reporter was used to monitor OC promoter activity because this fragment encompasses both the VDRE (−0.45 kb; Ref. 12) and the OCFRE (at −0.15 kb; Ref. 14). Expression of Msx2 decreases basal activity of the rat OC promoter by 80%; however, the promoter is still inducible 3-fold by calcitriol. By contrast, OC promoter induction by FGF2/FSK is completely abrogated by Msx2. Treatment of MC3T3-E1 cells with FGF2/FSK or calcitriol up-regulates specific DNA-protein interactions at the OCFRE or VDRE, respectively, detected by gel shift assay. Preincubation of crude nuclear extracts with recombinant GST-Msx2 dose-dependently inhibits OCFRE DNA binding activity, whereas GST has no effect. Msx2 residues 132–148 required for core suppressor function, as defined in transfection assays, are also required to inhibit OCFRE DNA binding activity. Immunologic probing of this complex with an antibody to the VDR demonstrated that the calcitriol-regulated VDRE binding complex contains the VDR. GST-Msx2 has no effect on calcitriol-regulated DNA-protein interactions at the VDRE. Using standard protein purification techniques, OCFRE DNA-binding protein (OCFREB) was purified to 50% homogeneity from MG63 osteosarcoma cells. Recombinant Msx2 inhibits the DNA binding activity of purified OCFREB, whereas the Msx2 variant lacking residues 132–148 is inactive. The addition of GST-Msx2 after OCFREB is bound to the OCFRE has no effect on DNA binding, indicating that Msx2 does not enhance the off-rate of OCFREB from the OCFRE in this assay. Thus, Msx2 abrogates transcriptional activation of the OC promoter by FGF2/FSK in part by selective inhibition of OCFRE binding to the osteocalcin FGF response element.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**MC3T3-E1 mouse calvarial osteoblasts (20) were grown as described previously (14). Tissue culture items were obtained from Fisher and Life Technologies, Inc. Molecular biology reagents were obtained from Promega (Madison, WI), Fisher, and Quagen (Chatsworth, CA). Forskolin was purchased from LC Laboratories, and calcitriol was obtained from BioMol (Plymouth Meeting, PA). FGF2 was purchased from Collaborative Research via Fisher. M2 anti-“FLAG” epitope antibody was purchased from IBI/Kodak via VWR Scientific (St. Louis, MO). Anti-Fos and Anti-VDR antibodies were obtained from Santa Cruz Biotechnology (San Diego, CA). Chemiluminescent Western blot detection reagents were obtained from Tropix (Bedford, MA). Protein was determined using the bichenichionic acid protein assay kit obtained from Pierce.

**Construction of FLAG-tagged Msx2 Expression Plasmids—**Synthetic oligodeoxynucleotides were obtained from the Washington University Protein and Nucleic Acid Laboratory or from Life Technologies, Inc. The NH2-terminal initiator methionine with the Asp-Tyr-Lys-Asp-Asp-Lys-FLAG motif and Msx2 deletions were introduced by sequential polymerase chain reaction with a Perkins-Elmer model 9600 thermal cycler (Foster City, CA), as described previously (19; for Msx2 residue numbering, see Ref. 21 and GenBank S60698). Msx2 variants were cloned into the KpnI-BamHI site in pcDNA3 (Invitrogen, Carlsbad, CA). All pcDNA3-Msx2 expression constructs in this study contain the CMV promoter upstream of the initiator methionine in good Kozak consensus (22) followed by the FLAG epitope tag and Msx2 coding sequence. All plasmids were purified by Qiagen column chromatography and sequenced to verify DNA sequence (Dye Terminator Cycle Sequencing Kit; Perkin-Elmer).

**Transfections, Luciferase Assays, and β-Galactosidase Assays—**MC3T3-E1 cells were plated into Costar six-well cluster dishes (35-mm diameter wells, 7 × 104 cells/well). Cells were transiently transfected the next day by calcium phosphate precipitation and 15% glycerol shock as detailed (19). The pcDNA3-Msx2 expression constructs were cotransfected with rat osteocalcin promoter fragment-luciferase reporter constructs (1050 OCLUC or 222 OCLUC as indicated; see Ref. 23). Empty pcDNA3 expression vector was added as required to maintain a constant amount of total plasmid DNA in each precipitation. CMV-β-galactosidase was used to monitor transfection efficiency. Two days after transfection, cells were re-fed with fresh medium (3% fetal calf serum) containing either vectors, 3 nM FGF2 and 10 μM FSK, or 25 nM calcitriol to stimulate OC promoter activity as described previously (14, 23). Three days after transfection, cell extracts were prepared and aliquots analyzed for luciferase, β-galactosidase, and FLAG-tagged Msx2 protein expression as detailed previously (14, 19). All data sets were repeated in a minimum of two independent experiments to verify results.

**Expression of Recombinant Msx2 Variants—**GST-Msx2S2/267, GST-Msx2S5/S208, and GST-Msx2S5/S208,132–148 expression plasmids were constructed as described previously (19). Recombinant proteins were isolated from transformed Escherichia coli extracts after isopropyl β-D-thiogalactopyranoside induction by affinity chromatography on glutathione-agarose and elution with 25 mM glutathione in 0.1 mM dithiothreitol and 50 mM Tris, pH 8.0 (19). Protein expression was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis fractionation and Coomassie Brilliant Blue visualization of purified proteins.

**Crude Nuclear Extract Preparation and Electrophoretic Mobility Gel Shift DNA Binding Assays—**MC3T3-E1 cells (five 150-mm diameter cultures/treatment, 90% confluent) were treated with either vehicles, 0.15 μM FGF2 and 10 μM FSK, or 50 μM calcitriol in 3% fetal calf serum in modified Eagle’s medium for 3 h (90% confluent) (19). Recombinant proteins were isolated from transformed E. coli extracts and used for gel shift assays (elements underlined in Fig. 5). OCFRE, GGAGCTGCGAATCCCGC and CCCCGGCTGACTGCGACTGCT; VDRE, GGAGCTGGTTAAGGACATTACCTCAGTAATCCTCAATTGGGTA; Sp1, GGAGTTGGCGGGGGGGGAG and CCGTGCCCGCGCGGATCT. For routine inhibition studies, crude nuclear extracts (or purified OCFREB) were preincubated for 20 min at 20 °C with GST (GST-Msx2, GST-Msx2S5/S208, or GST-Msx2S5/S208,132–148) as indicated before assessment of OCFRE or VDRE DNA binding activities by gel shift assay. OCFREB Purification—Using OCFREB gel shift assay and classical biochemical protein purification techniques, we have purified OCFREB to about 50% homogeneity (−50,000 fold) from MG63 osteoblastic osteosarcoma cells; this will be detailed in a separate manuscript. Briefly, after extraction of MG63 cells with HEPES-buffered 0.5 M NaCl, protease inhibitor mixture, 1 mM dithiothreitol, 0.5% Triton X-100 by shearing and partial adsorption of nucleic acid with glass beads, purification followed sequential ammonium sulfate fractionation, Sepharyl S-200 gel filtration chromatography, DE52 anion exchange chromatography, heparin-Sepharose chromatography, Mono Q FPLC anion exchange, and Hi-Trap heparin-agarose affinity purification. Erythropoietin inhibitor-sensitive, rapidly migrating minor complex arose during OCFRE purification as revealed by gel shift assay, suggesting that partial proteolysis occurred. Extraction of 3 × 109 MG63 cells yielded 20 μg of OCFREB that was about 50% homogeneous. Protein constituents of 80, 70, and 50 kDa were observed in this fraction by Coomassie staining of protein resolved on 11% polyacrylamide gels (see Fig. 5B, lane 5). The 70- and 80-kDa constituents marked in Fig. 5B coeluted precisely with OCFREB activity after heparin FPLC. The native size of OCFREB estimated by gel filtration chromatography was 150 kDa, suggesting that OCFREB is dimeric.
Preferably, we demonstrated that Msx2 suppresses basal OC promoter activity in MC3T3-E1 osteoblasts. We wished to determine whether hormonal regulation of the OC promoter is also influenced by Msx2. Therefore, we examined the effects of Msx2 expression on FGF2/FSK induction (via OCFRE at −0.15 kb; Ref. 14) and calcitriol induction (via VDRE at −0.45 kb; Refs. 11 and 12), using the rat OC promoter-luciferase reporter construct 1050 OCLUC (rat OC 2 kb; Ref. 23). As shown in Fig. 1A, treatment with FGF2/FSK for 24 h stimulates 1050 OCLUC activity ∼4-fold. Expression of Msx2 decreases basal OC promoter activity approximately 80% (Fig. 1A) as reported previously. Of note, Msx2 expression completely abrogates up-regulation of 1050 OCLUC by FGF2/FSK (Fig. 1A). By contrast, expression of Msx2 has no effect on induction of 1050 OCLUC by calcitriol, even though it completely abrogates induction by FGF2/FSK (Fig. 1B). Thus, Msx2 not only decreases basal OC promoter activity but selectively abrogates promoter responses to stimulants that control OC expression in osteoblasts.

**Recombinant Msx2 Inhibits OCFRE DNA Binding Activity**—We and others have identified that Msx homeodomain proteins do not require intrinsic homeodomain DNA binding activity for suppressor function (19, 25). To examine whether Msx2 might inhibit FGF2/FSK-induced OC promoter activity by regulating heterologous protein-DNA interactions assembled by the OC promoter, we assessed whether recombinant Msx2 influences DNA binding activity recognizing the OCFRE. As shown in Fig. 2 (lanes 1–5), increasing amounts of GST-Msx2 inhibit FGF2/FSK-regulated OCFRE DNA binding activity. Incubation with equivalent amounts of GST has no significant effect on OCFRE DNA binding activity (Fig. 2, lanes 6–10). Although competent to bind the HOXBOX cognate (Fig. 2, lane 13), GST-Msx2 does not directly bind the OCFRE (Fig. 2, lane 11). Thus, recombinant Msx2 inhibits OCFRE DNA binding activity.

**Recombinant Msx2 Does Not Inhibit VDRE DNA Binding Activity**—To test the specificity of Msx2 inhibition of OCFRE binding activity, we examined whether calcitriol-regulated DNA-protein interactions were influenced by recombinant

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**Fig. 1.** Msx2 abrogates FGF2/FSK up-regulation of the rat OC promoter without hindering calcitriol-induced transcriptional activity. MC3T3-E1 cells were cotransfected with 1050 OCLUC (rat OC promoter −1050 to +32; Ref. 23) and either pcDNA3 or pcDNA3-Msx2 expression plasmids as described under “Experimental Procedures.” Two days after transfection, cell cultures were treated for 24 h either with vehicles, 3 nM FGF2 and 10 μM FSK, or 25 nM calcitriol as indicated. Extracts were analyzed subsequently for luciferase activity as described previously (14, 19). Panel A, note that expression of Msx2 both decreases basal OC promoter activity and abrogates FGF2/FSK induction. Panel B, suppression of inducible 1050 OCLUC activity with increasing amounts of cotransfected pcDNA3-Msx2 expression plasmid (total plasmid DNA was kept constant in each transfection by adjustment with pcDNA3 vector). Note that even though Msx2 suppresses OC promoter activation by FGF2/FSK, calcitriol-inducible OC promoter activity is not inhibited by Msx2 expression. For details, see “Results.”

**Fig. 2.** Recombinant Msx2 inhibits OCFREB DNA binding activity in nuclear extracts from FGF2/FSK-treated MC3T3-E1 osteoblasts. MC3T3-E1 cells were cultured and treated with 3 nM FGF2, 10 μM FSK for 36 h as described under “Experimental Procedures.” Nuclear extracts were prepared, and 3-μg aliquots of MC3T3-E1 crude nuclear extracts were analyzed for OCFRE DNA binding activity as detailed previously (14) by gel shift analysis using the OCFRE duplex cognate (lanes 1–12) either in the presence (lanes 2–5) or absence (lanes 6–10) of recombinant Msx2 (see “Experimental Procedures”). 10 μg of bovine serum albumin is included in all gel shift binding reactions. Note that increasing amounts of Msx2 (0, 15, 30, 60, and 150 ng of recombinant protein) inhibit OCFRE activity (lanes 1–5), whereas equivalent amounts of recombinant GST do not inhibit binding (lanes 6–10). Note further that recombinant Max2 itself does not bind the OCFRE cognate (lane 11) even though it can recognize the HOXBOX homeodomain DNA cognate (lane 13). GST does not interact with either the OCFRE (lane 12) or the HOXBOX (lane 14).
Inhibition of OC Promoter Activity and OCFRE DNA Binding Activity Requires Msx2 Residues 132–148—Recent structure/function studies of Msx2 have identified residues 55–208 as the core suppressor domain and residues 132–148 within this minimal domain as crucial for maximal suppressor function (19). Like wild-type Msx2 (Fig. 1A), Msx2(2–208) suppresses the OC promoter in a dose-dependent manner (Fig. 4A). However, deletion of residues 132–148 within this context (Msx2(2–208;Δ132–148)) dramatically reduces Msx2 suppressor function (Fig. 4A and Ref. 19). Western blot analyses performed on these extracts demonstrate that the observed differences in suppressor function are not caused by differences in the level of Msx2(2–208) and Msx2(2–208;Δ132–148) protein accumulation (Fig. 4B). To investigate whether the core suppressor domain of Msx2 is also required for regulation of OCFRE DNA binding activity, we examined the effect of Msx2(2–208;Δ132–148) on OCFRE DNA binding activity. As shown in Fig. 5A (lanes 2–4), addition of GST-Msx2(255–208) completely inhibits binding of OCFREB to its DNA cognate. By contrast, a similar GST-Msx2 fusion protein (GST-Msx2(255–208;Δ132–148)) lacking residues necessary for core suppressor function (Fig. 4A) has no effect on OCFRE DNA binding activity (Fig. 2).
DNA binding activity (Fig. 5A, lanes 6–8). GST-Msx2(55–208) has no effect on calcitriol-induced binding to the VDRE (data not shown). Equal amounts of full-length GST-Msx2(55–208) and GST-Msx2(55–208;Δ132–148) fusion proteins were contained in these fractions, as shown by Coomassie Blue staining (Fig. 5B, lanes 3 and 4). Thus, the Msx2 core suppressor domain regulates DNA binding to the OCFRE, dependent upon residues 132–148 necessary for full suppressor function.

**Recombinant Msx2 Inhibits the DNA Binding Activity of Purified OCFREB**—We recently identified that OCFRE DNA-binding protein OCFREB (14) is constitutively expressed in MG63 cells, a human osteosarcoma cell line. Using standard biochemical techniques and OCFRE DNA binding as an assay, we have purified this protein to about 50% homogeneity2 (see “Experimental Procedures”). The native complex is 150 kDa and contains 80-kDa and 70-kDa constituents that copurify with activity (Fig. 5B, lane 5, and data not shown). To investigate whether inhibition of OCFRE DNA binding activity by Msx2 occurs through direct interaction with OCFREB itself or whether additional proteins are required, we assessed the effect of recombinant Msx2(55–208) on the activity of highly purified OCFREB. GST-Msx2(55–208) completely abrogates binding of purified OCFREB to its DNA cognate (Fig. 6, lanes 1–4), as observed with crude extracts from MC3T3-E1 cells. Again, GST-Msx2(55–208;Δ132–148) has no effect on OCFREB binding (Fig. 6, lanes 5–8). Thus, Msx2 inhibits the DNA binding activity of purified OCFREB, dependent upon residues 132–148 necessary for core suppressor function. To examine whether Msx2 inhibits OCFREB-OCFRE association (“on-rate”) or enhances OCFREB-OCFRE dissociation (“off-rate”), we altered conditions by binding purified OCFREB to the OCFRE before adding Msx2. As shown in Fig. 6, recombinant Msx2(55–208) cannot dissociate OCFREB that is prebound to

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the OCFRE (lanes 9–12) even though preincubation with Msx2 (55–208) profoundly inhibits OCFREB binding to the OCFRE (Fig. 6, lanes 1–4). Thus, Msx2 prevents association of purified OCFREB with the OCFRE. Because Msx2 does not bind the OCFRE (Fig. 2, lane 11), this strongly suggests that Msx2 directly inhibits formation of the OCFREB–OCFRE complex via protein–protein interactions with OCFREB.

DISCUSSION

The transcriptional hierarchy regulating skeletal gene expression in any particular mineralized tissue is dependent upon the morphogenetic, metabolic, and mechanical demands placed upon that ossicle (6, 9, 10). Studies of OC gene expression and function are now beginning to provide insights into how the osteoblast-specific transcriptional machinery integrates multiple regulatory cues provided by these physiologic demands (1, 10, 26, 27). For example, a Runt domain family member, core-binding factor A1 (7–9), globally controls skeletal mineralization, expression of type I collagen and alkaline phosphatase (promote mineralization), and OC (inhibits mineralization). By contrast, the homeodomain transcription factors Msx1 and Msx2 regulate mineralization in two specific morphogenetic fields (teeth and calvarial bone: Refs. 28–31) and repress OC gene expression in phenotypically immature calvarial osteoblasts (15–18). Signals initiated by FGF, transforming growth factor-β, glucocorticoids, and tumor necrosis factor-α all regulate OC expression via defined promoter elements (10, 26), and our understanding of how vitamin D regulates transcription largely derives from studies of OC regulation by calcitriol (10–13, 27). Thus, the OC gene integrates multiple FGF-activated gene targets is currently unknown; multiple regulatory cues provided by these physiologic programs that regulate mineralized tissue morphogenesis and the endocrine/paracrine signals that control mineral metabolism.

In this study, we examined the interactions between stimuli that induce OC promoter activity in calvarial osteoblasts and transcriptional repression by Msx2, a homeodomain protein that regulates calvarial morphogenesis. Msx2 inhibits not only basal OC promoter activity but also abrogates promoter induction by FGF2/FSK. This inhibition is selective because Msx2 neither abrogates nor attenuates calcitriol induction of the rat OC promoter. We also demonstrated that Msx2 inhibits formation of the FGF2/FSK-regulated OCFREB–OCFRE protein–DNA complex but not the calcitriol-regulated VDRE–VDRE complex. Domains of Msx2 required for transcriptional suppressor function are also required for inhibition of OCFREB–OCFRE complex formation. Msx2 itself does not bind the OCFRE cognate. Moreover, Msx2 inhibits the activity of highly purified OCFREB, strongly suggesting that Msx2 inhibits OC promoter DNA binding by a direct interaction with OCFREB that prevents OCFREB–OCFRE association. Msx2 may accomplish this by sterically shielding the DNA binding site of OCFREB or by preventing dimerization of OCFREB subunits. Once OCFREB has been cloned and characterized, it will be possible to test these mechanistic possibilities directly.

The observation that Msx2 selectively abrogates hormonally regulated inductive signals suggests that Msx2 can act as a transcriptional “filter,” allowing only certain signals to reach the OC promoter. Of note, genetic analyses of hereditary craniosynostosis syndromes have implied that Msx2 (31) and FGF receptor (32, 36) signaling both regulate calvarial osteogenesis. To our knowledge, OC regulation by Msx2 and OCFREB at the OC promoter. Of note, genetic analyses of hereditary craniosynostosis syndromes have implied that Msx2 (31) and FGF receptor (32, 36) signaling both regulate calvarial osteogenesis.

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