The Bone-specific Transcriptional Regulator Cbfa1 Is a Target of Mechanical Signals in Osteoblastic Cells*

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A primary goal of bone research is to understand the mechanism(s) by which mechanical forces dictate the cellular and metabolic activities of osteoblasts, the bone-forming cells. Several studies indicate that osteoblastic cells respond to physical loading by transducing signals that alter gene expression patterns. Accumulated data have documented the fundamental role of the osteoblast-specific transcription factor Cbfa1 (core-binding factor) in osteoblast differentiation and function. Here, we demonstrate that low level mechanical deformation (stretching) of human osteoblastic cells directly up-regulates the expression and DNA binding activity of Cbfa1. This effect seems to be fine tuned by stretch-triggered induction of distinct mitogen-activated protein kinase cascades. Our novel finding that activated extracellular signal-regulated kinase mitogen-activated protein kinase physically interacts and phosphorylates endogenous Cbfa1 in vivo (ultimately potentiating this transcription factor) provides a molecular link between mechanostressing and stimulation of osteoblast differentiation. Elucidation of the specific modifiers and cofactors that operate in this mechanotranscription circuitry will contribute to a better understanding of mechanical load-induced bone formation which may set the basis for nonpharmacological intervention in bone loss pathologies.

Mechanical stress has been long recognized to be an important regulatory factor in bone homeostasis and a determinant of skeletal morphology during development and in postnatal life (1). Given its influence on and interactions with all other modulators of bone growth, mineralization, and remodeling, there is great interest in understanding the effect of mechanical loading on osteoblast differentiation and function. Despite extensive investigations, information regarding the precise molecular events that govern transformation of mechanical signals into biochemical responses culminating in genetic reprogramming of bone cells still remains sparse and inconsistent.

The osteoblast is the bone-forming cell that originates from mesenchymal stem cells. A “master” regulator of osteoblast differentiation is the transcription factor Cbfa1 (core-binding factor), a member of the runt homology family of transcription factors (2). Cbfa1 binds to the osteoblast-specific cis-acting element 2 (OSE2) (3), which is found in the promoter regions of all the major osteoblast-specific genes (i.e. osteocalcin, type I collagen, bone sialoprotein, osteopontin, alkaline phosphatase, and collagenase-3) and controls their expression (2, 4, 5). Conceivably, Cbfa1 expression plays a key role during osteoblast differentiation and skeletogenesis (6–9). Members of the AP-1 (activator protein-1) family of homo/heterodimeric transcription factors are also instrumental in regulating genes activated early in osteoblast differentiation. Thus, the expression of several osteoblast phenotypic genes such as alkaline phosphatase, type I collagen, osteopontin, osteocalcin, and collagenase-3, which are under the control of Cbfa1, is also dependent on AP-1 proteins (10–12).

Several lines of evidence suggest that signaling through mitogen-activated protein kinases (MAPKs) is essential for the early stages of osteoblast differentiation (13–17). Moreover, it has been shown that extracellular signal-regulated kinase (ERK) MAPK signaling is involved in the stimulation of osteoblast-related gene expression by extracellular matrix-integrin receptor interaction as well as mechanostressing (18, 19). We have previously also shown, using an established system for applying calibrated stretch, that a low level continuous mechanical strain of human periodontal ligament (hPDL) osteoblastic cells (i.e. osteoblast-like cells capable of undergoing osteoblast differentiation in response to a variety of extracellular stimuli) (20–22) rapidly induces the principal AP-1 constituents, c-Jun and c-Fos, and enhances osteoblast-specific AP-1 binding activity via ERK/JNK (c-Jun N-terminal kinase) MAPK signaling (23, 24).

These observations, combined with recent data indicating that MAPK pathways can activate Cbfa1 in vitro (25) prompted us to explore whether and how the expression and activation profile of Cbfa1 are affected after mechanical loading of hPDL osteoblastic cells, as well as whether and to what extent MAPKs contribute to the process. We demonstrate that both the gene and Cbfa1 protein are key targets of mechanical stimulation in these cells, in a biologically relevant, ERK-catalyzed manner. These findings establish an important link between bone-specific transcription factor function and mechanotransduction in human osteoblasts which may be useful to plan strategies of noninvasive therapy based on mechanical forces 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. § Supported by a K. Karatheodoris postdoctoral research grant from the University of Patras Research Committee, Greece (to A. G. P.). ** To whom correspondence should be addressed. Tel.: 30-610-996-144; Fax: 30-610-996-110; E-mail: papavas@med.upatras.gr.

1 The abbreviations used are: OSE2, osteoblast-specific element 2; AP-1, activator protein-1; BMP, bone morphogenetic protein; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; hPDL, human periodontal ligament; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mut, mutant; wt, wild-type.
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Strain-facilitated osteoinduction in situations such as fracture healing and distraction osteogenesis. To our knowledge, this is the first indication that a physical stimulus such as mechanical stress elicits a direct effect on a tissue-specific transcriptional regulator.

**Experimental Procedures**

**General**—Cell culture media (including fetal serum, FBS) and reagents were from Invitrogen. 5,000 Ci/mmol [γ-32P]dATP, 3,000 Ci/mmol [α-32P]dATP, protein A-Sepharose CL-4B, and 14C-methylated molecular size protein markers were purchased from American BioScience. Mouse monoclonal antibodies against p-ERK (sc-7383; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin goat polyclonal antibody, reactive with phosphorylated JNK1 and JNK2, rabbit polyclonal anti-p-ERK (sc-7383; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-p-actin goat polyclonal antibody, reactive with phosphorylated JNK1 and JNK2, rabbit polyclonal anti-phospho-JNK (p54/p59) (sc-239, Santa Cruz Biotechnology, Santa Cruz, CA) were custom ordered from Microchemistry Laboratory, FORTH (Crete, Greece).

**Cell Cultures**—Human PDL osteoblast-like fibroblasts were obtained from explant cultures of PDL tissues as detailed previously (26). Human PDL cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS; all experiments were carried out with cells from the third to the sixth passage after being checked to reaffirm their osteoblastic characteristics. Human osteosarcoma Saos-2 cells and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and human T lymphoblastic Jurkat cells in RPMI 1640 medium supplemented with 10% FBS.

**Exposure of Cultured hPDL Cells to Mechanical Stretch and Preparation of Cell Extracts**—Approximately 4 × 10^6 cells were seeded onto 50-mm dishes with a flexible, hydrophilic growth surface (Petriperm™, Vivascience-Sartorius AG, Goettingen, Germany) and cultivated until they reached ~90% confluence. The medium was then changed to Dulbecco’s modified Eagle’s medium supplemented with 0.1% FBS, to remain quiescent. 24 h later the hPDL cell cultures were stretched continuously using the stretching apparatus described in our previous studies (23, 24, 27). Control (unstretched) cultures were treated with 4 mg/ml Cbfa1 protein. Immediately after electrophoresis, the gel was soaked twice for 45 min at room temperature in 200 ml of 20% 2-propanol, 50 mM HEPES-NaOH, pH 7.6, and twice for 60 min at room temperature in 200 ml of buffer A (50 mM HEPES-NaOH, pH 7.6, 5 mM β-mercaptoethanol). The gel was then incubated with gentle shaking twice for 1 h at room temperature in 200 ml of 6 mM guanidine HCl in buffer A, followed by five sequential renaturation steps (20 min each at 4°C) and then exposed to x-ray film at −80°C with an intensifying screen.

Production of Recombinant Cbfa1 Protein—The human full-length Cbfa1 cDNA was excised from pGEM-3Zf+ and subcloned into the pQ32 plasmid (Qiagen), to enable generation of a His6-N-terminal fusion protein. For protein purification, M13 (pREP4) E. coli phage infected with Cbfa1 cDNA were transformed with the pQ32-Cbfa1 phagemid, grown in E. coli DH5α to an A 600 of 0.6. After induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside (4 h at 30°C), the cells were harvested by centrifugation (4,000 × g for 20 min), and the cell pellet was subject to denaturation by treatment with 6 mM guanidine HCl, 0.1 mM sodium phosphate, pH 8.0, 10 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, and 0.1% Nonidet P-40. Cbfa1 in the lysate was purified by Ni²⁺-chelate affinity chromatography under denaturing conditions as described by Papavassiliou et al. (32).

**In-gel Kinase Assays**—In-gel kinase assays were performed by fractionating 100-μg SDS whole cell protein extracts or immunoprecipitates (HeLa cells; see below) onto routine SDS-10% polyacrylamide gels, in which recombinant full-length 100 μg/ml Cbfa1 protein was embedded. Immediately after electrophoresis, the gel was soaked twice for 45 min at room temperature in 200 ml of 20% 2-propanol, 50 mM HEPES-NaOH, pH 7.6, and twice for 60 min at room temperature in 200 ml of buffer A (50 mM HEPES-NaOH, pH 7.6, 5 mM β-mercaptoethanol). The gel was then incubated with gentle shaking twice for 1 h at room temperature in 200 ml of 6 mM guanidine HCl in buffer A, followed by five sequential renaturation steps (20 min each at 4°C) and then exposed to x-ray film at −80°C with an intensifying screen.

**Western Immunoblotting**—Protein extracts were resolved on 10% polyacrylamide gels followed by electrophoretic transfer onto polyvinylidene difluoride membranes (Amersham Biosciences). Membranes were blocked overnight with 5% lipid-free bovine serum albumin in Tris-buffered saline with Tween (TBST; 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and probed with the primary antibody at room temperature. After incubation with a horseradish peroxidase-conjugated secondary antibody, immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) kit (Amersham Biosciences). Detection of Cbfa1 protein was performed in nuclear extracts with anti-Cbfa1 antibody (1:1,000); detection of p-ERK, p-JNK, and ERK2 was performed in whole cell extracts using anti-p-ERK (1:1,000), anti-p-JNK (1:1,000), and anti-ERK2 (1:2,000) antibodies, respectively.

Electrophoretic Mobility Shift Assays (EMSAs)—The oligonucleotide sequences used as probe or as competitor are as follows. The wild-type Cbfa1 (wt Cbfa1), 5′-CCGGACTCCACACATCATCTT-3′ (top strand) is derived from the human osteocalcin promoter (−141 to −165) and contains an OE2 motif (AAACCAGA) (3). The mutant Cbfa1 (mut Cbfa1) sequence is 5′-CCGGACTCCACACATCATCTT-3′ (top strand).

The double-stranded wt Cbfa1 probe was end-labeled using [γ-32P]ATP and T4 polynucleotide kinase according to standard protocols. Nuclear extracts (5–10 μg of protein) were mixed with 3−5 × 10^4 cpm of radiolabeled probe in a total reaction volume of 20 μl containing 20 μM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA, 6% glycerol, 0.1% Nonidet P-40, 2 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 1 μg of unlabeled wt or mut Cbfa1 oligonucleotide. After incubation at room temperature for 20 min, DNA-protein complexes were resolved on 5% native polyacrylamide gels. Electrophoresis was carried out in 0.25 × TBE (Tris-borate-EDTA) buffer at 125 V at room temperature. Gels were dried and exposed to x-ray film at −80°C with an intensifying screen.

RNA Extraction and Northern Blot Analysis—Total RNA was extracted according to Chomczynski and Sacchi (30). RNA samples were fractionated on 1% agarose-formaldehyde gel and blotted onto nylon membrane (Hybond-N; Amersham Biosciences). The human full-length Cbfa1 cDNA was excised from pGEM-3Zf+ and purified from agarose gel prior to labeling with [α-32P]dATP using a random priming kit (Promega, Madison, WI). Hybridization with this probe was performed as described by Ausubel et al. (31).

Western Immunoblotting—Protein extracts were resolved on SDS-10% polyacrylamide gels followed by electrophoretic transfer onto polyvinylidene difluoride membranes (Amersham Biosciences). Membranes were blocked overnight with 5% lipid-free bovine serum albumin in Tris-buffered saline with Tween (TBST; 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and probed with the primary antibody at room temperature. After incubation with a horseradish peroxidase-conjugated secondary antibody, immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) kit (Amersham Biosciences). Detection of Cbfa1 protein was performed in nuclear extracts with anti-Cbfa1 antibody (1:1,000); detection of p-ERK, p-JNK, and ERK2 was performed in whole cell extracts using anti-p-ERK (1:1,000), anti-p-JNK (1:1,000), and anti-ERK2 (1:2,000) antibodies, respectively.

Electrophoretic Mobility Shift Assays (EMSAs)—The oligonucleotide
Cultured under similar conditions with the stretched cells for the maximum time, the cells were subjected to mechanical stretch, as described under "Experimental Procedures." A, at the indicated time points, total RNA was isolated, electrophoresed (20 μg/lane), and transferred to nylon membrane. Northern blots were hybridized with a radiolabeled human Cbfa1 cDNA probe. The bracket indicates the Cbfa1 mRNA isoforms. The ethidium bromide-stained agarose gel is included to verify loading of similar RNA amounts. B, nuclear extracts from quiescent hPDL cells exposed to mechanical stretch for the indicated times were fractionated by SDS-PAGE (20 μg protein/lane), proteins were electrotransferred onto polyvinylidene fluoride membrane, and Western blots were probed with an anti-Cbfa1 antibody. Recombinant Cbfa1 protein (Rec) is included to verify the antibody specificity and the size of human Cbfa1 protein. Lane 0 in both panels refers to unstretched (control) cells that were cultured under similar conditions with the stretched cells for the maximum period of stretch application (12 h).

RESULTS

Mechanical Stretch Induces Cbfa1 Expression in hPDL Osteoblastic Cells—It is well established that hPDL cells bear an osteoblastic potential (20–22). Consistently, Cbfa1 expression has been found in mammalian PDL tissue (33, 34). Furthermore, hPDL cells respond to mechanical stretch and express osteoblast phenotypic markers (23, 26, 27, 35). Therefore, we asked whether this mechanical stress affects Cbfa1 expression in hPDL cells at the mRNA and/or protein level. To this end, total RNA was isolated from cells mechanically stretched for different times (0–12 h) and subjected to Northern blot analysis. As depicted in Fig. 1A, unstretched cells (lane 0) exhibit marginally detectable levels of Cbfa1 mRNA. These levels are increased dramatically after 0.5 h of continuously applied mechanical load and remain elevated until 12 h. In agreement with previous reports, we also detected at least two isoforms of Cbfa1 which have been attributed to differential promoter usage or alternative splicing (36, 37). Although functional differences among these isoforms have not been yet clarified, it has been suggested that all are involved in the stimulatory action of osteoblast differentiation, but they exert different functions in the process of osteoblast differentiation (4, 38). Hence, mechanical stimulation evokes a rapid induction of Cbfa1 mRNA in hPDL osteoblastic cells, whose abundance remains high as long as these cells are exposed to mechanical stress.

Next, we examined whether this up-regulation in Cbfa1 mRNA is followed by an increase in Cbfa1 protein. Accordingly, quiescent hPDL cells were stretched for various times (0–12 h), nuclear extracts were prepared, and equivalent amounts of total protein were assayed by Western immunoblotting. Although not evident within 1 h poststretching (data not shown), a slight increase in Cbfa1 protein levels was observed after 3 h of mechanical loading; this increase peaked at 6 h and remained unaltered thereafter (Fig. 1B). These results demonstrate that in hPDL cells a physical stimulus, such as mechanical stretching, significantly augments the expression of the osteoblast-specific transcriptional regulator Cbfa1, at both the mRNA and protein levels.

Mechanical Stretch Stimulates the DNA Binding Activity of Cbfa1—Cbfa1 modulates osteoblast-related gene programing by binding to specific DNA elements in the regulatory region of target genes (9, 39). We thus asked whether mechanical stretch is capable of inducing the DNA binding activity of this transcription factor. To this end, a radiolabeled 25-bp synthetic oligonucleotide encompassing the Cbfa1 binding motif (OSE2) of the human osteocalcin promoter, a Cbfa1 inducible gene (3, 40), was used as probe in a standard EMSA employing nuclear extracts from hPDL cells exposed to mechanical stretch for 0–12 h (Fig. 2A). A weak DNA-protein complex was obtained in unstretched cells (lane 0), which markedly increased its abundance between 0.5 and 6 h of stretch application. The highest binding activity was observed at 6 h poststretching, a time point coinciding with maximal expression of the Cbfa1 protein (Fig. 1B). The specificity of this complex formation was verified by binding competition analyses with an excess of the relevant cold wt OSE2 and mut OSE2 oligonucleotides (Fig. 2B, lanes 2–4).

To demonstrate the presence of Cbfa1 in this specific DNA-protein complex, we performed a supershift EMSA (Fig. 2C). Addition of anti-Cbfa1 antibody in the binding reaction performed with nuclear extracts from 6-h stretched hPDL cells resulted in the appearance of a supershifted complex, concomitant with a remarkable decrease in complex formation (Fig. 2C, lanes 1 and 3). By contrast, an antibody against the ubiquitous transcription factor Sp1 failed to generate any effect (Fig. 2C, lanes 1 and 2). Notably, the Cbfa1-containing complex obtained in extracts from mechanically stretched cells displays the same electrophoretic mobility as that formed in extracts from human osteosarcoma (i.e. highly osteoblastic) Saos-2 cells (Fig. 2C, lanes 1 and 4). In marked distinction, extracts from Jurkat cells, known to lack Cbfa1 expression (41), do not exhibit the specific Cbfa1-DNA complex; instead, a faster migrating species is detected, whose electrophoretic profile remains unaffected by the addition of anti-Cbfa1 antibody (Fig. 2C, lanes 1, 5, and 6).

Induction of the MAPK Pathway in Mechanically Stretched hPDL Cells—We have shown recently that a brief period of mechanical load potentiates selective MAPK subtypes targeting AP-1 in hPDL osteoblastic cells (23). Conceivably, we proceeded to examine whether the observed up-regulation of Cbfa1 expression and DNA binding activity in mechanically stretched hPDL cells resulted from the appearance of a supershifted complex, concomitant with a remarkable decrease in complex formation (Fig. 2C, lanes 1 and 3). In marked distinction, extracts from Jurkat cells, known to lack Cbfa1 expression (41), do not exhibit the specific Cbfa1-DNA complex; instead, a faster migrating species is detected, whose electrophoretic profile remains unaffected by the addition of anti-Cbfa1 antibody (Fig. 2C, lanes 1, 5, and 6).
MAPK Activation Correlates with Increased Cbfa1 Binding Activity in Mechanically Stretched hPDL Cells—To address the role of MAPKs in the stretch-induced Cbfa1 binding activity in hPDL cells, we exploited the transmembrane protein kinase inhibition approach. For this purpose, two widely employed specific MAPK inhibitors were tested: U0126, a potent and selective inhibitor of MAPK/ERK kinase (MEK; an ERK1/2 upstream activating kinase) and thereby of ERK activation (42, 43); and curcumin, shown to hamper, albeit at different concentrations, the activation of both JNKs and ERKs (44). Serum-starved hPDL cells were incubated with the aforementioned inhibitors at doses that produced no cytotoxic effect on the cells for 1 h prior to applying mechanical stretch. Cells were then stretched for 6 h (where maximal Cbfa1 binding activity was observed; Fig. 2), and nuclear extracts were isolated and analyzed by EMSA as before. As demonstrated earlier (Fig. 2), formation of a Cbfa1-containing complex is enhanced markedly in extracts from stretched hPDL cells (Fig. 4, lanes 1 and 2). U0126 treatment produced a clear down-regulation of Cbfa1 binding activity in mechanically stimulated hPDL cells (Fig. 4, lanes 2 and 3), almost reaching the levels seen in unstretched cells (Fig. 4, lane 1). On the other hand, low dose (10 μM) curcumin treatment of cells led to only a mild reduction in complex formation, which was slightly more profound at higher concentrations (20 μM) (44). Taken together these results, along with previous reports that curcumin inhibits JNK with an IC50 of 5–10 μM whereas ERK at higher concentrations (IC50 = 20 μM) (44), imply that the above reduction in Cbfa1-DNA complex formation is mediated through an inhibitory effect on ERK rather than JNK activation. These data, in conjunction with the kinetics of JNK/ERK induction (Fig. 3), strongly implicate the ERK, and to a much lesser extent, the JNK MAPK cascades in the stretch-elicited up-regulation of Cbfa1 binding activity.

Phosphorylation of Cbfa1 by Stretch-stimulated ERK MAPKs in hPDL Cells—The effect of ERK (and perhaps of JNK) inhibition approach. For this purpose, two widely employed specific MAPK inhibitors were tested: U0126, a potent and selective inhibitor of MAPK/ERK kinase (MEK; an ERK1/2 upstream activating kinase) and thereby of ERK activation (42, 43); and curcumin, shown to hamper, albeit at different concentrations, the activation of both JNKs and ERKs (44). Serum-starved hPDL cells were incubated with the aforementioned inhibitors at doses that produced no cytotoxic effect on the cells for 1 h prior to applying mechanical stretch. Cells were then stretched for 6 h (where maximal Cbfa1 binding activity was observed; Fig. 2), and nuclear extracts were isolated and analyzed by EMSA as before. As demonstrated earlier (Fig. 2), formation of a Cbfa1-containing complex is enhanced markedly in extracts from stretched hPDL cells (Fig. 4, lanes 1 and 2). U0126 treatment produced a clear down-regulation of Cbfa1 binding activity in mechanically stimulated hPDL cells (Fig. 4, lanes 2 and 3), almost reaching the levels seen in unstretched cells (Fig. 4, lane 1). On the other hand, low dose (10 μM) curcumin treatment of cells led to only a mild reduction in complex formation, which was slightly more profound at higher concentrations (20 μM) (44). Taken together these results, along with previous reports that curcumin inhibits JNK with an IC50 of 5–10 μM whereas ERK at higher concentrations (IC50 = 20 μM) (44), imply that the above reduction in Cbfa1-DNA complex formation is mediated through an inhibitory effect on ERK rather than JNK activation. These data, in conjunction with the kinetics of JNK/ERK induction (Fig. 3), strongly implicate the ERK, and to a much lesser extent, the JNK MAPK cascades in the stretch-elicited up-regulation of Cbfa1 binding activity.

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STRETCH - + + + +
U0126 (μM) 0 0 10 0 0
Curcumin (μM) 0 0 0 10 20

Fig. 4. Effect of MAPK inhibitors on the stretch-induced Cbfa1 DNA binding activity in hPDL cells. Quiescent hPDL cells were treated with the MAPK inhibitors U0126 (lane 2) or curcumin (lanes 4 and 5) at the indicated concentrations (in μM). 1 h later the dishes were stretched for 6 h (+), nuclear extracts were isolated, and equal amounts (8 μg) were analyzed for Cbfa1 DNA binding activity by a standard EMSA, using the same probe as in Fig. 2. Lanes 1 and 2, DNA binding reactions performed with nuclear extracts from unstretched (−) and 6-h stretched cells, respectively, omitting kinase inhibitor treatment. Unstretched cells were cultured under similar conditions with the stretched cells for the entire period of stretch application (6 h).

Discussion

Bone has evolved primarily to serve mechanical needs. During evolution, bone has optimized its load-bearing role by adapting its architecture and function to mechanical forces. In the present study we provide evidence that Cbfa1, a pivotal transcriptional regulator of osteoblast differentiation and bone formation, is a key target of mechanical stimulation in hPDL osteoblastic cells, i.e., a cell type constantly “sensing” mechanical strain under physiological conditions and able to differentiate to participate actively in restoring the PDL and in remodeling of the surrounding bony tissue (45). We document that mRNA and protein levels, as well as the DNA binding activity of Cbfa1, are increased after a physical signal such as mechanical stretch. We also demonstrate that the force-ignited Cbfa1 induction is “funneled” via specific MAPK pathways. Our novel finding that Cbfa1 and activated ERK (most likely ERK2) physically associate in vivo further substantiates the critical role assigned to MAPK cascades in the osteoblast differentiation process in general (13–17).

Several studies have established the paramount importance of Cbfa1 in osteoblast differentiation and function (39, 46). Our data suggest that mechanical stimulation may promote the differentiation of osteoblasts by targeting this crucial transcription factor. Osteoblasts have been aptly called sophisticated fibroblasts (47), in regard for limited number of exclusive osteoblastic genes. Cbfa1 can stimulate the transcription of such osteoblast-specific genes through cognate binding sites in their promoter regions (2, 5, 48). Because mechanical stress results in augmentation of Cbfa1 levels and potentiation of its DNA binding capacity, it is plausible that the aforementioned genes may be physical end points of mechanical stimulation. This interpretation is supported by previous studies showing that many of these genes are induced under various conditions of mechanical stress (35, 49–51).
Induction of Cbfa1 expression in osteoblastic cells has been reported after stimulation with several growth factors, including transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP)-2, BMP-7, or the BMP-4/7 heterodimer (2, 52–54). All of these secreted molecules transduce their signals through receptor protein Ser/Thr kinases that promote the phosphorylation of SMAD proteins, leading to their nuclear accumulation whereby they regulate transcription of target genes (55). On the other hand, the kinetics of Cbfa1 mRNA expression after mechanical stimulation revealed that it increases sharply at 0.5 h and remains elevated thereafter. This immediate response of Cbfa1 expression is not likely to involve an indirect autocrine mechanism; the time required for synthesis, secretion, and ultimately action of the aforementioned growth factors is far beyond the time frame in which maximal Cbfa1 up-regulation was observed in our system. For instance, Lee et al. (53) have shown that Cbfa1 mRNA levels reach a maximum 2 h after transforming growth factor-β1 or BMP-2 stimulation and decrease gradually thereafter. The above consideration is in accordance with our previous studies indicating that mechanical stretch induces DNA synthesis in hPDL cells by a mechanism that seems to be direct and not mediated through autocrine growth factor action (27). We note, however, that our data cannot exclude a parallel contribution of autocrine growth factors which might be responsible for the sustained induction of Cbfa1 expression after mechanical stress.

Until recently, the only factor that has been shown to control Cbfa1 expression directly is Cbfa1 itself (39). This has been based on the fact that the Cbfa1 gene promoter contains functional Cbfa1 binding (OSE2) sites and, moreover, that overexpression of a dominant negative form of Cbfa1 in mature osteoblastic cells leads to a decrease of the endogenous protein (8, 56). Such an autoregulatory mechanism is consistent with our data and could explain to a large degree the observed induction of Cbfa1 after mechanical stimulation. Conceivably, the rapid activation of ERK MAPKs by the mechanical stimulus promotes the phosphorylation and hence activation of preexisting Cbfa1 molecules, which, in turn, bind to OSE2 elements in the Cbfa1 promoter thereby enhancing expression of its own gene. This scenario is supported by the kinetics of induction of Cbfa1 binding activity, which is indicative of an immediate-early response, as well as by the MAPK inhibition analyses. Modulation of Cbfa1 binding activity via phosphorylation has been documented recently in vitro (25). Our combined in-gel kinase and coimmunoprecipitation data provide strong evidence that phosphorylation of Cbfa1 by ERK MAPK(s) also occurs in vivo. Interestingly, it was reported that increased Cbfa1 binding activity after α₅-integrin-collagen interaction produced no significant alterations in Cbfa1 levels (57). This observation does not necessarily contradict our hypothesis. A possible explana-
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...the presence of MAPK inhibitors abrogates this inductive effect observed after mechanical stretching of these cells. Importantly, we have observed that the major AP-1 components (and immediate-early genes) are rapidly up-regulated after mechanical stretching of these cells. Importantly, the presence of MAPK inhibitors abrogates this inductive effect (24). The possibility of a mechanical signal-facilitated "cross-talk" is currently under investigation in our system.

Collectively, our study provides novel information for a molecular link between mechanical stimulation and osteoblast differentiation through the induction of expression and DNA binding potential of Cbfa1. Because Cbfa1 controls directly the rate of bone formation by differentiated osteoblasts, identifying stimuli that increase its expression and/or potency in these cells may lead to novel therapeutics to prevent or/and treat bone loss diseases. In this vein, the bone generating capacity of small physical signals, such as the low amplitude mechanical strain employed in the present study, suggests that biomechanical intervention might help to strengthen bone in bone degenerative diseases (e.g. osteoporosis) without the side effects associated with pharmacological treatment. In addition to being noninvasive and inducing a therapeutic response from the bone tissue itself, low intensity mechanical signals incorporate all aspects of a complex remodeling cycle ultimately improving bone quantity and quality (59).

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