Cloning and Characterization of a Novel Protein Kinase That Impairs Osteoblast Differentiation in Vitro*

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The bone morphogenic proteins (BMPs) play a key role in skeletal development and patterning. Using the technique of differential display polymerase chain reaction (ddPCR), we have identified a novel gene whose expression is increased during BMP-2-induced differentiation of the prechondroblastic cell line, MLB13MYC clone 17, to an osteoblastic phenotype. The 6.5-kilobase mRNA recognized by this ddPCR product is increased 10-fold by BMP-2 treatment of the MLB13MYC clone 17 cells. The mRNA recognized by this ddPCR product is also increased as MC3T3-E1 cells recapitulate the program of osteoblast differentiation during prolonged culture. The full-length transcript corresponding to this ddPCR product was cloned from a MLB13MYC clone 17 cell cDNA library. Analysis of the deduced amino acid sequence demonstrated that this gene encodes a novel 126-kDa putative serine/threonine protein kinase containing a nuclear localization signal. The kinase domain, expressed in *Escherichia coli*, is capable of auto-phosphorylation as well as phosphorylation of myelin basic protein. The gene was, therefore, named BIKe (BMP-2-Inducible Kinase). The BIKe nuclear localization signal is able to direct green fluorescent protein to the nucleus in transfected COS-7 cells. When stably expressed in MC3T3-E1 cells, BIKe significantly decreases alkaline phosphatase activity and osteocalcin mRNA levels and retards mineral deposition relative to vector control. This novel kinase, therefore, is likely to play an important regulatory role in attenuating the program of osteoblast differentiation.

Numerous investigations have been directed at elucidating factors that regulate osteoblast differentiation (1). The bone morphogenetic proteins (BMPs) are potent local factors that promote osteoblast differentiation during development as well as during bone remodeling (2). The molecular events downstream of BMP signaling that result in tissue-specific gene expression and skeletal development have only been partially elucidated. The binding of BMPs to their receptors leads to the assembly of a receptor complex in which the type II receptor phosphorylates and activates the type I receptor. As a result, pathway-restricted SMADs are phosphorylated, leading to interactions with the common mediator SMAD, smad4 (3). This complex is then translocated to the nucleus, where it modulates transcription of target genes. BMP signaling can also interfere with the effects of other growth and differentiation factors. It has been demonstrated that BMP-2 treatment of mesangial cells prevents phosphorylation of a transcription factor, Elk1, in response to platelet-derived growth factor (PDGF) signaling. This effectivly inhibits PDGF-induced Elk1-mediated transcription, and blocks PDGF-induced transcription of c-fos, an Elk-1 target (4).

BMP-7 has been shown to be a potent inducer of Cbfa1, a transcription factor belonging to the *runt*-domain gene family that, in turn, regulates the expression of several genes in the osteoblast (5). Although Cbfa1 expression is necessary, it alone is not sufficient for osteoblast differentiation (6). BMPs regulate the program of osteoblast differentiation at several levels. They play a critical role in the induction of several transcription factors that promote differentiation, such as *Cbfa1* (5) and *Dlx5* (7), as well as increasing the expression of negative regulators, including *Id* (8) and *Msx-2* (9). BMPs have also been shown to induce the expression of *follistatin* (10, 11) and *noggin* (2, 12), both of which are BMP-binding proteins that serve to modulate the actions of locally synthesized BMPs. A third level at which BMPs modulate osteoblast differentiation is exemplified by the induction of *Tob* by BMP-2. This protein negatively regulates osteoblast proliferation and differentiation at the level of BMP signaling by interacting with receptor-regulated SMADs (13). Like BMPs, growth factors may also play a dual role in regulating the program of osteoblast differentiation. Notable in this respect is the observation that fibroblast growth factor-1 stimulates the proliferation of immature osteoblasts, whereas it limits the number of osteoblasts undergoing terminal differentiation by promoting apoptosis in this latter cell population (14).

Studies of cranial suture closure have provided critical *in vivo* correlates for the *in vitro* studies demonstrating the effects of growth and transcription factors on osteoblast differentiation. Haploinsufficiency of *Cbfa1* delays intramembranous bone formation and ossification of cranial sutures, which is consistent with the role of this transcription factor in promoting osteoblast differentiation (15, 16); however, gain of function mutations in *Msx2* cause craniosynostosis (17). Craniosynostosis is also seen in patients with mutations that increase the activity of fibroblast growth factor receptor-2, notably Aperts and Crouzons syndromes (18, 19).
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Although the induction of Cbfal by BMPs has been shown to be a pivotal event in endochondral bone formation, the identification of genes induced prior to and after Cbfal will be essential to elucidate other factors, the interaction of which is required for normal skeletal differentiation. These factors are likely to play an important role in skeletal homeostasis post-natally as well. Fracture healing models have demonstrated that BMPs play a key role in skeletal remodeling, and studies in transgenic mice have suggested an important role for Cbfal in skeletal homeostasis (20). In an analogous fashion, it is likely that other factors involved in osteoblast differentiation will play a pivotal role in the maintenance of skeletal homeostasis (20). In an analogous fashion, it is likely that other factors involved in osteoblast differentiation will play a pivotal role in the maintenance of skeletal homeostasis (20). We, therefore, undertook studies to identify novel genes that are induced during the cascade of molecular events that occur as a cell acquires the markers of a mature osteoblast, using an in vitro cellular model. We have identified a novel protein kinase containing a nuclear localization signal and a glutamine-rich region characteristic of many transcription factors. When stably expressed, this kinase markedly attenuates the program of osteoblast differentiation recapitulated during prolonged culture of MC3T3-E1 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—The MBL135MYC clone 17 cells and rhBMP-2 were kindly provided by Dr. Vicki Rosen (Genetics Institute, Cambridge, MA). The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. At confluence, cells were treated with 200 mg/ml rhBMP-2 in Dulbecco’s modified Eagle’s medium with 1% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin, adding fresh medium and rhBMP-2 daily as described previously (21). Conditionally immortalized murine bone marrow stromal cells, osteoblasts, and osteocytes (Dr. F. R. Bringhurst, Massachusetts General Hospital, Boston, MA) were isolated and cultured as reported previously (22, 23). Primary calvarial osteoblasts form 18.5-days post-coital embryos were cultured as described previously (22). MC3T3-E1 cells (ATCC) were cultured in α-minimum Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. For experiments directed at addressing the role of BIKe (BMP-2-Inducible Kinase) on mineralization, primary calvarial osteoblasts were cultured in α-minimum Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. MC3T3-E1 cells were stably transduced with BIKe. COS-7 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.

Differential Display PCR—Total RNA was isolated from parallel cultures of MBL135MYC clone 17 cells, untreated, and treated with 200 ng/ml rhBMP-2 for 72 h using Trizol reagent (Life Technologies, Inc.). After phenol extraction and ethanol precipitation, RNA was dissolved in DEPC-treated water. cDNA was synthesized from total RNA using random hexamers with SuperScript II reverse transcriptase (24). COS-7 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.

Northern Analysis—Total RNA was isolated from cultured cells or mouse tissues using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. northern Blot hybridization was performed with a Sepharose-4B-agarose gel. Fractions containing cDNAs greater than 1 kb in length were collected and pooled for these cells to acquire markers of osteoblast differentiation, using an in vitro cellular model. We have identified a novel protein kinase containing a nuclear localization signal and a glutamine-rich region characteristic of many transcription factors. When stably expressed, this kinase markedly attenuates the program of osteoblast differentiation recapitulated during prolonged culture of MC3T3-E1 cells.

RESULTS

Confluent MBL135MYC clone 17 cells were induced to differentiate by treatment with 200 ng/ml rhBMP-2 every 24 h for 72 h. This concentration and duration of treatment is sufficient for these cells to acquire markers of osteoblast differentiation, including expression of the osteocalcin gene (21). RNA isolated from cells, treated with rhBMP-2 for 72 h, or left untreated was used for differential display PCR. After overnight autoradiography, several bands representing differentially expressed...
mRNAs were identified. A prominently up-regulated band (Fig. 1A) was chosen for further characterization. This band was excised from the gel and used as a probe for Northern blotting analyses following reamplification. The mRNA encoded by the ddPCR product was ~7 kilobases in size, barely detectable in untreated MLBMYC clone 17 cells and markedly increased following 72 h of rhBMP-2 treatment (Fig. 1B). The peak level of expression of this transcript post-BMP-2 treatment was later than that observed for Chba1 in this cell line (peak at 48 h, data not shown) and earlier than induction of osteocalcin (first detected at 48 h post-treatment (Ref. 21 and data not shown).

To investigate whether the expression of this transcript was specific to bone or was expressed in other mouse tissues, multissite Northern blotting analysis was performed. The mRNA corresponding to this ddPCR product was expressed in spleen, kidney, lung, brain, heart, diaphragm, and calvaria but not in liver (data not shown). The transcript was also expressed in conditionally immortalized osteocytes (C59), osteoblasts (F10), and marrow stromal cells (MS1) as well as in primary osteoblasts (POB) isolated from 18.5-days post-coital mouse embryos (Fig. 1C).

Since the original 322-base pair sequence of the ddPCR product was not present in the GenBank data base, a cDNA library was constructed using mRNA isolated from rhBMP-2-treated MLBMYC clone 17 cells and screened to obtain the full-length cDNA sequence. Six positive phages were isolated following which the cDNA inserts and pBluescript were excised using ExAssist helper phage. The four clones containing the longest cDNA inserts were further characterized. Sequence analysis was obtained from both strands of at least two independent cDNA inserts, and alignment was performed using SeqMan. The 6.5-kilobase pair cDNA has an open reading frame of 1138 amino acids (Fig. 2) with a predicted molecular size of 126 kDa. Because the N-terminal region contains a putative serine/threonine kinase domain, the novel gene was named BIKe. Protein sequence analysis (PROSITE, Swiss Institute of Bioinformatics) predicts a nuclear localization signal in the C-terminal region of the peptide (underlined). Analysis of the BIKe protein also reveals a glutamine-rich region, analogous to that found in several transcription factors (27, 28) and thought to be important for protein-protein interactions (29). A search of the BLAST protein data base (NCBI) identified significant homology between BIKe and partial human cDNA clones of unknown function. Furthermore, the kinase domain of BIKe was evolutionarily conserved with significant homology to Xenopus and Drosophila kinases (U58205 and AP197910). Drosophila Numb-associated kinase was identified as the most homologous protein of known function. However, the absence of significant homology between the nonkinase domains of BIKe and Numb-associated kinase suggests that BIKe is unlikely to be a vertebrate homolog of this Drosophila kinase.

To demonstrate that the BIKe kinase domain was functional, in vitro kinase assays were performed. A fusion protein of GST and the BIKe kinase domain (GST-BIKeKD) was expressed in E. coli and purified. Following incubation of GST-BIKeKD with myelin basic protein and [γ-32P]ATP, labeled proteins corresponding to GST-BIKeKD and myelin basic protein were resolved by SDS-polyacrylamide gel electrophoresis. Autoradiography of the gel demonstrated the ability of BIKe to autophosphorylate (Fig. 3B, lanes 2 and 4; GST-BIKeKD) as well as to phosphorylate myelin basic protein (Fig. 3B, lane 2; MBP), a general protein kinase substrate. A GST fusion protein encoding a transcription factor (30) was unable to phosphorylate itself or myelin basic protein (Fig. 3B, lane 6). After thrombin cleavage to remove GST, the BIKeKD retained its ability to autophosphorylate; however, heat inactivation of GST-BIKeKD prevented phosphorylation (Fig. 3B, lane 3).
To evaluate the function of the bipartite nuclear localization signal (NLS) in the C-terminal region of BIKe, the cDNA sequences encoding amino acids 906–1025 were inserted in frame to that encoding GFP. As shown in Fig. 4B, the NLS of BIKe can direct GFP expression (left upper panel) to the nucleus of COS-7 cells (visualized with Hoescht dye in the right upper panel), whereas GFP expressed without the BIKeNLS remains diffuse (Fig. 4A, left upper panel).

To identify a role for this novel kinase in osteoblast differentiation, MC3T3-E1 cells were transfected with either pcDNA3.1/BIKe Fig. 5 (panels B and C, lane B) or pcDNA3.1 lacking insert (lane EV). This cell line was chosen because when plated at low density, the MC3T3-E1 cells have features of a preosteoblastic cell. When left in culture for approximately a month, these cells acquire characteristics of a fully differentiated osteoblast without the addition of exogenous differentiating agents such as BMPs. It is also notable that the endogenous mRNA encoding BIKe in this cell line increases as the program of osteoblast differentiation is recapitulated with prolonged culture, as assessed by alizarin red-S staining (not shown) and quantitation of calcium deposited into the matrix by methylthymol blue analysis (26) (Fig. 7).

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**FIG. 3.** Kinase activity of GST-BIKeKD. **A,** Coomassie stain of the SDS-polyacrylamide gel shown in panel B. **B,** autoradiograph of SDS-polyacrylamide gel resolving products of kinase reactions. Lane 1, molecular size markers in kDa to left; lane 2, GST-BIKeKD and myelin basic protein (MBP); lane 3, GST-BIKeKD heated to 95 °C prior to incubation with MBP; lane 4, GST-BIKeKD alone; lane 5, MBP alone; lane 6, a GST fusion protein encoding the transcription factor BTEB3 incubated with MBP.

**FIG. 4.** The BIKe nuclear localization signal can direct GFP to the nucleus. COS-7 cells were transfected with a vector containing a GFP control plasmid (A) or GFP fused to the BIKeNLS (B). Upper left panel, fluorescent confocal microscopy. Nuclei were visualized with Hoescht dye (upper right panel). Lower panels, light microscopy.

**FIG. 5.** Northern analyses of mRNA isolated from MC3T3-E1 cells. At the indicated time (days) post-plating, RNA was isolated from the MC3T3-E1 cells; 10 μg was subjected to Northern analysis and probed with the BIKe cDNA. Panel A, levels of mRNA encoding the endogenous BIKe transcript increase with differentiation. Panel B, the mRNA encoding Cbfa1 is not altered in the MC3T3-E1-BIKe (lane B) pooled clones relative to the MC3T3-E1-EV (lane EV) pooled clones (B). Panel C, the expression of osteocalcin (OC) mRNA is decreased in MC3T3-E1-BIKe (lane B) relative to MC3T3-E1-EV (lane EV) pooled clones, including the peak level of expression seen at 20 days. Autoradiographs are representative of results obtained in three independent experiments.
cells, supporting the hypothesis that its induction by BMP-2 in the MLB13MYC clone 17 cells parallels its expression during osteoblast differentiation. However, BIKe attenuates rather than promotes the osteoblast differentiation program of the MC3T3-E1 cells, most dramatically affecting the level of alkaline phosphatase activity and osteocalcin mRNA as well as inhibiting mineral deposition into the cultures. Several studies of osteoblast differentiation have focused on factors that promote the differentiation of pluripotent mesenchymal stromal cells into osteoblasts. It has been shown that Chfα1 is required for the development of an osseous skeleton and regulates the expression of many osteoblast genes (5, 15, 16, 33). However, stable transfection of BIKe did not change the pattern of Chfα1 expression in the MC3T3-E1 cells, suggesting that the mechanism of BIKe action is independent of or downstream to Chfα1.

Little is known about the factors that prevent or retard the commitment of pluripotent mesenchymal stromal cells to the osteoblast lineage; however, the mechanisms by which these cells are diverted into alternative pathways has largely been clarified. It has been demonstrated that the nuclear receptor peroxisome proliferator-activated receptor-γ plays a pivotal role in directing these stromal cells to the adipocyte lineage (34) and that several helix-loop-helix transcription factors are involved in the differentiation of these cells along the myogenic pathway (35). Our studies, however, were performed in cells that had already acquired markers of chondroblasts (the MLB13MYC clone 17 cells are prechondroblastic) or osteoblasts (MC3T3-E1 cells). These studies were not directed at addressing the effect of BIKe on the commitment of stromal cells to the osteoblast versus adipocyte or myocyte pathway; however, our finding that BIKe is expressed in marrow stromal cells does not exclude this possibility. The data presented here are consistent with BIKe playing a role in attenuating differentiation of and mineral deposition by the maturing osteoblast. The induction of BIKe during osteoblast differentiation may serve as a brake to control the rate of osteoblast differentiation, which is critical for normal skeletal development and morphogenesis. Negative regulation almost certainly plays a key role in skeletal development. The program of osteoblast differentiation involves an early proliferative phase not associated with the expression of markers of terminal differentiation or mineralized matrix formation (36–38). Although the laying down of mineralized matrix is considered to be the terminal phase of osteoblast differentiation, this is not the major in vivo role of the lining osteoblasts or osteocytes. Therefore, factors that seem to attenuate osteoblast differentiation in traditional assays, such as the MC3T3-E1 and rat calvarial cells, may actually play a role in the transition from an active matrix-synthesizing osteoblast to a cell that serves a different function in vivo. Notable in this respect is the observation that conditionally immortalized osteocytes express the BIKe transcript.

The kinase activity, nuclear localization signal, and glutamine-rich domain of BIKe suggest potential molecular mechanisms by which this novel protein influences osteoblast differentiation. Phosphorylation is a critical posttranslational modification that regulates the activity of several proteins involved in signal transduction, cellular proliferation, and gene transcription. The presence of a nuclear localization signal and a glutamine-rich domain raises the interesting question as to whether BIKe acts as a transcription factor. Alternatively, the nuclear localization of this protein could reflect its role as a kinase involved in phosphorylation of histones and/or transcription factors with protein-protein interactions mediated by the glutamine-rich region. In addition to being present in numerous transcription factors, glutamine-rich regions have a high propensity to form self-

**FIG. 7.** Calcium deposition into the cultures of pooled clones. Mineral deposition into the cultures, as assessed by calcium content, is markedly delayed and diminished in the MC3T3-E1-BIKe pooled clones compared with the MC3T3-E1-EV (control) pooled clones (p < 0.003 at all points indicated). Data are representative of those obtained in three independent experiments.

**FIG. 6.** Assessment of alkaline phosphatase activity in pooled clones. Alkaline phosphatase activity is decreased from days 7 to 28 in culture (p < 0.008 at all points indicated) as is the peak level of expression in MC3T3-E1-BIKe compared with MC3T3-E1-EV (control) pooled clones. Data are representative of those obtained in three independent experiments. *wt*, wild type; PNPP, p-nitrophenyl phosphate.

**DISCUSSION**

To identify factors regulated by BMP-2 that contribute to the development and maintenance of a normal skeleton, we performed differential display PCR analyses, which led to the identification of a gene encoding a novel protein kinase. Investigations aimed at identifying BMP-regulated genes have been performed in various cellular systems. Studies directed at identifying immediate early genes regulated by BMPs in embryonic stem cells have demonstrated that several regulators of skeletal development are involved in the differentiation of these cells along the myogenic pathway (35). Our studies, however, were performed in cells that had already acquired markers of chondroblasts (the MLB13MYC clone 17 cells are prechondroblastic) or osteoblasts (MC3T3-E1 cells). These studies were not directed at addressing the effect of BIKe on the commitment of stromal cells to the osteoblast versus adipocyte or myocyte pathway; however, our finding that BIKe is expressed in marrow stromal cells does not exclude this possibility. The data presented here are consistent with BIKe playing a role in attenuating differentiation of and mineral deposition by the maturing osteoblast. The induction of BIKe during osteoblast differentiation may serve as a brake to control the rate of osteoblast differentiation, which is critical for normal skeletal development and morphogenesis. Negative regulation almost certainly plays a key role in skeletal development. The program of osteoblast differentiation involves an early proliferative phase not associated with the expression of markers of terminal differentiation or mineralized matrix formation (36–38). Although the laying down of mineralized matrix is considered to be the terminal phase of osteoblast differentiation, this is not the major in vivo role of the lining osteoblasts or osteocytes. Therefore, factors that seem to attenuate osteoblast differentiation in traditional assays, such as the MC3T3-E1 and rat calvarial cells, may actually play a role in the transition from an active matrix-synthesizing osteoblast to a cell that serves a different function in vivo. Notable in this respect is the observation that conditionally immortalized osteocytes express the BIKe transcript.

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The cell line used for our studies was a prechondroblastic cell line that responds to BMP-2 with an increase in *Chfα1* mRNA levels, similar to that seen in BMP-2-treated immortalized articular and primary costal chondrocyte cultures (32) and pluripotent mesenchymal cells (5). During prolonged treatment with BMP-2, the MLB13MYC clone 17 cell line loses markers of a prechondroblast and acquires markers characteristic of an osteoblast (21). The mRNA encoding the novel kinase we identified increases as the osteoblast differentiation program is recapitulated during prolonged culture of MC3T3-E1
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propagating amyloid fibrils (29). They are also hot spots for the trinucleotide repeat expansions involved in the pathogenesis of several human diseases, including Huntington’s disease, Kennedy’s disease, and several spinocerebellar ataxias. Currently, however, there are no diseases linked to chromosome 4q21 (the locus for the human homolog of BIKe, NCBI) to suggest that BIKe is involved in the pathogenesis of human disease. The identification of potential BIKe substrates and characterization of its other functional domains will serve to characterize the molecular mechanism by which this novel kinase modulates the program of osteoblast differentiation.

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