Erk Is Essential for Growth, Differentiation, Integrin Expression, And Cell Function in Human Osteoblastic Cells*

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*Dedicated to the memory of Dr. Louis V. Avioli.

#Deceased

Running Title: MAPK is essential for integrin expression and osteoblast function.

Key Words: MAPK, osteoblast, integrin, growth, differentiation, adhesion, migration

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Abstract

Extracellular signal-regulated kinases (Erks), members of the MAPK superfamily, play an important role in cell proliferation and differentiation. In this study we employed a dominant negative approach to determine the role of Erks in the regulation of human osteoblastic (HOB) cell function. HOB cells were transduced with a pseudotyped retrovirus encoding either a mutated Erk1 protein with a dominant negative action against both Erk1 and Erk2 (Erk1DN cells) or the LacZ protein (LacZ cells) as a control. Both basal and growth factor-stimulated MAPK activity and cell proliferation were inhibited in Erk1DN cells. Expression of Erk1DN protein suppressed both osteoblast differentiation and matrix mineralization by decreasing alkaline phosphatase activity and the deposition of bone matrix proteins. Cell adhesion to collagen, osteopontin, and vitronectin was decreased in Erk1DN cells as compared with LacZ cells. Cell spreading and migration on these matrices were also inhibited. In Erk1DN cells, expression of αβ1, αvβ3, and αvβ5 integrins on the surface was decreased. Metabolic labeling indicated that the synthesis of these integrins was inhibited in Erk1DN cells. These data suggest that Erks are not only essential for the growth and differentiation of osteoblasts but also are important for osteoblast adhesion, spreading, migration, and integrin expression.
Introduction

For bone formation to occur, osteoblast precursor cells must migrate from bone marrow compartment to bone surface where they differentiate and deposit bone matrix. Several growth factors including FGF-2, TGF-β, and BMP-2 not only are potent chemotactic agents for osteoblasts but also have profound effects on their growth and differentiation (1-5). In addition to growth factors, the interaction between integrins and the extracellular matrix proteins also governs cell migration, adhesion, proliferation, and differentiation (6-10). Human osteoblastic (HOB) cells express a repertoire of integrins, which interact with bone matrix proteins such as osteopontin, bone sialoprotein, vitronectin, type I collagen, and fibronectin (11-15). These interactions have been shown to regulate osteoblast growth and differentiation (16-23).

Numerous signal transduction pathways are induced by integrin-matrix interactions in a variety of cell systems (6-10). The Ras/MAPK signal transduction pathway is one of the best-characterized pathways. Adhesion of cells to collagen, fibronectin, or vitronectin has been shown to induce MAPK activity in many cell systems (24,25). Growth factors have also been shown to activate MAPK activity (26,27). Although both integrins and growth factors propagate their signals via MAPK, the induction of MAPK by integrin-matrix interaction is prolonged whereas growth factor-stimulated MAPK is more transient and requires integrin-mediated cell adhesion (24,28,29). Conversely, growth factors can regulate integrin expression (30,31). Thus, MAPK mediates two important signal transduction pathways, one initiated by integrins and the other by growth factors, which cross talk to each other and further emphasize the importance of MAPK in regulating a variety of cell functions.
It has been documented that MAPK activity is stimulated in osteoblasts when they adhere to type I collagen (32). However, no data is currently available with regard to the MAPK activity when osteoblasts adhere to other bone matrix proteins. We have previously shown that MAPK is activated by growth factors in osteoblasts (27). Since integrin-matrix interaction and growth factors regulate osteoblast growth and differentiation, MAPK may play important role in mediating these osteoblastic activities in bone formation. Therefore, in this study, we analyzed the role of Erks of the MAPK superfamily in the regulation of osteoblast growth and differentiation by overexpressing a dominant negative Erk1 (Erk1DN) protein in these cells. We also examined the effects of Erk1DN protein on the expression of integrins and the migration, adhesion, and spreading properties of osteoblasts.

**Abbreviations:** Erks, extracellular signal-regulated kinases; Erk1DN, dominant negative Erk1; HIFBS, heat inactivated fetal bovine serum; HOB cells, human osteoblastic cells; MAPK, mitogen-activated protein kinases; α-MEM, α-minimum Eagle’s medium; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PT, PBS with 0.1% Triton X-100; PTB, PT with 1% BSA; PTA, PBS with 0.5% Tween 20 and 0.02% NaN3; PCT, PBS containing 1% casein and 0.04% Tween 20; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VSV-G, vesicular stomatitis virus G glycoprotein; CLS, Coffin-Lowry Syndrome.
Experimental Procedures

Materials

Type I collagen (Vitrogen 100) was from Cohesion (Palo Alto, CA). Recombinant osteopontin was purified as described previously (33). [125I]NaI and Tran-35S-Label were from ICN (Costa Mesa, CA). [3H]Thymidine, [γ-32P]ATP, ECL kit, and protein-G Sepharose were from Amersham Pharmacia Biotech (Piscataway, NJ). AP-1 consensus oligonucleotide (5′-CGCTTGATGAGTCAGCCGGAA-3′) and T4 polynucleotide kinase were from Promega (Madison, WI). Recombinant Protein A-Sepharose 4B was from Zymed Laboratories, Inc. (San Francisco, CA). Polyclonal antibodies against type I collagen α chain (LF-67), osteopontin (LF-7) and bone sialoprotein (LF-6) were kindly provided by Dr. Larry W. Fisher (National Institute of Health, Bethesda, MD). Vitronectin and monoclonal antibody against human αvβ5 (P1F6) were from Life Technologies (Gaithersburg, MD). Monoclonal antibodies against human αvβ3 (LM609) and human β1 were purchased from Chemicon International Inc. (Temecula, CA). The anti-pan-Erk antibody, which recognizes the C-terminal half of Erk1 and Erk2 molecules, was from Transduction Laboratories (Lexington, KY). Polyclonal antibody against phosphorylated Erk (p-Erk) was from New England Biolabs, Inc. (Beverly, MA). Monoclonal antibodies against p38, phosphorylated p38 (p-p38), and phosphorylated JNK (p-JNK), and goat anti-RSK2 antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MAPK assay kit was from Stratagene (La Jolla, CA). S6 kinase assay kit was from Upstate Biotechnology (Lake Placid, NY). Transwells with polycarbonate membrane containing 8.0 μm pores were from Costar (Cambridge, MA). Phosphatase inhibitor cocktails I (p2850) and II (p5726),
protease inhibitor cocktail (p8340), and anti-tubulin antibody were from Sigma Chem. Comp. (St. Louis, MO). Leukostat kit was from Fisher Scientific (Pittsburgh, PA).

Cell cultures

Human osteoblasts and bone marrow stromal cells were isolated from ribs as described previously (33,34). Briefly, the ribs were cleaned of any extraneous tissue and then split. The trabecular bone chips were recovered by bone curette and were subjected to a two-hour collagenase (1 mg/ml) digestion. After digestion, bone chips were plated into culture flasks. Osteoblasts outgrew from bone chips were subcultured. Bone marrow stromal cells, which represent relatively immature preosteoblastic cells, were also isolated from bone marrow using Histopaque-1077 gradient followed by adhesion to culture flasks. Both osteoblasts and bone marrow stromal cells were subcultured in α-minimum Eagle’s medium (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum (HIFBS). Since the results obtained with either osteoblasts or bone marrow stromal cells were similar, we defined these two cell types together as human osteoblastic (HOB) cells for convenience.

Generation of pseudotyped retrovirus carrying a dominant negative Erk1 cDNA

A kinase-defective Erk1 cDNA (35,36), in which the AA nucleotides at 211 and 212 positions were mutated to CG resulting in replacing amino acid 71 Lys with Arg (Erk1 K71R), was kindly provided by Dr. Melanie H. Cobb (University of Texas, Southwestern Medical Center, Dallas, TX). Erk1 K71R has been shown to function in a dominant negative fashion and can block both Erk1 and Erk2 activities (37). The Erk1 K71R cDNA was cloned into the NcoI
and BamH1 sites of SFG retroviral vector as described previously (38,39). For generation of retroviral particles pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G), SFG-Erk1 K71R viral vector was transfected using LipofectAmine into the 293GPG packaging cell line, which expresses the MuLV gag-pol, and also expresses the VSV-G protein under tetracycline regulation (38,39). The conditioned medium was harvested daily after the withdrawal of tetracycline, and the media harvested from day 3 to day 7 containing the highest titer of viral particles (≥ 5 x 10^6 cfu/ml) were combined and used for transduction. Before transduction, the medium was filtered through a 0.45 µ membrane and polybrene (hexadimethrine bromide, 8 µg/ml) was added to the virus medium. As a negative control, a virus carrying the SFG-LacZ cDNA was generated in the same fashion. This pseudotyped retrovirus has been shown to have no effect on osteoblast differentiation (40).

Transduction of human osteoblastic cells with pseudotyped retroviruses

The day before transduction, HOB cells were seeded in 150 mm culture dishes (1x10^6/dish). The next day, 25 ml of conditioned medium containing Erk1DN or LacZ virus was added to each dish, and the incubation continued for another 24 h. At the end of the incubation period, the virus medium was removed, and cells were grown to confluence in α-MEM with 10% HIFBS.

Immunostaining of the transduced cells for Erk proteins

HOB cells were seeded onto cover slips and allowed to recover overnight. Cells were then transduced with either LacZ or Erk1DN virus for 24 h followed by further incubation in the
growth medium for additional 48 h. Cells were washed twice with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 15 min. After washing twice with PBS, cells were treated with ammonium chloride to quench any remaining paraformaldehyde. Cells were again washed with PBS and then permealized with 0.1% Triton X-100 in PBS (PT), followed by incubation with anti-pan Erk antibody (1:400) in PT containing 1% bovine serum albumin (BSA) (PTB) for 1 h at room temperature. After washing 3 times with PT, cells were incubated with Cy3-conjugated goat anti-mouse antibody (1:200) in PTB for 1 h. Finally, after extensive washing with PT, the cover slips were mounted on slides, and fluorescence was observed using an epifluorescence microscope.

MAPK activity assay

Confluent cells in 100 mm culture dishes were incubated in α-MEM containing 0.2% HIFBS for 24 h followed by treatment with designated growth factor for 10 min at 37°C. After two PBS washes, the cell layers were extracted with 0.5 ml of MAPK assay buffer consisting of 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 5 mM NaF, phosphatase inhibitor cocktail II (0.4 ml/10 ml), and protease inhibitor cocktail (0.4 ml/10 ml). Extracts were homogenized by passing through 26 G needles 10 times. After micro-centrifugation, the supernatant was transferred to a new tube. MAPK activity in the cell extract (20 µg protein) was measured by using the MAPK assay kit with PHAS-1 protein as the substrate and [γ-32P]ATP according to the protocol supplied by the manufacturer (Stratagene).

Western blot analysis
Cell extracts (40 µg protein), isolated as described above, were subjected to SDS-PAGE and transblotted to Immobilon-P membranes. The membranes were dried completely and then incubated with the appropriate primary antibody (1:1000) in PBS containing 1% casein and 0.04% Tween 20 (PCT) for 1 h at room temperature. After three PBS washes, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000 in PCT) for 30 min followed by another four PBS washes. Protein bands were visualized by chemiluminescence using an ECL kit.

**Nuclear extract preparation and electrophoresis mobility shift assay (EMSA)**

Nuclear extracts were prepared as previously described (31). Briefly, HOB cells were lysed in ice-cold buffer containing 10 mM Hepes-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.6% NP-40. After centrifugation, nuclear pellets were extracted with a high salt buffer (20 mM Hepes-KOH, pH 7.9, 1.2 mM MgCl₂, 420 mM NaCl, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 10 µg/ml each of leupeptin and pepstatin, and 25 µg/ml aprotinin) for 1 h. For EMSA, radioactive synthetic double-stranded AP-1 consensus oligonucleotide, labeled with T4 polynucleotide kinase and [γ-³²P]ATP, was incubated with nuclear extracts (2 µg) for 20 min in binding buffer which consisted of 50 mM Tris HCl, pH 7.5, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM DTT, 20% glycerol, and 0.25 mg/ml poly(dI-dC). The binding reaction was terminated by the addition of 1 µl of 5 X TBE buffer (0.445 M Tris borate, pH 8.3 and 10 mM EDTA) containing 0.03% bromphenol blue, 0.03% xylene cyanol, and 30% glycerol. The mixtures were separated on 4-
20% polyacrylamide gradient gels in 0.3 X TBE. The gels were dried and subjected to autoradiography.

**RSK2 phosphotransferase activity assay**

Immunocomplex kinase assay was employed to measure the phosphotransferase activity of RSK2. Cells were washed in ice-cold PBS three times and extracted with lysis buffer consisted of PBS, pH 7.4, 1% Triton X-100, 1% NP-40, 0.5% sodium deoxycholate, 0.5 mM DTT, 1 mM Na$_3$VO$_4$, 2 mM EGTA, 2 mM EDTA, 5 mM β-glycerophosphate, 25 µg/ml aprotinin, 10 µg/ml each of leupeptin and pepstatin, 50 µg/ml benzamidine, 1 mM PMSF, and phosphatase inhibitor cocktail I (1:100). Supernatants (0.6 mg) obtained after micro-centrifugation were incubated with goat anti-RSK2 antibody (1:200) together with protein G-Sepharose at 4º C overnight. The beads were collected by micro-centrifugation, washed twice with lysis buffer, followed by kinase buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na$_3$VO$_4$, and 1 mM DTT). RSK2 phosphotransferase activity in the beads was measured by using S6 Kinase Assay Kit and [γ-32P]ATP according to the protocols provided by the manufacturer (Upstate Biotechnology).

**Cell proliferation assay**

Viral-transduced HOB cells were seeded in a 24 well plate (20,000 cells/well) and allowed to recover overnight in α-MEM with 10% HIFBS. The medium was removed, and cells were incubated in 0.2% HIFBS medium for another 24 h. $[^3]$HThymidine (2 µCi/well) in α-
MEM containing 0.2 % HIFBS was added with the appropriate growth factor and the incubation continued for another 24 h. [3H]Thymidine incorporated into DNA was measured after trichloroacetic acid precipitation, ethanol washing, and alkali extraction as described previously (34).

**Alkaline phosphatase activity assay**

Confluent viral-transduced cells in 24 well plates were washed three times with TBS (Tris buffered saline, 20 mM Tris HCl, pH 7.4, and 0.9% NaCl), scraped into 0.5 ml of 50 mM Tris HCl, pH 7.4, and sonicated in a Fisher Dismembranetor (30-40% of maximum strength). Alkaline phosphatase activity in the sonicate was measured as described previously (34).

**Matrix mineralization assay**

Mineralized matrix was detected by alizarin red-S staining for deposited calcium. Osteoblasts in 6 well culture plates were incubated for 28 days in mineralization medium consisted of α-MEM, 10% HIFBS, ascorbic acid (50 µg/ml) and β-glycerophosphate (10 mM). At the end of incubation, cells were washed 3 times with PBS and fixed in ice-cold 70 % ethanol for 1 h. After washing with water 3 times, the cells were incubated with 0.4% alizarin red-S in water for 10 min at room temperature followed by 5 washes with water and final incubation in PBS for 15 min.

**Cell adhesion and spreading assays**

Adhesion to type I collagen, osteopontin, or vitronectin was performed as previously
described (33). Briefly, single cell suspensions were obtained after collagenase and
trypsin/EDTA digestion. After rinsing with serum-free α-MEM supplemented with 0.1% BSA,
cells were seeded to wells pre-coated with either BSA or a matrix protein (4 x 10^4 cells/cm^2).

After 1 h incubation for adhesion measurement or 2 h for spreading assay at 37°C, the wells
were washed with PBS to remove the non-adherent cells. Adherent cells were fixed and stained
with 0.5% toluidine blue in 4% paraformaldehyde. To obtain the adherent cell number, stained
cells were extracted with 1% SDS, and the absorbance at 630 nm was measured. For analysis of
cell spreading, cell morphology was recorded by photography using phase contrast microscope.

For adhesion-induced MAPK assays, cells were adhered to matrices for 20 min and lysed in
MATERIALS AND METHODS

**Cell migration assay**

The day before the assay, Costar transwell membranes were coated with collagen (100
ng), osteopontin (250 ng), or vitronectin (250 ng) for 3 h at room temperature. Single cell
suspensions obtained as described above for the adhesion assay were delivered into the
transwells (100,000 cells in 0.1 ml/transwell) suspended inside the bottom wells containing 0.6
ml of α-MEM with 0.1% BSA and 25 ng/ml FGF-2. After overnight migration in an incubator,
cells on the membranes were fixed and stained using Leukostat kit. The non-migrated cells on
top of the membranes were removed with cold PBS-soaked cotton swabs and the migrated cells
on the bottom side of the membranes were counted under the microscope with the aid of a grid.
A total of 9 separate fields (3 x 10^{-4} cm²/field) were counted for each sample and the average
number of cells per field was calculated.

Cell surface and metabolic labeling and immunoprecipitation of integrins

Cells in 150 mm culture dishes were surface-labeled with $[^{125}\text{I}]$NaI as described previously (33). After labeling, cells were extracted with cell lysis buffer consisted of 10 mM Tris HCl, pH 8.5, 0.15 M NaCl, 1 mM CaCl$_2$, 0.02% NaN$_3$, 2% Renex 30, and 1 mM AEBSF. Aliquots (approximately 400 µl) of equal trichloroacetic acid- precipitable radioactivity were incubated overnight with monoclonal antibodies against $\beta_1$, $\alpha_v\beta_3$, or $\alpha_v\beta_5$ and Protein A-Sepharose at 4°C on a Nutator. Pellets obtained after micro-centrifugation were washed twice with RIPA buffer, followed by PBS containing 0.5% Tween 20 and 0.02% NaN$_3$ (PTA) in addition to 1 mg/ml ovalbumin, and finally with PTA. Immunoprecipitated integrins in the beads were extracted with sample buffer and applied to SDS-PAGE. Integrin bands were visualized by autoradiography. For measurement of integrin biosynthesis, cells were incubated in methionine- and cystine- deficient medium containing 5% dialyzed HIFBS and 25 µCi/ml of Tran$^{35}$S-Label for 24 h. After lysis, $^{35}$S-labeled integrins were analyzed by immunoprecipitation and SDS-PAGE as described above.

Protein measurement and quantification of band intensity on X-ray films

Protein concentration was measured by using the BioRad protein DC assay kit. To quantify the band intensity, X-ray films were subjected to image analysis using ISS SepraScan 2001 (Integrated Separation Systems, Natick, MA).
Statistics

Statistical analyses were performed using Student’s unpaired t-test. Each experiment was performed at least twice and the representative data were presented as mean ± SEM.
Results

**Erk/MAPK activity is induced upon adhesion of human osteoblastic cells to bone matrix proteins**

HOB cells have been shown to adhere to numerous bone matrix proteins, including type I collagen, osteopontin, vitronectin, fibronectin, and bone sialoprotein, in an integrin-dependent fashion (12,15,33). It has been well established in various cell systems that Erk/MAPK activity is stimulated by cell adhesion to matrix (6,8-10). When HOB cells adhered to type I collagen, fibronectin, osteopontin, or vitronectin, both Erk-2 [major band at 42 kDa, the main Erk expressed in normal human osteoblasts (27)] and Erk-1 (minor band at 44 kDa) activities were consistently enhanced (4.9- to 7.4-fold after normalization with the corresponding tubulin protein level) as determined by Western blot analysis using antibody against the active phosphorylated Erks (p-Erk) (Figure 1). Likewise, the total MAPK enzymatic activity, determined by the incorporation of $^{32}$P into PHAS-1 substrate, was stimulated 4- to 6-fold upon HOB cell adhesion to these matrix proteins (Figure 1).

**Generation of human osteoblastic cell lines expressing a dominant negative Erk1 protein**

Since Erks mediate both growth factor- and integrin- induced signal transduction, it is likely that they play important role in the regulation of osteoblast function. We employed the dominant negative Erk1 K71R (Erk1DN) protein, which can inhibit both Erk1 and Erk2 activities (37), to analyze the role of Erks in osteoblast function. Using a pseudotyped retrovirus as a carrier for Erk1DN cDNA, 70-80 % of transduced HOB cells expressed high level of Erk1
mutant protein as compared with cells transduced with virus encoding the LacZ protein (Figure 2). Western blot analysis using anti-pan Erk antibody confirmed the overexpression of Erk1 K71R in Erk1DN cells (Figure 3, top panel, top band at 44 kDa). Interestingly, the total amount of Erk2 was decreased to 68% of LacZ level in Erk1DN cells (Figure 3, top panel, bottom band at 42 kDa). Analysis of individual Erk activity by Western blotting for p-Erk protein levels indicated that the concentration of active p-Erk2 was further decreased to 30% of LacZ level in Erk1DN cells (Figure 3, 2nd panel, bottom band). Consistent with earlier reports (35,37), Erk1 K71R in Erk1DN cells could be phosphorylated by MEK despite its defect in kinase activity (Figure 3, 2nd panel, top band). Since p38 and JNK are also members of the MAPK superfamily, we analyzed the activity of these two kinases by Western blotting using antibodies that recognize the activated forms of these enzymes. As shown in Figure 3 (bottom 3 panels), active p38 (p-p38) and JNK (p-JNK) were not inhibited, but were, in fact, stimulated (2-3 fold) by the expression of Erk1DN protein when normalized with the total p38 levels. The stimulation of p38 activity in Erk1DN cells is consistent with an earlier report that p38 activity can be inhibited by Erk (41).

Analysis of total MAPK activity indicated that cells expressing Erk1DN protein had lower (55%) basal MAPK activity as compared with LacZ virus transduced cells (Figures 4A and 4B, top left 2 lanes). Since FGF-2, TGF-β, and BMP-2 can stimulate MAPK activity in osteoblastic cells (27,42,43, and unpublished data), we also analyzed the MAPK activity in transduced cells after stimulation with these growth factors. As shown in Figure 4A, the FGF-2-stimulated MAPK activity in Erk1DN cells was only 22% of LacZ cell level under the same conditions (Figure 4A, top right 2 lanes). Similarly, the stimulation of MAPK activity by BMP-
2 and TGF-β in Erk1DN cells was only 30% of the values obtained from stimulated LacZ cells (Figure 4B, top right 4 lanes). The bottom panels of Figures 4A and 4B showed the total Erk proteins in the extracts, further confirming the expression of Erk1DN protein in cells transduced with Erk1DN viruses. Since activation of Erks leads to increased c-fos transcription and AP-1 activity (26), we examined the effect of Erk1DN protein on AP-1 activity in osteoblasts. Results of EMSA demonstrated that the expression of Erk1DN protein strongly inhibited AP-1 binding activity to its consensus oligonucleotide to 10% of LacZ control cell level (Figure 4C). Moreover, the activity of RSK2, another downstream effector of Erk (44) was inhibited by 54% in Erk1DN cells as compared with the LacZ cells after normalization for protein loading, both under basal condition and after FGF-2 stimulation (Figure 5). These combined data indicated that Erk1 K71R cDNA introduced into HOB cells by using pseudotyped retrovirus was expressed and was functional in both basal and growth factor-stimulated Erk1DN cells.

Expression of Erk1DN protein inhibited the proliferation and differentiation of human osteoblastic cells

After establishing the expression of functional Erk1DN protein, we examined the effect of this protein on HOB cell proliferation. As shown in Figure 6, the basal DNA synthesis, as measured by [3H]thymidine incorporation, was slightly but significantly decreased (86% of LacZ level) in Erk1DN cells. Since both FGF-2 and TGF-β stimulate the proliferation of normal human osteoblastic cells (4,45), we analyzed the effects of Erk1DN protein on the proliferation induced by these factors. As expected, FGF-2 stimulated the incorporation of [3H]thymidine into DNA in LacZ cells (135% of the basal level), while no stimulation of DNA
synthesis was detected in Erk1DN cells (Figure 6). Similarly, TGF-β augmented DNA synthesis in LacZ cells (186% of the basal level) whereas this stimulation was diminished in Erk1DN cells (129% of the basal level) (Figure 6).

Since MAPK can regulate cell differentiation (26), we examined the effects of Erk1DN protein on HOB cell differentiation by analyzing differentiation markers including alkaline phosphatase activity, matrix protein deposition, and matrix mineralization. Expression of Erk1DN protein reduced alkaline phosphatase activity to 37% of the LacZ control cell level (Figure 7). In addition, the protein levels of type I collagen, osteopontin, and bone sialoprotein in the cell layers of Erk1DN cells were decreased to 47%, 63%, and 64%, respectively, of the corresponding LacZ level (Figure 8). Consistently, matrix mineralization was inhibited in Erk1DN cells after long-term culture in the presence of ascorbic acid and β-glycerophosphate (Figure 9). Thus, expression of Erk1DN protein inhibits both proliferation and differentiation in HOB cells.

Expression of Erk1DN protein inhibited cell adhesion, spreading, migration, and integrin expression

Since Ras has been implicated to regulate integrin activities, such as cell adhesion, spreading, and migration, in an “inside-out” fashion (46-48) and since Erk is one of the major downstream effectors of Ras, we examined the effect of Erk1DN protein on these activities. Expression of Erk1DN protein inhibited cell adhesion to type I collagen, osteopontin, and vitronectin to 25%, 35%, and 31%, respectively, of the corresponding LacZ level (Figure 10). Cell spreading on these matrices was also curtailed in Erk1DN cells (Figure 11). Similarly, the
migration of Erk1DN cells on collagen, osteopontin, and vitronectin was reduced to 67%, 28%, and 26%, respectively, of the LacZ cell level (Figure 12). Since integrins mediate cell adhesion, spreading, and migration on these matrices, we examined the surface integrin levels in Erk1DN and LacZ cells. As shown in Figure 13, Erk1DN cells expressed lower levels of $\alpha\beta_1$ (43%), $\alpha\beta_3$ (54%), and $\alpha\beta_5$ (51%) integrins when compared with the LacZ cell levels. Metabolic labeling with Tran$^{35}$S-Label indicated that the synthesis of $\alpha\beta_1$, $\alpha\beta_3$, and $\alpha\beta_5$ integrins in Erk1DN cells was only 77%, 70%, and 64%, respectively, of the corresponding LacZ cell level (Figure 14). These results suggested that the inhibition of integrin expression on the cell surface was derived, at least in part, by the inhibition of integrin synthesis.
Discussion

We have demonstrated that the expression of an Erk1 dominant negative protein inhibited Erk/MAPK activity, resulting in decreased proliferation and differentiation of human osteoblastic cells. The expression of Erk1DN protein also inhibited osteoblast adhesion, spreading, migration, and integrin expression. These data indicate that Erks of the MAPK superfamily play an important role in “outside-in” signal transduction regulating osteoblast growth and differentiation and in “inside-out” signal transduction modulating integrin levels on cell surface and, hence, cell adhesion, spreading and migration. The inhibition of osteoblast function by Erk1DN protein was not a result of global apoptosis since only a few dead cells (<0.01%) were detected in Erk1DN cells by TUNEL assay, which was not significantly different from LacZ cells (data not shown).

The role of MAPK in cell proliferation and differentiation has been well documented in a variety of cell systems (26). Our data demonstrate that Erk/MAPK mediates FGF-2- and TGF-β-stimulated proliferation in human osteoblastic cells. In addition, our observation that Erk/MAPK is activated by osteoblast adhesion to several bone matrix proteins, such as type I collagen, fibronectin, vitronectin, and osteopontin, is consistent with earlier reports using other cell systems (6,8-10). In osteoblast differentiation, alkaline phosphatase activity is increased early in the process, and it is this increase that commits the cells to differentiate into osteoblast lineage (49). A shortage or complete deficiency in alkaline phosphatase activity delays or prevents matrix mineralization in osteoblasts (50). In line with these reports, inhibition of Erk activity in osteoblasts decreases not only alkaline phosphatase activity but also matrix

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mineralization. Thus, Erk appears to be essential for the commitment of osteoblasts to differentiate, and our results are consistent with the role of alkaline phosphatase in matrix mineralization. It has been reported that the stimulation of MAPK activity upon adhesion of osteoblasts to collagen is via FAK signaling and that it is this stimulation of FAK that leads to elevated alkaline phosphatase mRNA level (32). Since Erk/MAPK is stimulated by adhesion of HOB cells to various bone matrix proteins, and since Erk/MAPK is one of the down-stream effectors of FAK, the decreased alkaline phosphatase activity observed in Erk1DN cells may stem from the modulation of FAK/MAPK signaling.

In addition to alkaline phosphatase, Erk/MAPK regulates the deposition of bone matrix proteins (type I collagen, osteopontin, and bone sialoprotein) and matrix mineralization by HOB cells. Type I collagen and bone sialoprotein have been shown to serve as mineralization scaffolds and hydroxyapatite initiation sites, respectively while osteopontin functions as the modulator of matrix mineralization (51-54). Therefore, the inhibition of the deposition of these matrix proteins by dominant negative Erk1 will retard matrix mineralization. Recently, MEK, the up-stream effector of MAPK, has been shown to regulate the activity of Cbfa1, a transcription factor that mediates osteoblast differentiation (55). Stimulation of MAPK by constitutively active MEK enhances the expression of osteocalcin, another osteoblast differentiation marker, while the dominant negative MEK or MEK inhibitor PD98059 inhibits osteocalcin expression (55). These observations suggest that MAPK is essential for osteoblast differentiation and matrix mineralization.

Bone is a dynamic tissue that undergoes constant remodeling. For bone formation to occur, osteoblast precursor cells must migrate from residing bone marrow compartment to the
remodeling site where they differentiate. The inhibition of integrin expression by Erk1DN suggests that the Erk/MAPK pathway is critical for osteoblast integrin expression, which, in turn, dictates cell migration, adhesion, and spreading on matrices since integrins play important role in these processes. MAPK has previously been shown to be associated with the up-regulation of integrins (56,57). Our data provide direct evidence linking Erk/MAPK signaling to the expression of $\alpha\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins. Although the reduction of surface integrin levels in Erk1DN cells can be attributed in part to the decreased synthesis, the latter is only to a moderate degree (23-36% inhibition) as compared with the former (46-57% reduction). Therefore, other mechanism(s) may also be regulated by Erk/MAPK to affect the integrin levels on cell surface. The extent of inhibition on cell adhesion and migration in Erk1DN cells (approximately 70% inhibition with the exception of migration on collagen) cannot be fully accounted for by the degree of reduction in surface integrin levels. These data suggest that Erk may affect the integrin activity. Additional studies will be required to confirm these speculations. Since cell migration is preceded by cell adhesion, the inhibition of cell migration in Erk1DN cells may simply be the consequence of decreased cell adhesion. In addition, cells need to spread directionally toward chemokine during migration, the decreased ability of Erk1DN cells to spread will hinder cell migration.

Several mechanisms may mediate the inhibition of osteoblast activity by Erk1DN protein. Among these, the inhibition of AP-1 activity appears to be a critical factor (58). It is important to note that the expression of type I collagen, osteocalcin, and osteopontin has been shown to be dependent on AP-1 (59-61). Other transcription factors may also contribute to the Erk/MAPK effects, especially the aforementioned Cbfal since the activation of Cbfal is dependent on
Erk/MAPK (26,44,55). Cbfa1 can regulate the expression of bone sialoprotein, type I collagen, osteocalcin, and osteopontin in osteoblasts (62-65). Therefore, the inhibition of Erk activity in Erk1DN cells will prevent the activation of Cbfa1 leading to the inhibition of bone matrix protein deposition.

Recently, the mutated gene for Coffin-Lowry Syndrome (CLS) has been identified as Rsk2, ribosomal S6 kinase 2 (66,67). This syndrome is characterized by a variety of clinical symptoms including mental retardation, delayed closure of fontanelles, digital dysmorphism, progressive skeletal deformations, osteopenia, and delayed bone age (68-71). Since Rsk2 is a substrate that is phosphorylated and activated by Erk (44), the skeletal defects observed in CLS patients suggest that Rsk2 is one of the important down-stream effectors in the regulation of osteoblast function by Erk. The inhibition of RSK2 activity in Erk1DN cells is consistent with this hypothesis. Our data also provide an explanation for the skeletal abnormalities observed in patients with CLS since lack of RSK2 may result in inhibition of osteoblast growth, differentiation, adhesion, spreading, and migration.
Acknowledgements

We thank Dr. Melanie H. Cobb for providing us the Erk1 K71R plasmid, Dr. Larry W. Fisher for antibodies against bone matrix proteins, and Genetics Institute (Cambridge, Massachusetts) for BMP-2. This work was supported by grants from the National Institutes of Health, AR32087 and AR07033.
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Figure Legends

Figure 1. Activation of Erk/MAPK activity by adhesion of HOB cells to bone matrix proteins. HOB cells were adhered to the indicated bone matrix protein or remained in suspension for 20 min. Cells were extracted and aliquots containing equal amount of protein were subjected to Western blot analysis for measurement of activated Erk (p-Erk) and tubulin levels. MAPK activity was also assayed by using PHAS-1 as the substrate and \( [\gamma^{32}\text{P}] \) ATP followed by SDS-PAGE and autoradiography.

Figure 2. Expression of Erk1 K71R in HOB cells. HOB cells were transduced with a pseudotyped retrovirus encoding either LacZ protein (top) or Erk1 K71R protein (bottom). After 48 h, cells were fixed and stained with anti-pan-Erk antibody, which recognizes both native Erk and Erk1K71R proteins. Similar results were obtained when anti-Erk1 antibody was employed.

Figure 3. Effect of Erk1DN on Erk2, JNK, and p38 activity in HOB cells. Cells were transduced with virus encoding either LacZ or Erk1 K71R (Erk1DN) protein. After confluence, cells were extracted and Western blot analysis performed using antibodies against pan-Erk, p-Erk, p-p38, p38, or p-JNK.

Figure 4. Inhibition of growth factor-activated MAPK activity and AP-1 activity by Erk1DN. HOB cells were transduced with virus encoding either LacZ (L) or Erk1DN (E) protein.
confluence, cells were stimulated with vehicle, FGF-2 (50 ng/ml), BMP-2 (100 ng/ml), or TGF-β (1 ng/ml) for 10 min. Cell layers were extracted and MAPK activity measured by the phosphorylation of PHAS-1. Total Erk concentration in the extracts was measured by Western blot analysis using anti-pan Erk antibody. A: MAPK activity before and after stimulation with FGF-2. B: MAPK activity before and after stimulation with BMP-2 or TGF-β. C: nuclear extracts derived from LacZ (L) or Erk1DN (E) cells were incubated with radiolabelled AP-1 consensus oligonucleotide and EMSA performed.

Figure 5. Inhibition of RSK2 activity by Erk1DN. Cells transduced with virus encoding either LacZ or Erk1DN protein were stimulated with either vehicle (Control) or FGF-2 (50 ng/ml) for 15 min. RSK2 activity in the cell lysate was measured by using the S6 Kinase Assay Kit and [γ-32P]ATP after immunoprecipitation with anti-RSK2 antibody. Inset: Western blotting of total RSK2 for loading variation in kinase assay. L, LacZ cells; E, Erk1DN cells. *p<0.01 when compared with the corresponding LacZ level.

Figure 6. Erk1DN inhibits osteoblast proliferation. HOB cells transduced with virus encoding either LacZ or Erk1DN protein were stimulated with vehicle (Basal), FGF-2 (50 ng/ml), or TGF-β (1 ng/ml). Cell proliferation was measured by [3H]thymidine incorporation into DNA. a: p< 0.05 when compared with the corresponding LacZ cell level. b: p < 0.05 when compared with the corresponding basal level.

Figure 7. Erk1DN inhibits alkaline phosphatase activity. HOB cells transduced with virus
encoding either Erk1DN or LacZ protein were allowed to grow to confluence. Alkaline phosphatase activity in the cell layer was measured and normalized with total cellular protein concentration.

**Figure 8.** The bone matrix protein levels are reduced in Erk1DN cells. HOB cells transduced with virus encoding either LacZ or Erk1DN protein were allowed to grow to confluence. The levels of type I collagen, osteopontin, and bone sialoprotein in the cell layer were measured by Western blot analysis and quantified by densitometry analysis of the bands on X-ray films.

**Figure 9.** Inhibition of matrix mineralization by Erk1DN. HOB cells transduced with virus encoding either LacZ or Erk1DN protein were cultured in the presence of β-glycerophosphate and ascorbic acid for 28 days. Mineralized matrix was visualized by alizarin red-S staining.

**Figure 10.** Erk1DN inhibits osteoblast adhesion to bone matrix proteins. HOB cells transduced with virus encoding either LacZ or Erk1DN protein were adhered to type I collagen, osteopontin, or vitronectin for 1 h. The number of adherent cells was measured by absorbance at 630 nm after fixation, staining with toluidine blue, and solubilization of the blue dye with SDS solution. a: p < 0.001 when compared with the corresponding LacZ level.

**Figure 11.** Erk1DN inhibits osteoblast spreading on bone matrix proteins. HOB cells transduced with virus encoding either LacZ or Erk1DN protein were allowed to adhere and spread on type I collagen, osteopontin, or vitronectin for 2 h. Cells were fixed and visualized under microscope.
**Figure 12.** Erk1DN inhibits osteoblast migration on bone matrix proteins. HOB cells transduced with virus encoding either LacZ or Erk1DN protein were allowed to migrate on type I collagen, osteopontin, or vitronectin in the presence of FGF-2 (25 ng/ml) for 24 h in Costar transwells. Migrated cells at the bottom of the membranes were counted. a: p < 0.001 when compared with the corresponding LacZ level.

**Figure 13.** Reduction of surface integrin levels by Erk1DN. Cells transduced with virus encoding either LacZ (L) or Erk1DN (E) protein were allowed to grow to confluence. Levels of $\alpha\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins on cell surface were measured after labeling with $^{125}\text{I}$ followed by immunoprecipitation with specific antibody and protein A-Sepharose pull down. Immunoprecipitated integrins were separated by SDS-PAGE, visualized by autoradiography and quantified by scanning the autoradiograms.

**Figure 14.** Erk1DN inhibits integrin synthesis. Cells transduced with virus encoding either LacZ (L) or Erk1DN (E) protein were allowed to grow to confluence. The day before harvest, cells were metabolically labeled with Tran$^{35}$S-Label. Newly synthesized $\alpha\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins were measured after immunoprecipitation, SDS-PAGE, and autoradiography.
Figure 2

Lac Z

Erk1DN
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

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- Type I collagen
- Osteopontin
- Bone Sialoprotein
Figure 9

Lac Z

Erk1DN
Figure 10

Adhesion (Absorbance at 630 nm)

- LacZ
- Erk1DN

Matrix

Collagen  Osteopontin  Vitronectin

Bars with 'a' indicate significant differences.
Figure 11

LacZ

ErK1DN

Collagen  Osteopontin  Vitronectin
Figure 12

Number of Cells Migrated / Field

Matrix

Collagen  Osteopontin  Vitronectin

LacZ  Erk1DN

0  5  10  15  20  25  30

a
Figure 13
Figure 14
Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells
Chung-Fang Lai, Lala Chaudhary, Aurora Fausto, Linda R. Halstead, Daniel S. Ory, Louis V. Avioli and Su-Li Cheng

J. Biol. Chem. published online January 29, 2001

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