BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 134–139 (1997)
ARTICLE NO. RC977256

Activation of Extracellular Signal-Regulated Kinases 1 and 2 (ERK1 and ERK2) by FGF-2 and PDGF-BB in Normal Human Osteoblastic and Bone Marrow Stromal Cells: Differences in Mobility and In-Gel Renaturation of ERK1 in Human, Rat, and Mouse Osteoblastic Cells

Lala R. Chaudhary[1] and Louis V. Avioli

Department of Internal Medicine, Division of Bone and Mineral Diseases, Washington University School of Medicine at Barnes-Jewish Hospital, North Campus, St. Louis, Missouri 63110

Received August 1, 1997

We recently demonstrated that basic fibroblast growth factor (FGF-2) and platelet-derived growth factor-BB (PDGF-BB) mainly activated extracellular signal-regulated kinase 2 (ERK2) in normal human osteoblastic (HOB) and bone marrow stromal (HBMS) cells by an "in-gel" MAP kinase assay, although both ERK1 and ERK2 proteins were present. In the present study, we examined whether ERK1 is also activated by growth factors by using three different MAPK assay procedures, an "in-gel MAP kinase assay," an immune-complex kinase assay, and western blotting with anti-active MAPK antibody which recognizes specifically activated forms of both ERK1 and ERK2. Results have demonstrated that in addition to ERK2, ERK1 is activated by FGF-2 and PDGF-BB in normal HOB and HBMS cells. The human ERK1 moved faster on SDS-polyacrylamide gel compared to rat and mouse, revealing differences in the apparent molecular weight of ERK1 in normal human osteoblastic and bone marrow osteoprogenitor cells, human (TE-85) and rat (ROS 17/2.8 and UMR-106) osteosarcoma, and mouse (MC3T3-E1) osteoblastic cells. ERK1 is less stable in the in-gel renaturation process compared to ERK2; thus, in-gel MAP kinase assay does not provide an accurate estimation of ERK1 activity. Results also showed that anti-active MAPK antibody can be used reliably and accurately to measure the activation of ERK1 and ERK2 in osteoblastic cells. © 1997 Academic Press

Basic fibroblast growth factor (FGF-2) (1-5) and platelet-derived growth factor (PDGF) (6) play important roles in bone cell biology and exert their biological effects through their respective tyrosine kinase receptors. However, the signaling pathways used by these growth factors for transmitting signals in the cytoplasm and to the nucleus from cell surface in normal human osteoblastic (HOB) and bone marrow stromal (HBMS) cells have not been fully characterized. Extracellular signal-regulated kinase 1 (ERK1) and ERK2 (also referred to as mitogen-activated protein (MAP) kinase $p44^{mapk}$ and $p42^{mapk}$) are terminal serine/threonine protein kinases in the three-kinase cascade, where each enzyme sequentially phosphorylates and thereby activates the next enzyme in the sequence in response to a variety of extracellular stimuli (7-11). ERK1 and ERK2 are the best characterized MAP kinases(12,13) and phosphorylation at both threonine and tyrosine is required for their maximal activation (14,15). MAP kinases are activated by receptor tyrosine kinases such as the receptors for insulin, epidermal growth factor (EGF) and PDGF, nerve growth factor (NGF) and FGF-2 (7-11,16) as well as by G-protein-coupled receptors and protein kinase C (11). We have recently demonstrated the presence of both ERK1 and ERK2 proteins in normal HOB and HBMS cells and also in rat and mouse osteoblastic cells (16). Using "in-gel MAP kinase assay", we also showed the activation of ERK2 by IGF-I, FGF-2 and PDGF-BB. Although both ERK1 and ERK2 proteins were present in HOB and HBMS cells, activation in reponse to growth factors in human and mouse osteoblastic cells was limited primarily to ERK2. This response could have resulted from the fact that the relative amounts of ERK1 were low when compared to ERK2. ERK1 could have also been less stable than ERK2 and thus may not have renatured completely in the gel during renaturation process. Finally, the mobility of ERK1 may have been

[1] Corresponding author. Fax: (314) 454-5047.

0006-291X/97 $25.00

different for human as compared to rat or mouse and as a consequence separated poorly on minigel.

In the present report, we have addressed these questions by using different MAPK assay methods which include "in-gel MAP kinase assay", immune-complex kinase assay, and western blotting with anti-active MAPK antibody which specifically recognizes activated forms of both ERK1 and ERK2. The accumulated results reveal that both ERK1 and ERK2 are activated by FGF-2 and PDGF-BB in normal human osteoblastic and bone marrow stromal cells, in addition to human and rat osteosarcoma and mouse osteoblastic cells. Although differences in the mobility of ERK1 in rat and mouse cells were also observed when compared to human osteoblastic cells. We also observed that ERK1 was less stable and did not completely renature.

MATERIALS AND METHODS

Materials. Myelin basic protein (MBP), sodium orthovanadate, 2-mercaptoethanol, Dulbecco's phosphate-buffered saline (PBS), crude bacterial collagenase, trypsin-EDTA, Histopaque-1077, fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM): Ham's F-12 medium (1:1) were obtained from Sigma Chemical Company (St. Louis, MO.). Protein G-Sepharose 4 fast flow was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). Human recombinant PDGF-BB was purchased from R&D Systems (Minneapolis, MN). Polyclonal rabbit antisera against ERK2 (ERK2-CT) were generated by immunizing rabbits with synthetic peptides (15 amino acid residues C-terminal sequences) and affinity purified using columns prepared with the respective peptides as described previously (17) and generously provided by Dr. John C. Lawrence, Jr., Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, VA. Polyclonal anti-ERK1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-active MAPK antibody which recognizes activated forms of both ERK1 and ERK2 was purchased from Promega (Madison, WI). FGF-2 was a generous gift from Amgen Corporation (Thousand Oaks, CA). Human osteosarcoma cell lines TE85 and MG-63, and mouse osteoblast-like cell line, MC3T3-E1 were obtained from American Tissue Culture Collection (Rockville, MD). Rat osteosarcoma cell lines ROS 17/2.8 and UMR-106 were generously provided by Dr. Gideon A. Rodan (Merck, West Point, PA) and Dr. Nicola C. Partridge (St. Louis University, St. Louis, MO) respectively.

Human bone marrow stromal (HBMS) cell culture. Human ribs obtained from surgery patients were transported to the laboratory in tissue culture flasks containing DMEM/Ham's F-12 medium and were processed immediately or after storage overnight in the refrigerator. The bone marrow stromal cells were isolated as described previously (18). Briefly, the bone marrow was harvested by gently flushing the marrow compartment with DMEM/F-12 medium containing heparin (10 units/ml) and DNase (1μg/ml). Marrow cells were pelleted by centrifugation, re-suspended in 20 ml of α-MEM containing 10% FBS and subjected to Histopaque-1077 (Sigma) gradient. The marrow cell layer at the interface was harvested and washed three times with the medium. Cells were seeded in T-175 culture flasks at a density of 4×10^5 cells/cm^2 and grown to confluency.

Human bone cell culture. Human ribs used for bone marrow collection as described above were used to scrape out trabecular bone with a size 4 bone currette. Human osteoblast-like (HOB) cells were cultured from trabecular bone explants by the method of Robey and Termine (19) with some modifications (20). Briefly, bone chips were washed several times with DMEM:Ham's F-12 medium and digested with collagenase (Boehringer Mannheim, 250 units/ml) and DNase type I (Sigma, 1 μg/ml) in DMEM:F-12 medium for 2 hours at 37°C. After digestion, chips were washed with DMEM:F-12 (calcium-free) containing 10% FBS and plated in calcium-free DMEM:F-12 medium containing 10% FBS and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively) in T-175 culture flasks. Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The first passage HBMS and HOB cells were used in all experiments. Human, rat and mouse osteoblastic cell lines were cultured in appropriate media to confluency and then used for experiments.

Cell lysate preparation. After cells reached confluency, normal HOB and HBMS cells and human, rat and mouse osteoblastic cell lines were trypsinized and seeded in P-100 culture dishes at a density of one million cells/dish. Cells were grown in P-100 cell culture petri dishes to confluency and then made quiescent in α-MEM containing 0.1% BSA for 24 h. The required amounts of concentrated stock solutions of growth factors were directly added to the medium for specified time. Cells were rinsed with cold PBS containing 100 μM sodium vanadate. Cell lysates were prepared by adding 0.5 ml of lysis buffer A (50 mM β-glycerophosphate, pH 7.4, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin, 3 mM benzamidine, 1% NP-40, 2 mM EDTA, pH 8.0, 1 mM EGTA) to each dish, transferred to microcentifuge tubes, and centrifuged for 15 min at 14,000 $\times$ g in microcentrifuge. The supernatants were removed, stored at -80°C and used as the source of enzyme. Proteins in cell lysates were measured with the Bio-Rad DC protein assay kit (Bio-Rad Labs., Hercules, CA).

Immune-complex kinase assay. ERK1 and ERK2 were immunoprecipitated by incubation of cell lysates (100 μg protein) at 4°C for 1 h with appropriate antibodies as described previously (21) with slight modifications. This was followed by incubation of the immune-complexes with 30 μl of protein G-Sepharose beads for another 1 h at 4°C. The beads were then were washed with cold RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 0.2 mM sodium orthovanadate) and a cold kinase washing buffer B (25 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 20 μM ATP, 500 μg/ml MBP, 10 μg/ml aprotinin, 1 mM benzamidine). ERK1 and ERK2 activities were determined by resuspending the immunecomplexes in 40 μl of kinase assay buffer C (25 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 20 μM ATP, 500 μg/ml MBP, 10 μg/ml aprotinin, 1 mM benzamidine 0.5 mM EDTA, 2 μCi of [γ-^{32}P]ATP and incubating at 30°C for 30 min. Reactions were stopped by adding 20 μl of 3 $\times$ SDS sample buffer, samples boiled for 5 min and electrophoresed on 12.5% SDS-polyacrylamide gels. The phosphorylated substrate bands were detected by autoradiography and quantitated by densitometry.

In-gel MAP kinase assay. The MAP kinase activity was determined by using the in-gel assay method as described (22, 23) with some modifications. Samples of cell lysates were subjected to electrophoresis on a SDS-polyacrylamide gel (10%) formed by polymerizing with myelin basic protein (0.3 mg/ml) in the gel matrix. After electrophoresis, gels were washed with 20% isopropanol in 50 mM Tris-HCl, pH 8.0 for 1 h to remove SDS, then incubated for 1 h in buffer D (50 mM Tris-HCl, pH 8.0 and 5 mM β-mercaptoethanol) with continuous agitation. Proteins were denatured in 6 M guanidine-HCl in buffer D for 1 h and renatured by incubating in buffer D containing 0.04% Tween-20 at 4°C with continuous agitation for 16 h. To detect MAPK activity, gels were incubated in kinase buffer E (40 mM Hepes-NaOH, pH 8.0, 2 mM DTT, 0.1 mM EGTA, 100 μM sodium orthovanadate, 10 mM $MgCl_2$, 25 μM ATP and 50 μCi [γ-^{32}P]ATP for 1 h at room temperature. The gels were extensively washed with 5% TCA containing 1% sodium pyrophosphate with continuous agitation, dried and subjected to autoradiography. All incubations were performed at room temperature unless otherwise stated.

Western blotting (immunoblotting). Cell lysates were subjected to SDS-PAGE (24) and proteins were electrophoretically transferred

to polyvinylidene fluoride (PVDF)/Immobilin-P membrane (Millipore Corp., Medford, MA). The membranes were immersed in blocking buffer [Tris-buffered saline-0.1% Tween-20 (TBST) containing 5% Carnation nonfat dry milk] for ERK1 and ERK2 or TBST +1% BSA for anti-active MAPK at room temperature for 1 h, then incubated with appropriate antibodies according to the protocols of the manufacturers (Promega and Santa Cruz) and washed with TBST. Antibody binding was detected by enhanced chemiluminescence (ECL) western blotting detection system as directed by the manufacturer (Amersham, Arlington Heights, IL).

Densitometric analysis. Autoradiographs were scanned and quantitated by using a computerized ISS SepraScan 2001 (Integrated Separation System-Enprotech, Natick, MA). The data presented are representative of two or more individual experiments with similar results.

RESULTS

Activation of ERK1 and ERK2 by FGF-2 and PDGF-BB in normal HOB and HBMS cells. HOB and HBMS cells were treated with FGF-2 (50 ng/ml) and PDGF-BB (20 ng/ml) for 10 min and Cell lysates were prepared by extracting cells in lysis buffer. Cell lysates were immunoprecipitated with ERK1 and ERK2 antibodies and enzyme activity was determined by incorporation of [^{32}P] from [γ-^{32}P]ATP into myelin basic protein (MBP). As shown in Fig. 1, incorporation of ^{32}P was increased in MBP in the presence of ERK1 and ERK2 immune-complexes by FGF-2 and PDGF-BB when compared to controls demonstrating an activation of both ERK1 and ERK2 in normal HOB and HBMS cells. Autoradiographs were quantitated by densitometry. Results showed an activation of ERK1 in HBMS (approximately 12-fold by FGF-2 and 23-fold by PDGF-BB) and HOB (approximately 5-fold by FGF-2 and 4-fold by PDGF-BB) cells. ERK2 activation was approximately 5-fold in HBMS and approximately 10-fold in HOB cells in the presence of either FGF-2 or PDGF-BB.

Comparison of in-gel renaturation stability, mobility, and relative amounts of ERK1 in human, mouse, and rat osteoblastic cells. We previously observed mainly the activation of ERK2 in human osteoblastic cells despite the presence of both ERK1 and ERK2 proteins (16). Thus, these follow up experiments were performed to determine whether there is a difference in the mobility of ERK1 and ERK2 from different species and whether ERK1 and ERK2 activation can be observed by "in-gel assay" using regular gel instead of the minigel used previously (16). Results showed that the band corresponding to ERK2 was activated by FGF-2 or PDGF-BB in all cell types while relatively a faint band corresponding to ERK1 was observed in HOB, HBMS, MG-63 and ROS 17/2.8 cells (Fig. 2A). ERK1 activation was not detectable in TE-85, UMR-106 and MC3T3-E1cells. Data also showed that the mobility of ERK1 in ROS 17/2.8 cells was different than that of human cells. Since a small activation MAP kinases was observed in UMR-106 and MC3T3-E1 cells, the amount of cell lysate protein was increased for subsequent experiments. To further confirm whether ERK1 was activated in different osteoblastic cells we used anti-active MAPK antibodies which specifically identify activated forms of ERK1 and ERK2 in response to growth factors. As shown in Fig. 2B, activated forms of ERK1 and ERK2 were recognized by the antibody which were in-

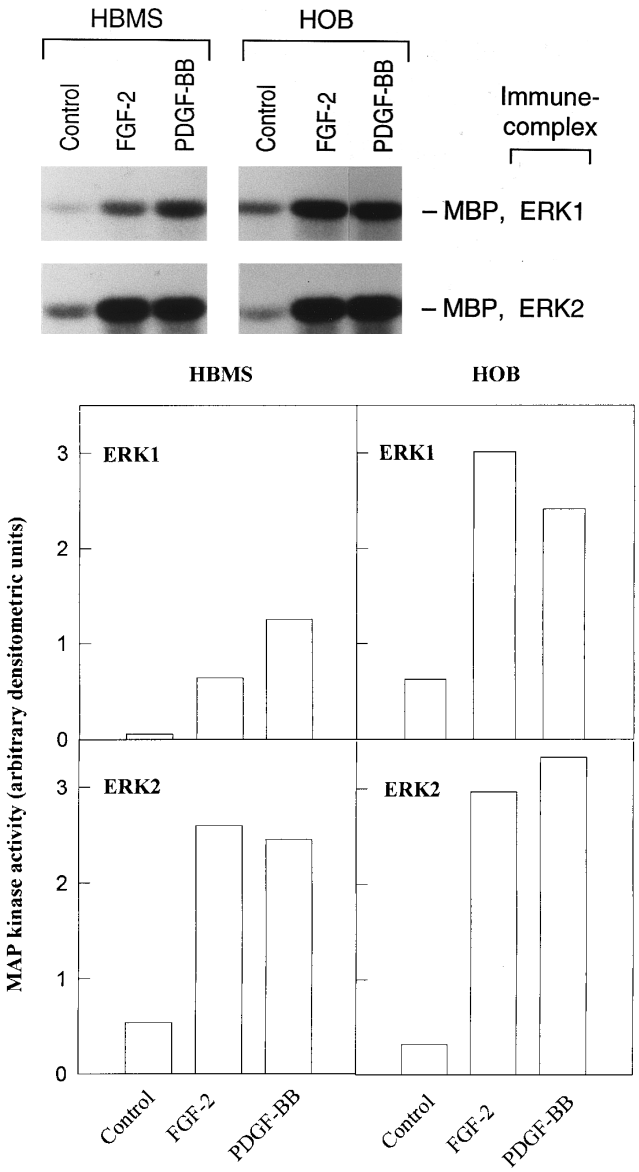

FIG. 1. Activation of ERK1 and ERK2 in normal HOB and HBMS cells. Cell lysates (100 μg protein) were immunoprecipitated with ERK1 and ERK2 antibodies and enzyme activities determined in the immunecomplex as described in Materials and Methods. Incubations were carried out for 30 min at 30°C, reactions were stopped with SDS sample buffer, and samples subjected to SDS-PAGE (12.5% gel). Gels were dried and exposed to x-ray film at −80°C for appropriate time periods. Autoradiographs (upper panel) were scanned and quantitated by densitometry (lower panel).

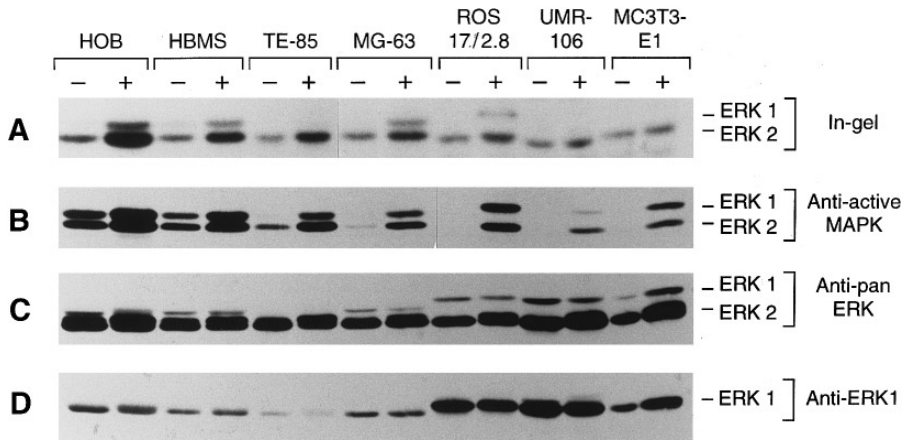

FIG. 2. Comparison of ERK1 and ERK2 activation by FGF-2 and PDGF-BB in different human, rat, and mouse osteoblastic cells. Cells were incubated in the absence (−) or presence (+) of FGF-2 (50 ng/ml) or PDGF-BB (20 ng/ml) for 10 min. In cell lysates (35 μg protein for all cell types except for UMR-106 and MC3T3-E1, 51 μg protein), MAP kinase activity was determined by "in-gel MAP kinase assay" using myelin basic protein (MBP) as the substrate (A). Equal amounts of proteins (32 μg protein for all cell types except for UMR-106, 83 μg; MC3T3-E1, 23 μg control; and treated, 41 μg protein) from non-treated controls and treated cells were applied on 10% SDS polyacrylamide gel, and proteins were transferred to Immobilin-P membrane and incubated with anti-active MAPK antibodies (B). The same membrane was stripped and incubated with anti-pan ERK (C) and stripped again and incubated with anti-ERK1 antibodies (D). Proteins were detected by the ECL western blotting detection system (Amersham). All cell types were treated with FGF-2 but MG-63 with PDGF-BB.

creased in response to growth factors as compared to non-stimulated control cells. The mobility of ERK1 was also retarded in rat and mouse osteoblastic cells as compared to human suggesting differences in the apparent moleculat weights of ERK1 across species. These observations also supported the utility of anti-active MAPK antibody to reliably detect and measure ERK1 and ERK2 activation. The pan ERK antibody which reconizes both the active and basal forms of the ERK1 and ERK2, serves as an important control to demonstrate the amount of total ERK protein in each lane, thereby allowing accurate interpretation of the corresponding signal obtained with the anti-active MAPK antibody. Therefore, the same membrane was stripped and then incubated with pan-ERK antibody to check the amounts of ERK1 and ERK2 proteins. As shown in Fig. 2C, amounts of ERK1 and ERK2 proteins were same in control and treated samples. Results also showed differences in the mobility of rat and mouse ERK1 when compared to human ERK1 confirming the results shown in Fig. 2A and 2B. Since results shown in Fig. 2B and 2C were obtained with the antibodies which recognized both ERK1 and ERK2, experiments were performed to further confirm the differences in the mobility of ERK1 across species by using an antibody which recognizes only ERK1. As shown in Fig. 2D, incubation of membranes with ERK1 antibody detected only one bandcorresponding ERK1 with a difference in the mobility of ERK1 in human, and rat and mouse osteoblastic cells further confirming data obtained with two different antibodies which recognized both ERK1 and ERK2. Results also showed that relative amounts of ERK1 were lower in all cell types but this difference was greater in human cells (Figs. 2C and Fig. 2D).

DISCUSSION

In the present study we provide more definite answers to the questions of whether ERK1is activated by growth factors in normal human osteoblastic cells, and whether there are differences in the mobility and stability of ERK1 in different species. We addressed these questions by analyzing ERK1 and ERK2 activity by an in-gelMAP kinase assay, an immune-complex kinase assay and western blotting with anti-active MAP kinase antibody which specifically reconizes activated forms of ERK1 and ERK2. Using an immune-complex assay, our results have clearly demonstrated that in addition to ERK2, ERK1is also activated by FGF-2 and PDGF-BB in normal HOB and HBMS cells. Similarly, ERK2 activation by epidermal growth factor (EGF) and FGF-2 has been reported in rat UMR-106 and ROS 17/2.8 cells respectively (25). Activation of MAP kinase by PMA has also been demonstrated in mouse MC3T3-E1 cells (26). Our results further demonstrate that the relative amounts of ERK1 were lower when compared to ERK2 in human, mouse and rat osteoblastic cells. This difference was much greater in human cells as compared to rat and mouse osteoblastic cells. These data are consistent with observations in other human cells where ERK2 protein has been shown to be predominant as compared to ERK1 (27). Our results also reveal that both FGF-2 and PDGF-BB increased the amounts of activated forms of ERK1 and ERK2 in normal human HOB and HBMS cells, observations which are consistent with ERK1 and ERK2 activation observed with immune-complex assay. Furthermore, activation of ERK1 and ERK2 by FGF-2 or PDGF-BB was

also demonstrated in rat (ROS 17/2.8 and UMR-106) and mouse (MC3T3-E1) osteoblastic cells. The ERK2 basal activity was higher in HOB, HBMS and TE-85 cells whereas very little or no activity was observed in MG-63, ROS 17/2.8, UMR-106 and MC3T3-E1 osteoblastic cells. Similar results were obtained with in-gel MAP kinase and immune-complex kinase assays where the ERK1 basal activity was either very low or non-detectable as compared to ERK2. These observations strongly suggest that ERK1 and ERK2 remain activated at low levels under basal conditions and are greatly activated in response to extracellular stimuli. The basal activity and the magnitude of activation by growth factors also depend on the cell type. The fact that the activation of ERK1 could be demonstrated with the use of a specific anti-active MAPK antibody in all cell types although minimal if any activation was observed by in-gel MAP kinase assay strongly suggests that ERK1 does not renature completely in the gel and/or is less stable during the renaturation process. To our knowledge this is the first demonstration regarding the in-gel stability of ERK1. The accumulated data are consistent with the fact that lower stability and relative lower amounts of ERK1 may be responsible for a small or no activation observed by in-gel MAP kinase assay, thus, supporting that this method can not be used to measure accurately ERK1 in osteoblastic cells. The apparent molecular weight of human ERK1 is different than that of mouse and rat ERK1. The results of our study also offer the first clear demonstration of a difference in the apparent molecular size of human (HOB, HBMS, TE-85, MG-63), rat (Ros 17/2.8 and UMR-106) and mouse (MC3T3-E1) osteoblastic cell ERK1. These observations are consistent with the findings of others wherein similar differences in the mobility of MAP kinases have been reported in the human osteosarcoma cell line G292 and in primary rat calvarial osteoblast cell cultures (28). Whether this difference is due primarily to species difference and/or has any physiological significance is conjectural at this time.

FGF-2 is a potent mitogen for mesenchymal cells including normal human bone marrow stromal cells (1) and osteoblasts (2,3), and stimulates *in vivo* endosteal bone formation in young and adult rats (4,5). Overexpression of FGF-2 (29), mutations in FGF receptors 1 and 2 (FGFR1 and FGFR2) (30-31) and lack of FGFR3 (32) cause skeleton deformities further emphasizing the significance of FGF-2 and its signaling in the regulation of bone development. PDGF stimulates thymidine incorporation in normal human osteoblastic cell culture (6) and *in vivo* bone density and skeleton strength (33). Although the precise physiological role of MAP kinases in osteoblastic cells is currently not well established our results have demonstrate that the actions of FGF-2 and PDGF-BB are mediated by MAP kinase signal transduction pathways.

In summary, we have demonstrated that both ERK1 and ERK2 are activated by FGF-2 and PDGF-BB in normal human osteoblastic cells, human and rat and mouse osteoblastic cell lines. Results have also shown that ERK1 is less stable than ERK2 and does not always renature completely; and the degree of the stability of ERK1 in-gel also depends on the cell type. Thus, the " in-gel MAP kinase assay " method may not provide an accurate measurement of ERK1 activity in osteoblastic cells. Furthermore, human ERK1 moves faster on SDS polyacrylamide gel as compared to mouse and rat demonstrating a species diffrence in its apparent molecular weight.

ACKNOWLEDGMENTS

Authors thank Aurora Fausto for excellent technical assistance in isolating and maintaining HOB and HBMS cells and also human and rat osteosarcoma and mouse MC3T3-E1 cells. We are extremely thankful to Dr. John C. Lawrence, Jr. for the generous gift of the ERK2 antibody. This research was supported in part by NIH Grant PO1 AR32087 to L.V.A.

REFERENCES

1. Robinson, D., Bab, I., and Nevo, Z. (1995) *J. Bone Miner. Res.* **10,** 690–696.
2. Globus, R. K., Patterson-Buckendahl, P., and Gospodarowicz, D. (1988) *Endocrinology* **123,** 98–105.
3. Rodan, S. B., Wesolowski, G., Thomas, K. A., Yoon, K., and Rodan, G. A. (1989) *Connect. Tissue Res.* **20,** 283–288.
4. Mayahara, H., Ito, T., Nagai, H., Miyajima, H., Tsukuda, R., Taketomi, S., Mizoguchi, J., and Kato, K. (1993) *Growth Factors* **9,** 73–80.
5. Nakamura, T., Hanada, K., Tamura, M., Shibanushi, T., Nigi, H., Tagawa, M., Fukumoto, S., and Matsumoto, T. (1995) *Endocrinology* **136,** 1276–1284.
6. Zhang, L., Leeman, E., Carnes, D. C., and Graves, D. T. (1991) *Am. J. Physiol.* **261,** C348–C354.
7. Ahn, N. G., Seger, R., and Krebs, E. G. (1992) *Curr. Opin. Cell Biol.* **4,** 992–999.
8. Pelech, S. L., and Sanghera, J. S. (1992) *Science* **257,** 1355–1356.
9. Blenis, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5889–5892.
10. Davis, R. J. (1993) *J. Biol. Chem.* **268,** 14553–14556.
11. Cobb, M. H., and Goldsmith, E. J. (1995) *J. Biol. Chem.* **270,** 14843–14846.
12. Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinto, R. A., Panayotatos, N., Cobb, M. H., and Yancopoulos, G. D. (1991) *Cell* **65,** 663–675.
13. Rossomando, A. J., Sanghera, J. S., Marsden, L. A., Weber, M. J., Pelech, S. L., and Sturgill, T. W. (1991) *J. Biol. Chem.* **266,** 20270–20275.
14. Anderson, N. G., Maller, J., Tonks, N. K., and Sturgill, T. W. (1990) *Nature (London)* **343,** 651–653.
15. Seger, R., Ahn, N. G., Boulton, T. G., Yancopoulos, G., Panayotatos, N., Radziejewska, E., Ericsson, L., Bratlien, R. L., Cobb, M. H., and Krebs, E. G. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6142–6146.
16. Chaudhary, L. R., and Avioli, L. V. (1997) *Mol. Cell. Biochem.*, in press.

17. Sevetson, B. R., Kong, X., and Lawrence, J. C., Jr. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10305–10309.
18. Chaudhary, L. R., and Avioli, L. V. (1996) *J. Biol. Chem.* **271,** 16591–16596.
19. Robey, P. G., and Termine, J. D. (1985) *Calcif. Tissue Int.* **37,** 453–460.
20. Rifas, L., Halstead, L. R., Weck, W. A., Avioli, L. V., and Welgus, H. G. (1989) *J. Clin. Invest.* **84,** 248–254.
21. Chen, Q., Lin, T. H., Der, C. J., and Juliano, R. L. (1996) *J. Biol. Chem.* **271,** 18122–18127.
22. Kameshita, I., and Fujisawa, H. (1989) *Anal. Biochem.* **183,** 139–143.
23. Chao, T.-S. O., Byron, K. L., Lee, K.-M., Villereal, M., and Rosner, M. R. (1992) *J. Biol. Chem.* **267,** 19876–19883.
24. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
25. Verheijen, M. H. G., and Defize, H. K. (1995) *Endocrinology* **136,** 3331–3337.
26. Siddhanti, S. R., Hartle II, J. E., and Quarles, L. D. (1995) *Endocrinology* **136,** 4834–4841.
27. Boulten, T. G., and Cobb, M. H. (1991) *Cell Regulation* **2,** 357–371.
28. Zhang, W., Dziak, R. M., and Aletta, J. M. (1995) *J. Cell. Physiol.* **162,** 348–358.
29. Coffin, J. D., Florkiewicz, R. Z., Neumann, J., Mort-Hopkins, T., Dorn II, G. W., Lightfoot, P., German, R., Howles, P. N., Kier, A., O'Toole, B. A., Sasse, J., Gonzalez, A. M., Baird, A., and Doetschman, T. (1995) *Mol. Biol. Cell* **6,** 1861–1873.
30. Muenke, M., Schell, U., Hehr, A., Robin, N. H., Losken, H. W., Schinzel, A., Pulleyn, L. J., Rutland, J., Reardon, W., Malcolm, S., and Winter, R. M. (1994) *Nature Genetics* **8,** 269–274.
31. Jabs, E. W., Li, X., Scott, A. F., Meyers, G., Chen, W., Eccles, M., Mao, J. I., Charnas, L. R., Jackson, C. E., and Jaye, M. (1994) *Nature Genetics* **8,** 275–279.
32. Colvin, J. S., Bohne, B. A., Harding, G. W., McEwen, D. G., and Ornitz, D. M. (1996) *Nature Genetics* **12,** 390–397.
33. Mitlak, B. H., Finkelman, R. D., Hill, E. L., Li, J., Martin, B., Smith, T., D'Andrea, M., Antoniades, H. N., and Lynch, S. E. (1996) *J. Bone Miner. Res.* **11,** 238–247.