New Signaling Pathway for Parathyroid Hormone and Cyclic AMP Action on Extracellular-regulated Kinase and Cell Proliferation in Bone Cells

CHECKPOINT OF MODULATION BY CYCLIC AMP

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Cyclic AMP signaling, activated by extracellular stimuli such as parathyroid hormone, has cell type-specific effects important for cellular proliferation and differentiation in bone cells. Recent evidence of a second enzyme target for cAMP suggests divergent effects on extracellular-regulated kinase (ERK) activity depending on Epac/Rap1/B-Raf signaling. We investigated the molecular mechanism of the dual functionality of cAMP on cell proliferation in clonal bone cell types. MC3T3-E1 and ATDC5, but not MG63, express a 95-kDa isoform of B-Raf. cAMP stimulated Ras-independent and Rap1-dependent ERK phosphorylation and cell proliferation in B-Raf-expressing cells, but inhibited growth in B-Raf-lacking cells. The mitogenic action of cAMP was blocked by the ERK pathway inhibitor PD98059. In B-Raf-transduced MG63 cells, cAMP stimulated ERK activation and cell proliferation. Thus, B-Raf is the dominant molecular switch that permits differential cAMP-dependent regulation of ERK with important implications for cell proliferation in bone cells. These findings might explain the dual functionality of parathyroid hormone on osteoblastic cell proliferation.

In mammals, parathyroid hormone (PTH) is the most important hormone affecting bone growth and resorption. It shares the PTH/PTH-related protein (PTHRP) receptor (PPR) with PTHrP (1–8), the only other known endogenous ligand for PPR. A series of gene disruption studies of PTHrP and/or PPR in mice (9–12) has indicated that PTH action on PPR can generate either bone-forming or bone-resorbing signaling, respectively. MLO-Y4 and MLO-A5 subclones 1 and 2 (MLO1 and MLO2) from Dr. Hidetsugu Abe (National Cancer Institute, Frederick, MD) (13–17). However, how cAMP activates ERK is still unknown. The most important target of cAMP is protein kinase A (PKA), but activation of PKA is known to counteract the Ras/Raf-1/MEK signaling pathway (18–20) that is essential for activation of ERK and stimulation of cell proliferation (21–23). Thus, we have been in search of the pathway linking the cAMP signal to ERK activation.

Recently, a second enzyme target of cAMP, cAMP-guanine nucleotide exchange factor (cAMP-GEF)/Epac, emerged as a Rap1-specific GEF (26, 27), indicating that cAMP can modulate ERKs via the Epac/Rap1/B-Raf pathway in a PKA- and Rap1-independent manner (24–27). We now show that a variety of bone cell lines, clonal and primary, constitutively express the components of this pathway in a cell type-specific manner. Modulation of cell proliferation through cAMP signaling is regulated primarily by the expression pattern of B-Raf splice variants, and B-Raf appears to function as a molecular switch in this signaling system. The decisive role of B-Raf first identified in this study may explain the long-known dual functionality of PTH signaling in bone.

EXPERIMENTAL PROCEDURES

Materials—MC3T3-E1, C3H10T1/2, C2C12, and ATDC5 were purchased from RIKEN Cell Bank (Ibaraki, Japan). ROS17/2.8 was obtained from Dr. Gideon Rodan (Mereck, NJ) (28), MG63 from Dr. Akifumi Togari (Aichi-Gakuen University, Nagoya, Japan), and MLO-Y4 and MLO-A5 from Dr. Lynda Bonewald (Texas Health Science Center, San Antonio, TX) (28), MC3T3-E1 subclone4 (MC4) from Dr. Renny T. Franceschi (University of Michigan School of Dentistry, Ann Arbor, MI) (29), and PC12 cells from Dr. Akemichi Baba (Osaka University, Osaka, Japan). Human PTH(1–34) was a gift from Suntory Ltd. (Osaka, Japan). HA-tagged N17Ras, V12Ras, N17Rap1, and V12Rap1 were from Dr. Daniel Altschuler of University of Pittsburgh (Pittsburgh, PA) (30). HA-tagged Epac 1 and -2 cDNA were obtained from Dr. Johannes L. Bos (University Medical Center Utrecht, Utrecht, The Netherlands) (31). Flag-tagged B-Raf vector was kindly provided by Dr. Deborah Morrison (National Cancer Institute, Frederick, MD).

Cell Culture—Primary osteoblastic cells were cultured as described previously (32). MC3T3-E1 was cultured in 10% fetal calf serum (FCS)/α-minimal essential medium (αMEM); C3H10T1/2 was cultured in 10% FCS/modified Eagle medium (MEM); C2C12 and MG63 were cultured in 10% FCS/Dulbecco’s modified Eagle medium (DMEM); ROS17/2.8 and ATDC5 were in 10% FCS and 5% MEM, F12, respectively. MLO-Y4 and MLO-A5 were cultured in 5% FBS, 5% FCS/α-MEM on collagen type I-coated plates as described previously (33).
RNA Analysis—Total RNA was extracted using guanidinium thiocyanate/phenol/chloroform method as reported by Chomczynski and Sacchi (34). Brain and calvaria were isolated from male ddY mice (Shimizu Experimental Supplies, Kyoto, Japan) and RNA samples were extracted. Northern blot analysis was performed under high stringency condition as described previously (32). cDNA probe was electrophoresed in 1.2% agarose-formaldehyde gels, transferred on nylon membrane filters (Hybond N+, Amersham Biosciences, Buckinghamshire, UK), and hybridized with μ-labeled cDNA probes. cDNAs encoding PPR and gyceraldehyde-3-phosphate dehydrogenase cloned by polymerase chain reaction (PCR) were used as probes (32). After the final wash, the membrane was exposed to a BAS imaging plate (Fuji Film, Tokyo, Japan), and the relative signal intensity was estimated. For reverse transcription-PCR, a 0.5-μg RNA aliquot was reverse transcribed at 37 °C for 2 h in a 20-μl reaction volume containing 200 units of Superscript II (Invitrogen, Gaithersburg, MD), 4 μM random primers, 500 μM dNTPs, and 5 mM dithiotreitol. PCR was performed using one-tenth of the reverse transcription reaction volume and 30 pmol of following oligonucleotides. For Epac1, Epac1-F, 5′-GGCTTCCTCAACAACTCTC-3′, Epac1-R, 5′-AAGCCTGCCATCGCATCTCTC-3′ (AN: NM_006105); for Epac2, Epac2-F, 5′-AGGCTTTAGGCTCCAGGTT-3′, Epac2-R, 5′-CTGACTGTATTCGCCTCCAC-3′, and a 100-fold dilution of cDNA samples. Each PCR contained 200 μM dNTPs, and 5 μM random primers, and 5 mM dITH. PCR was performed using a TAKARA PCR thermal cycler MP system (Shiga, Japan). PCR thermocycling conditions were performed with 0.1% bromphenol blue and 0.05% 2-mercaptoethanol, boiled for 5 min, and then electrophoresed on a 1% agarose gel in Tris-acetate-EDTA buffer. The number of cycles selected for each primer pair produced a linear relationship between the amount of input RNA and resulting PCR products.

cAMP Assay—Cells were washed twice with incubation buffer (αMEM containing 0.5 mM 1-methyl-3-isobutylxanthine (IBMX) and 1 mg/ml bovine serum albumin) and incubated for 30 min at 37 °C in the same buffer containing various concentrations of test agents. The reaction was terminated with trichloroacetic acid. CAMP was measured by radioimmunoassay (Amersham Biosciences) and the protein concentrations were estimated using a BCA protein assay kit (Pierce).

Cell Growth Assay—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) activity was measured using a colorimetric assay (35). Cells were plated in 96-well plates at a density of 30,000 cells/well. Cells were treated with or without various agents for 4 days. The cells were then gently washed twice with 100 μl of phosphate-buffered saline and then further incubated for 2 h after the addition of 100 μl of MTT. Then, 50 μl of solubilizing solution containing 20% sodium dodecyl sulfate (SDS), 50% dimethylformamide, 2% acetic acid, and 2.5% of 1 M HCl, pH 4.7, was added to extract the dark blue crystals. After complete extraction, the absorbance was measured on a Bio-Rad Model 550 microplate reader (Bio-Rad, Hemel Hempstead, UK), using a test wavelength of 570 nm and a reference wavelength of 655 nm. Bromodeoxyuridine (BrdUrd) incorporation assay was performed using a colorimetric BrdUrd Cell proliferation Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s protocol as described previously (36). There was no difference in either assay in the number of dead cells between the cell lines determined by a trypan blue exclusion assay. FCS was reduced to 1% for all treatment conditions.

Establishment of Stable Transformants—The day before transfection, cells were plated on 96-well plates at a density of 10^5 cells/well, and then transfected by FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s protocol as described previously (37). The vector containing both the green fluorescent protein and neomycin-resistant genes, pEGFP-N1 (CLONTECH, Valencia, CA), and the respective vectors were co-transfected in the cells. Subconfluent cells were trypsinized and plated at low density before selection. Subsequently, cells were selected by culturing them in the presence of 400 μg/ml G418 for 3 weeks. Three clones of each mutant from MC4 and MG63 were isolated. In this study, number 004, N17Rap1 MC4; number 005, V12 Rap1 MC4; number 001, N17 Ras MC4; number 021, N17Rap1 MG63; number 008, V12 Rap1 MG63; number 015, B-Raf MG63 were used.

Intracellular cAMP was measured by the competition assay. Cells were incubated in Tris-HCl buffer, pH 7.4, containing 3% SDS and 10% glycerol and the protein concentrations were estimated using a BCA protein assay kit. The sample was mixed with 0.1% bromphenol blue and 0.05% 2-mercaptoethanol, boiled for 5 min, and then loaded (equal amounts of protein/lane) on 10% gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes for Western blotting, using antibodies against Epac1 (C-17), Epac2 (M-18), B-Raf (e19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Rap1 (clone3; Transduction Labs, Lexington, KY), and phospho-ERK1/2, ERK1/2 (New England Biolabs, Beverly, MA), and horseradish peroxidase-conjugated antibody as described previously (37). As a positive control, PC12 cell lysates were used. For selection of clones, anti-HA antibody (Y-11; Santa Cruz Biotechnology, Inc.) or anti-FLAG antibody (M15; Sigma) was added to the media.

Statistical Analysis—Unless otherwise described, statistical analyses were performed using Student’s t test. A p value of less than 0.05 was considered to be significant. Two-way analysis of variance was performed to determine statistical differences between cultures according to time in culture.

RESULTS

Epac, Rap1, and B-Raf Expression—B-Raf exists as a number of isoforms that are expressed primarily in neural and endocrine tissues (26, 38). Although the 95-kDa B-Raf isoform is found in both brain and spinal cord, its presence in bone cells has not been systematically explored. We measured B-Raf protein expression in cultures of fibroblast C3H10T1/2, osteoblast MC4, chondrocyte ATDC5, two osteocytic clones MLO-Y4 and MLO-A5, two osteosarcoma cell lines, ROS17/2.8 and MG63, and myoblast C2C12 cells by Western blotting using an antibody specific for B-Raf (Fig. 1, A, lower panel). ATDC5, MC4, C3H10T1/2, and the two types of MLO cells had both the 95- and 62-kDa B-Raf splice variants, and C2C12, MG63, and ROS17/2.8 cells expressed only the 62-kDa isoform. Additionally, Epac1 and Epac2, two isoforms of Epac, and Rap1 protein expression were investigated (Fig. 1). In vivo bone expressed Epac mRNA (Fig. 1A). Similar Epac mRNA expression was observed in brain and PC12 cells. Epac2 expression was detected in all cells (Fig. 1B). In contrast, Epac1 was not detected in ATDC5, C2C12, and ROS17/2.8, and Rap1 expression was limited and weak in C2C12 and ROS17/2.8 cells. Either expression pattern of 95-kDa B-Raf, Rap1, or Epac, however, failed to show any notable change during maturation (Fig. 1D, right), while ERKs (p44 and p42) were gradually decreased (Fig. 1D, left). Taken together, these results suggest that, among cell types tested, only primary calvaria cells predominantly express the 95-kDa B-Raf isoform while the other limited lines the 62-kDa isoform alone and this allows cell type-specific cAMP signal transduction and diverse proliferative reactions.

PPR Expression and cAMP Accumulation Triggered by PTH—(1–34)—Next, we performed Northern blot analysis using specific probes for PPR and examined CAMP accumulation levels triggered by PTH-(1–34) in some clonal cells (Fig. 2). PPR mRNA expression was detected in ATDC5, MC3T3-E1, and MC4 cells. The MC4 cells expressed 10-fold more PPR mRNA compared with other lines (Fig. 2A). As expected, PTH-(1–34) dose-dependently stimulated CAMP accumulation in these cells (Fig. 2B). In MC4, CAMP accumulation levels were 100-fold compared with ATDC5 and MC3T3-E1. On the other hand, the increase in cAMP induced by PTH-(1–34) was negligible and barely detectable in MG63, C3H10T1/2, and the two MLO cell lines (data not shown), although high enough to suppress ERK activity and cell proliferation (refer to Figs. 3B and 6A). In development model using primary osteoblastic cells, Northern blot analysis showed that PPR mRNA expression levels were gradually increased up to 21 days and then gradually decreased (Fig. 2C). Similar results were reported in the MC3T3-E1 and ATDC5 developmental culture system (17, 39). In contrast, expression of ERK had a peak at the start of culture. Since the Epac pathway is expressed constantly, the ERK level peaked at the start may be an important variable to assure proliferative response toward cAMP signal that increases the cell number, as the main determinant of bone mass. These results suggest that increased intracellular cAMP is perhaps the major PTH-induced signaling mechanism in these osteoblastic cells during cell proliferation.
cAMP Stimulates ERK Phosphorylation in 95-kDa B-Raf-expressing Bone Cells—To determine the consequences of elevated intracellular cAMP on bone cellular ERK activity, we measured ERK activity using antibodies specific for the active phosphorylated ERK. For these experiments, a variety of agents that can increase intracellular levels of cAMP or act as...
a cAMP analogue, forskolin, dibutyryl-cAMP (Bt2cAMP), and 8-(4-chlorophenylthio)-cyclopencyladenosine, were used. Only clonal cells expressing 95-kDa B-Raf, PTH-(1–34), forskolin, Bt2cAMP, and 8-(4-chlorophenylthio)-cyclopencyladenosine (8-CPT) increased the activity of ERK 2–12-fold more than in unstimulated cells (Fig. 3, B and C, and data not shown). Maximal activation by 1 nM PTH-(1–34) occurred rapidly, within 5–30 min and its activation was reduced by a higher concentration of the peptide (Fig. 3 A). IBMX increases cAMP levels by inhibiting its degradation by cAMP phosphodiesterase. IBMX also potentiated ERK activity (Fig. 3 C), indicating that the potentiation was due to increased intracellular cAMP. PTH-(1–34)-induced ERK phosphorylation was completely inhibited by the ERK pathway inhibitor PD98059. When cells were treated with H89, low concentrations of PTH-(1–34)-induced ERK activation were not affected, while increased ERK activation was observed with high concentrations of the peptide (Fig. 3 D). These results suggest that PTH stimulates intracellular cAMP accumulation, cAMP then activates Rap1 via GEF molecules, both Epac dependently and PKA independently, which in turn leads to B-Raf activation and results in ERK activation. Therefore, PKA might inhibit cAMP-mediated ERK activation when excess intracellular cAMP accumulates. In our preliminary experiments, calcitonin also stimulated cAMP-ERK signaling mechanisms in two MLO cell lines that express functional calcitonin receptors.2 Therefore, cAMP-induced ERK activation by other hormones and factors was thought to be a common regulatory pathway in bone cells.

**cAMP Stimulates ERK Phosphorylation and Cell Proliferation**

In neuronal cells, cAMP signaling has an important role in cell differentiation and survival through a Rap1-B-Raf expression-dependent mechanism (24). Activated ERK provides a mitogenic and a differentiating signal in many cell types (22, 23). To determine whether cAMP stimulates cell proliferation, we examined cell proliferation of bone cells assessed by MTT and BrdUrd incorporation assays. MTT activity and BrdUrd incorporation were directly correlated with the counted cell number of MC4 and MG63 cells in our preliminary experiments, thus growth was estimated using the MTT method. There was no correlation, however, between MTT activity and BrdUrd incorporation in ATDC5 and gene-transduced cells. Therefore, the BrdUrd method was used to measure cell proliferation (data not shown). PTH-(1–34), Bt2cAMP, and forskolin stimulated BrdUrd incorporation in two B-Raf-expressing bone cell lines in a low concentration range (ATDC5 and MC4; Fig. 4, A and B). With increased ERK activity, IBMX also potentiated cell proliferation in basal and cAMP-stimulated conditions (Fig. 4 C), indicating that growth potentiation was due to the increase in intracellular cAMP. The ERK pathway inhibitor PD98059 inhibited cAMP-triggered ERK activation and cell proliferation (Figs. 3 D and 4 D). Basal cell proliferation of MC4 was not affected by 1 μM PD98059. The PKA inhibitor H89 (1 μM) did not affect low concentration cAMP-induced cell growth (Fig. 4 D), indicating that if the PKA signaling pathway was inhibited, the mitogenic action of cAMP was not blocked because the signaling pathway of the Epac remained active. In contrast to the low concentration effects of cAMP, high concentration cAMP-induced cell proliferation was normalized to control levels. H89 did not affect the low concentration cAMP stimulation, whereas it potentiated the high concentration cAMP-mediated
activation of cell growth. Thus, PKA might function to inhibit cell proliferation via ERK signal-induced mechanisms only with high concentrations of intracellular cAMP.

**Regulation by Rap1 in 95-kDa B-Raf-expressing Cells**—Because Rap1 is a transducer of cAMP-mediated regulation of ERK, we next tested the hypothesis that the cAMP effect on ERK activity and MC4 proliferation is the result of Rap1 activation. We established MC4 overexpressing dominant negative N17Rap1 or the constitutively active V12Rap1 mutants, and mutants of Ras were generated and several independently isolated clones analyzed for Rap1 expression by Western blotting using HA antibody. All clones demonstrated elevated Rap1 or Ras expression compared with vector-transfected control lines (data not shown). ERK activation by PTH-(1–34), Bt2cAMP, and forskolin was observed in Ras mutant clones, whereas it was completely blocked in N17Rap1-transduced MC4 cells, and accelerated in V12Rap1-transduced cells (Fig. 5A). Thus, cAMP actions on ERK were mediated via Rap1 activation and activated Rap1 induced ERK activation. We next tested the hypothesis that the mitogenic effects of cAMP were the result of Rap1 activation. The increased cAMP-induced cell growth was blocked in N17Rap1 but not in N17Ras clones, and accelerated growth was observed in V12Rap1 (Fig. 5B), demonstrating that Rap1 functions to stimulate ERK activation and cell proliferation in B-Raf-expressing cells.

cAMP Suppresses ERK Phosphorylation and Cell Proliferation in 95-kDa B-Raf-lacking Cells—Ras-dependent signaling activates ERK, but can be blocked by cAMP-dependent activation of Rap1 in many cell types (19). Thus, cAMP should decrease ERK activity through a negative Rap1 effect on Ras signaling in 95-kDa B-Raf-lacking cells. Consistent with this idea, cAMP reduced ERK activity in B-Raf-lacking cells. In MG63 and ROS17/2.8 cells, PTH-(1–34), forskolin, and Bt2cAMP suppressed ERK activation (Fig. 3B and data not shown). To determine the effect of cAMP-mediated down-regulation of ERK activity on cell proliferation in B-Raf-lacking cells, MITT activity was measured in MG63 cultures exposed to treatments that modify ERK activity. Consistent with our prior observation that cAMP decreased ERK activity, cell proliferation was inhibited by cAMP and its inhibition was not blocked by H89 in either a low or high concentration of forskolin (Fig. 6, A and B, and data not shown), suggesting that PKA does not participate in the modulation of cell growth in B-Raf-lacking skeletal cells. PD98059 only blocked proliferation of MG63 cells to 70% of control levels (Fig. 6B), suggesting that ERK is an important mitogenic signal in MG63 cells.

**Regulation by Rap1 in MG63**—In B-Raf-lacking MG63 cells, cAMP suppressed ERK activation. Because Rap1 is activated by cAMP, we tested whether the inhibitory effects of cAMP on ERK activity and cell proliferation were the result of Rap1 activation. We established MG63 cells that overexpress mutant Rap1 proteins. Clones were generated and several independently isolated clones were analyzed for HA-Rap1 proteins. All clones had elevated Rap1 expression compared with vector-
To determine how overexpression of mutant Rap1 would affect ERK activity and proliferation, the clones were analyzed by phospho-ERK Western blot analysis and BrdUrd incorporation assay. Basal levels of ERK activity were decreased in V12Rap1-transduced cells (Fig. 6C). Increased ERK activation, however, was observed in
clones containing the N17Rap1 mutation. These results suggest that Rap1 suppresses ERK activity. To elucidate whether N17Rap1-induced ERK activation results in increased cell proliferation, cell growth of the clones was analyzed (Fig. 6D). In N17Rap1 clones, increased cell proliferation correlated with increased ERK activity and suppressed growth in V12Rap1, directly demonstrating that Rap1 functions to inhibit ERK activation and cell proliferation in B-Raf-lacking MG63 cells.

**Conversion to B-Raf-expressing MG63 Cells**—As Rap1 appears to mediate the cAMP growth inhibitory signal for B-Raf-lacking MG63 cells, we determined whether the critical difference in Rap1/cAMP signaling was dependent on the presence or absence of B-Raf. For these experiments, we generated stable MG63 clones expressing the 95-kDa isoform of B-Raf. Basal levels of ERK activity were similar in vector- and B-Raf-transduced cells (Fig. 6C). ERK activation by forskolin, however, was observed only in B-Raf-transduced clones. Thus, introduction of the B-Raf protein alone is sufficient to convert cAMP from a negative to a positive regulator of ERK. For this reason, we generated stable MG63 clones expressing the 95-kDa isoform of B-Raf. Basal levels of ERK activity were similar in vector- and B-Raf-transduced cells (Fig. 6C). ERK activation by forskolin, however, was observed only in B-Raf-transduced clones. Thus, introduction of the B-Raf protein alone is sufficient to convert cAMP from a negative to a positive regulator of ERK. Finally, we determined whether the increased levels of ERK activity resulting from B-Raf overexpression were associated with increased cell proliferation. Basal growth levels were increased in B-Raf clones compared with vector-transduced control cells, and dramatically increased cell proliferation by forskolin were observed in B-Raf-transduced clones (Fig. 6D). These data indicate that one functional outcome of the molecular switch provided by B-Raf is increased ERK activation and cell proliferation in MG63 cells.

**DISCUSSION**

**cAMP Signaling Pathway to Induce PTH-induced Proliferation in Bone Cells**—In a wide variety of tissues (40), cAMP signaling is known to activate the ERK signaling pathway, thereby up-regulating cell proliferation and/or viability. As confirmed with cells of bone origin expressing PPR in this study, PTH action on PPR generates a cAMP signal and stimulates cell proliferation. Because activation of the classic target of cAMP, PKA, is inhibitory to cell proliferation (18–21), and consistent with the inhibition of cell proliferation observed with a high concentration of forskolin (Fig. 4D), this suggests that the cAMP signal activates a hitherto unidentified cascade upstream of the ERK junction to stimulate cell proliferation. Our most striking observation was that, in mouse bone cells, the cAMP signal was propagated through a new route to the ERK junction to control cell proliferation, not via other classic pathways (e.g. Ras-Raf, PKA-CREB). The functional outcome, stimulated or inhibited proliferation of bone cells, solely depended on the cell type-specific expression of the ratio of two B-Raf splicing variants. In this new route, cAMP directly and specifically activates Epac (27), a cAMP-GEF, which then acts on Rap1, an antagonist of Ras-dependent signaling (41–43), and blocks Ras-dependent activation of Raf-1 in the presence or absence of the short form of B-Raf (41, 44–46). In cells that express the Raf isoform, B-Raf, cAMP is known to activate ERK via the activation of Rap1 (24, 47–52). Thus, two splice variants of B-Rafs function as a molecular switch in the activation or inhibition of the MEK-ERK pathway, and in our study, cAMP-mediated inhibition of proliferation of MG63 cells was due to the predominant expression of 62-kDa B-Raf. This is the first report to show that molecules in this pathway are constitutively expressed in bone clonal cell lines and possibly in bone tissue in vivo and that these molecules play a major role in directing the cell reaction to the cAMP signal that is generated by PPR stimulation.

**Relationship of New Signaling Pathway to Other Known Pathways**—Quite recently, Swarthout et al. (53) reported that in the UMR106 rat osteosarcoma cell line and calvarial osteoblasts, subnanomolar PTH-(1–34) increased ERK activity in a manner sensitive to PKC inhibitor (GF109203) and MEK in-
inhibitor (PD98059) but resistant to PKA inhibitor (H-89), suggesting the participation of the PKC pathway in the activation of ERK. During the course of our study (data not shown), we also tested whether PKC lies upstream along the pathway leading to ERK activation. However, we were unable to inhibit cAMP-induced ERK activation with PKC inhibitors (calphostin and staurosporine) although they prevented cell proliferation. These differences may reflect the significant degree of hetero-

FIG. 6. Regulation of ERK and growth by Rap1 and B-Raf in MG63 cells. A, PTH-(1–34), Bt, cAMP (Db-cAMP), and forskolin (FK) suppressed cell proliferation in MG63. B, effects of H89 and PD98059 on cell proliferation. Growth was inhibited by PD98059, while H89 did not affect either basal or stimulated cell growth. C, ERK activity in V12Rap1-, N17Rap1-, or B-Raf-transduced cells. D, there was increased BrdUrd incorporation in N17Rap1 and B-Raf clones, but BrdUrd incorporation was suppressed in V12Rap1 clones. The inhibition of BrdUrd incorporation by forskolin (10 μM) was diminished in N17Rap1. Vec, vector-transduced control line. #, versus B-Raf; *, versus control: *, p < 0.05; **, p < 0.01; and ***, p < 0.005; ##, p < 0.01. n = 6. Similar results were obtained from four additional experiments.
In conclusion, we have presented evidence indicating that normal bone cells of mouse calvaria as well as a variety of bone-related clonal cell lines constitutively express a signal transduction pathway including Epac, Rap1, and B-Raf upstream of the MEK-ERK cascade. This pathway integrates the cAMP signal to promote or inhibit cell proliferation in a PKA- and Ras-independent manner. The expression pattern of the members of the pathway, especially the B-Raf splice variants (95 and 62 kDa), varies in a cell type-specific manner in clonal cell lines and possibly in normal osteoblasts, and it determines whether the final effect of cAMP is to increase or decrease cell proliferation.

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New Signaling Pathway for Parathyroid Hormone and Cyclic AMP Action on Extracellular-regulated Kinase and Cell Proliferation in Bone Cells: CHECKPOINT OF MODULATION BY CYCLIC AMP

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