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cAMP-response Element-binding Protein Is not Essential for Osteoclastogenesis Induced by Receptor Activator of NF-κB Ligand

Ha-Neui Kim, Hyunil Ha, Jong-Ho Lee, Han Bok Kwak, Hong-Hee Kim, and Zang Hee Lee*
Department of Cell and Developmental Biology, DRI, BK21 Program, School of Dentistry, Seoul National University, Seoul 110-749, Korea

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Osteoclasts are multinucleated cells with bone resorbing activity and differentiated from hematopoietic cell lineages of monocyte/macrophages in the presence of receptor activator of NF-κB ligand (RANKL) and M-CSF. However, the exact molecular mechanisms through which RANKL stimulates osteoclastogenesis remain to be elucidated. Here we report that activation of cAMP-response element-binding protein (CREB) is not involved in osteoclastogenesis from osteoclast precursors in response to RANKL. RANKL induced CREB activation in osteoclast precursors. Using pharmacological inhibitors, we found that RANKL-induced CREB activation is dependent on p38 MAPK pathways. We also found that ectopic expressions of wild type and dominant negative forms of CREB in osteoclast precursors did not affect RANKL-induced osteoclast formation and bone resorbing activity. Furthermore, dominant negative forms of CREB did not alter the expression levels of osteoclast-specific marker genes. Taken together, these data suggest that CREB is dispensable for differentiation and resorbing activity of osteoclasts.

Keywords: Osteoclast, RANKL, CREB

Introduction

Bone remodeling is highly regulated processes that involve synthesis of bone formation by osteoblasts and coordinated bone resorption by osteoclasts (Karsenty *et al.*, 2002). Unbalanced osteoclast and osteoblast differentiation and activation can be caused by a variety of inflammatory cytokines and growth factors, and result in skeletal abnormalities that are characterized by decreased (osteoporosis) or increased (osteopetrosis) bone mass (Rodan *et al.*, 2000; Karsenty *et al.*, 2002).

Osteoclasts, multinucleated giant cells that resorb bone, originate from hematopoietic cells of monocyte/macrophage lineage (Suda *et al.*, 1992). Osteoclasts and macrophages are known to have several morphological, cytochemical, and functional characteristics in common, and are derived from the same committed population of hematopoietic precursors (Suda *et al.*, 1992). These osteoclasts have known to be formed directly from precursor cell populations of monocytes and macrophages (Udagawa *et al.*, 1990). Osteoblasts express two cytokines essential for osteoclast differentiation, macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) (Suda *et al.*, 1999; Teitelbaum *et al.*, 2003). M-CSF, which is imperative for macrophage maturation, binds to its receptor on early osteoclast precursors, thereby providing signals required for their survival, proliferation, and differentiation (Udagawa *et al.*, 1990; Li *et al.*, 2002). RANKL, on the other hand, binds to receptors for activation of NF-κB (RANK) and induces signals necessary for both differentiation and activation of osteoclasts (Kobayashi *et al.*, 2000). Binding of RANKL to RANK recruits adaptor molecules and leads to activation of MAPKs and NF-κB. Several transcription factors including c-Fos and nuclear factor activated T cells c1 (NFATc1) are known to be important down-stream of osteoclast formation (Teitelbaum *et al.*, 2003). However, the signaling pathways through which RANKL induces these transcription factors remain unknown.

cAMP-response element-binding protein (CREB) is a 43 kDa-basic/leucine zipper transcription factor and binds to

*Corresponding author: Zang Hee Lee, Department of Cell and Developmental Biology, School of Dentistry, Seoul National University. Tel.: +82-02-740-8672; Fax.: +82-02-747-6589, E-mail: zang1959@snu.ac.kr

the octanucletide cAMP response element (CRE) consensus sequence as homo- or hetero-dimers in conjunction with other members of the CREB/ATF superfamily of transcription factors (Hoeffler *et al.*, 1988; Gonzalez *et al.*, 1989; Habener *et al.*, 1990). In resting cells, CREB exists in an unphosphorylated state that can bind to DNA but is transcriptionally inactive. The transcriptional activity of CREB is regulated by phosphorylation of Ser133 (Brindle *et al.*, 1993). Phosphorylated CREB activates its transcriptional activity at least in part by promoting recruitment of the transcriptional coactivator CREB binding protein (CBP) (Kwok *et al.*, 1994). CREB has been shown to be activated by several kinases, including PKA, PKC, RSK2, MAPKs, CaMKII, and Akt (Sun *et al.*, 1994; Tan *et al.*, 1996; Du *et al.*, 1998; Muthusamy *et al.*, 1998). CREB has been shown to functions in glucose homeostasis growth factor-dependent cell survival, learning, memory and immune regulation (Mayr *et al.*, 2001).

In this study, we investigated whether CREB is essential for osteoclast formation and activation induced by RANKL. Here we show that RANKL induces phosphorylation of CREB via p38 MAPK in osteoclast precursors. To study the contribution of the CREB to differentiation and activation of osteoclasts, we have used dominant-negative cDNA constructs of CREB, K-CREB mutated in the DNA-binding domain (Woloshin *et al.*, 1992) and CREB(S133A) mutated at Ser 133 (Barton *et al.*, 1996). Ectopic expressions of wild type and dominant negative forms of CREB in osteoclast precursors did not affect osteoclast formation and bone resorbing activity. These results suggest that CREB does not mediate osteoclastogenesis and resorbing activity induced by RANKL, even though CREB acts as a downstream component of RANKL-induced p38 MAPK signaling pathways.

Materials and Methods

Reagents

Recombinant human soluble RANKL and human M-CSF were purchased from PeproTech EC (London, United Kingdom). Anti-phospho-CREB, anti-CREB, and anti-Actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). SB203580, PD98059, SP600125, LY294002, Go6983, U0126, and H89 were purchased from Calbiochem (La Jolla, CA, USA).

Cell culture

Bone marrow cells were obtained by flushing tibiae from 6 week-old ICR mice, suspended in an α-minimum essential medium (α-MEM: Invitrogen Life Technologies, Carlsbad, USA) containing 10% FBS and antibiotics (Invitrogen) and incubated for 24 h in the presence of M-CSF (10 ng/ml). The non-adherent cells were collected and separated on the Histopaque (Sigma, St. Louis, USA) gradient. Bone marrow-derived macrophages (BMMs) were prepared as previously described (Ha *et al.*, 2003).

Retroviral gene transduction

Retroviral vectors pMX-CREB, pMX-K-CREB and pMX-CREB(S133A) were constructed by inserting CREB, K-CREB and CREB(S133A), purchased from BD Biosciences (Bedford, MA, USA) into pMX-puro vector. Retrovirus packaging was performed by transient transfection of these pMX vectors into Plat-E retroviral packaging cell line. After incubation in fresh medium for 2 days, supernatants of the retrovirus producing cells were collected. Nonadherent bone marrow cells were cultured in M-CSF (30 ng/ml) for 48 h. Medium was then removed and replaced with supernatants of pMX empty vector, pMX-CREB, pMX-K-CREB and pMX-CREB(S133A) virus-producing Plat-E cells together with 6 μg/ml polybrene and M-CSF (30 ng/ml) for 8 h. Infected cells were then cultured for 1 day in the presence of M-CSF and further cultured for 2 days with puromycin (2 μg/ml) and M-CSF to remove uninfected cells.

Osteoclast formation in the cell cultures

Stably infected cells were cultured in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 days. For osteoclast formation assay, tartrate-resistant acid phosphatase (TRAP) cytochemistry was performed using the Leukocyte Acid Phosphatase Assay Kit (Sigma), following the manufactures procedure. TRAP-positive multinucleated cells were counted as osteoclasts.

Bone resorption assay

Stably infected cells were replated on serum-coated dentin slices and cultured in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 6 days. After the incubation, attached cells were completely removed by abrasion with a cotton tip, and resorption pits were visualized by staining with 0.3% toluidine blue.

Immunoblotting analysis

Cells were lysed in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors. Protein concentrations of cell lysates were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Twenty to thirty micrograms cellular protein were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). The blots were probed with each primary antibody and finally developed by using horseradish peroxidase-conjugated secondary antibodies and visualized by using ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK).

RT-PCR analysis

Total RNA was prepared using TRI Reagent (Invitrogen) following the manufacturer's instructions, and cDNA was

synthesized from total RNA (2 μg) by reverse transcriptase (Superscript II Preamplification System; Invitrogen). For PCR amplification, the following primers were used : mouse TRAP (sense 5′-ACTTCCCCAGCCCTTACTAC-3′, antisense, 5′-TCAGCACATAGCCCACACCG-3′); mouse cathepsin K (cath k; sense 5′-CTTGTGGACTGTGTGACT-3′, antisense, 5′-ACCACTGCATGGTTCACA-3′); mouse β3 integrin (sense 5′-TGACTCGGACTGGACTGGCTA-3′, antisense, 5′-CACTCAGGCTCTTCCACCACA-3′); mouse c-Src (sense 5′-CCAGGCTGAGGAGTGGTACT-3′, antisense, 5′-CAGCTTGCGGATCTTGTAGT-3′); mouse calcitonin receptor (CTR; sense 5′-GACAACTGCTGGCTG AGTG-3′, antisense, 5′-GAAGCAGTAGATAGTCGCCA-3′); mouse MMP-9 (sense 5′-CTGTCCAGACCAAGGGTACA GCCT-3′, antisense, 5′-GAGGTATAGTGGGACACATAG TGG-3′); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense 5′-ACCACAGTCCATGCCATCAC-3′, antisense, 5′-TCCACCACCCTGTTGCTGTA-3′). The amplified cDNA fragments were run on 1.5% agarose gel, stained with ethidium bromide, and detected under ultraviolet light.

Statistical analysis

All quantitative data are presented as mean ± SD. Each experiment was performed 4-5 times, and results from one representative experiment are shown. Statistical differences were analyzed by Student's *t*-test. $P<0.05$ was considered statistically significant.

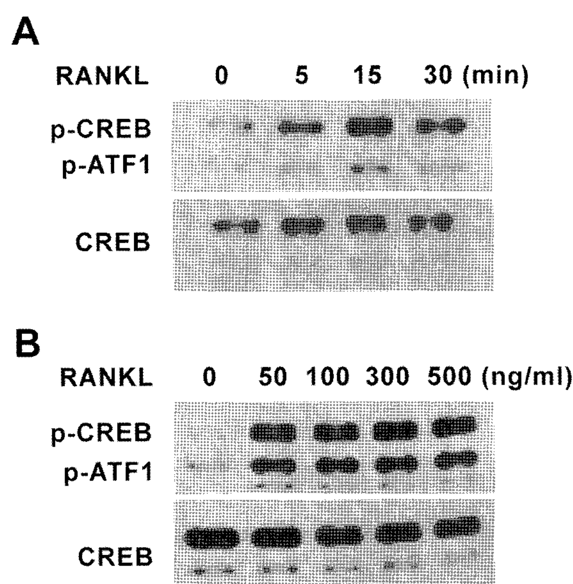

Fig. 1. Phosphorylation of CREB by RANKL in osteoclast precursors. (A) Time course analysis of CREB phosphorylation by RANKL in BMMs. BMMs were stimulated with RANKL (100 ng/ml) for the indicated times. (B) Dose-response experiments of CREB phosphorylation. BMMs were stimulated with various concentrations of RANKL (50-500 ng/ml) for 15 min. Whole cell lysates were immunoblotted with anti-phospho-CREB (p-CREB) antibody. The membranes were stripped and reprobed with anti-CREB antibody.

Results

RANKL induces the phosphorylation of CREB in BMMs

A variety of cytokines and growth factors have been reported to activate CREB as signaling mediators in various cells (Bernhard *et al.*, 2001). Thus, we first investigated whether RANKL induces activation of CREB in osteoclast precursors. We found that RANKL induced significant increase in activation of CREB (p-CREB), as analyzed by immunoblotting with a phospho-CREB (Ser133) antibody, in BMMs. The peak activation of CREB occurred at 15 min (Fig. 1A). The phospho-CREB antibody used for these studies recognizes the phosphorylated form of CREB and its related family members, ATF-1 and CREM. RANKL also increased phosphorylation of ATF-1 with similar pattern of p-CREB (Fig. 1A). Dose-response experiments indicated that 50 ng/ml RANKL efficiently induced maximum induction of p-CREB (Fig. 1B).

RANKL-induced CREB phosphorylation is dependent on p38 MAPK pathway

We next examined which signaling pathways are involved in RANKL-induced activation of CREB. We pretreated with several inhibitors for signaling molecules thought or known to activate CREB. A p38 inhibitor SB203580, MEK (ERK upstream kinase) inhibitors PD98059 and U0126, a JNK inhibitor SP600125, a PI3K inhibitor LY204002, a PKC inhibitor Go6983, or a PKA inhibitor H89 was pretreated before RANKL stimulation. As shown in Fig. 2, activation of CREB induced by RANKL was inhibited by pretreatment of p38 inhibitor, SB203580. But inhibition of JNK, ERK, PI3K and PKC pathway had no effect on RANKL-induced CREB activation, whereas inhibition of PKA pathway resulted in a slight decrease in the phosphorylated CREB protein level (Fig. 2). These results indicate that p38 MAPK pathways play a crucial role in RANKL-induced CREB activation.

CREB is not essential for osteoclast formation induced by RANKL

To address whether activation of CREB is necessary to promote osteoclast differentiation, CREB, CREB(S133A) and K-CREB were transduced into BMMs using retrovirus-mediated gene transfer. Overexpression of wild type and dominant negative forms of CREB had no effect on osteoclast formation from BMMs induced by M-CSF plus RANKL (Fig. 3A, B). Ectopic expression of these genes was confirmed by immunoblotting with CREB antibody (Fig. 3C). These results indicate that CREB is not essential for osteoclast differentiation from precursors.

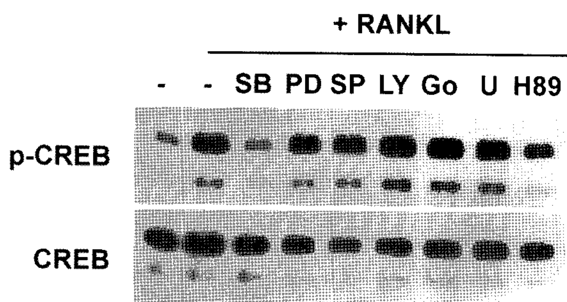

Fig. 2. p38 MAPK-dependent CREB phosphorylation in response to RANKL. BMMs were pretreated with SB203580 (SB; 10 μM), PD98059 (PD; 10 μM), SP600125 (SP; 10 μM), LY294002 (LY; 10 μM), Go6983 (Go; 0.5 μM), U0126 (U; 5 μM) or H89 (20 μM) for 30 min and then stimulated with RANKL (100 ng/ml) for 15 min. Whole cell lysates were immunoblotted with anti-phospho-CREB (p-CREB) and CREB antibodies.

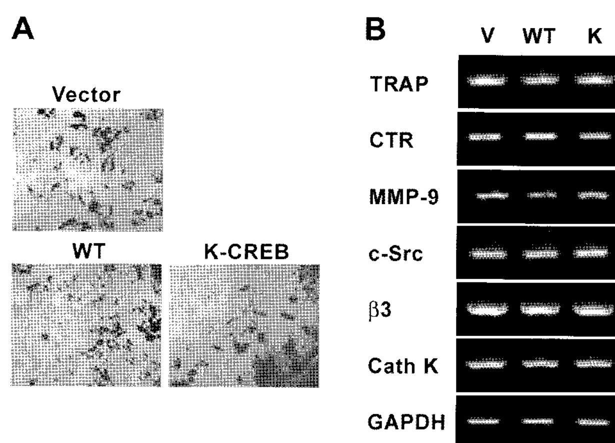

Fig. 4. Effect of CREB on bone resorption activity of osteoclasts. (A) BMMs infected with retroviruses expressing pMX-puro empty vector (V), wild type (WT), K-CREB (K) were cultured on dentin slices in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 6 days, and dentin slices were stained with 0.3% toluidine blue. (B) BMMs were cultured as in A for 4 days. Total RNA from the cells was isolated, and the expression levels of TRAP, calcitonin receptor (CTR), MMP-9, c-Src, β3 integrin (β3), cathepsin K (Cath K) and GAPDH mRNA were analyzed by RT-PCR.

CREB is not required for bone resorbing function of osteoclasts

Differentiated multinuclear osteoclasts have the capacity of resorption on the mineralized bone surface. We next assayed whether CREB affect the bone resorption activity of osteoclasts. BMMs infected with wild type and dominant negative form of CREB were cultured on dentin slices in the presence of M-CSF plus RANKL. As shown in Fig. 4A, the area and the numbers of resorption pit were not changed by overexpression of each gene. In lines with this, wild type as well as dominant negative form of CREB had no effects on the levels of osteoclastogenic markers including genes

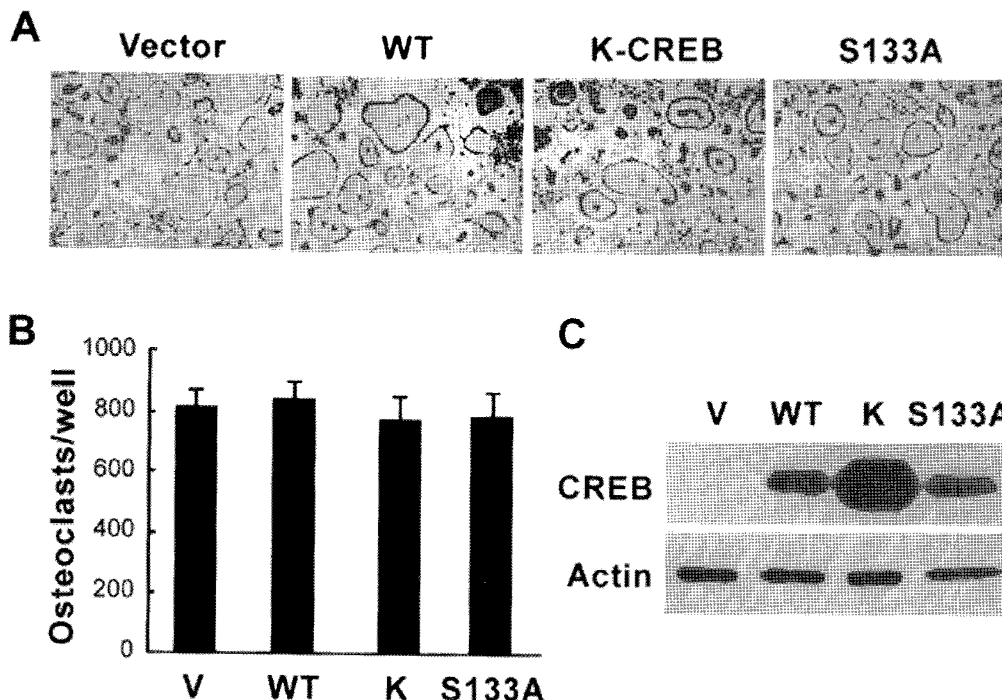

Fig. 3. Effect of CREB on RANKL-induced osteoclast formation. BMMs were infected with retroviruses expressing pMX-puro empty vector (V), wild type (WT), K-CREB (K) or CREB (S133A) as described under "Materials and Methods." Infected cells were cultured in the presence of M-CSF (30 ng/ml) plus RANKL (100 ng/ml) for 4 days. (A) Cells fixed and stained for TRAP. (B) TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. (C) Ectopic expression of each constructs was analyzed by immunoblotting.

required for bone resorbing activity (Fig. 4B). These results suggest that CREB do not play a major role in the process of bone resorption by osteoclasts.

Discussion

Osteoclasts are responsible for physiological and pathological bone resorption, and RANKL is critically involved in the differentiation, activation, and survival of these cells (Tanaka *et al.*, 2005). The cytoplasmic tail of RANK can directly bind TNF receptor-associated factors (TRAFs), which in turn trigger multiple downstream signaling pathways including three-well-known MAPKs (Erk, JNK and p38) and NF-κB (Teitelbaum *et al.*, 2003).

In this study, we found that RANKL induce phosphorylation of transcription factor CREB through p38 MAPK in osteoclast precursors (Fig. 1, 2). CREB activation has been reported to be involved in cell differentiation such as adipogenesis (Reusch *et al.*, 2000). In addition, p38 MAPK-mediated pathways are required for inducing osteoclast differentiation. Matsumoto *et al.* showed that SB203580, a specific inhibitor of the p38 MAPK, markedly suppresses not only RANKL-induced cathepsin K gene expression in osteoclasts but osteoclast differentiation (Matsumoto *et al.*, 2004). They also found that p38 MAPK phosphorylates NFATc1 and thereby invokes enhancement of nuclear accumulation of NFATc1 and of the transcriptional activation of the cathepsin K gene promoter. We found that SB203580 dramatically inhibits RANKL-induced mRNA and protein expression levels of c-Fos (data not shown) which known to be an essential transcription factor for osteoclast differentiation. Furthermore, the full upstream regulatory region of the c-Fos gene contains binding sties for CREB-ATFs, C/EBP, AP-1, SRF, p62TCF, STATs, and other stimulus-sensitive transcription factors (Edwards, 1994). It was also suggested that CREB or its closely related family members are general mediators of stimulus-dependent transcription of c-Fos (Ahn *et al.*, 1998). These results suggest that CREB might mediate RANKL-induced expression of c-Fos and osteoclastogenesis.

In this study, ectopic expressions of wild type and dominant negative forms of CREB have no effects on RANKL-induced osteoclast formation (Fig. 3), bone resorbing activity and the expression levels of osteoclast-specific marker genes (Fig. 4). Thus, these results indicate that CREB activation is not essential for RANKL-induced cellular functions. Interestingly, although nerve growth factor (NGF) and agents that trigger an increase in intracellular levels of cAMP or Ca^{2+} induce the phosphorylation of CREB Ser133 to a similar extent, these stimuli influence the transcription of genes containing CREs in distinct ways. A dominant negative inhibitor of CREB (A-CREB) which prevent the basic region of wild type CREB from binding to DNA completely inhibited cAMP-mdeiated, but only partially inhibited Ca^{2+}- and NGF-mediated, transcription of a reporter gene containing c-Fos promoter (Ahn *et al.*, 1998). In addition, induction of c-Fos transcription by UV light was not inhibited by A-CREB (Ahn *et al.*, 1998). Thus, CREB may partially mediate RANKL induction of c-Fos, though RANKL induces the phosphorylation of CREB.

M-CSF provides signals required for survival, proliferation and differentiation of osteoclast precursors (Udagawa *et al.*, 1990; Li *et al.*, 2002). We found that M-CSF induces the phosphorylation of CREB Ser133 (data not shown). However, dominant negative forms of CREB had no effects on survival and proliferation during osteoclastogenesis from BMMs induced by M-CSF plus RANKL, implying that CREB do not mediate M-CSF-induced these cellular responses.

In summary, we have shown that CREB is not required for RANKL-induced cellular function including differentiation and resorbing activity, though CREB acts as a target of RANKL-induced p38 MAPK pathways.

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