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Activation of c-Jun N-Terminal Kinase and Activator Protein 1 by Receptor Activator of Nuclear Factor κB

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ABSTRACT

Receptor activator of nuclear factor κB (RANK), a lately identified member of the tumor necrosis factor receptor superfamily, plays important roles both in osteoclastogenesis and in lymph node development. Previously, we and others showed that RANK could stimulate the activity of c-Jun N-terminal kinase (JNK). In this study, we investigated the mechanism by which RANK activates JNK. We found that N-terminal deletion mutants of tumor necrosis factor receptor-associated factor 2 and 6 were inhibitory to RANK activation of JNK. The JNK activation by RANK was also reduced by cotransfection of kinase-inactive mutants of apoptosis signal-regulating kinase 1, MAPK/ERK kinase kinase 1, and nuclear factor κB-inducing kinase. In addition, dominant negative mutants of Rac and Ras decreased the RANK stimulation of JNK activity. Furthermore, we determined whether the RANK engagement of JNK signaling pathways could lead to the activation of the activator protein 1 (AP-1) transcription factor, one of the potential downstream targets of activated JNK. RANK was found to activate AP-1 in a manner dependent on the signaling molecules involved in the JNK activation by this receptor. Furthermore, the activation of JNK and ERK, but not that of p38, appeared to be involved in the AP-1 activation by RANK. Thus, RANK may use both JNK and ERK pathways to signal to the AP-1 transcription factor.

Receptor activator of nuclear factor κB (RANK) is a recently cloned member of the tumor necrosis factor receptor (TNFR) superfamily, showing 20 to 40% amino acid identity to other TNFR family proteins in its extracellular domain (Anderson et al., 1997). Several studies have implicated physiological functions of this receptor in the regulation of bone metabolism and the immune system. Stimulation of RANK with its ligand, RANKL [also called ODF (osteoclast differentiation factor), OPGL (osteoprotogerin ligand), and TRANCE (TNF-related activation induced cytokine)], has been shown to induce the differentiation of osteoclasts from hematopoietic progenitors and to deliver activating and survival signals in mature osteoclasts (Fuller et al., 1998; Lacey et al., 1998; Yasuda et al., 1998; Burgess et al., 1999). The RANKL/RANK interaction has been reported to increase the allostimulatory activity of dendritic cells in a mixed lymphocyte reaction and to provide the costimulation for T helper cell activation in the absence of CD40, which was assumed to be mediated by enhanced dendritic cell function (Anderson et al., 1997; Bachmann et al., 1999). The mechanism for the RANK-dependent increase of dendritic cell function may be explained by the observation that RANKL/TRANCE up-regulated Bcl-xL expression and inhibited apoptosis of isolated dendritic cells (Wong et al., 1997).

Many of the TNFR family members share certain biochemical consequences, such as the activation of nuclear factor κB (NF-κB) and that of c-Jun N-terminal kinase (JNK). In both of these events, direct or indirect recruitment of TNF receptor-associated factor (TRAF) family molecules to the receptor in the plasma membrane appears to be an initial step. We and others have shown that RANK can directly associate with TRAF proteins and activate NF-κB and JNK (Anderson et al., 1997; Darnay et al., 1998; Wong et al., 1998; Kim et al., 1999).

The JNK activation pathway is a cascade of phosphorylation events that involve JNK-activating kinases (JNKK1/SEK1/MKK4/MEK4 and JNKK2), which, in turn, are activated by JNKK-activating kinases [MEKK1 and MEKK5/apoptosis signal-regulating kinase 1 (ASK1)]. The JNK

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ABBREVIATIONS: RANK, receptor activator of nuclear factor κB (NF-κB); RANKL, RANK ligand; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; JNK, c-Jun N-terminal kinase; AP-1, activator protein 1; ASK1, apoptosis signal-regulating kinase 1; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEKK, MAPK/ERK kinase kinase; NIK, NF-κB-inducing kinase; SEK, SAPK (JNK)/ERK kinase; GST, glutathione *S*-transferase; TNFR, tumor necrosis factor receptor; PI, phosphatidylinositol; TRANCE, TNF-related activation induced cytokine; β-gal, β-galactosidase.

activation cascade has been shown to be positively regulated by small GTPases Rac, Cdc42, and Ras (Coso et al., 1995; Minden et al., 1995), and MEKK1 has been demonstrated to bind those small GTPase proteins (Russell et al., 1995; Fanger et al., 1997). In some cases, PI 3-kinase is implicated in the Rac-mediated JNK activation (Timokhina et al., 1998). MEKK1 and ASK1 have been shown to participate in the TNF-induced or TRAF2-mediated JNK activation (Liu et al., 1996; Song et al., 1997; Nishitoh et al., 1998). For the activation of JNK by TNF-α and CD40, TRAF2 has been demonstrated to be essential (Lee et al., 1997; Yeh et al., 1997). However, the role of TRAF proteins, JNK- and JNKKK-activating kinases, small GTPases, and PI 3-kinase for the RANK activation of JNK has not been studied to date.

JNK regulates gene expression by phosphorylating and thus activating the transactivation domain of c-Jun in activator protein (AP)-1 complexes, homo- and heterodimers of Jun and Fos family proteins (Derijard et al., 1994). In addition to NF-κB activation, AP-1 activation appears to participate in the TNF regulation of gene expression (Westwick et al., 1994). However, AP-1 activation by other TNFR family receptors, including RANK, has not been reported, and whether AP-1 activation is a common feature of TNFR family proteins that activate JNK has not been addressed. Because both the *rank* and the c-*fos* genes seem to be essential for osteoclast development and RANK can activate JNK, it was intriguing to know whether the RANK signaling to JNK extends to AP-1 activation.

In this study, we determined the involvement of TRAF proteins, JNK-regulating kinases, small GTPases, and PI 3-kinase in the RANK-induced JNK activation and explored the potential linkage of JNK pathway to AP-1 in RANK signaling. The RANK-induced JNK activation appeared to be mediated by TRAF2 and to a less extent by TRAF6. The involvement of ASK1, MEKK1, NIK, and SEK1 was indicated, because the kinase-inactive mutants of these kinases showed dominant negative effects. The dominant negative mutants of small GTPases Rac and Ras, but not Rho, also attenuated RANK activation of JNK. In addition, RANK was found to cause AP-1 activation that seemed to be linked to JNK pathways, because the signaling molecule mutants with dominant negative effects on RANK activation of JNK also exerted inhibitory effects on AP-1 activation by RANK. Furthermore, the catalytic activities JNK and ERK, but not that of p38, was found to be required for the RANK induction of AP-1 activity.

Materials and Methods

Expression Plasmids. Mammalian expression vectors for T7-tagged human RANK, Flag-tagged TRAFs, GST-JNK1, and GST-SEK-KR, and prokaryotic GST-c-Jun-77 constructs were previously described (Kim et al., 1999). Expression plasmids for NIK-KK/AA$^{429–430}$ and ASK1-KM were generous gifts from Drs. H. Y. Song (Lilly Co. Center, Indianapolis, IN) and H. Ichijo (Tokyo Medical and Dental University, Tokyo, Japan), respectively. MEKK-K432A (Xia et al., 1995), JNK-APF (Derijard et al., 1994), ERK1-KR (Butch and Guan, 1996), and ERK2-KR (Her et al., 1993) have been described. The dominant negative mutants Rac1-N17, RhoA-N19, and Ras-N17 were kindly provided by Dr. J. S. Gutkind (National Institutes of Health, Bethesda, MD) and have been described (Coso et al., 1995). The luciferase reporter constructs pAP1-Luc and pNF-κB-Luc were from Dr. Y. D. Yun (Mogam Biotechnology Research Institute, Yongin, Korea). N-terminal deletion mutant ΔTRAF2 (amino acids 87–501) was kindly provided by Dr. H. Y. Song. ΔTRAF5 (amino acids 251–548) and ΔTRAF6 (amino acids 273–522) were generated by polymerase chain reaction.

JNK Activity Assays. 293-EBNA cells (Invitrogen, San Diego, CA) cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum were plated into six-well plates (8×10^5/well). The next day, cells were transfected with 0.3 to 0.5 μg of pEBG-JNK and other indicated DNAs plus 8 μl of SuperFect reagent (Qiagen, Chatsworth, CA) following the manufacturer's instruction. The total amounts of DNA were kept constant by adding control vector DNAs. Twenty to 36 h after transfection, cells were lysed in a lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 2 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4) and centrifuged for 10 min at 10,000*g*. Cleared lysates (300 μg) were incubated with glutathione beads for 2 h at 4°C. The precipitated beads were extensively washed and subjected to kinase reactions as previously described (Kim et al., 1998).

RANK Ligand Stimulation. RANKL stimulation was performed using stable cell lines established by transfecting the full-length human RANKL (pCEP4-RANKL) or the control (pCEP4) vector into 293-EBNA and selecting cells in medium containing 250 μg/ml hygromycin and G418. Cells were lysed in a hypotonic lysis buffer (10 mM Tris-HCl, pH7.4, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM sodium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride), and unbroken cells and nuclei were removed by centrifugation for 5 min at 500*g*. The supernatants were centrifuged for 10 min at 10,000*g*, and the pellet fractions were used as crude membrane preparations to stimulate RANK-transfected 293-EBNA cells. RANK stimulation was also carried out with recombinant RANKL proteins immobilized on agarose beads. The entire extracellular domain of RANKL was amplified by polymerase chain reaction and subcloned into pET21a (Novagen, Madison, WI). The transformed *Escherichia coli* were cultured and induced with 0.1 mM isopropyl β-D-thiogalactoside for 2 h at 37°C. Cell pellets were disrupted by sonication, and the His-tagged RANKL was allowed to bind His·Bind resins (Novagen). The beads were extensively washed and used for stimulation of cells.

Reporter Gene Assays. Cells (1.5×10^5) were plated onto 24-well plates 1 day before transfection with 100 to 200 ng of pAP1-Luc or pNF-κB-Luc and indicated amounts of various constructs, keeping the ratio of DNA:SuperFect reagent (Qiagen) at 1:2 or 1:3. Fifty nanograms of a β-gal vector was cotransfected for normalizing transfection efficiencies. The total amounts of DNA were kept constant by supplementing with control vector DNAs. Sixteen to 20 h after transfection, cells were lysed with 150 μl of Reporter Lysis Buffer (Promega, Madison, WI), and 20 μl of lysates were used for detection of luciferase activity with Luminometer (EG&G Berthold, Bad Wildbad, Germany). In experiments with the p38 inhibitor SB 202190 (Calbiochem, San Diego, CA) and the PI 3-kinase inhibitor wortmannin (Sigma, St. Louis, MO), cells were incubated with the inhibitor 16 or 4 h after transfection for 2 to 16 h and harvested at the time point of 20 to 24 h post-transfection.

Western Blotting Analyses. Cell lysates were prepared as above, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 1 h in TBS-T (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) plus 3% skim milk, incubated with anti-RANK, anti-T7 (Novagen), anti-RANKL, anti-Flag M2 (Sigma), anti-GST, or anti-hemagglutinin (12CA5), washed for 1 h in TBS-T, and incubated with anti-mouse Ig- or anti-rabbit Ig-horseradish peroxidase for 1 h. The immune complexes were detected by the ECL system (Amersham). Generation of anti-RANK has been described (Kim et al., 1999), and anti-RANKL rabbit serum was generated by a similar method using GST fusion proteins of the extracellular domain of human RANKL as the immunogen.

Results

Induction of JNK Activity by RANK Expression in 293-EBNA Cells. To assess the signaling potential of RANK to the JNK activation cascade, RANK was transiently transfected into human embryonic kidney cells (293-EBNA), and JNK activity was evaluated. As shown in Fig. 1A, an elevated level of JNK activity was observed in the RANK-transfected cells. The RANK-transfected cells showed further activation of JNK upon stimulation with RANKL, presented on cell membranes (Fig. 1B, lane 2) or immobilized on beads (lane 4), when compared with the levels of JNK activity stimulated with the control membranes or beads (lanes 1 and 3). The RANKL-induced JNK activation was observed within 5 min and reached a maximum at approximately 30 min (Fig. 1C). The expression of RANKL in the membrane preparations used for stimulation of the RANK-transfected cells was confirmed by Western blotting with anti-RANKL sera raised against the extracellular domain of RANKL (Fig. 1D).

Inhibition of the RANK Activation of JNK by Dominant Negative Mutants of TRAF2, TRAF6, ASK, MEKK, NIK, Rac, and Ras. To identify the signaling components involved in the RANK-induced JNK activation, dominant negative mutant forms of signaling molecules that have been implicated in JNK activation pathways for other receptors were utilized. Studies with dominant negative TRAF2 (amino acids 87–501, lacking the N-terminal ring finger motif) in

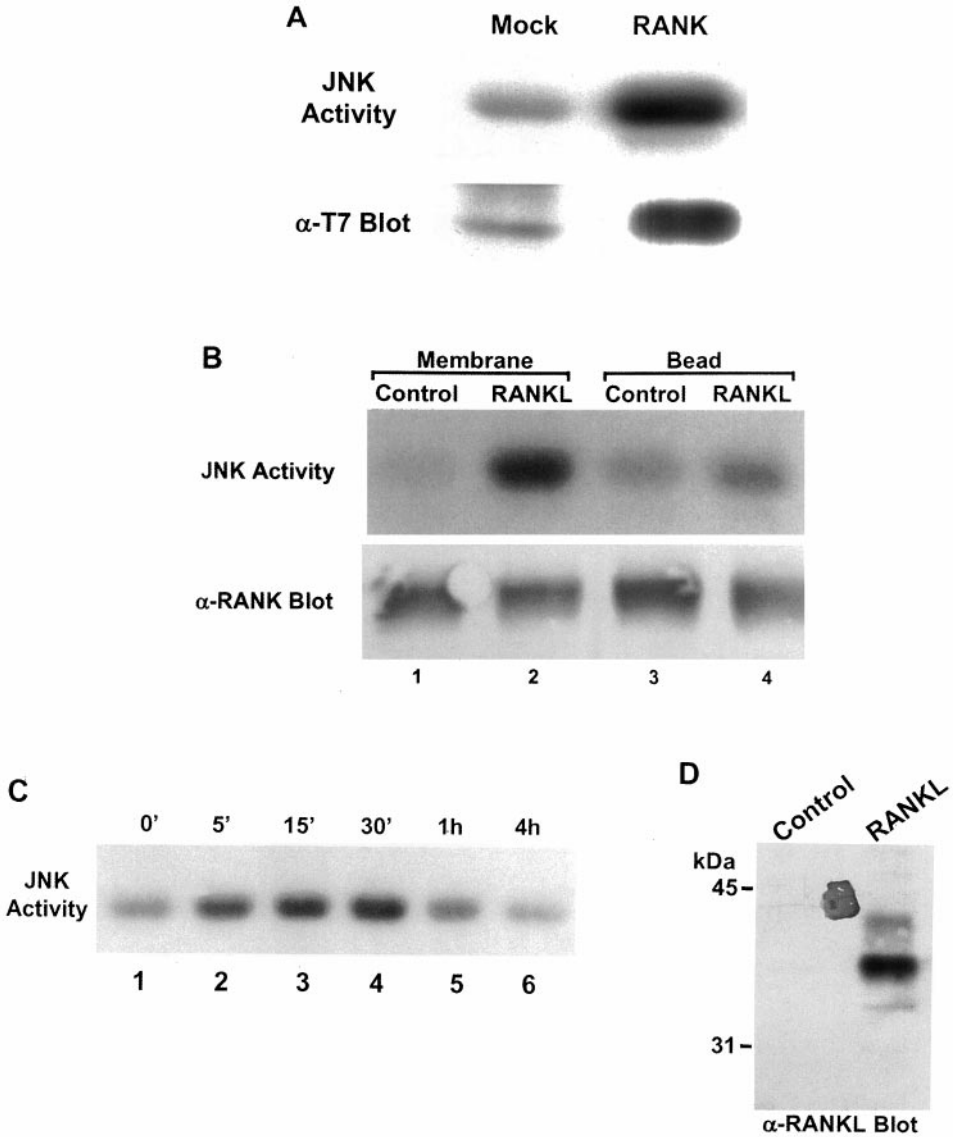

Fig. 1. Induction of JNK activation by ectopic expression of RANK in 293-EBNA cells. A, cells in six-well plates were transfected with 2 μg of pSRα vector control or pSRα-RANK-T7 plus 0.5 μg of pEBG-JNK. Cell lysates were prepared 36 h after transfection, and JNK activity was measured (top). Aliquots of cell lysates were subjected to Western blotting with anti-T7 to verify RANK expression (bottom). B, cells were transfected as in A and the next day were stimulated for 40 min at 37°C either with cell membranes containing RANK ligand (lane 2) or the control cell membranes (lane 1) or with recombinant RANKL immobilized to agarose beads (lane 4) or the control beads (lane 3). Cell lysates were analyzed for JNK activity (top), and RANK expression was confirmed by Western blotting with anti-RANK (bottom). C, cells were transfected with pSRα-RANK-T7 and pEBG-JNK and then stimulated with RANKL membrane for the indicated times as described in B. Cell lysates were prepared, and JNK activity was determined. D, to verify the presence of RANKL in membranes used for stimulation in B and C, fractions of membrane preparations from the RANKL- or the control vector-transfected stable cells were subjected to Western blotting with anti-RANKL rabbit serum raised against the extracellular domain of RANK ligand. Data shown are representative of several independent experiments.

transgenic mice and cultured cell line systems have indicated that TRAF2 is essential for TNF-stimulated JNK activation (Lee et al., 1997; Natoli et al., 1997). When the dominant negative TRAF2 was cotransfected with RANK, the RANK-induced JNK activation was abolished (Fig. 2A, top). The TRAF6 mutant (amino acids 273–522), in which both the ring and zinc finger motifs are deleted, also showed some inhibitory effect. The expression of the wild-type TRAF1, the TRAF member naturally lacking the ring finger motif, almost completely inhibited the JNK activation by RANK. A dominant negative TRAF5 (amino acids 251–548) had little effect, and the full-length TRAF3 had a marginal effect (Fig. 2A, top). The lack of inhibitory effect of TRAF5 mutant was not due to any defect of the plasmid, because its expression was detected at a level similar to all other TRAF plasmids used (Fig. 2A, bottom), and also the cotransfection of the TRAF5 mutant resulted in the inhibition of RANK-induced NF-κB activation (data not shown).

ASK1 has been demonstrated to be necessary for the TNF- and TRAF2-induced JNK activation (Nishitoh et al., 1998).

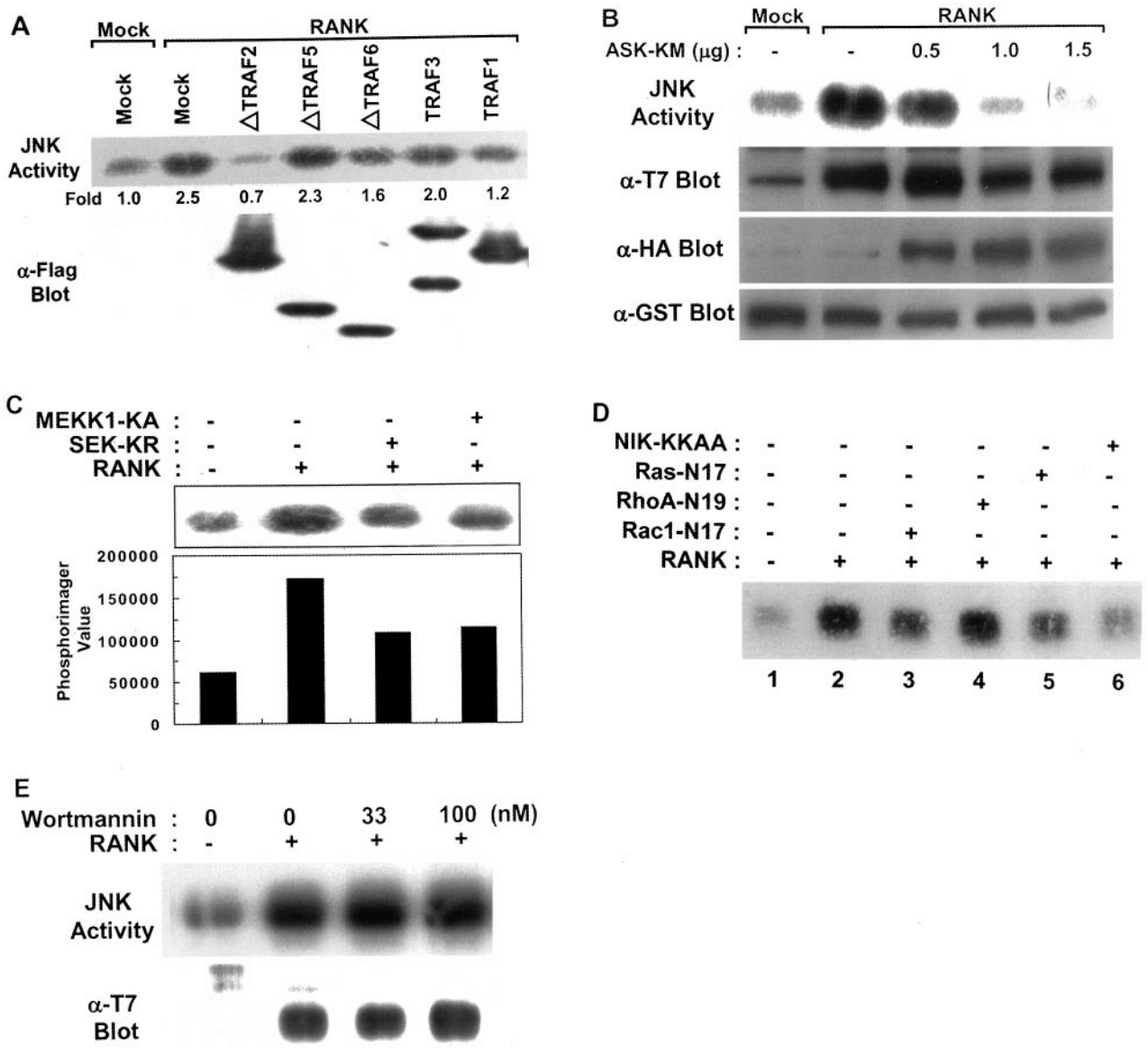

Fig. 2. Regulation of the RANK-induced JNK activation by multiple signaling adaptors, kinases, and GTP-binding proteins. A, 293-EBNA cells were transfected with 2 μg of pSRα-RANK-T7, 2 μg of the indicated Flag-tagged TRAF expression vector, and 0.3 μg of pEBG-JNK. JNK was precipitated with glutathione beads from cell lysates, and the kinase activity was measured (top). Fold indicates the relative JNK activity assessed by phosphoimager. Aliquots of cell lysates were analyzed for TRAF expression levels by Western blotting with anti-Flag (bottom). B, cells were transfected with 1.0 μg of pSRα-RANK-T7, indicated amounts of HA-tagged ASK1-KM, and 0.5 μg of pEBG-JNK, and JNK activity was measured as in A (top). Aliquots of cell lysates were subjected to Western blotting with anti-T7, anti-HA, and anti-GST to verify expression of the transfected RANK, ASK1, and JNK, respectively. C, cells were transfected with 2.0 μg of pSRα-RANK-T7, 2.0 μg of kinase-deficient mutant plasmid of MEKK1 or SEK1, and 0.5 μg of pEBG-JNK. JNK assay was performed as in A. The autoradiogram of (top) and the histogram of phosphoimager values from the dried gel are shown (bottom). D, 293-EBNA cells were transfected with 2.0 μg of pSRα-RANK-T7, 2.0 μg of the indicated mutant of Rac, RhoA, Ras, or NIK, and 0.3 μg of pEBG-JNK. The next day, cells were harvested, and JNK activity was measured in glutathione precipitates of cell lysates. E, cells were transfected with 3.0 μg of pSRα vector or pSRα-RANK-T7 and 0.5 μg of pEBG-JNK. Thirty-six hours after transfection, cells were treated with the indicated concentrations of wortmannin or the control vehicle for 2 h at 37°C. JNK activity was determined as in A. Data presented are representative results of two or three independent experiments.

In addition, the MEKK1-SEK1-JNK cascade has been demonstrated to be activated upon TNF stimulation (Yuasa et al., 1998). To test the possibility of ASK1 involvement in the JNK activation by RANK, the kinase-inactive ASK1 mutant (ASK-KM) was cotransfected with RANK, and the JNK activity was assessed. Overexpression of ASK-KM significantly decreased the RANK-induced JNK activation (Fig. 2B). When the catalytically inactive MEKK1 and SEK1 were tested for effects on the RANK-dependent JNK activation, both of them suppressed the kinase activity (Fig. 2C). JNK activity has also been shown to be regulated by small GTPases Rac, Cdc42, and Ras (Coso et al., 1995; Minden et al., 1995). Whether the small GTP-binding proteins are involved in the RANK-induced JNK activation was assessed by cotransfecting the dominant negative forms of Rac1, RhoA, and Ras. Dominant negative Rac1-N17 and Ras-N17 inhibited RANK stimulation of JNK activity (Fig. 2D, lanes 3 and 5), whereas cotransfection of the RhoA-N19 mutant did not have significant effect (lane 4). Cross-talk between two major TNF signaling pathways, the NIK-IKK-NFκB and MEKK-SEK-JNK pathways, has been suggested, and overexpression of NIK was shown to result in JNK activation (Karin and Delhase, 1998). The possibility that the RANK-induced JNK activation is regulated by NIK was explored by cotransfecting the kinase-inactive NIK mutant (NIK-KKAA$^{429-430}$) that suppressed RANK activation of NF-κB under similar transfection conditions (data not shown). The expression of NIK-KKAA$^{429-430}$ significantly reduced the JNK activation by RANK (Fig. 2D, lane 6). PI 3-kinase has been shown to play a role in the JNK activation pathway for receptor tyrosine kinases, perhaps through Rac (Logan et al., 1997; Timokhina et al., 1998). To determine whether PI 3-kinase is involved in the RANK-induced JNK activation, wortmannin, an inhibitor specific for PI 3-kinase at concentrations below 200 nM, was used. As shown in Fig. 2E, wortmannin had no effect on RANK activation of JNK in these cells at doses known to be sufficient for PI 3-kinase inhibition.

Induction of AP-1 Activity by RANK and Involvement of TRAF Proteins. JNK activation has been linked to transactivation of the AP-1 transcription complexes (Derijard et al., 1994). The observation that RANK can stimulate JNK signaling pathways in which TRAF proteins, serine/threonine kinases, and GTP-binding proteins are involved prompted us to determine the AP-1-activating potential of RANK and to evaluate the role of these signaling components. When overexpressed in human embryonic kidney cells, RANK caused induction of an AP-1-responsive reporter gene as efficiently as TRAF2 (Fig. 3A). This AP-1 activation by RANK correlated with the amounts of transfected RANK DNA (Fig. 3B). We next included N-terminal truncation mutants of TRAFs that have been shown to have dominant negative effects on NF-κB activation. The TRAF2 and TRAF5 mutants abolished the RANK-induction of AP-1 activity, and the TRAF6 mutant had a significant inhibitory effect (Fig. 3C). In these cells the TRAF mutants also inhibited RANK activation of NF-κB (Fig. 3D). In the absence of RANK, the TRAF6 deletion mutant itself exerted some activation of AP-1, albeit to a lower extent than the wild-type TRAF6 (data not shown). The cotransfection of wild-type TRAF1 or TRAF3 also resulted in reduction of the AP-1 activation by RANK (data not shown).

Involvement of ASK1 and NIK in RANK-Induced AP-1 Activation. To determine signaling components downstream of TRAF proteins in RANK activation of AP-1, kinase-inactive mutants of ASK1, NIK, and MEKK1, which all showed inhibitory effects on RANK stimulation of JNK activity, were tested for the AP-1-derived reporter gene induction. As shown in Fig. 4A, the kinase-null mutants ASK1-KM and NIK-KKAA showed significant inhibitory effect on RANK-induced AP-1 activation. The role of MEKK1 in AP-1 activation by RANK could not be assessed, because transfection of the kinase domain mutant of MEKK1 itself evoked a high level of AP-1 activation under the experimental conditions (data not shown). Transfection of various amounts of ASK1-KM and NIK-KKAA showed dose-dependent inhibitory effects (Fig. 4, B and C). The role of immediate upstream JNK kinase SEK was also evaluated by using the kinase-inactive mutant SEK-KR that was previously shown to inhibit RANK-induced JNK activation (Kim et al., 1999). The cotransfection of SEK-KR mutant resulted in suppression of the AP-1 activation by RANK (Fig. 4D).

Differential Regulation of RANK Activation of AP-1 by MAPK Family Enzymes. The inhibitory effect of ASK1-KM and SEK1-KR on RANK-induced AP-1 activation (Fig. 4, B and D) directed us to assess the involvement of JNK in AP-1 activation of RANK. Two additional MAPK family enzymes, ERK and p38, were also examined. As might be expected, cotransfection of JNK1-APF, a JNK1 mutant containing substitutions in the TPY residues that are to be phosphorylated by upstream activating kinases upon stimulatory signals, into 293-EBNA cells led to the reduction in RANK-induction of AP-1 activity (Fig. 5A). In addition, kinase-inactive mutants of ERKs were found to attenuate the AP-1 activation by RANK in these cells, and the ERK2 mutant was more effective than the ERK1 mutant (Fig. 5B). The ERK2-KR also efficiently suppressed the AP-1 activation induced by NIK or TRAF6 (data not shown). The role of p38 MAPK family kinase was determined by treating RANK-transfected cells with a specific p38 inhibitor SB202190. Treatment of the cells with the p38 inhibitor for 4 h had no effect on the RANK-induced AP-1 activation even at a dose 57-fold higher than the IC_{50} (350 nM) (Fig. 5C). Variation in the treatment time did not affect the result (data not shown). These observations indicate that the RANK-to-AP-1 signaling pathway is independent of p38 MAPK activity.

Inhibition of RANK Activation of AP-1 by Interference of Rac and Ras but Not That of Rho and PI 3-Kinase. We next evaluated the role of Ras family of GTP-binding proteins and PI 3-kinase in the RANK-induced AP-1 activation. The dominant negative mutant of Rac1, RhoA, or Ras was cotransfected with RANK and AP-1-dependent reporter gene, and the reporter activity was examined. Transfection of dominant negative Rac1 and Ras resulted in reduction in the RANK activation of AP-1 by 33 and 50%, respectively (Fig. 6A). In contrast, dominant negative RhoA showed no inhibition under the same experimental conditions (Fig. 6A). Treatment of cells with a specific PI 3-kinase inhibitor wortmannin also did not inhibit the RANK activation of AP-1 at concentrations up to 40-fold higher than IC_{50} (5 nM), which suggested that the catalytic activity of PI 3-kinase is not involved in RANK activation of the AP-1 transcription factor in these cells (Fig. 6B).

Discussion

Activation of JNK is one of the major biochemical consequences of stimulation of TNF receptor family proteins such as TNFR1, TNFR2, CD95 (Fas/Apo-1), CD40, and CD27. For TNF-stimulation of JNK activity, TRAF2 seems to be essential, as demonstrated by studies with TRAF2 knock-out and dominant negative TRAF2 transgenic mice (Lee et al., 1997; Yeh et al., 1997), whereas Fas-induced JNK activation was shown to be mediated by the Fas-binding protein Daxx (Yang et al., 1997). Both TRAF2 and TRAF5 were reported to be involved in CD27-induced JNK activation (Akiba et al., 1998; Gravestein et al., 1998). In B cells, CD40 activation of JNK has been shown to be mediated by TRAF3 (Grammer et al., 1998). Recently, TRAF1 was implicated in prolonging TNF-induced JNK activation (Schwenzer et al., 1999). Overexpression of TRAF2, -5, or -6 could also evoke JNK activation in 293 cells (Song et al., 1997). Taken together different TRAF family proteins and perhaps complexes of them may have capacity to transduce JNK activating signals from various TNFR family members in different cellular environments. The role of TRAF family proteins in RANK-induced JNK activation has not been reported to date. In this study, we found that expression of N-terminal deletion mutant of TRAF2, and to a lesser extent that of TRAF6, inhibited RANK activation of JNK (Fig. 2A), implicating these TRAF members in JNK activation by RANK. These TRAF proteins as well as TRAF1, −3, and −5 were shown to associate with RANK in cultured cells (Darnay et al., 1998; Kim et al., 1999), and N-terminal deletion mutants of TRAF2, -5, and -6 have been demonstrated to have the dominant negative effect on NF-κB activation (Wong et al., 1998; Kim 1999). However, the relative contribution of TRAF2 and TRAF6 to the RANK-induced JNK and NF-κB activation pathways appears to be different, because the dominant negative TRAF2 mutant was more effective than the dominant negative TRAF6 in blocking JNK activation, whereas with the same mutant constructs the reverse was observed in the context of NF-κB activation by RANK under similar experimental conditions (Figs. 2A and 3D).

Several MAPK cascade enzymes have been implicated in the signaling of TNF family receptors to JNK. Some of these JNK-activating kinases have been suggested to work immediately downstream of TRAF proteins. The ASK1 and NIK proteins were shown to bind TRAF1, -2, -3, -5, and -6 and the

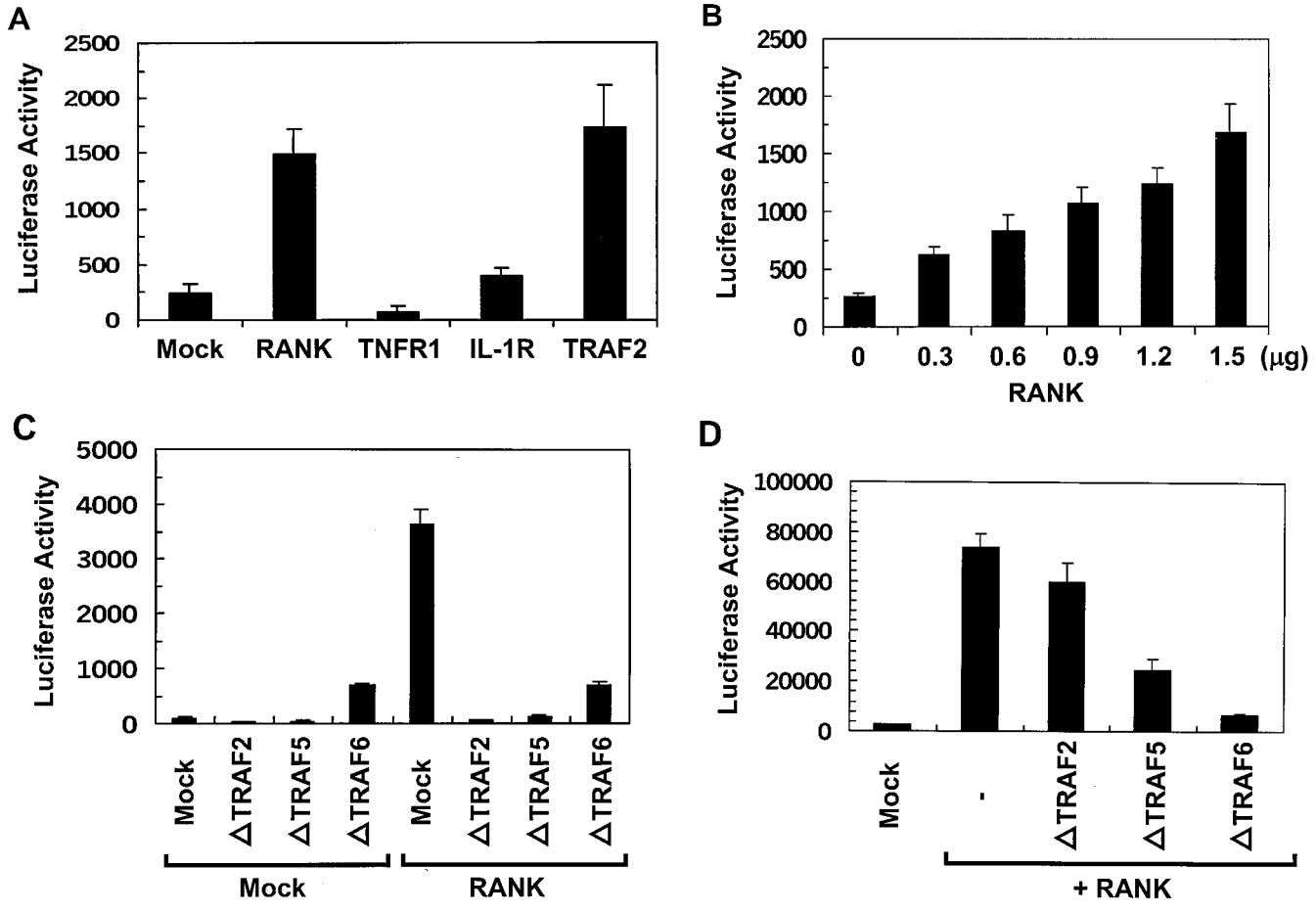

Fig. 3. Activation of AP-1 by RANK and its inhibition by N-terminal deletion mutants of TRAF2, -5, and -6. A, 293-EBNA cells in 24-well plates were transfected with 0.5 μg of the indicated plasmids, 0.1 μg of pAP1-Luc, and 50 ng of β-gal. Sixteen hours post-transfection, cell lysates were prepared and luciferase activity was measured. IL-1R, interleukin-1 receptor. B, cells were transfected as in A with the indicated amounts of pSRα-RANK-T7. The total amounts of DNA were kept constant by adding pSRα vector DNA. Cell lysates were prepared, and the AP-1 activity was determined. C, cells were cotransfected with 1.0 μg of pSRα or pSRα-RANK-T7 and 1.0 μg of the indicated TRAF mutant together with 0.2 μg of pAP1-Luc and 50 ng of β-gal. The next day, cell lysates were prepared, and luciferase activity was assessed. D, cells were transfected with 0.5 μg of pSRα or pSRα-RANK-T7 and 0.5 μg of the TRAF mutant together with 0.1 μg of pNF-κB-Luc and 50 ng of β-gal. The next day, luciferase activity was measured from cell lysates. Error bars indicate standard deviations between triplicate samples and the data represent one of three independent experiments.

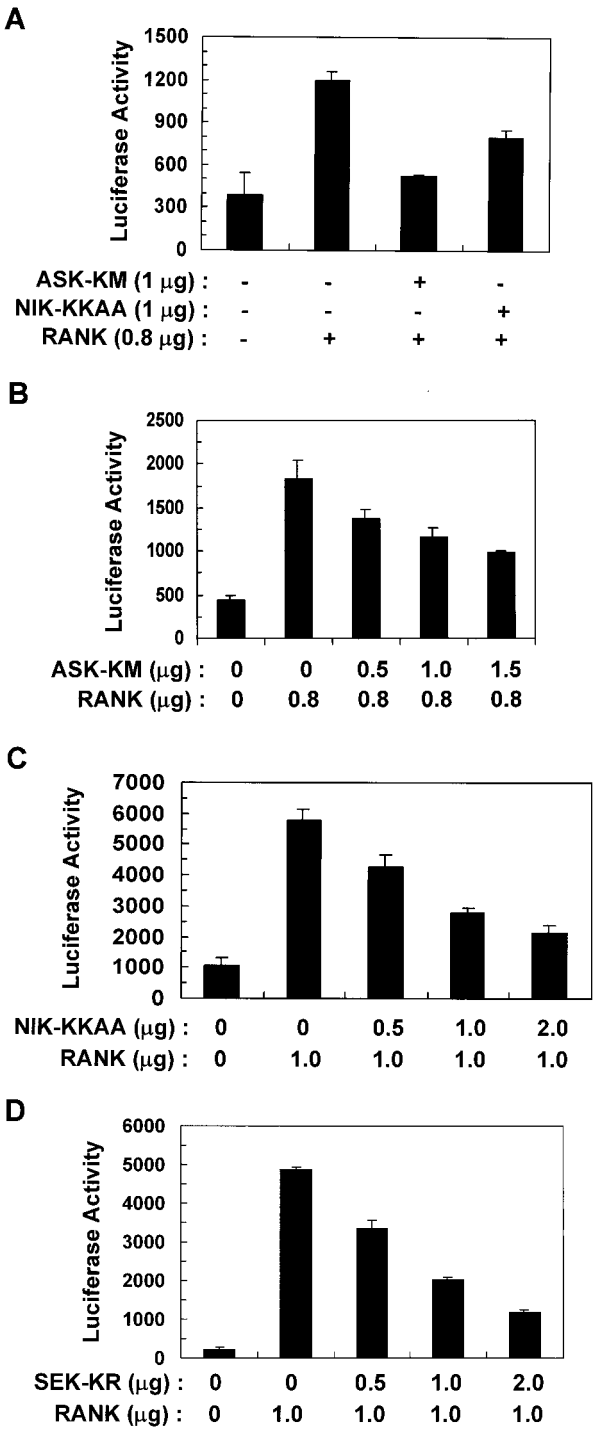

Fig. 4. Involvement of ASK, NIK, and SEK in AP-1 activation by RANK. A, 293-EBNA cells were transfected with indicated amounts of expression vectors for ASK1-KM, NIK-KKAA, and RANK, and 0.1 μg pAP1-Luc. Sixteen hours after transfection, cells were lysed, and luciferase activity was measured. B, cells were transfected with indicated amounts of the RANK and the kinase-inactive ASK-KM mutant plasmids and 0.1 μg of pAP1-Luc. The next day, cell lysates were prepared, and luciferase assays were performed. C, indicated amounts of the RANK and the kinase-deficient mutant NIK-KKAA plasmids were cotransfected with 0.2 μg of pAP1-Luc into 293-EBNA cells, and luciferase assays were carried out. D, cells were transfected with indicated amounts of SEK-KR and RANK expression plasmids and 0.2 μg of pAP1-Luc DNA. Twenty hours post-transfection, cell lysates were prepared, and AP-1 activity was assessed by luciferase assays. The data are presented as the mean ± S.D. of triplicate samples. The results of one experiment are shown, and similar results were observed in two other experiments.

MEKK1 and GCK proteins to TRAF2 (Song et al., 1997; Nishitoh et al., 1998; Yuasa et al., 1998). All of these kinases were shown to mediate JNK activation through TRAF2 for TNF or CD27 signaling (Akiba et al., 1998; Nishitoh et al., 1998; Yuasa et al., 1998). The expression of kinase-inactive mutants of ASK1, MEKK1, or NIK inhibited RANK-induced JNK activation (Fig. 2), suggesting that RANK could engage, perhaps through TRAF2, these kinases for JNK pathway signaling. MEKK1 has been shown to bind the JNK-activating small GTP-binding proteins Rac, Cdc42, and Ras (Russell et al., 1995; Fanger et al., 1997), which have been implicated in cytokine- and growth factor-induced JNK activation (Coso et al., 1995; Minden et al., 1995). In RANK signaling, Rac and Ras, but not Rho, seem to be involved, because the dominant negative mutants of the former, but not the latter, interfered RANK-induced JNK activation (Fig. 2D). Although PI 3-kinase has been implicated in the Rac-mediated JNK activation in some cases (Timokhina et al., 1998), the JNK activation by RANK appeared to be independent of PI 3-kinase. Taken together, these results would suggest that RANK signaling to JNK is mediated by TRAF2-ASK1/MEKK1/NIK-SEK cascades with the TRAF2-to-ASK1/MEKK1/NIK steps possibly being modulated by Rac and Ras. However, more questions, such as the relative contributions of ASK1, MEKK1, and NIK, the participation of other TRAF proteins in linking RANK to the kinases, the immediate downstream target of NIK for JNK activation, and the exact target of Rac and Ras in modulation of the RANK-to-JNK signaling, remain to be addressed.

JNK regulates the AP-1 transcription factor by phosphorylating the c-Jun component on Ser-63 and Ser-73 residues (Derijard et al., 1994). We found that RANK could evoke AP-1 transactivation, which was regulated by TRAF2, ASK1, NIK, SEK1, Rac, and Ras, signaling components involved in the RANK activation of JNK (Figs. 3, 4, and 6). The role of NIK in the activation of JNK and AP-1 for TNF family receptors has not been clearly resolved. In some studies, NIK appeared to have no effect on TNF- and TRAF2-induced JNK activity (Song et al., 1997; Karin and Delhase, 1998) and to cause AP-1 activation in JNK-independent manner (Natoli et al., 1997), whereas in other studies, NIK seemed to mediate JNK activation by CD27 (Akiba et al., 1998) and have JNK-activating potential under high expression conditions (Karin and Delhase, 1998). The role of the JNK pathway in RANK-induced AP-1 activation was further supported by the interference of the JNK-APF mutant, which is incapable of receiving activating signals (Fig. 5A). Another MAPK pathway also seemed to participate in RANK activation of AP-1. The impedance of ERK, but not that of p38, was found to affect AP-1 activation (Fig. 5, B and C). ERK activity can positively regulate AP-1 by phosphorylating target transcription factors. Phosphorylation of Elk-1 by ERK increases formation of the ternary complex between Elk-1 and a homodimer of serum response factor that binds the serum response element to induce c-fos (Gille et al., 1995), and the c-fos protein itself has been shown to be stabilized upon phosphorylation by ERK (Okazaki and Sagata, 1995). ERK has been reported to be activated by CD40 through TRAF6 (Kashiwada et al., 1998). AP-1 activation through the ERK-dependent pathway may also account for the inhibitory effect of TRAF5 mutant on AP-1 activation (Fig. 3C) in the absence of an effect on JNK activation by RANK (Fig. 2A). Whether RANK can

activate ERK and whether TRAF5 and TRAF6 are involved in that are under investigation.

Activation of Ras can lead to stimulation of both the ERK and JNK subfamily enzymes of MAPKs (Minden et al., 1994). The Ras stimulation of ERK activity is mediated by the Ras-Raf-MEK pathway, whereas the JNK activation signal from Ras seems to be transduced by the MEKK1-SEK cascade. Ras may regulate the RANK induction of AP-1 through both JNK and ERK pathways. This notion may be supported by the finding that the dominant negative Ras was more effective than that of Rac in attenuating the RANK activation of AP-1 (Fig. 3B), whereas both mutants inhibited the RANK-induced JNK activation to the same extent (Fig. 2A).

Our results provide information based on which a potential mechanism for signaling from RANK to JNK and to AP-1 is proposed (Fig. 7). It is postulated that RANK is linked to redundant JNK-activating pathways through TRAF2 and possibly TRAF6. The TRAF proteins can associate with ASK1, MEKK1, and NIK to activate SEK, which, in turn, activates JNK. The small GTP-binding proteins Rac and Ras regulate JNK and AP-1 activation, perhaps by modulating the step at which TRAFs activate MAPKKKs. RANK also couples to ERK for AP-1 activation, although the pathway remains to be unraveled. For better understanding of RANK signal transduction, further studies of the signaling components involved in AP-1 activation through ERK and the pos-

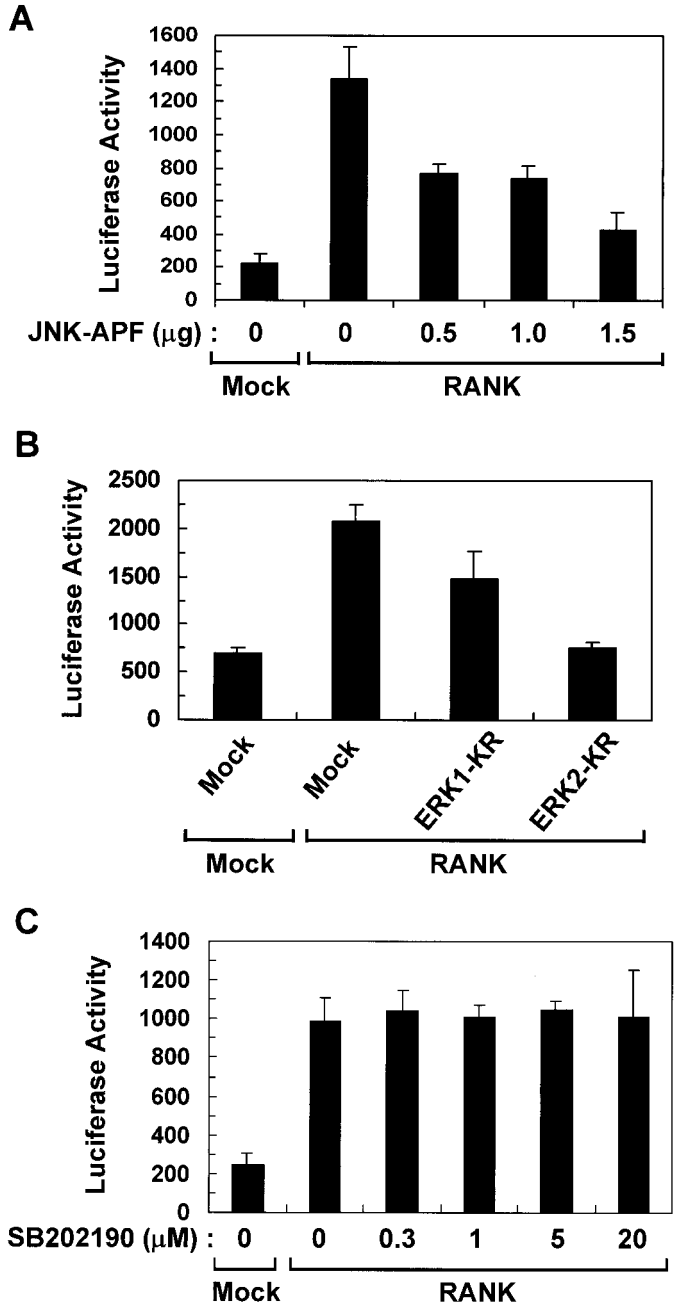

Fig. 5. Requirement of JNK and ERK, but not p38, for the RANK activation of AP-1. A, JNK-APF, a JNK mutant unable to be activated by upstream JNK kinases, was cotransfected with 0.8 μg of RANK, 0.1 μg of pAP1-Luc, and 50 ng of β-gal DNAs into 293-EBNA cells. The next day, cells were harvested, and luciferase assays were performed. B, cells were transfected with 0.8 μg of RANK and 1 μg of ERK mutant as in A. Twenty hours post-transfection, the AP-1 activity was assessed by luciferase assay. C, 293-EBNA cells were transfected with 0.8 μg of RANK, 0.1 μg of pAP1-Luc, and 50 ng of β-gal DNAs. Sixteen hours after transfection, cells were treated with the indicated concentrations of the p38 inhibitor SB 202190 or the control vehicle containing 0.8% dimethyl sulfoxide for 4 h at 37°C. Lysis of cells and luciferase assays were followed. The averages and standard deviations of triplicate samples are shown. These results are representative of three independent experiments.

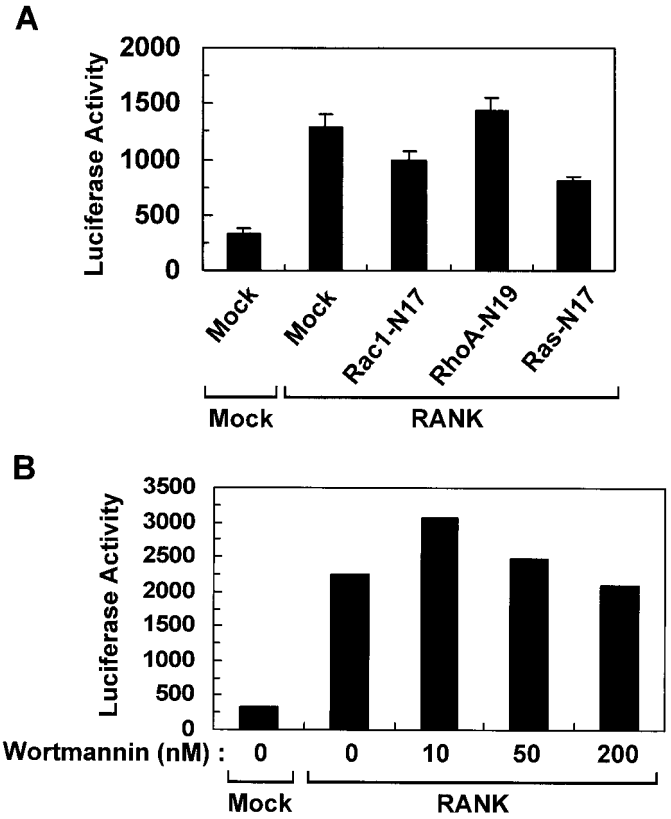

Fig. 6. Regulation of the RANK-induced AP-1 activation by Rac and Ras but not Rho and PI 3-kinase. A, 1 μg of the indicated dominant negative mutant of GTP-binding protein plasmid was cotransfected with 0.8 μg of RANK, 0.1 μg of pAP1-Luc, and 50 ng of β-gal DNAs into 293-EBNA cells. Cells were harvested the next day, and the level of reporter gene induction was measured. B, cells were transfected with 0.8 μg of RANK and 0.1 μg of pAP1-Luc. Sixteen hours after transfection, cells were treated with the indicated concentrations of the PI 3-kinase inhibitor wortmannin for 4 h. Cells were lysed, and luciferase activity was assessed. Data from one experiment are shown, and similar results were obtained in a replicate experiment. Error bars in A denote standard deviations between triplicate samples.

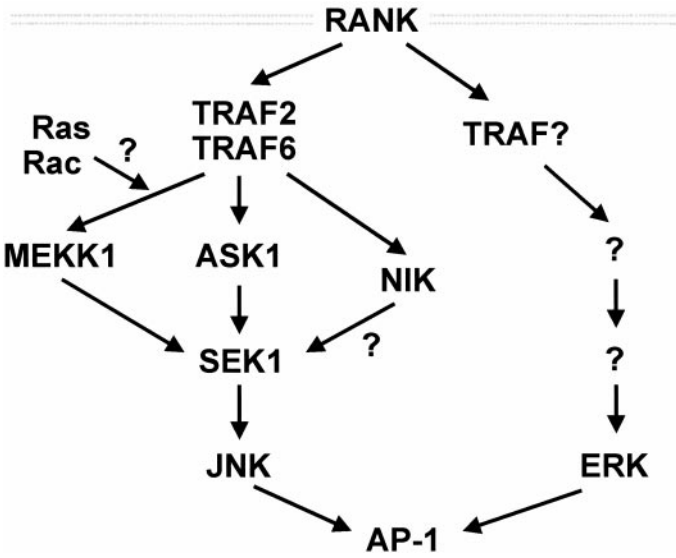

Fig. 7. A scheme for RANK signal transduction to JNK and AP-1. Possible mechanisms for RANK activation of JNK and AP-1 based on the findings of this study are diagrammed. RANK recruits TRAF adaptor proteins that bind and activate ASK1, MEKK1, and NIK. Activation of SEK, which in turn phosphorylates and activates JNK, follows. Phosphorylation of c-Jun in AP-1 by the activated JNK transactivates the transcription factor complexes, which are also induced and regulated by ERK.

sibility of cross-talk between pathways for the activation of JNK, ERK, and NF-κB will be required. Also, verification of the pathways that have been identified to mediate RANK signaling using overexpression systems under more normal conditions, such as in dendritic cells or osteoclasts, will facilitate the linking of these signaling pathways to physiological functions of RANK.

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