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TAK1-dependent Activation of AP-1 and c-Jun N-terminal Kinase by Receptor Activator of NF-κB

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The receptor activator of nuclear factor kappa B (RANK) is a member of the tumor necrosis factor (TNF) receptor superfamily. It plays a critical role in osteoclast differentiation, lymph node organogenesis, and mammary gland development. The stimulation of RANK causes the activation of transcription factors NF-κB and activator protein 1 (AP1), and the mitogen activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK). In the signal transduction of RANK, the recruitment of the adaptor molecules, TNF receptor-associated factors (TRAFs), is an initial cytoplasmic event. Recently, the association of the MAPK kinase kinase, transforming growth factor-β-activated kinase 1 (TAK1), with TRAF6 was shown to mediate the IL-1 signaling to NF-κB and JNK. We investigated whether or not TAK1 plays a role in RANK signaling. A dominant-negative form of TAK1 was discovered to abolish the RANK-induced activation of AP1 and JNK. The AP1 activation by TRAF2, TRAF5, and TRAF6 was also greatly suppressed by the dominant-negative TAK1. The inhibitory effect of the TAK1 mutant on RANK- and TRAF-induced NF-κB activation was also observed, but less efficiently. Our findings indicate that TAK1 is involved in the MAPK cascade and NF-κB pathway that is activated by RANK.

Keywords: Activator protein 1, Receptor activator of NF-κB, Transforming growth factor-β-activated kinase 1, TNF receptor-associated factor

Introduction

The receptor activator of nuclear factor kappa B (RANK) is a recently cloned member of the tumor necrosis factor receptor (TNFR) superfamily (Anderson *et al*., 1997, Kim *et al*., 2001). The ligand for RANK, RANKL, was cloned by several groups and is synonymous with ODF (osteoclast differentiation factor), TRANCE (TNF-related activation-induced cytokine), and OPGL (osteoprotegerin ligand) (Anderson *et al*., 1997; Wong *et al*., 1997; Lacey *et al*., 1998; Yasuda et al., 1998). The gene knock-out mice of RANK and RANKL revealed features of severe osteopetrosis. This was the result of a blockade in osteoclast differentiation, defects in early differentiation of lymphocytes, deficient B cell population in the spleen, and lack of peripheral lymph nodes. Therefore, there is evidence for the essential role of the RANK-RANKL signal in osteoclastogenesis, lymphocyte development, and lymph node organogenesis (Dougall *et al*., 1999, Kong *et al*., 1999). In addition, RANK-/- and RANKL-/- mice also showed defects in lobulo-alveolar mammary structures, suggesting another important function of the RANK-RANKL pair (Fata *et al*., 2000).

The TNF receptor-associated factor (TRAF) proteins function as signaling adaptor molecules for TNFR family receptors by directly or indirectly binding to the receptor proteins and transducing the receptor signals to downstream targets (Arch *et al*., 1998; Inoue *et al*., 2000). The association of TRAF proteins with RANK has been demonstrated. The dominant negative forms of TRAF2, TRAF5, and TRAF6 were also shown to block the RANK-induced activation of the transcription factors nuclear factor kappa B (NF-κB) and activator protein 1 (AP1), as well as the mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK) (Darney *et al*., 1998; Galibert *et al*., 1998; Wong *et al*., 1998; Kim *et al*., 1999; Lee *et al*., 2000).

The MAPK cascade is a sequential process of phosphorylation and activation of Ser/Thr protein kinases. It consists of MAPK, MAPK kinase (MAP2K), and MAPK kinase kinase (MAP3K) (Bogoyevitch *et al*., 2001). The transforming growth factor b-activated kinase 1 (TAK1) is a MAP3K that can phosphorylate and activate MAP2Ks MKK3 and MKK6. It, in turn, can phosphorylate and activate MAPKs JNK and p38 (Yamaguchi *et al*., 1995; Moriguchi *et*

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al., 1996). TAK1 mediates the MAPK cascade and NF-κB activation in IL-1 signaling pathways by associating with TARF6 complexes (Ninomiya-Tsuji *et al*., 1999; Wang *et al*., 2001).

In this study, we investigated the potential role of TAK1 in the RANK signaling to AP1 and NF-κB transcription factors and JNK. The interference of the kinase activity by use of a kinase-defective dominant-negative form of TAK1 greatly reduced the activation of AP1 and RANK by RANK. In addition, the RANK-induced NF-κB activation was significantly attenuated by the dominant-negative TAK1. The results of our study suggest that TAK1 is involved in RANK signaling pathways.

Materials and Methods

Expression plasmids Mammalian expression vectors for T7-tagged human RANK and TRAFs were previously described (Kim *et al*., 1999). The expression plasmid for TAK1-DN (K63W) was a generous gift from Dr. K. Matsumoto (Nagoya University, Nagoya, Japan). The luciferase reporter constructs pAP1-Luc and pNF-κB-Luc were kind gifts from Dr. Y.D. Yun (Ewha Womens University, Seoul, Korea).

Luciferase reporter assays The 293-EBNA (Invitrogen, Carlsbad, USA) were plated onto 24-well plates at 1.5×10^5 cells/well. On the next day, the cells were transfected with 100-200 ng of pAP1-Luc or pNF-κB-Luc and indicated amounts of various constructs. This kept the ratio of DNA : SuperFect reagent (Qiagen, Stanford Valencia, USA) at 1 : 2 or 1 : 3. 50 ng of a b-gal vector that was co-transfected for normalizing transfection efficiencies. The total amount of DNA was kept constant by supplements from the control vector DNAs. After 16-20 h of transfection, the cells were incubated with or without TNF-α (R&D Systems, Minneapolis, USA) or IL-1 (R&D Systems) for 4-6 h. They were lysed with 150 μl of Reporter Lysis Buffer (Promega, Madison, USA). The luciferase activity from 20 μl of lysate was measured with a Luminometer (EG&G Berthold, Wildbad, Germany).

JNK activity assays The 293-EBNA cells were seeded on 6-well plates at 8×10^5 cells/well. On the next day, the cells were transfected with 0.3-0.5 μg of pEBG-JNK and other indicated DNAs, plus 8 μl SuperFect reagent (Quiagen). The total amount of DNA was kept constant by adding control vector DNAs. After 20-36 h of transfection, the cells were lysed in a lysis buffer (20 mM Tris-HCl, 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 $10,000 \times g$. Three hundred mg of cleared lysates were incubated with glutathione beads for 2 h at 4°C. The precipitated beads were extensively washed and subjected to kinase reactions, using GST-c-Jun as the substrate as previously described (Kim *et al*., 2000).

Western blotting analyses The Cell lysates were prepared as described previously using a lysis buffer that contained 1% Triton X-100. Lysate proteins were separated by SDS-PAGE 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, and incubated with anti-T7 (Novagen, Madison, USA), anti-TAK1 (Santa Cruz, Santa Cruz, USA). They were washed for 1 h in TBS-T, and incubated with anti-mouse or anti-rabbit Ig-horseradish peroxidase (Amersham, Piscataway, USA) for 1 h. The immune complexes were detected by the ECL system (Amersham).

Results and Discussion

Suppression of RANK-induced AP1 activation by dominant-negative TAK1 We previously showed that RANK signaling leads to the activation of the AP1 transcription factor (Lee *et al*., 2000). The RANK activation of AP1 was inhibited by the kinase-defective mutants of ASK1 and NIK (Kim *et al*., 2000). In this study, we explored the possibility of the involvement of TAK1 in the AP1 activation by RANK. The 293-EBNA cells were transiently transfected with RANK and an AP1-responsive luciferase reporter plasmids. The overexpression of RANK resulted in a significant increase in luciferase activity (Fig. 1). Co-transfection of equal amounts of RANK and dominant-negative TAK1 (TAK1-DN) plasmid DNAs suppressed the RANK-induced activation of AP1 by ~80% (Fig. 1A). The TAK1-DN plasmid transfection alone showed no significant luciferase activity (Fig. 1A). The TAK1-DN transfection also reduced the TNF-α and IL-1 activation of AP1 to the same extent (Fig. 1B). Transfection with increasing amounts of the TAK1-DN plasmid revealed the dose-dependent suppression of the RANK-induced AP1 stimulation (Fig. 1C). These results implicate protein kinase TAK1 in the signal pathway from the cell surface receptor RANK to the transcription factor AP1.

Inhibition of TRAF-induced AP1 stimulation by dominant-negative TAK1 TRAF proteins associate with RANK and mediate the RANK-induced activation of NF-κB and JNK (Darnay *et al*., 1998; Wong *et al*., 1998; Kim *et al*., 1999). Therefore, we examined whether or not the TAK1-DN that suppressed the AP1 activation by RANK (Fig. 1) would also affect the AP1 activation by TRAF proteins. TRAF2, TRAF5, and TRAF6 were transiently transfected with the AP1-dependent luciferase reporter. The overexpression of TRAF2, TRAF5, and TRAF6 increased luciferase activity, reflecting AP1 stimulation (Fig. 2). The co-transfection of TAK1-DN reduced the TRAF-induced AP1 activation by 65-85% (Fig. 2). This result suggests that TAK1 may play a role for AP1 activation that is downstream of the TRAF proteins in the RANK-signaling pathway.

Involvement of TAK1 in JNK activation by RANK The AP1 transcription factor complexes are composed of homo- and heterodimers of the Jun and Fos family proteins. The phosphorylation of c-Jun by JNK can stimulate AP1 activity

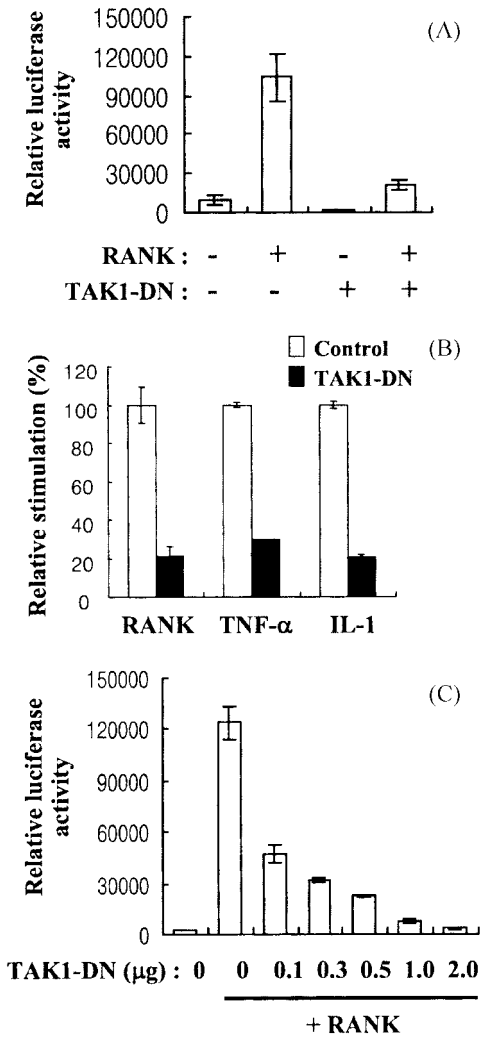

Fig. 1. Involvement of TAK1 in the activation of AP1 by RANK. (A) The 293-EBNA cells were transfected with 0.2 μg AP1-Luc, 0.5 μg RANK, and 0.5 μg TAK1-DN plasmids as described in *Materials and Methods*. Twenty-six hours after transfection, the cells were lysed and the luciferase activity was measured with 20 μl of cell lysate. The relative luciferase activities are shown. (B) The cells were transfected with 0.5 μg RANK (RANK lanes) or the control vector DNA (TNF-α and IL-1 lanes) as in *A*. Twenty hours after transfection, the cells were incubated with the control vehicle (RANK lanes), 50 μg/ml TNF-α (TNF-α lanes), or 10 ng/ml IL-1 (IL-1 lanes) for 6 h. The cells were lysed and luciferase assays were performed. The relative levels of luciferase activity from the TAK1-DN transfected cells that were compared with that from the control vector transfected cells are presented. (C) The cells were transfected with 0.2 μg AP1-Luc, 0.5 μg RANK, and the indicated amount of TAK1-DN plasmids. The luciferase activity was measured with 20 μl of cell lysates 24 h after transfection. The relative luciferase activities are presented.

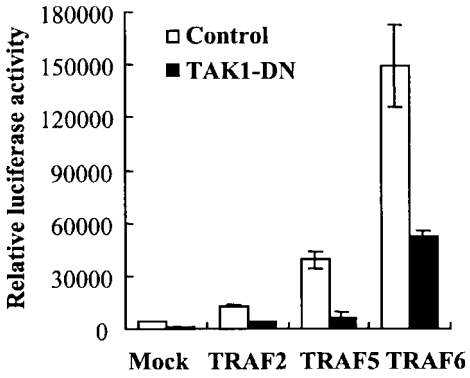

Fig. 2. Suppression of TRAF-induced AP1 activation by TAK1-DN. The 293-EBNA cells were transfected with 0.2 μg AP1-Luc, 0.5 μg TRAF, and 0.5 μg TAK1-DN plasmids, described in *Materials and Methods*. Twenty-six hours after transfection, the cells were lysed and the luciferase activity was measured.

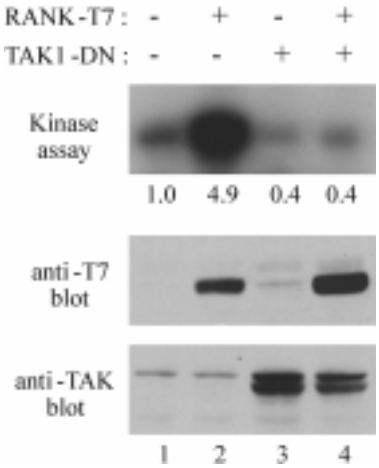

Fig. 3. Inhibition of the RANK stimulation of JNK by TAK1-DN. The 293-EBNA cells were transfected with 2 μg RANK-T7, 2 μg TAK1-DN, or 4 μg control vectors, as indicated, and cultured for 24 h. The cells were lysed and the kinase activity assay was performed with GST-c-Jun as the substrate (top panel). The values that were determined by phosphoimager are indicated below the autoradiogram. Aliquots of the cell lysates were subjected to Western blotting analyses with anti-T7 for RANK expression (middle panel) and anti-TAK (bottom panel), described in Materials and Methods.

(Derijard *et al*., 1994). The signal transduction of RANK leads to the activation of JNK (Kim *et al*., 1997; Wong *et al*., 1997; Darney *et al*., 1998). Whether or not the inhibition of RANK-induced AP1 activation by TAK1-DN (Fig. 1) is due to the effects of TAK1-DN on JNK activity was determined. RANK was transfected with or without TAK1-DN, and the kinase activity of JNK was measured. As shown in Fig. 3, the expression of RANK resulted in a 4.9 fold stimulation of JNK activity (top panel, lane 2). The co-transfection of TAK1-DN completely blocked the RANK activation of JNK (top panel, lane 4). The effect of the TAK1-DN co-transfection was not from the reduced expression of RANK, as verified by Western blotting analyses (middle panel, lanes 2 and 4). The expression of both endogenous TAK1 (bottom panel, upper

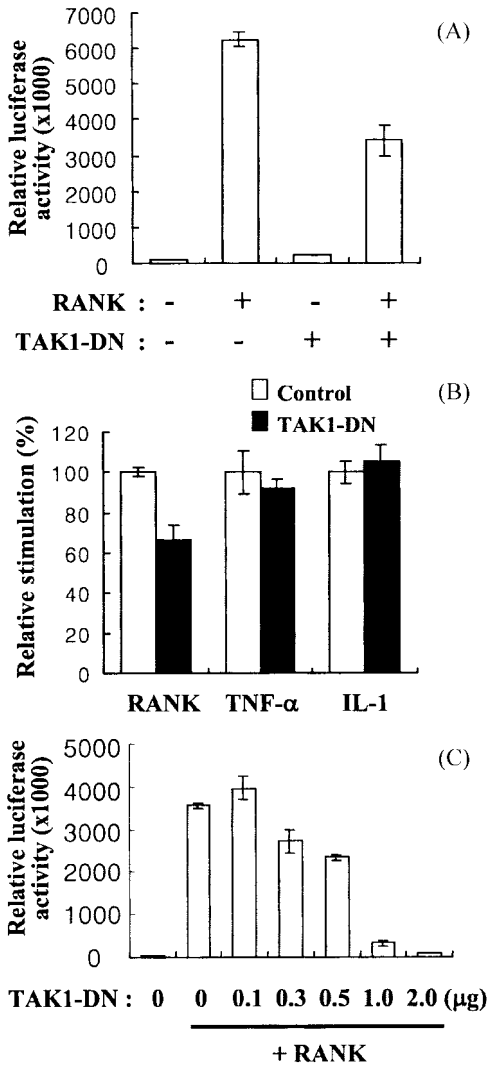

Fig. 4. Effect of TAK1-DN on NF-κB activation by RANK. (A) The 293-EBNA cells were transfected with 0.1 μg NF-κB-Luc, 0.5 μg RANK, and 0.5 μg TAK1-DN plasmids. Twenty hours after transfection, the cells were lysed and luciferase activity was assessed with 20 μl of cell lysates. The relative luciferase activities are shown. (B) The cells were transfected with RANK (RANK lanes) or the control vector (TNF-α and IL-1 lanes) as in *A*. Twenty-four hours after transfection, the cells were incubated with the control vehicle (RANK lanes), 50 μg/ml TNF-α (TNF-α lanes), or 10 ng/ml IL-1 (IL-1 lanes) for 6 h. The luciferase activity was measured with 20 μl of cell lysate. The relative levels of the luciferase activity from TAK1-DN transfected cells when compared with that from the control vector transfected cells are shown. (C) The cells were transfected with 0.1 μg NF-κB -Luc, 0.5 μg RANK, and the indicated amount of TAK1-DN. The luciferase activity was measured with 20 μl of cell lysates 24 h after transfection. The relative luciferase activities are presented.

band) and transfected TAK-DN (lower band) was also detected in these cells. This result indicates that the MAP3K protein TAK1 mediates the RANK activation of JNK.

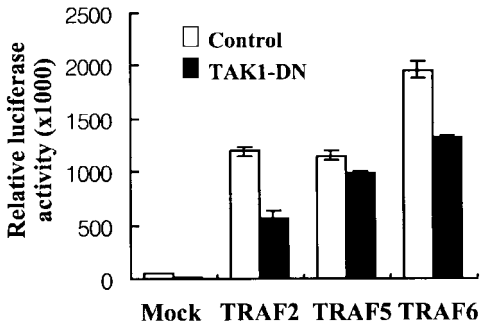

Fig. 5. Effect of TAK1-DN on NF-κB activation by TRAFs. The 293-EBNA cells were transfected with 0.1 μg NF-κB-Luc, 0.5 μg TRAF, and 0.5 μg TAK1-DN plasmids. Twenty-six hours after transfection, the cells were lysed and a luciferase assay was carried out with 20 μl of cell lysates.

Effects of TAK1-DN on NF-κB activation by RANK and TRAFs The activation of NF-κB by RANK is widely documented (Anderson *et al.*, 1997; Galibert *et al.*, 1998). TAK1 plays a role in NF-κB activation by inflammatory cytokines, such as IL-1 and LPS (Ninomiya-Tsuji *et al.*, 1999; Wang *et al.*, 2001). Therefore, we investigated whether or not TAK1 is also involved in the RANK activation of NF-κB. A NF-κB-responsive luciferase reporter plasmid was transfected into 293-EBNA cells in combination with RANK and TAK1-DN. The co-transfection of equal amounts of TAK1-DN suppressed RANK-induced NF-κB activation by 30-50% (Fig. 4 A and B). In contrast to the inhibitory effect of TAK1-DN on the TNF-α and IL-1-induced AP1 activation (Fig. 1B), the TNF-α and IL-1-induced NF-κB activation was not reduced by TAK1-DN in these cells (Fig. 4B). Ninomiya-Tsuji *et al.* suggested that TAK1 mediates the IL-1-induced NF-κB activation by activating NIK in 293IL-1RI cells (Ninomiya-Tsuji *et al.* 1999). The IL-1 activation of NF-κB was relatively weak in the 293-EBNA cells that were used in our study, suggesting that the number of IL-1R in these cells might be low. Whether or not the difference between our study and the study of Ninomiya-Tsuji *et al.* with regard to the effect of TAK1-DN on the IL activation of NF-κB reflects the difference in the receptor level, or other differences in the intrinsic characteristics of the cell lines, remains unknown. The extent of inhibition by TAK1-DN of the RANK-induced NF-κB activation increased with increased amounts of TAK1-DN (Fig. 4C). TAK1-DN also reduced the NF-κB activation by TRAF2 and TRAF6 by 30-60% (Fig. 5). Only a weak inhibition was observed with the TRAF5-induced NF-κB activation (Fig. 5). The differential effect of TAK1-DN may imply that different mechanisms are in operation for the NF-κB activation that is induced by the overexpression of TRAF proteins. Many studies have shown the association of TAK1 with TRAF6 in the IL-1 signal transduction; whereas, the relationship between TAK1 and TRAF2 or TRAF5 has not been reported (Ninomiya-Tsuji *et al.*, 1999; Takaesu *et al.*, 2000; Qian *et al.*, 2001).

In summary, we investigated whether or not MAP3K TAK1 plays a role in the signal transduction of RANK by employing a dominant-negative form of the kinase. The dominant-negative TAK1 greatly suppressed AP-1 activation by RANK and TRAF proteins (Figs. 1 and 2). The inhibitory effect of the RANK activation on AP-1 was accompanied with the inhibition of JNK activation by the TAK1 mutant (Fig. 3). The dominant-negative TAK1 also reduced the NF-κB activation by RANK, TRAF2, and TRAF6, but to a lesser degree (Figs. 4 and 5). Our findings implicate TAK1 in the RANK signaling pathway to AP1 through JNK, and suggest a potential regulatory function of TAK1 in the NF-κB activation by RANK. During the preparation of our paper, Mizukami *et al*. reported a complex formation of RANK, TRAF6, TAK1, and TAB2 and the activation of TAK1 upon RANKL stimulation (Mizukami *et al*., 2002). Our results, and the observations of Mizukami *et al*., suggest that TAK1 may play an essential role in the RANK signaling pathways that are downstream of TARF6.

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