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The scaffold protein RACK1 mediates the RANKL-dependent activation of p38 MAPK in osteoclast precursors

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Abstract

The E3 ubiquitin ligase TRAF6 and the associated kinase TAK1 are key components of the signaling pathways that activate nuclear factor κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs) in response to various stimuli. The cytokine RANKL (receptor activator of NF- κ B ligand) is essential for the differentiation of bone marrow cells into bone-resorbing osteoclasts through the activation of NF- κ B and MAPK. We found that the scaffold protein RACK1 (receptor for activated C kinase 1) selectively mediated the RANKL-dependent activation of p38 MAPK through the TRAF6-TAK1 axis by interacting with the MAPK kinase MKK6, which is upstream of p38 MAPK. RACK1 was necessary for the differentiation of bone marrow cells into osteoclasts through the stimulation of p38 MAPK activation. Osteoclast precursors exposed to RANKL exhibited an interaction among RACK1, RANK, TRAF6, TAK1, and the kinase MKK6, thereby leading to the activation of the MKK6-p38 MAPK pathway. Experiments in which RACK1 or TAK1 were knocked down in osteoclast precursors indicated that RACK1 acted as a bridge bringing MKK6 to the TRAF6-TAK1 complex. Furthermore, local administration of RACK1-specific siRNA into mice calvariae reduced the RANKL-induced loss of bone by reducing the numbers of osteoclasts. These findings suggest that RACK1 specifies the RANKL-stimulated activation of p38 MAPK by facilitating the association of MKK6 with TAK1, and may provide a molecular target for a new therapeutic strategy to treat bone diseases.

Introduction

Bone-resorbing osteoclasts are multinucleated cells that are derived from CD11b⁺ hematopoietic progenitor cells of the monocyte-macrophage lineage (1, 2). Two critical cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear

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factor κ B (NF- κ B) ligand (RANKL), are essential for the generation and function of osteoclasts (3, 4). Upon binding its ligand RANKL, the receptor RANK recruits the adaptor protein and E3 ubiquitin ligase TRAF6 [tumor necrosis factor (TNF) receptor (TNFR)-associated factor 6 (TRAF6)] through three TRAF6-binding sites in its cytoplasmic tail (5). Although other TRAF family members, including TRAF2 and TRAF5, can bind to RANK, studies of the phenotype of knockout mice identified TRAF6 as the major adaptor molecule that mediates signals activated by RANKL (6–8). TRAF6 facilitates the synthesis of nondegradative, lysine-63–linked polyubiquitin chains to recruit and activate transforming growth factor- β (TGF- β)–activated kinase 1 (TAK1) (9). The TRAF6-TAK1 complex then activates several downstream kinase cascades, such as those mediated by inhibitor of κ B (I κ B) kinase (IKK) and mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (10, 11). Activation of the NF- κ B and MAPK signaling pathways results in the increased abundance of the transcription factor nuclear factor of activated T cells cytoplasmic 1 (NFATc1) and the expression of its target genes, including those encoding cathepsin K, osteoclast-associated receptor (OSCAR), the v-ATPase V0 subunit d2 (Atp6v0d2), and tartrate-resistant acid phosphatase (TRAP) (12, 13).

Activation of the MAPKs is one of the important signaling events downstream of RANK. Among the three MAPKs, p38 MAPK, most notably p38 α , constitutes a distinct MAPK subfamily that plays an essential role in mediating osteoclast differentiation, but not osteoclast function (14, 15). Treatment of bone marrow–derived macrophages (BMMs) with SB203580, a specific inhibitor of p38 α and p38 β , or expression of dominant-negative forms of p38 α and MKK6 in RAW264.7 cells (a mouse macrophage cell line) suppresses the RANKL-induced differentiation of bone marrow cells into osteoclasts (16). A kinase cascade consisting of TAK1 and MKK6 activates p38 MAPK in response to RANKL (17). These studies indicate that the signaling cascade downstream of RANK, which involves TRAF6, TAK1, and MKK6, is responsible for the activation of p38 MAPK. Previous studies also demonstrated that the pathway consisting of TRAF6, TAK1, MKK6, and p38 MAPK is involved in other signaling pathways, such as interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR) signaling (18), as well as in skeletal muscle differentiation (19). Therefore, there may exist stimulus-specific mechanisms that enable the regulation of common signaling pathways with different biological outcomes depending on the stimuli received.

Receptor for activated C-kinase 1 (RACK1) is a member of the Trp-Asp⁴⁰ (WD40)-repeat protein family, and has homology with the G protein β subunit of transducin (20). RACK1 was originally identified as an anchor protein of protein kinase C (PKC) (21). As a multifunctional scaffold protein, it interacts with PKC, the kinase Src, and phosphodiesterase the PDE4D5 isoform, as well as the cytoplasmic domains of several membrane-bound receptors, including integrin β subunits (such as β_1 , β_2 , and β_5), the N-methyl-D-aspartic acid (NMDA) receptor, and insulin-like growth factor receptor I (IGF-IR), thereby integrating signals from various pathways (22–27). Other studies demonstrated that RACK1 may serve as a scaffolding protein for JNK signaling (28, 29), and that it is also associated with the activation of MEK-ERK signaling (30); however, whether RACK1 is involved in RANKL signaling is unknown.

We found that RACK1 was necessary for the generation of osteoclasts by selective activation of p38 MAPK in osteoclast precursors. RACK1 acted as a scaffold protein to link the TRAF6-TAK1 complex with MKK6, but not MKK3, in response to RANKL. Our findings provide insight into a stimulus-specific regulatory mechanism of p38 MAPK, a critical step in RANKL-initiated differentiation of monocytes and macrophages from hematopoietic progenitor cells into osteoclasts.

Results

RACK1 selectively mediates RANKL-mediated p38 MAPK

To examine whether RACK1 mediated the activation of RANKL-dependent MAPK and NF- κ B signaling, primary BMMs infected with empty or RACK1-expressing retroviruses were stimulated with RANKL, and their activation was analyzed by examining the phosphorylation of p38, ERK, and JNK, as well as the degradation of I κ B α . The increased abundance of RACK1 in transfected cells substantially increased the extent of phosphorylation of p38, but not ERK and JNK (Fig. 1A). No substantial differences in the degradation of I κ B α were observed between the control and RACK1-overexpressing BMMs. These results were further confirmed in experiments with stable RAW264.7 cell lines expressing hemagglutinin (HA)-tagged RACK1 (fig. S1A). Consistent with these findings, we observed that knockdown of endogenous RACK1 with a small hairpin RNA (shRNA) or a small interfering RNA (siRNA) attenuated only the RANKL-dependent activation of p38 (Fig. 1B and fig. S1B). The phosphorylation of p38 and its downstream substrates, including ATF2 (activating transcription factor 2), were maximal at 15 min after stimulation with RANKL and gradually declined thereafter (fig. S2).

Because the stimulation of BMMs with M-CSF or LPS also activates MAPKs and NF- κ B (31–33), we next examined whether an increase in the abundance of RACK1 affected the M-CSF- or LPS-dependent activation of MAPKs or NF- κ B. As expected, stimulation of BMMs with M-CSF activated MAPKs, but not NF- κ B (Fig. 1C), whereas stimulation with LPS activated MAPKs and NF- κ B (Fig. 1D). However, unlike for RANKL signaling, the increased abundance of RACK1 in BMMs had no effect on the M-CSF- or LPS-stimulated activation of MAPKs or NF- κ B (Fig. 1, C, and D). Similarly, the protein kinase cascades initiated by IL-1 β were not abrogated by the overexpression of RACK1 in BMMs (Fig. 1E).

RACK1 acts as an adaptor for the PKC-mediated activation of JNK (29). In addition, PKC β stimulates the formation of osteoclasts (34, 35). Therefore, we investigated whether RACK1 was linked to PKC β in RANKL signaling. Consistent with a previous report (29), we found that exogenous RACK1 interacted with PKC β in human embryonic kidney (HEK) 293T cells (fig. S3A). However, immunoprecipitation assays showed that endogenous RACK1 did not associate with PKC β in response to stimulation of RAW264.7 cells with RANKL (fig. S3B). Furthermore, neither knockdown of PKC β nor pharmacological inhibition of PKC had any apparent effect on the RANKL-dependent activation of MAPKs or NF- κ B (fig. S3, C and D). Given that RACK1 acts as a ribosome-associated protein that promotes efficient translation of mRNA in yeast and mammalian cells (27, 36), we tested whether the knockdown of RACK1 affected protein synthesis in BMMs. We found that knockdown of RACK1 had no effect on either basal rates of protein synthesis (fig. S4, A and B) or

RANKL-stimulated protein synthesis (fig. S4, C and D) compared to that in matched control cells. Together, these results suggest that RACK1 selectively mediates the activation of p38 in response to RANKL.

RACK1 promotes RANKL-dependent osteoclastogenesis

Stimulation of BMMs with RANKL during osteoclastogenesis led to a gradual increase in RACK1 mRNA and protein abundances (fig. S5, A and B). The 0.6-kb *GNB2L1* (encoding RACK1) promoter fragment (fig. S5C), linked to a luciferase reporter construct, is activated in response to RANKL, whereas this induction was abolished when the putative NF- κ B binding site of this promoter was mutated (fig. S5D), suggesting that RACK1 is a direct transcriptional target of NF- κ B during osteoclastogenesis.

Considering the importance of p38 in RANKL-dependent osteoclastogenesis (14, 15), we next examined whether RACK1 regulated this process. To accomplish this, primary BMMs infected with empty or RACK1-expressing retroviruses were cultured with M-CSF and RANKL and then were stained for TRAP, a marker of osteoclasts. Although the increased abundance of RACK1 had no effect on cell proliferation in the presence of M-CSF and RANKL (fig. S6), RANKL-stimulated osteoclast formation was substantially enhanced in RACK1-overexpressing cells compared to that in control cells (Fig. 2A). The number of TRAP⁺ multinucleated cells (MNCs) (> 3 nuclei) was greater for RACK1-overexpressing BMMs than for control cells (Fig. 2B). Moreover, the abundance of NFATc1, a transcription factor that is essential for osteoclastogenesis, as well as that of Atp6v0d2, which is encoded by a well-known target gene of NFATc1, were also increased in RACK1-overexpressing BMMs compared to those in control BMMs (Fig. 2C). The importance of RACK1 in osteoclast formation was further investigated in experiments with RAW264.7 cells stably expressing hemagglutinin (HA)-tagged RACK1. Compared to control cells, RAW264.7 cells expressing HA-RACK1 showed substantially increased numbers of TRAP⁺ MNCs and greater amounts of NFATc1 and Atp6v0d2 in response to RANKL (fig. S7, A to C). Note that the p38 inhibitor SB203580 suppressed the RACK1-stimulated increase in the phosphorylation of p38 and ATF2, the abundance of NFATc1, and osteoclast formation, suggesting that RACK1 acted upstream of p38 (fig. S8, A to D).

To further confirm the effects of RACK1 on RANKL-induced osteoclastogenesis, we knocked down endogenous RACK1 in BMMs with shRNA. Similar to the effects of overexpression of RACK1, its knockdown in BMMs did not affect cell proliferation in the presence of M-CSF and RANKL (fig. S9), suggesting that RACK1 is not involved in the proliferation of osteoclast precursors in response to M-CSF and RANKL. However, knockdown of RACK1 in BMMs with shRNA or siRNA markedly suppressed osteoclast formation and also attenuated the RANKL-dependent increase in the abundances of NFATc1 and Atp6v0d2 (Fig. 2, D to F and fig. S10, A to C). Consistent with these findings, shRNA-mediated knockdown of RACK1 caused a marked inhibition of osteoclast formation over time, but did not affect the viability of BMMs (fig. S11, A to C). Together, these results suggest that RACK1 acts as a stimulator of RANKL-induced osteoclastogenesis.

RACK1 associates with TRAF6 and TAK1

Upon stimulation of BMMs with RANKL, the binding of TRAF6 to RANK causes p38 activation through TAK1 (5, 37). Given our data indicating that RACK1 selectively modulated the RANKL-dependent activation of p38, we investigated whether RACK1 interacted with TRAF6. HEK 293T cells were co-transfected with plasmids encoding HA-RACK1 and Flag-tagged TRAF family members, including TRAF2, TRAF5, and TRAF6. RACK1 bound most abundantly to TRAF6, and minimally bound to TRAF2 and TRAF5 (Fig. 3A). Because TRAF6 is recruited to the cytoplasmic tail of RANK (38), we next investigated whether RACK1 was recruited to RANK through its interaction with TRAF6. We found that wild-type RANK interacted with TRAF6 and with RACK1 (Fig. 3B); however, the RANK E3A (E342A/E375A/E449A) mutant, which has mutations in the three TRAF6-binding sites, failed to interact with either TRAF6 or RACK1 (Fig. 3B). The recruitment of RACK1 to RANK was substantially increased in the presence of TRAF6 (Fig. 3B), suggesting that the binding of RACK1 to RANK is mediated through an interaction with TRAF6.

We next analyzed the regions of RACK1 and TRAF6 that might be involved in their interaction by generating several deletion mutants (fig. S12A) followed by co-immunoprecipitation analysis of transfected HEK 293T cells. RACK1 interacted with the TRAF6 N derivative, which contains just the coiled-coil N-terminal domain and the conserved C-terminal domain of TRAF, whereas RACK1 failed to interact with the TRAF6 C derivative, which contains the N-terminal ring and zinc finger domains of TRAF6 spanning amino acid residues 1 to 289 (fig. S12B). On the other hand, experiments with serial deletion constructs of RACK1 revealed that RACK1 mutants lacking either the WD2 domain (WD2) or the WD6 domain (WD6) showed reduced association with TRAF6 (fig. S12C). Consistent with these findings, the RACK1 WD2^Δ6 mutant, which contains the WD2 and WD6 domains of RACK1, interacted with TRAF6, whereas the RACK1 WD2,6 mutant, which lacks both the WD2 and WD6 domains, failed to interact with TRAF6 (fig. S12D). To test whether RANKL stimulated an association between TRAF6 and RACK1, we performed co-immunoprecipitation experiments with an anti-TRAF6 antibody and extracts from RAW264.7 cells that were treated with RANKL for various times. In response to RANKL, there was an increase in the extent of interaction between endogenous RACK1 with TRAF6 (Fig. 3C). These results suggest that RACK1 is involved in RANKL signaling through its association with TRAF6.

TAK1, a MAP3K family member, is a critical effector downstream of TRAF6 (39), and the TRAF6-TAK1 axis activates p38 in response to RANKL (37). We therefore investigated whether RACK1 interacted with TAK1 in the presence or absence of TAK1 binding protein 1 (TAB1), a known activator of TAK1 (40). RACK1 associated with TAK1 more efficiently in TAB1-expressing cells (in which TAK1 was activated) than it did in TAB1-deficient cells (Fig. 4A). Similarly, a mutant TAK1, TAK1 K63W, which is devoid of kinase activity (41), was unable to bind to RACK1 (Fig. 4A). This suggests that the kinase activity of TAK1 is required for its interaction with RACK1. These results were confirmed in experiments with RAW264.7 cells treated with RANKL to activate TAK1. RANKL stimulated the phosphorylation of TAK1, greatly increasing the association between RACK1 and TAK1

(Fig. 4B). These data suggest that once TAK1 was activated in response to RANKL, it associated with RACK1.

RACK1 mediates RANKL-dependent p38 MAPK activation through an interaction with MKK6

The association between RACK1 and TAK1 led us to examine whether this interaction influenced the activation of MAPKKs, through an interaction with MKK3 or MKK6, which, in turn, promotes the activation of p38. The amounts of phosphorylated MKK3 and MKK6 (designated as pMKK3/6) were substantially increased in control cells in response to RANKL (Fig. 5A). However, pMKK3/6 abundance was markedly reduced in RANKL-stimulated cells in which RACK1 was knocked down (Fig. 5A), suggesting that RACK1 is required for the phosphorylation of MKK3 and MKK6. We next examined whether RACK1 interacted with MKK6 or MKK3. The results of co-immunoprecipitation experiments showed that RACK1 interacted with MKK6, but not MKK3 (Fig. 5B). Moreover, a dominant-negative MKK6 mutant (MKK6-DN) showed markedly decreased binding to RACK1 compared to that of wild-type MKK6, suggesting that MKK6 phosphorylation is important for its binding to RACK1. Unlike the interaction between RACK1 and MKK6, an interaction between RACK1 and p38 α was not detected (fig. S13).

We next investigated whether the interaction between RACK1 and MKK6 affected the activation of p38. Overexpression of RACK1 in a HEK 293 cell line expressing RANK receptor (HEK 293-RANK cells) caused an increase in the abundance of phosphorylated p38 (pp38) in response to RANKL (Fig. 5C). However, the abundance of pp38 was subsequently decreased in cells in which MKK6-DN, but not MKK3-DN, was overexpressed (Fig. 5C). To further confirm these results, we examined the effects of RACK1 knockdown on RANKL-stimulated activation of p38 in HEK 293-RANK cells. In the control cells, RANKL stimulated an increase in the amount of pp38, and the overexpression of MKK6 further increased the extent of RANKL-stimulated p38 phosphorylation (Fig. 5D). In contrast, any increase in pp38 abundance was substantially attenuated in RACK1-knockdown cells (Fig. 5D). Together, these results suggest that the RACK1-MKK6 interaction contributes to the activation of p38.

RACK1 is required for the association between the TRAF6-TAK1 complex and MKK6

Given that RACK1 is a scaffold protein, we hypothesized that it might function as a critical scaffold protein to form a TRAF6-TAK1-MKK6 complex in response to RANKL. We first found that TAK1 co-immunoprecipitated with RACK1 and MKK6 in transfected HEK 293T cells (Fig. 6A). However, knockdown of RACK1 decreased the association between TAK1 and MKK6, suggesting that RACK1 bridges TAK1 and MKK6 to promote p38 activation (Fig. 6A). To further investigate whether this scaffolding event occurred in response to RANKL, we performed co-immunoprecipitation experiments of endogenous proteins in primary BMMs. After stimulation of BMMs with RANKL, endogenous TRAF6 was immunoprecipitated with an anti-TRAF6 antibody (Fig. 6B). The association of endogenous TRAF6 with TAK1 and MKK6, as well as RACK1, was substantially increased in the cells stimulated by RANKL; however, TRAF6 failed to co-immunoprecipitate with MKK6 in RACK1-knockdown cells (Fig. 6B). Knockdown of RACK1 did not disrupt the association

between TRAF6 and TAK1, suggesting that RACK1 is required for the interaction between TAK1 and MKK6, but not TRAF6. Similar effects of RACK1 knockdown were observed when endogenous TRAF6 was immunoprecipitated with an anti-TRAF6 antibody in HEK 293-RANK cells (fig. S14). We also found that the siRNA-mediated loss of TAK1 reduced the association between TRAF6 and RACK1, as well as MKK6 (Fig. 6C). Collectively, these data suggest that RACK1 is required for the association of the TRAF6-TAK1 complex with MKK6, thereby leading to p38 activation.

RACK1 is involved in RANKL-mediated cytokine production in dendritic cells (DCs)

Osteoclasts differentiate from precursor cells of the monocyte and macrophage lineage, which also give rise to dendritic cells (DCs) (1). In addition, RANKL-RANK signaling is involved in the survival of DCs and their production of cytokines (1, 42). Therefore, we sought to test whether RACK1 regulated the RANKL-mediated survival of DCs or their cytokine production. As expected, blocking the RANKL-RANK interaction with osteoprotegerin (OPG) impaired the survival of DCs; however, knockdown of RACK1 did not affect the survival of DCs (fig. S15, A and B). Furthermore, knockdown of RACK1 resulted in a substantial reduction in the amounts of IL-6 and IL-12p40 produced by DCs in response to RANKL (fig. S15, C and D), which suggests that RACK1 mediates the RANKL-stimulated production of cytokines by DCs.

Inhibitory effects of RACK1-specific siRNA on RANKL-induced bone loss

To investigate the role of RACK1 in a RANKL-induced model of bone loss, subcutaneous tissue over the periosteum of mouse calvaria was injected with vehicle alone [phosphate-buffered saline (PBS)], RANKL with scrambled siRNA, or RANKL with RACK1-specific siRNA at 2-day intervals. Whole calvaria were fixed in 4% paraformaldehyde and used for TRAP staining (Fig. 7A). The formation of mature TRAP⁺ osteoclasts on the bone surface was suppressed in the mice treated with RACK1-specific siRNA (Fig. 7B), and led to a substantial reduction in the extent of bone erosion (Fig. 7C), suggesting that knockdown of RACK1 inhibited RANKL-induced bone loss in vivo.

Discussion

It is widely thought that the TRAF6-TAK1 axis is a key component of NF- κ B and MAPK signaling pathways in response to various stimuli, including IL-1R, TLRs, and RANKL (10, 11). However, the mechanism underlying the context- or stimulus-specific control of the TRAF6-TAK1 axis has remained elusive. In this context, we propose that the scaffolding protein RACK1 is a key component of the p38 pathway, linking the cascade to RANKL. RACK1 associated with multiple components of the RANK-dependent p38 activation pathway, including TRAF6, TAK1, and MKK6, and selectively facilitated the activation of p38. Furthermore, experiments in which RACK1 and TAK1 were knocked down provided evidence that RACK1 is a component in the activation of p38 in response to RANKL, acting as a bridge for the binding of MKK6 to the TRAF6-TAK1 complex.

The MAPK signaling pathway is activated in response to a broad spectrum of extracellular stimuli (43); however, it is unclear how the signal from the ubiquitously activated MAPK

cascade is transmitted to generate distinct biological responses. For example, the MAPK signaling pathway in BMMs is activated by different stimuli, including RANKL and M-CSF, which are the two critical cytokines for osteoclast generation (31), as well as by LPS, which is a potent activator of macrophages (32), and IL-1 β , a representative mediator of the inflammatory response (33). Whereas the signaling pathway by which M-CSF activates MAPKs is still obscure, it is well-documented that MAPKs can be activated by RANKL, LPS, or IL-1 β through the TRAF6-TAK1 axis (44). Here, because we found that RACK1 selectively mediated the phosphorylation (and thus activation) of p38 in response to RANKL, but not M-CSF, LPS, or IL-1 β , the molecular mechanism by which RACK1 regulates the p38 pathway seems to be stimulus-specific. In this context, it is possible that a scaffold protein(s) acts as a critical regulatory component that selectively assembles signaling modules and links them to specific stimuli. Similar mechanisms of controlling distinct biological outcomes through the use of different stimuli, such as growth, differentiation, or activation factors, despite their use in overlapping signaling cascades, have been reported (38, 45, 46). For example, RACK1 enhances the activity of JNK and thereby promotes the growth of hepatocellular carcinoma through direct binding to MKK7, increasing MKK7 activity (28). In neurons, the scaffold protein JNK-interacting protein 1 (JIP-1) is necessary for the activation of JNK when cells are exposed to anoxic conditions or excitotoxic stress, but JIP-1 is dispensable when cells are treated with anisomycin or ultraviolet (UV) light (47). That RACK1 is used in a stimulus-specific manner to facilitate p38 activation raises the possibility that it selectively controls a subset of p38-dependent RANKL responses for osteoclast generation.

A previous study showed that RANK forms complexes with TRAF6, TAK1, and TAB2 in HEK 293 cells overexpressing RANK (9, 48). Moreover, TAK1 and MKK6 constitute the p38 signaling pathway in the RANKL-induced differentiation of bone marrow cells into osteoclasts (9, 17, 46); however, how the TRAF6-TAK1-MKK6 axis is linked to RANK to activate p38 is unclear. Here, we demonstrated that RACK1 interacted with RANK in a TRAF6-dependent manner. Further experiments showed that once TAK1 was activated in response to RANKL, active TAK1 associated with RACK1. We also found that RACK1 interacted with MKK6, but not MKK3. Therefore, it is possible that the p38 pathway mediated by the RANK signaling complex that contains TRAF6, RACK1, TAK1, and MKK6 is specific for RANKL, but not other stimuli. Similarly to RANK, CD40 binds to members of the TRAF family, such as TRAF2, TRAF3, TRAF5, and TRAF6 (49). Despite such similarities, the stimulation of CD40 in osteoclast progenitor cells does not result in osteoclast formation (38). These findings suggest that RANK, but not CD40, transmits specific signals leading to osteoclastogenesis (50). Based on our data, it is interesting to speculate that the involvement of RACK1 in the RANK signaling complex may be required for signaling specificity or fine-tuning of signaling, such as the RANKL-stimulated activation of p38. Hence, we suggest that a difference in p38 activation, manifesting from the involvement of RACK1 in RANK signaling, could be one of the key mechanisms that distinguishes RANK from other receptors that use TRAF6-TAK1 axis, in terms of osteoclastogenic potential.

Although RACK1 is highly abundant in all mammalian cells and remains at relatively constant amounts (27, 51), the mRNA and protein expression profiles of RACK1 indicated

that the expression of RACK1 gradually increases during osteoclastogenesis. Further experiments showed that NF- κ B is involved in the transcription of *GNB2L1* (52). Here, we characterized the role of RACK1 during the early phase of stimulation with RANKL. Upon stimulation of osteoclast precursors with RANKL, MAPKs and NF- κ B were rapidly activated within 1 hour of RANKL stimulation in our cell culture system (Fig. 1, A and B). Considering the expression pattern of RACK1 during osteoclast formation, it is possible that RACK1 is also involved in the signaling pathways that participate in the resorption activity of osteoclasts. A number of studies have shown that the interaction of RACK1 with the β_1 and β_2 integrin subunits and Src promotes cell adhesion and cell spreading in cancer cells (27, 30, 51). Because the adhesion and spreading of osteoclasts is important for their resorption activity (2, 3), the role of RACK1 in regulating osteoclast function is worthy of further investigation in the future.

In conclusion, we propose that RACK1 is a scaffold protein in the p38 pathway, which links p38 to the RANKL signaling cascade. Because TRAF6 and TAK1 have diverse cellular functions (53), the direct targeting of TRAF6 or TAK1 may increase the risk of adverse effects, especially causing susceptibility to infections. Future studies to determine the molecular interactions between RACK1 and TRAF6, TAK1, as well as MKK6, could lead to new selective strategies for potential therapeutics that target bone loss.

Materials and Methods

Primary cells and cell lines

Bone marrow-derived macrophages (BMMs) from murine bone marrow precursors of 6- to 8-week-old C57BL/6 mice (The Jackson Laboratory) were prepared as described previously (54). RAW264.7 cells were used for the generation of stable cells expressing RACK1. HEK 293T cells were used for protein-protein interaction experiments. The generation of HEK 293-RANK stable cells was described previously (48).

Plasmids and RNA interference

The cDNA encoding HA-RACK1 in the pcDNA3.1 vector was provided by Michael J. Weber (University of Virginia Health System, USA). shRNA targeting human RACK1 was provided by Mutsuhiro Takekawa (University of Tokyo, Tokyo, Japan). The target sequences of human shRACK1 are as follows: 5'-gcttaaaaGGATGAGACCAACTATGGAATtctcttgaaATTCCATAGTTGGTCTCATCCggg-3'. The nucleotides in uppercase letters represent nucleotides 141 to 161 of the open reading frame of human *GNB2L1*, which forms a hairpin in the mRNA. The lowercase letters are the sequences recommended by the protocol for designing shRNA oligonucleotides (55). RNAi oligonucleotides targeting mouse PKC β (5'-TCTGCTGCTTTGTTGTACA-3') were synthesized by Bioneer and were subcloned into the retroviral shRNA vector pSuper-retro Puro (OligoEngine) according to the manufacturer's protocol (35). The cDNAs encoding wild-type (WT) MKK6 (MKK6-WT), MKK6-DN, MKK3-WT, and MKK3-DN were provided by H-H Kim (Seoul National University, Seoul, Korea). shRNA targeting mouse RACK1 (shRACK1 R3) was provided by Lalita Ramakrishnan (University of Washington). Recombinant retroviral vectors encoding HA-RACK1 and mouse shRACK1 were generated

by subcloning of the cDNAs encoding HA-RACK1 and shRACK1 R3 into the retroviral pMX-puro vector. The target sequences of mouse shRACK1 are as follows: 5'-gatccccGCAAGATCATTGTAGATGattcaagagaTCATCTACAATGATCTTGCTtttgaaa-3' (56). The 0.6-kb mouse *GNB2L1* promoter was amplified from mouse genomic DNA by PCR with the following primers. Sense: 5'-GAA GGT ACC CTT CGC TAC ATG AAA TTT TG-3'; antisense: 5'-CCC GTA CCT CGA GAC GCA GAC A GG ATC ATG T-3'. The PCR fragment was subcloned into the pGL3-Basic luciferase reporter vector (Promega). Mutation of the NF- κ B-binding site within the WT *GNB2L1* promoter luciferase reporter construct was generated through the Quick Change method of site-directed mutagenesis (Stratagene). The plasmids encoding RANK-E3A, TRAF6, and TAK1 were described previously (38). The siRNAs targeting mouse RACK1 and TAK1 were designed and synthesized by Bioneer (Taejon, Republic of Korea). The corresponding target mRNA sequences for the siRNAs are as follows. si-RACK1: 5'-GCAAGATCATTGTAGATGAuu-3' (56); si-TAK1: 5'-GGCAAAGCAACA GAGUGAAUCUGGAuu-3' (57); scrambled non-targeting siRNA (negative control): 5'-ACGTGACACGTTCCGGAGAAuu-3'. BMMs were transfected with the siRNAs (20 nmol) with Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. After transfection, the BMMs were cultured with M-CSF and RANKL for osteoclast generation or protein analyses.

Reagents

Recombinant human M-CSF and granulocyte macrophage colony-stimulating factor (GM-CSF) were purchased from R&D Systems Inc. RANKL was obtained from Peprotech EC. Anti-RACK1 antibody was obtained from BD Transduction Laboratories, and a monoclonal anti-Flag antibody (M2) and G α 6976 were obtained from Sigma-Aldrich. Antibodies specific for HA, Myc, GST, ubiquitin, TRAF6, NFATc1, PKC β , and actin were obtained from Santa Cruz Biotechnology Inc. Antibodies specific for p38, pp38, JNK, pJNK, ERK, pERK, I κ B, pMKK3/6, MKK6, TAK1, pTAK1, ATF2, and pATF2 were purchased from Cell Signaling Technology. The anti-puromycin antibody 12D10 was purchased from Millipore. Anti-Atp6v0d2 antibody was provided by Y. Choi (University of Pennsylvania, USA).

Generation of RAW264.7 cells that stably express RACK1

RAW264.7 cells were respectively transduced with the plasmid pMX-puro-HA-RACK or the empty plasmid pMX-puro by microporation (Invitrogen). Cells were selected in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and puromycin (4 μ g/ml). The puromycin-resistant clones were collected and examined by Western blotting analysis.

Osteoclastogenesis

Osteoclasts were generated from BMMs as described previously (42). In brief, isolated BMMs were treated for 3 to 4 days with M-CSF (30 ng/ml) in the presence or absence of RANKL (100 ng/ml). The cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) with a TRAP staining kit (Sigma). Pink-colored, TRAP-positive, multinucleated (>

3 nuclei) cells (MNCs) were counted as osteoclast-like cells. The values presented are the averages of two separate experiments, each performed in triplicate, $\pm$ the standard deviation (SD) of the means.

Transfections and protein analysis

Cells were transfected with expression plasmids with the calcium phosphate precipitation method. For protein interaction assays, HEK 293T cells were transfected with the combinations of expression plasmids indicated in the figure legends. The transfected cells were lysed and analyzed by Western blotting, as indicated in the figure legends. Equal amounts of cell lysates from the indicated time periods were subjected to Western blotting analysis with specific antibodies against p38, pp38, JNK, pJNK, ERK, pERK, I κ B, pMKK3/6, MKK6, TAK1, pTAK1, and β -actin. For immunoprecipitation analysis, cell lysates were subjected to immunoprecipitation with the antibodies indicated in the figure legends and then were analyzed by Western blotting, as described previously (58).

Retrovirus preparation

To prepare retroviruses, the PLAT-E packaging cell line was transfected with pMX-puro empty vector, pMX-puro-HA-RACK1, pMX-puro control shRNA, and pMX-puro-shRACK1 with Lipofectamine 2000 (Invitrogen). The retroviruses were used to infect BMMs as previously described (54, 58). The pMX-puro vector and PLAT-E cells were kindly provided by T. Kitamura (University of Tokyo, Tokyo, Japan). After retroviral infection, the BMMs were cultured overnight, detached with trypsin and EDTA, and further cultured in the presence of M-CSF (100 ng/ml) and puromycin (2 μ g/ml) for 2 days. Puromycin-resistant BMMs were induced to differentiate by culturing them with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for an additional 4 to 5 days, or were used for stimulation by RANKL, M-CSF, or LPS, followed by further analysis.

Protein synthesis assay

Protein synthesis assays were performed with the SUNSET method, as previously described (59). Briefly, transduced BMMs were incubated with puromycin (10 μ g/ml) in the presence or absence of cycloheximide (CHX) for 30 min. For RANKL stimulation, cells were starved for 4 hours and then stimulated with RANKL (200 ng/ml), which was added 10 min before the cells were treated with puromycin (10 μ g/ml). The cells were incubated for an additional 20 min before they were harvested. Puromycin-treated samples were identified by Western blotting analysis with an anti-puromycin antibody.

Quantitative real-time PCR (qPCR) analysis

BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for the times indicated in the figure legends. Total RNAs from the cultured cells were extracted with TRIzol (Kakara). 5 μ l of complementary DNA (cDNA) template (1/20 dilution) was used for quantitative real-time polymerase chain reaction (qPCR) assays, which were performed with the KAPA SYBR FAST Universal qPCR Kit (green) (Kapa Biosystems) and an ABI Prism 7300 sequence detection system (Applied Biosystems). Real-time PCR primers were as follows. RACK1 sense: 5'-GCC TCT GGG ATC TCA CAA C-3'; RACK1 antisense: 5'-

AAC TTT ATG GTC TTG TCT CGG G-3'; NFATc1 sense: 5'-CAA GTC TCT TTC CCC GAC ATC-3'; NFATc1 antisense: 5'-CTG CCT TCC GTC TCA TAG TG-3'.

Luciferase assays

RAW264.7 cells were transfected with the RACK1 luciferase reporter constructs with Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. Six hours after transfection, the cells were treated with RANKL (100 ng/ml). After an additional 36 hours, the cells were lysed in reporter lysis buffer. Luciferase activity was measured with a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. For each sample, the firefly luciferase activity was corrected by *Renilla* luciferase activity to account for differences in cell numbers and transfection efficiency.

Survival assays for bone marrow-derived dendritic cells (BMDCs) and measurement of cytokines

BMDCs were derived from BM suspensions prepared from femurs and tibiae as previously described (42). On day 7 of culture, BMDCs were transfected with control scrambled siRNA or RACK1-specific siRNA with lipofectamin RNAiMAX (Invitrogen). Twenty-four hours later, the transfected BMDCs were washed and were left untreated or were stimulated with RANKL (400 ng/ml) in the presence or absence of OPG (100 ng/ml). BMDC survival was quantified by the LIVE/DEAD reduced-biohazard viability/cytotoxicity test (Invitrogen). Staining and analysis were performed as recommended by the manufacturer. Mouse IL-6 and IL-12p40 in culture media were measured with ELISA kits obtained from BD OptEIA systems according to the manufacturer's instructions.

RANKL-induced bone destruction

All animal study protocols were approved by the Animal Care Committee of Ewha Laboratory Animal Genomics Center. Either 30 μ l of control scrambled siRNA or RACK1-specific siRNA, mixed with 10 μ l of Lipofectamine RNAiMAX (Invitrogen) or an equal volume of PBS, was injected onto the calvariae of six-week-old C57BL/6 mice three times at 2-day intervals. One day after the first injection, RANKL (2 mg/kg body weight) or an equal volume of PBS was injected onto the calvariae twice, at 2-day intervals. One day after the final injection, all of the mice were sacrificed and analyzed as previously described (54). The numbers of osteoclasts per millimeter of calvarial bone surface and the eroded surface per bone surface (ES/BS, %) were determined with Osteomeasure XP version 1.2 software (OsteoMetrics Inc.).

Statistical analysis

Results are expressed as means $\pm$ SD from at least three independent experiments. Values with $P < 0.05$ were considered statistically significant as determined by a two-tailed Student's *t* test (SigmaPlot version 12; Systat). Three or more comparisons were analyzed by one way analysis of variance (ANOVA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. RACK1 selectively mediates the RANKL-stimulated activation of p38 MAPK

(A) BMMs were transduced with the pMX-puro retrovirus (control) or with retrovirus expressing HA-tagged RACK1 (RACK1). The transduced BMMs were then serum-starved overnight, incubated in the absence or presence of RANKL (200 ng/ml) for the indicated times, and analyzed by Western blotting with antibodies specific for the indicated proteins. Right: The ratios of pp38 to total p38, pERK to total ERK, and pJNK to total JNK were quantified from three independent experiments. * $P < 0.05$. ns, not significant. (B) BMMs were transduced with retroviruses expressing scrambled shRNA (control) or RACK1-specific shRNA (shRACK1). The transduced BMMs were then serum-starved overnight, incubated in the absence or presence of RANKL (200 ng/ml) for the indicated times, and analyzed by Western blotting with antibodies against the indicated proteins. Western blots were first analyzed to detect phosphorylated forms of p38, ERK, and JNK. The blots were

then stripped and analyzed for the presence of total p38, ERK, and JNK proteins. (**C to E**) BMMs were transduced with the pMX-puro retrovirus (control) or with retrovirus expressing HA-tagged RACK1 (RACK1). The transduced BMMs were then serum-starved overnight, incubated with or without (C) M-CSF (30 ng/ml), (D) LPS (100 ng/ml), or (E) IL-1 β (10 ng/ml) for the indicated times, and analyzed by Western blotting with antibodies specific for the indicated proteins. Western blots in all panels are representative of three independent experiments.

Fig. 2. Analysis of the role of RACK1 in the RANKL-induced formation of osteoclasts

(A to C) BMMs were transduced with the pMX-puro retrovirus (control) or with retrovirus expressing HA-tagged RACK1 and then were cultured for 3 to 4 days in the presence of M-CSF and RANKL. (A) Cultured cells were fixed and stained for TRAP. Scale bar: 200 μ m. (B) The numbers of TRAP⁺ MNCs (> 3 nuclei) were counted. Data are means $\pm$ SD of three independent experiments. * P < 0.01 versus control. (C) Cells were harvested and lysates were analyzed by Western blot analysis with antibodies specific for the indicated proteins. (D to F) BMMs were transduced with retroviruses expressing control shRNA (control) or RACK1-specific shRNA (shRACK1) and then were cultured for 4 to 5 days in the presence of M-CSF and RANKL. (D) Cultured cells were fixed and stained for TRAP. Scale bar: 200 μ m. (E) The numbers of TRAP⁺ MNCs (> 3 nuclei) were counted. Data are means $\pm$ SD of three independent experiments. * P < 0.01 versus control. (F) Cells were harvested and lysates were analyzed by Western blot analysis with antibodies specific for the indicated proteins. Images in (A) and (D) and Western blots in (C) and (F) are representative of three independent experiments.

Fig. 3. RACK1 associates with RANK in a TRAF6-dependent manner

(A) HEK 293T cells were transfected with plasmid encoding HA-RACK1 alone or together with plasmids encoding the indicated Flag-tagged TRAF molecules. Cell lysates were then subjected to immunoprecipitation (IP) with an anti-Flag antibody followed by Western blotting with antibodies against the indicated proteins. Right: The expression of the indicated plasmids was confirmed by Western blot analysis of the cell lysates (Input). (B) HEK 293T cells were transfected with the indicated combinations of plasmids encoding HA-TRAF6, HA-RACK1, and Flag-tagged WT RANK or the E3A mutant RANK. Cell lysates were then subjected to immunoprecipitation (IP) with an anti-Flag antibody followed by Western blotting with antibodies against the indicated proteins. Right: The expression of the indicated plasmids was confirmed by Western blot analysis of the cell lysates (Input). (C) RAW264.7 cells were serum-starved before being left unstimulated or stimulated with

RANKL (400 ng/ml) for the indicated times. Cell lysates were subjected to immunoprecipitation with an anti-TRAF6 antibody and Western blotting analysis with anti-RACK1 and anti-TRAF6 antibodies. Right: The ratios of RACK1 to TRAF6 proteins at each time were quantified from three independent experiments. $*P < 0.05$. Western blots in (A) to (C) are representative of three independent experiments.

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Fig. 4. RACK1 interacts with the active form of TAK1

(A) HEK 293T cells were transfected with the indicated combinations of plasmids. Top: Cell lysates were prepared and subjected to immunoprecipitation (IP) with an anti-HA antibody, followed by Western blotting analysis with antibodies against the indicated proteins. Bottom: The expression of the indicated plasmids was confirmed by Western blotting analysis of the cell lysates (Input). Note that overexpression of TAK1 and TAB1 resulted in production of the active form of TAK1, which was detectable as pTAK1 (lane 4). (B) RAW264.7 cells were serum-starved before being left unstimulated or stimulated with RANKL (400 ng/ml) for 5 min. Cell lysates were then prepared and subjected to immunoprecipitation (IP) with anti-TRAF6 or control IgG antibodies, followed by Western blotting analysis with antibodies specific for RACK1, total TAK1, and pTAK1. Western blots in (A) and (B) are representative of three independent experiments.

Fig. 5. The interaction between RACK1 and MKK6 is required for enhancing the RANKL-dependent activation of p38 MAPK

(A) BMMs were transfected with control siRNA or RACK1-specific siRNA and then were cultured for 2 days. The transfected BMMs were serum-starved for 6 hours and then left unstimulated or were stimulated with RANKL for 10 min. Whole-cell lysates were analyzed by Western blotting with antibodies specific for the indicated proteins. Right: The ratios of pMKK3/6 to total MKK6 proteins for each condition were quantified from three independent experiments. $*P < 0.05$. (B) HEK 293T cells were cotransfected with plasmids encoding HA-RACK1 together with plasmids encoding the indicated Flag-tagged MKK6 or MKK3 constructs. Samples were then subjected to immunoprecipitation (IP) with an anti-Flag antibody and Western blotting analysis with antibodies against the indicated proteins. (C) Tetracycline-dependent, RANK-expressing HEK 293 cells (293-RANK) were transfected with plasmids encoding Flag-tagged RACK1 and MKK6(DN) or MKK3(DN) constructs, as indicated, and then were incubated with tetracycline (1 $\mu\text{g/ml}$) for 24 hours. The cells were then serum-starved for 6 hours before being left unstimulated or stimulated with RANKL (200 ng/ml) for 15 min. Whole-cell lysates were analyzed by Western blotting

with antibodies specific for the indicated proteins. Bottom: The ratios of pp38 to total p38 proteins were quantified from three independent experiments. * $P < 0.05$. **(D)** Tetracycline-dependent, RANK-expressing HEK 293 cells (293-RANK) were transfected with scrambled shRNA (control) or RACK1-specific shRNA (shRACK1), with or without MKK6-encoding plasmid, as indicated. Whole-cell lysates were analyzed by Western blotting with antibodies specific for the indicated proteins. Western blots in (A) to (D) are representative of three independent experiments.

Fig. 6. RACK1 is required for the association of the TRAF6-TAK1 complex with MKK6
(A) HEK 293T cells were transfected with the indicated combinations of expression plasmids. Whole-cell lysates were subjected to immunoprecipitation (IP) with an anti-Myc antibody and Western blotting analysis with antibodies specific for the indicated proteins. Right: The expression of the indicated constructs was confirmed by Western blotting analysis of the cell lysates (Input). **(B)** BMMs transfected with control siRNA or RACK1-specific siRNA (siRACK1) were cultured for 2 days before being left unstimulated or stimulated with RANKL (200 ng/ml) for 10 min. Whole-cell lysates were subjected to immunoprecipitation (IP) with an anti-TRAF6 antibody, and then were analyzed by Western blotting with antibodies specific for the indicated proteins. Bottom: The ratios of MKK6 to TRAF6 proteins were quantified from three independent experiments. * $P < 0.05$. Right: The expression of the indicated constructs was confirmed by Western blotting analysis of whole-cell lysates (Input). **(C)** BMMs transfected with control siRNA or TAK1-specific siRNA were cultured for 2 days before being left unstimulated or stimulated with RANKL (200 ng/ml) for 10 min. Whole-cell lysates were subjected to immunoprecipitation (IP) with an anti-TRAF6 antibody, and then were analyzed by Western blotting with antibodies specific

for the indicated proteins. Right: The expression of the indicated constructs was confirmed by Western blotting analysis of whole-cell lysates (Input). Western blots in (A) to (C) are representative of three independent experiments.

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A

B

C

Fig. 7. Knockdown of RACK1 attenuates RANKL-induced osteoclast formation and bone loss in mice

(A) Mice calvariae were injected with PBS, control siRNA, or RACK1-specific siRNA as described in the Materials and Methods. TRAP staining was performed on sections of calvarial bone. (B and C) The number of osteoclasts (B) and the extent of the eroded surface (C) were analyzed by bone morphometric analyses with OsteoMeasure software. Data are means $\pm$ SD from five mice for each condition. $n = 5$. The experiments were performed twice with similar results. Scale bar: 800 μ m. * $P < 0.01$, ** $P < 0.05$ versus control.