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

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The E3 ubiquitin ligase TRAF6 [tumor necrosis factor (TNF) receptor (TNFR)–associated factor 6] and the associated kinase TAK1 [transforming growth factor–β (TGF-β)–activated kinase 1] are key components of the signaling pathways that activate nuclear factor–κB (NF–κB) and mitogen-activated protein kinases (MAPKs) in response to various stimuli. The cytokine RANKL (receptor activator of NF–κB ligand) is essential for the differentiation of bone marrow cells into bone-resorbing osteoclasts through the activation of NF–κB 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 (MAPK kinase kinase 6), 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 was 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 small interfering RNA (siRNA) into mice calvariae reduced the RANKL-induced bone loss 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 (MNCs) 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 factor–κB (NF–κB) 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] 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, Lys63-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 κB (IκB) 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–κB and MAPK signaling pathways results in the increased abundance of the transcription factor nuclear factor–κB (NFκB) and the expression of its target genes, including those encoding cathepsin K, osteoclast-associated receptor (OSCAR), the v-ATPase V0 subunit δ2 (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 p38α, 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 p38α and p38β, or expression of dominant-negative forms of p38α and MKK6 (MAPK kinase kinase 6) 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-Asp(WD40) repeat protein family, and has homology with the G protein (heterotrimeric guanine nucleotide–binding 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 the phosphodiesterase PDE4D5 isofrom, as well as the cytoplasmic domains of several membrane-bound receptors, including integrin β subunits (such as β1, β2, and β3), the N-methyl-D-aspartic acid (NMDA) receptor, and insulin-like growth factor receptor 1 (IGF-1R), 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 MAPK

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kinase (MEK)–ERK signaling (30); however, whether RACK1 is involved in RANKL signaling is unknown.

We found that RACK1 is 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-κB 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 IkBα. 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 IkBα were observed between the control and RACK1-overexpressing BMMs. These results were further confirmed in experiments with stable RAW264.7 cells 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), was maximal at 15 min after stimulation with RANKL and gradually declined thereafter (Fig. S2).

Because the stimulation of BMMs with M-CSF or lipopolysaccharide (LPS) also activates MAPKs and NF-κB (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-κB. As expected, stimulation of BMMs with M-CSF activated MAPKs, but not NF-κB (Fig. 1C), whereas stimulation with LPS activated MAPKs and NF-κB (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-κB (Fig. 1, C and D). Similarly, the protein kinase cascades initiated by IL-1β 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, PKCβ stimulates the formation of osteoclasts (34, 35). Therefore, we investigated whether RACK1 was linked to PKCβ in RANKL signaling. Consistent with a previous report (29), we found that exogenous RACK1 interacted with PKCβ in human embryonic kidney (HEK) 293T cells (Fig. S3A). However, immunoprecipitation assays showed that endogenous RACK1 did not associate with PKCβ in response to stimulation of RAW264.7 cells with RANKL (Fig. S3B). Furthermore, neither knockdown of PKCβ nor pharmacological inhibition of PKC had any apparent effect on the RANKL-dependent activation of MAPKs or NF-κB (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-κB–binding site of this promoter was mutated (fig. S5D), suggesting that RACK1 is a direct transcriptional target of NF-κB 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 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+ 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 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 cotransfected with plasmids encoding HA-RACK1 and Flag-tagged TRAF family members, including TRAF2, TRAF5, and TRAF6. RACK1 bound most abundantly to TRAF6 and with RACK1 (Fig. 3B). The 0.6-kb binding site of this promoter was mutated (fig. S5D), linked to a luciferase reporter construct, is activated in response to RANKL, whereas this induction was abolished when the putative NF-κB–binding site of this promoter was mutated (fig. S5D), suggesting that RACK1 is a direct transcriptional target of NF-κB during osteoclastogenesis.
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. 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-1β (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.
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 coimmunoprecipitation analysis of transfected HEK 293T cells. RACK1 interacted with the TRAF6^DN 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^DC 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 (ΔWD2) or the WD6 domain (ΔWD6) showed reduced association with TRAF6 (fig. S12C). Consistent with these findings, the RACK1 WD2^6 mutant, which contains the WD2 and WD6 domains of RACK1, interacted with TRAF6, whereas the RACK1 ΔWD2,6 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 coimmunoprecipitation 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 TAB1-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 MAPKs, 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 coimmunoprecipitation 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 was not detected (Fig. S13).

We next investigated whether the interaction between RACK1 and MKK6 affected the activation of p38. Overexpression of MKK1 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 coimmunoprecipitated 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 coimmunoprecipitation 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 coimmunoprecipitate 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

Osteoclasts differentiate from precursor cells of the monocyte and macrophage lineage, which also give rise to dendritic cells (DCs) (I). In addition,
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 was 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-κB 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-1β, 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-1β 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-1β, 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 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 to activate the p38 pathway.
signals leading to osteoclastogenesis (50). On the basis of 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-κB 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-κB 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 β1 and β2 integrin subunits and Src promotes cell adhesion and cell spreading in cancer cells (27, 30, 51). Because the adhesion and spreading of osteoclasts are important for their resorption activity (2, 3), the role of RACK1 in regulating osteoclast function is worthy of further investigation in the future.
The experiments were performed twice with similar results. Scale bars, 800 versus control. Analyses with OsteoMeasure software. Data are means ± SD from five mice for each condition. Of osteoclasts (OCs) (B) and the extent of the eroded surface (C) were analyzed by bone morphometric vector was provided by M. J. Weber (University of Virginia Health System).

The complementary DNA (cDNA) encoding HA-RACK1 in the pcDNA3.1 (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).

shRNA targeting human RACK1 was provided by M. Takekawa (University of Tokyo). The target sequences of human shRACK1 are as follows: 5′-gcttaaaaaGG-ATGAGACCAACATATGGAAACATctctggaat-ATTCCCATGTTGTCATCCGggg-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). RNA interference (RNAi) oligonucleotides targeting mouse PKCβ (5′-TCTGGTGTTCCTTGGTGTACA-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 MKK6 (MKK6-WT), MKK6-DN, MKK3-WT, and MKK3-DN were provided by H.-H. Kim (Seoul National University). shRNA targeting mouse RACK1 (shRACK1 R3) was provided by L. 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′-cataccccGCAAGATCATCATGTGATTTCatagaaTGAATCATTGGTGTTTTTG-3′ (56). The 0.6-kb mouse GNB2L1 promoter was amplified from mouse genomic DNA by polymerase chain reaction (PCR) with the following primers: sense: 5′-GAAAGTTACCCTCTTGCTTACATGAAATTTTG-3′ and antisense: 5′-CCTGCTGACGCAAAGACGACGACGACGTATTGTTTTTG-3′. The PCR fragment was subcloned into the pGL3-Basic luciferase reporter vector (Promega). Mutation of the NF-κB-binding site within the wild-type GNB2L1 promoter luciferase reporter construct was generated through the QuickChange 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. The corresponding target mRNA sequences for the siRNAs are as follows: si-RACK1: 5′-GCAAGATCATCATGTGATTTCatagaaTGAATCATTGGTGTTTTTG-3′ (56), si-TAK1: 5′-GCAAGATCATCATGTGATTTCatagaaTGAATCATTGGTGTTTTTG-3′ (56), and scrambled nontargeting siRNA (negative control): 5′-ACGTGACACGTTCGGAGAAuu-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

MATERIALS AND METHODS

Primary cells and cell lines 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 complementary DNA (cDNA) encoding HA-RACK1 in the pcDNA3.1 vector was provided by M. J. Weber (University of Virginia Health System).
antibody (M2) and Gö6976 were obtained from Sigma-Aldrich. Antibodies specific for HA, Myc, glutathione S-transferase, ubiquitin, TRAF6, NFATc1, PKCβ, and actin were obtained from Santa Cruz Biotechnology Inc. Antibodies specific for p38, pp38, JNK, pJNK, ERK, pERK, IκB, 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 previously described (54).

**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 microinjection (Invitrogen). Cells were selected in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and puromycin (4 μg/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 TRAP with a TRAP staining kit (Sigma). Pink-colored, TRAP⁺ MNCs were counted as osteoclast-like cells. The values presented are the averages of two separate experiments, each performed in triplicate, ± 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, IκB, 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 Platinum-E (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 provided by T. Kitamura (University of Tokyo). After retroviral infection, Puromycin-resistant BMMs were induced to differentiate by culturing them with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 2 days. Puromycin-resistant BMMs were transduced with the plasmid pMX-puro empty vector, pMX-puro HA-RACK1, or the empty plasmid pMX-puro by microporation (Invitrogen). Cells were selected in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and puromycin (4 μg/ml). The puromycin-resistant clones were collected and examined by Western blotting analysis with the antibodies indicated in the figure legends, and then were analyzed by Western blotting, as previously described (54).

** 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 DCs and measurement of cytokines**

Bone marrow–derived DCs (BMDCs) were derived from bone marrow 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 Lipofectamine 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-12 p40 in culture medium were measured with BD OptEIA enzyme-linked immunosorbent assay (ELISA) kits 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 μl of control scrambled siRNA or RACK1-specific siRNA, mixed with 10 μl of Lipofectamine RNAiMAX (Invitrogen), or an equal volume of PBS was injected onto the calvariae of 6-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 ± 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 pairs were analyzed by one-way analysis of variance (ANOVA).

SUPPLEMENTARY MATERIALS
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Fig. S1. RACK1 is required for the RANKL-dependent activation of p38 MAPK.
Fig. S2. Effects of RACK1 knockdown on RANKL-stimulated signaling in BMMs.
Fig. S3. PKCδ has no effect on RANKL signaling.
Fig. S4. Knockdown of RACK1 has no effect on protein synthesis in BMMs.
Fig. S5. RACK1 abundance is increased during RANKL-dependent osteoclastogenesis.
Fig. S6. An increase in the abundance of RACK1 has no effect on the proliferation of BMMs.
Fig. S7. The increased abundance of RACK1 enhances osteoclast formation and the expression of marker genes.
Fig. S8. The p38 inhibitor SB203583 counteracts the effects of RACK1 on NFATc1 abundance and osteoclast formation.
Fig. S9. Knockdown of RACK1 does not affect the proliferation of BMMs.
Fig. S10. Knockdown of RACK1 inhibits osteoclast formation.
Fig. S11. Effects of RACK1 knockdown on osteoclast formation and cell viability.
Fig. S12. Mapping of the RACK1 and TRAF6 interaction regions.
Fig. S13. RACK1 does not interact with p38α.
Fig. S14. Effects of RACK1 knockdown on the association of TRAF6 with TAK1 and MKK6.
Fig. S15. Effects of RACK1 knockdown on BMDc survival and cytokine production.

REFERENCES AND NOTES

Acknowledgments: We thank T. Kitamura (University of Tokyo, Tokyo, Japan) for the Plat-E cells and pMX vectors. We thank M. J. Weber (University of Virginia Health System, USA), M. Takekawa (University of Tokyo, Tokyo, Japan), H.-H. Kim (Seoul National University, Seoul, South Korea), and L. Ramakrishnan (University of Washington, USA) for sharing plasmids and antibody. We thank J. Kim (Ewha Womans University, Seoul, South Korea) for the critical review of the manuscript. Funding: This work was supported by the National Research Foundation of Korea (grants funded by the Government of South Korea [Ministry of Science, ICT and Future Planning (MSIP)] (nos. 2013R1A2A1A05005153, 2012R1A5A1048236, and 2012M3A9C5048708) and the National Research Council of Science and Technology through the Degree and Research Center program (DRC-14-3-KBSI). Y.C. was supported in part by grants from the NIH (AI064909 and AI08627). Author contributions: J.L. designed and performed experiments, analyzed data, and contributed to the writing of the manuscript; D.L. and Y.C. designed the study and analyzed data; and S.Y.L. designed the study, analyzed data, and wrote the manuscript. Competing interests: The authors declare that they have no competing interests.

Submitted 2 September 2014 Accepted 14 May 2015

The scaffold protein RACK1 mediates the RANKL-dependent activation of p38 MAPK in osteoclast precursors
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Sci. Signal. 8 (379), ra54.
DOI: 10.1126/scisignal.2005867

Directing osteoclast precursors with RACK
Bone density depends on the balance between bone-forming osteoblasts and bone-resorbing osteoclasts; excessive bone resorption leads to osteoporosis. The cytokine RANKL (receptor activator of NF-κB ligand), which activates multiple members of the mitogen-activated protein kinase (MAPK) family, stimulates the production of osteoclasts. Lin et al. found that precursor cells exposed to RANKL recruited the scaffold protein RACK1 (receptor for activated C kinase 1) to the RANKL receptor complex, thereby directing the signal to proceed through a kinase that activates p38 MAPK, the MAPK family member required for osteoclast formation. A portion of mouse skulls in which RACK1 was reduced had reduced osteoclasts and less bone loss in response to RANKL. Thus, RACK1 directs RANKL signaling to activate the MAPK necessary to specify osteoclasts, providing a potential therapeutic target for the treatment of osteoporosis.

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