A Novel Receptor Activator of NF-κB (RANK) Cytoplasmic Motif Plays an Essential Role in Osteoclastogenesis by Committing Macrophages to the Osteoclast Lineage*

Receptor activator of nuclear factor κB (RANK), also known as OPG ligand (OPGL)/osteoprotegerin (OPG) ligand/ODF (osteoclast differentiation factor)/TRANCE (tumor necrosis factor (TNF)-related activation-induced cytokine), was identified as a member of the TNF superfamily independently by two bone groups (1, 2) and two immunology groups (3, 4) in the late 1990s. To date, RANKL has been shown to play pivotal roles in regulating various biological processes such as bone homeostasis (2, 5), immune function (3, 6) and mammary gland development (7).

In bone, RANKL is not only an essential regulator of osteoclast formation (osteoclastogenesis) but also a potent modulator of osteoclast function and survival (5, 8, 9). RANKL exerts its effects on osteoclast function and formation by binding to its receptor RANK (receptor activator of nuclear factor κB) expressed on osteoclast precursors and mature osteoclasts (1, 10, 11). RANKL also has a decoy receptor, OPG, which inhibits RANKL action by competing with RANK for binding RANKL (5, 12). The essential role of both RANKL and RANK in the osteoclastogenic process has been unambiguously demonstrated by the findings that mice lacking the gene for either protein develop osteoporosis due to failure to form osteoclasts (13–15).

Because the unraveling of the RANKL/RANK system, enormous efforts have been focused on elucidating RANK-initiated intracellular signaling. RANK was identified as a member of the TNF receptor (TNFR) family (3). Members of the TNFR family lack intrinsic enzymatic activity, and hence, they transduce intracellular signals by recruiting various adaptor proteins including TNF receptor-associated factors (TRAFs) through specific motifs in the cytoplasmic domain (16, 17). Six TRAF proteins (TRAF1, -2, -3, -4, -5, and -6) have been identified so far, and they play important but distinct roles in TNFR member-induced intracellular signaling (17). Numerous in vitro studies demonstrated that RANK indeed interacts with various TRAF proteins (10, 18–22). Our recent functional study revealed that RANK contains three functional TRAF-binding sites (669PFQEP673, 559PVQET564, and 604PVQEQG609) that redundantly play a role in osteoclast formation and function (23). In particular, the 366PVQEP73 site mediates osteoclastogenesis by recruiting TRAF6 (24). Collectively, through these functional TRAF binding motifs, RANK activates six major signaling pathways NF-κB, JNK, ERK, p38, NFATc1, and Akt, which play important roles in osteoclast formation, function and/or survival (8, 9, 23, 25).

However, several lines of evidence suggest that RANK may also activate novel pathways to regulate osteoclastogenesis. For example, although it has been established that both RANK and interleukin 1 (IL-1) receptor utilize TRAF6 to activate intracellular signaling pathways (26), administration of IL-1 to RANK−/− mice fails to promote osteoclast formation (15). The complete absence of osteoclasts in the RANK−/− mice administrated with IL-1 suggests that RANK activates other novel signaling pathways essential for osteoclastogenesis. Similarly, two other reports demonstrated that IL-1 fails to stimulate osteoclastogenesis in vitro in the presence of M-CSF (27, 28). Furthermore, RANK has a very long cytoplasmic domain (mouse RANK is 391-aa long, and human RANK is 383-aa long) that shares no homology with any known members of the TNFR family (3), suggesting that it may activate downstream signals different from those arising from its cousins.

In the present study we set out to identify the novel RANK signaling pathways essential for osteoclastogenesis by carrying out a detailed structure/function study of the RANK cytoplasmic domain. Using numerous internal deletion and point mutation constructs, we have identified a novel RANK motif (235VYV258) that is essential for osteoclastogenesis. We further demonstrate that this novel RANK motif plays a crucial role in osteoclastogenesis by committing macrophages to the osteoclast lineage. In line with this notion, this RANK motif is not implicated in osteoclast function or survival. In addition, we have also shown that this motif is not involved in activating the signaling path-
Novel RANK Motif Essential for Osteoclastogenesis

The RANK (Receptor Activator of NF-κB) receptor is critical for osteoclastogenesis. A novel RANK motif mediates novel signaling pathways, essential for osteoclastogenesis. This motif mediates pathways that may serve as specific and potent therapeutic targets for bone disorders such as postmenopausal osteoporosis, inflammatory bone loss, and tumor-induced osteolysis.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Chemicals were purchased from Sigma unless indicated otherwise. Synthetic oligonucleotides were purchased from Sigma Genosys (The Woodlands, TX). Bacterial was from EMD Biosciences, Inc. (San Diego, CA). Antibodies against the external domain of mouse TNFR1 (for flow cytometry) (TNF-R1, sc-12746PE) and c-fos (sc-253) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant mouse TNFα (410-TRNC-050) was from R&D Systems (Minneapolis, MN). The following antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA): antibodies against IκBα (#9242), phospho-IκBα (#9241), p44/42ERK (#9102), phospho-p44/42ERK (#9101), JNK (#9252), phospho-JNK (#9251), p38 (#9212), phospho-p38 (#9211), Akt (#9272), and phospho-Akt (#9271).

Preparation of Retrovirus and Infection of Bone Marrow Macrophages (BMM) — We used the chimeric receptor construct (pMX-puro-TNFRI-RANK) and the retrovirus system described in Liu et al. (23) for the present study. Plat-E cells were cultured in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum supplemented with puromycin and lactic acid as previously described (29). pMX-puro-TNFRI-RANK and its various deletion/point mutation mutants were transiently transfected into Plat-E cells using Lipofectamine Plus reagent (Invitrogen). Virus supernatant was collected at 48, 72, and 96 h after transfection.

Bone marrow cells were isolated from long bones of 4–8-week-old TNFR1−/−R2−/− double knock-out mice (The Jackson Laboratory, Bar Harbor, ME) or wild-type mice (Harlan Industries, Indianapolis, IN) as described (30). BMMs were prepared by culturing isolated bone marrow cells in α-minimal essential medium containing 10% heat-inactivated fetal bovine serum supplemented with puromycin and lactic acid as previously described (29). pMX-puro-TNFRI-RANK and its various deletion/point mutation mutants were transiently transfected into Plat-E cells using Lipofectamine Plus reagent (Invitrogen). Virus supernatant was collected at 48, 72, and 96 h after transfection.

In Vitro Osteoclastogenesis Assays — Retrovirally infected BMMs were cultured in 24-well tissue culture plates (5 × 10^5 cells/well) in α-minimal essential medium containing 10% heat-inactivated fetal bovine serum in the presence of 0.02 volume of culture supernatant of M-CSF-producing cells for 2 days as previously described (31). Cells were then infected with virus for 24 h in the presence of 0.01 volume of culture supernatant of M-CSF-producing cells and 8 μg/ml Polybrene. Cells were further cultured in the presence of M-CSF and 2 μg/ml puromycin for selection and expansion of transduced cells. Selected cells were subsequently used for various studies.

In Vitro Osteoclastogenesis Assays — Retrovirally infected BMMs were cultured in 24-well tissue culture plates (5 × 10^5 cells/well) in α-minimal essential medium containing 10% heat-inactivated fetal bovine serum in the presence of 0.02 volume of culture supernatant of M-CSF-producing cells (final M-CSF concentration is 44 ng/ml) and 100 ng of glutathione S-transferase-RANKL (32). In osteoclast formation assays involving the use of the chimeric receptor, TNFα was added at concentrations as indicated in individual assays. Osteoclasts began to form on day 3, and cultures were stained for tartrate-resistant acid phosphatase activity on day 6 using a commercial kit (Sigma, 387-A).

In Vitro Bone Resorption and Osteoclast Survival Assays — To perform bone resorption assays, osteoclasts were generated on bovine cortical bone slices from infected or uninfected BMMs with the stimulation of M-CSF (44 ng/ml) and RANKL (100 ng/ml) for 4 days. After osteoclasts were formed, the cultures were then treated with different factors as indicated in the individual assays and then continued for 3 more days. Then, bone slices were harvested, and cells were removed from the bone slices with 0.25 M ammonium hydroxide and mechanical agitation. Bone slices were then subjected to scanning electron microscopy. The data were quantified by counting and statistically analyzing pits in 5 random resorption areas (at 30 × magnification) and by measuring the percentage of the pits areas in 4 random resorption sites. The percentage of the pits area was determined using Metamorph software from Molecular Devices Corporation (Downingtown, PA).

The osteoclast survival assays were performed by generating osteoclasts in tissue culture dishes from infected or uninfected BMMs with the stimulation of M-CSF (44 ng/ml) and RANKL (100 ng/ml) for 4 days. After osteoclasts were formed, the cultures were then treated with different factors as indicated in the individual assays and then continued for 6 more hours. The cultures were then stained for tartrate-resistant acid phosphatase activity using a commercial kit (Sigma, 387-A). Survived osteoclasts were determined as those cells with strong tartrate-resistant acid phosphatase activity and intact plasma membrane.

Flow Cytometric Analysis — Infected BMMs (up to 1 × 10^6 cells) were suspended in 200 μl phosphate-buffered saline/azide. Cells were then blocked with 1 μg of 2.4G2 antibody (33) for 30 min on ice. Under dim light, 20 μl of TNFR1 antibody conjugated with phycoerythrin (Santa Cruz, sc-12746PE) or control IgG was added to the cell suspension, and cells were incubated on ice for 30 min. Cells were washed twice with 1 ml of cold phosphate-buffered saline (PBS)/azide and resuspended in 300 μl of cold PBS/azide. 200 μl of cold 0.5% parafomaldehyde solution was added to fix the cells. Flow cytometric analysis was performed using a BD Biosciences FACScan (BD Biosciences Immunocytometry Systems, Mountain View, CA).

Construction of Deletion Mutants and Mutagenesis — Construction of internal deletion was performed based on a method described in by Barnhart (34). Briefly, to delete a region in the RANK cytoplasmic tail in TNFR1-RANK chimera, we used SK-TNFRI-RANK plasmid described in our previous study (23) as the template and two primers to perform a PCR with Pfu polymerase (high fidelity polymerase). Both primers contain a Mlu restriction site at its 5’ end and a sequence complementary to a desired region of the template. The PCR reaction generated a linear full-plasmid-length product lacking the desired sequence, and the product contained a Mlu site at each of its ends. The PCR product was cut with MluI, religated, and sequenced to confirm that no mutations were introduced during PCR amplification. Then, the TNFR1-RANK construct containing the internal deletion was cut out and subcloned into pMX-puro retrovirus vector.

Mutations were generated in SK-TNFRI-RANK using the Quick-Change™ Site-directed mutagenesis kit (Stratagene). The mutated sites were confirmed by sequencing, and other regions in the chimera cDNA were sequenced to confirm that no mutations were introduced by PCR amplification during the mutagenesis. The mutant chimera cDNAs were then subcloned into pMX-puro plasmids.

Western Analysis — BMMs infected with retrovirus or control BMMs (uninfected) were cultured in serum-free α-minimal essential medium in the absence of M-CSF for 16 h for IκB, JNK, ERK, and p38 assays or 4 h for Akt assay before treatment with RANKL or TNF-α for various times as indicated in individual experiments. Cells were washed twice with ice-cold phosphate-buffered saline and then lysed in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM NaF, and 1× protease inhibitor mixture 1 (Sigma, P-2850) and 1× protease inhibitor mixture 2 (Sigma, P-5726). Lysates were then subjected to Western analysis as described in our previous study (23). Membranes were washed extensively, and an
FIGURE 1. Osteoclastogenesis assays with 20 deletion mutants. A, schematic diagrams of 20 deletion mutants, D1–D20. Ext, external domain; TM, transmembrane domain; WT, wild-type chimeric receptor. Regions deleted in each mutant are indicated in parentheses. B, osteoclastogenesis assays with the mutants. TNFR1L2/H11002/H11002R2/H11002 BMMs were either uninfected or infected with virus encoding WT chimera/mutants (D1–D20) and were selected with puromycin for 2 days. Then uninfected BMMs or infected BMMs were treated with M-CSF (44 ng/ml) and TNF-α (10 ng/ml). Osteoclasts began to form at day 3, and the cultures were stained for tartrate-resistant acid phosphatase activity at day 6. C, flow cytometric analysis showing the surface expression of the chimeras on infected BMMs. The flow cytometric analysis was performed using TNFR1 antibody conjugated with phycoerythrin (Santa Cruz, sc-12746PE). Uninfected BMMs were used as the control.
enhanced chemiluminescence (ECL) detection assay was performed using SuperSignal West Dura kit from Pierce. Quantification of the blots was performed using ImageJ analysis software obtained from NIH (rsb.info.nih.gov/ij).

RESULTS

Identification of a 40-aa RANK Intracellular Segment Essential for Osteoclastogenesis—Previously, we reported the characterization of three functional TRAF binding motifs in the RANK cytoplasmic domain by using a chimeric receptor consisting of mouse TNFR1 external domain linked to the transmembrane and intracellular domains of mouse RANK (23). In the current study we use the same chimeric receptor approach to identify novel RANK motifs involved in osteoclastogenesis. Mouse RANK has a 391-aa cytoplasmic domain. Initially, we generated 20 deletion mutants (D1–D20) (Fig. 1A). In D1, an 18-aa segment (from aa 235 to aa 252) was deleted. In D2-D19, a 20-aa segment was deleted. In D20, the last 13-aa of the RANK cytoplasmic domain was removed (Fig. 1A). Deletion of such small segments (20 aa or smaller) is to minimize the potential effect of the deletions on the three-dimensional structural integrity of the RANK cytoplasmic domain, which is critical for its function.

To determine whether these mutants are capable of mediating osteoclastogenesis, we expressed wild-type chimera (WT) and the mutants in primary bone marrow macrophages (BMMs, namely, primary osteoclast precursors) derived from TNFR1−/− R2−/− double knock-out mice using a retroviral system as described in our previous study (23). Infected BMMs were selected with 2 μg/ml puromycin for 2 days, then selected BMMs expressing WT chimera or the mutants were treated with M-CSF (44 ng/ml) and TNF-α (10 ng/ml). The data indicate that all mutants except for D15 and D16 are able to mediate osteoclastogenesis, revealing that the regions deleted in D15 and D16 are functionally critical for osteoclastogenesis. Fig. 1B shows the cultures of negative control (uninfected BMMs), positive control (BMMs expressing WT chimera), D15, D16, and several representatives of ineffective mutants (D1, D5, D10, D14, D17, and D18). To rule out the possibility that the failure of D15 and D16 to form osteoclasts is caused by the inability of these two mutants to be expressed on cell surface, we performed flow cytometric analysis with antibody against the external domain of TNFR1, and our data demonstrated that both D15 and D16 were expressed on the cell surface of infected BMMs (Fig. 1C). Fig. 2A shows the schematic location and aa sequence of the segments deleted in D15 and D16. In support of the critical role of the segments deleted in D15 and D16 in osteoclastogenesis, these two segments are located within a RANK cytoplasmic region that is highly conserved between human and mouse (Fig. 2B). Moreover, these segments are not overlapped with the three functional TRAF binding motifs identified previously (23) (Fig. 2B).

Identification of a 4-aa Motif in the RANK Intracellular Region Essential for Osteoclastogenesis—To further identify specific motifs in the 40-aa RANK segment that are involved in osteoclastogenesis, we gen-
generated 10 more internal deletion mutants designated as SD1-SD10 (Fig. 3A). In each mutant four amino acids in the 40-aa segment were deleted (Fig. 3A). Then we performed osteoclastogenesis assays with these 10 mutants. As shown in Fig. 3B, when infected BMMs expressing these mutants were treated with M-CSF (44 ng/ml) and TNF-α (10 ng/ml), SD4, SD5, SD6, and SD7 failed to mediate osteoclastogenesis, indicating that the residues deleted in these mutants are crucial for osteoclastogenesis. We repeated the assays with higher doses of TNF-α. The assays were performed as described in Fig. 1B except that infected BMMs were treated with 44 ng/ml M-CSF plus 30 ng/ml of TNF-α. D, flow cytometric analysis showing the surface expression of the mutants on infected BMMs. The assay was performed as described in Fig. 1C.

To identify specific amino acid residues in the eight-aa RANK segment (DIIVYVS) mediating osteoclastogenesis, we prepared four point mutation mutants PM1, PM2, PM3, and PM4 (Fig. 4A). In each mutant, two residues are mutated. To minimize the effect of the point mutations on the three-dimensional structure of the RANK cytoplasmic domain, we converted each of the eight residues to another one with similar polarity, charge, and chemical structure. Osteoclastogenesis assays with these point mutants showed that only PM2 and PM3 failed to mediate osteoclast formation, revealing that four amino acid residues (IVY) are essential for osteoclastogenesis. Flow cytometric analysis confirmed that PM2 and PM3 were expressed on the cell surface (Fig. 4C).

**The Novel RANK Motif Plays a Crucial Role in Osteoclastogenesis by Committing Macrophages to the Osteoclast Lineage**—A previous study demonstrated that the RANKL/RANK system plays an important role in committing macrophages to the osteoclast lineage (32). Next, we
investigated whether the novel RANK motif is involved in committing macrophages to the osteoclast lineage. To this end we sought to devise an experimental approach that enables us to activate the novel motif-mediated signal at the beginning of the osteoclastogenic process for various times while maintaining the activation of other RANK signals (i.e. the TRAF-dependent signals) for the entire osteoclastogenic process.

The chimeric receptor approach possesses a unique feature that permits such study. As highlighted in Fig. 5A, BMMs infected with virus encoding PM3 express not only PM3 but also endogenous RANK. In PM3, the novel motif is mutated and non-functional (Fig. 4). However, the novel motif in endogenous RANK is functional. More importantly, the chimeric receptor and the endogenous RANK on the infected BMMs can be independently activated by TNF-α and RANKL, respectively (23). As such, we can use this feature to choose to activate either the endogenous RANK or the chimeric receptor for any length of time in any order during the osteoclastogenic process.

In initial experiments BMMs infected with virus encoding PM3 were treated with RANKL (100 ng/ml) and M-CSF (44 ng/ml) for 1 day (Experiment 1), 2 days (Experiment 2) or 3 days (Experiment 3), and then RANKL (100 ng/ml) was removed. After the removal of RANKL, the cultures were continued with TNF-α (10 ng/ml) plus M-CSF (44 ng/ml) until the end of the assays (day 4). Thus, in Experiment 1 both the novel motif-mediated signal and other RANK signals (i.e. the TRAF-mediated signal) were activated during the first 24-h of the culture,
FIGURE 4. Identification of a 4-aa segment essential for osteoclastogenesis. A, schematic diagrams of four point mutation mutants (designated as PM1–PM4). Ext, external domain; TM, transmembrane domain; WT, wild-type chimeric receptor. B, osteoclastogenesis assays with PM1–PM4. The assays were performed as described in Fig. 1B. C, flow cytometric analysis showing the surface expression of the mutants on the infected BMMs. The assay was performed as described in Fig. 1C.
whereas only the TRAF-mediated signals were activated during the subsequent 3 days. Similarly, in Experiments 2 and 3, both the TRAF-mediated signals and the novel motif-mediated signal were activated during the first 2 or 3 days of the culture, respectively. Then the osteoclastogenic process was continued only with the activation of the TRAF-mediated signals for the remaining days. These initial experiments indicated that osteoclasts formed in all three assays, revealing that the novel motif-mediated signal is indeed involved in priming macrophages to the osteoclast lineage since it is not required for the entire osteoclastogenic process. Moreover, these initial experiments also demonstrated that it takes no more than 24 h for the novel motif-mediated signal to commit macrophages to the osteoclast lineage.

To further elucidate the precise time window of the novel signaling pathway required for the commitment, we performed an additional 5 osteoclastogenesis assays (Assays 1–5) which are detailed in Fig. 5A. In Assay 1, the infected or uninfected cells (as control) were treated with M-CSF (44 ng/ml) and TNF-α (10 ng/ml) throughout the 4 days of the osteoclastogenic process. In Assays 2–5, cells were treated with M-CSF (44 ng/ml) and RANKL (100 ng/ml) for 4, 8, 16, or 24 h, respectively. Then the cultures were switched to TNF-α (10 ng/ml) and M-CSF (44 ng/ml) for the rest of the osteoclastogenic process. These assays demonstrated that the treatment of the infected BMMs with RANKL for only 4 h can partially commit macrophages to the osteoclast lineage (Assay 1, Fig. 5B). Moreover, a 16-h or longer treatment of the infected BMMs with RANKL can fully commit BMMs to the osteoclast lineage (Assays 4–5, Fig. 5B). These data indicate that the novel motif-activated signaling pathway is only required for committing BMMs to the osteoclast lineage but not the entire osteoclastogenic process.
As shown in the left panel in Fig. 5B, we also performed the assays using uninfected BMMs. None of these assays involving uninfected BMMs gave rise to osteoclasts, revealing that although the novel motif-mediated signal is able to commit macrophages to the osteoclast lineage, it is not sufficient for osteoclastogenesis. For instance, in Assay 4 involving uninfected BMMs (left panel in Fig. 5B), although both the novel motif-mediated signal and the TRAF-mediated signals were activated during the first 16-h M-CSF/RANKL treatment, once the cultures were switch to TNF-α/H9251, neither the novel motif-mediated signal nor the TRAF-mediated signals were activated since uninfected BMMs do not express PM3. Thus, the failure of uninfected BMMs to form osteoclasts in this assay is because the TRAF-mediated signals were not activated for the remaining days after the osteoclast lineage commitment. Taken together, the assays in Fig. 5 showed that osteoclastogenesis requires not only the novel motif-mediated signal for the initial lineage commitment but also the TRAF-mediated signals to complete the remaining process after the lineage commitment.

The Novel RANK Motif Is Not Involved in Osteoclast Function or Survival—Above we have shown that the novel motif plays an essential role in osteoclastogenesis by committing macrophages to the osteoclast lineage, suggesting that this novel motif may not be involved in osteoclast function and survival. To experimentally prove this, we next performed osteoclast function and survival assays using an experimental strategy described in Fig. 6A. Once again, this strategy takes advantage of the fact that infected BMMs express both chimeric receptor (PM3) and endogenous RANK (Fig. 6A). This feature allowed us to activate

![FIGURE 6: The novel RANK motif plays no role in osteoclast function. A, diagram depicting the experimental procedures employed to examine the role of the novel RANK motif in osteoclast function. B, bone resorption assays using the experimental procedures detailed in A. Uninfected BMMs, infected BMMs expressing WT construct (WT), or infected BMMs expressing PM3 (PM3) were plated on bovine cortical bone slices and treated with M-CSF (44 ng/ml) and RANKL (100 ng/ml) for 4 days to form osteoclasts. Then M-CSF and RANKL were removed from one set of assays, and these cultures were continued with M-CSF (44 ng/ml) and TNF-α (10 ng/ml) for 3 more days (bottom panel). As control, another set of assays was continued with M-CSF and RANKL for 3 more days (top panel). A representative view area (at 100× magnification) of the bone resorption assays from each condition is shown. C, quantification of the number of pits in the bone resorption assays in B. The number of pits in 5 random resorption areas (at 30× magnification) was determined. The data show the averages of the five counts ± S.D. U, uninfected BMMs; WT, infected BMMs expressing the WT construct; PM3, infected BMMs expressing PM3. D, quantification of the pits areas in the bone resorption assays in B. The percentage of pits areas in 4 random resorption areas (at 30× magnification) was determined. The data show the averages of the four measurements ± S.D.](image-url)
to examine the role of the novel RANK motif in osteoclast survival. After osteoclasts were formed, we remove RANKL and M-CSF and then examined the capacity of PM3 in modulating osteoclast function and survival by treating the mature osteoclasts with TNF-α.

To investigate the role of the novel motif in bone resorption, uninfected BMMs or BMMs infected with virus encoding WT chimera or mutant PM3 were plated on bovine cortical bone slices and treated with M-CSF (44 ng/ml) and RANKL (100 ng/ml) (Fig. 6B). Once osteoclasts were formed at day 4, the cultures were then treated with M-CSF plus TNF-α for 3 more days to determine whether PM3 is able to mediate bone resorption. As a positive control, separate cultures were continued with M-CSF (44 ng/ml) and RANKL (100 ng/ml) for 3 days. Representative bone resorption pits are shown in Fig. 6B. The resorption assays were quantified in Figs. 6, C and D. The data showed that osteoclasts expressing both WT and PM3 are highly effective in mediating bone resorption. As the negative control, osteoclasts derived from uninfected BMMs failed to survive in response to TNF-α stimulation.

Investigation of the Involvement of the Novel RANK Motif in Known RANK Signaling Pathways—It has been established that RANK mediates osteoclast formation, function, and/or osteoclast survival by activating various intracellular signaling pathways including NF-κB (10, 35), JNK (10, 36), ERK (10, 37), p38 (37–39), and Akt (35). In addition, RANKL also stimulates the expression of c-fos (25), an essential factor for osteoclastogenesis (40). Notably, activation of these signaling pathways by RANK primarily involves the recruitment of various TRAF proteins such as TRAF6 to the TRAF binding motifs (8, 9).

Upon the elucidation of this novel RANK motif, we investigated whether the novel motif is implicated in activation of these signaling pathways. To this end, we infected TNFR1-/-R2-/- BMMs with virus encoding either WT or PM3. Infected cells were then treated with TNFα for 0, 5, and 10 min, and the activation of the four pathways, NF-κB, JNK, ERK, and p38, was determined by Western analysis. The assays indicate that both WT and PM3 are able to activate 1kB, JNK, ERK, and p38 (Fig. 8, A–D). In addition, we also treated the infected cells with TNFα for 0, 15, and 30 min and determined the activation of Akt. The data indicate that the mutation of the novel motif does not abrogate Akt activation (Fig. 8E). Next, we examined whether PM3 is involved in the RANK-dependent induction of c-fos expression. In this assay, infected BMMs were treated with TNFα for 0 and 24 h. Western analysis indicates that WT and PM3 have similar effects on stimulation of c-fos expression (Fig. 8F). Taken together these data indicate that the endogenous RANK by treating infected BMMs with RANKL in the presence of M-CSF to stimulate osteoclast formation. After osteoclasts were formed, we remove RANKL and M-CSF and then examined the capacity of PM3 in modulating osteoclast function and survival by treating the mature osteoclasts with TNF-α.
novel RANK cytoplasmic motif regulates osteoclastogenesis by activating a novel signaling pathway(s) that remains to be identified.

DISCUSSION

The discovery of RANKL and its receptor RANK in the late 1990s has advanced our understanding of the molecular mechanism underlying several biological processes such as osteoclast differentiation and function (5, 8) and immune response (41, 42) as well as mammary gland development (7). In particular, the unraveling of the important role for the RANKL/RANK system in osteoclast formation, function, and survival has been recognized as a milestone in osteoclast biology (43). Moreover, the functional establishment of the crucial role for the RANKL/RANK system in osteoclast biology has also generated great interest in elucidating the RANK-activated intracellular signaling involved in osteoclast differentiation, function, and survival. However, despite enormous previous efforts, we have still not completely understood the signaling mechanism by which the RANKL/RANK system regulates osteoclast differentiation (osteoclastogenesis).

Primarily based on the fact that RANK belongs to the TNFR family, the previous efforts have been largely focused on examining the capacity of RANK to interact with various TRAF proteins in osteoclast formation and function, and survival has been recognized as a milestone in osteoclast biology (43). Moreover, the functional establishment of the crucial role for the RANKL/RANK system in osteoclast biology has also generated great interest in elucidating the RANK-activated intracellular signaling involved in osteoclast differentiation, function, and survival. However, despite enormous previous efforts, we have still not completely understood the signaling mechanism by which the RANKL/RANK system regulates osteoclast differentiation (osteoclastogenesis).

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Primarily based on the fact that RANK belongs to the TNFR family, the previous efforts have been largely focused on examining the capacity of RANK to utilize the signaling complex/mechanisms known to be employed by other members of the TNFR family to transmit intracellular signaling. Given that members of the TNFR family mainly utilize intracellular signaling molecules TRAFs to convey non-apoptotic signals (16, 44), the early studies have been devoted to investigating whether RANK also employs TRAF proteins to transmit signaling. Six TRAF proteins (TRAF1, -2, -3, -4, -5, and -6) have been identified and characterized (17, 26). TRAF1, -2, -3, -5, and -6 have been shown to play a role in TNFR family member-induced intracellular signaling (17, 26). Because TRAF4 appears to be a nuclear protein, its role in TNFR signaling has not been unambiguously established (17, 26). As expected, several lines of evidence support RANK as indeed employing various TRAF proteins to transmit downstream signaling in osteoclastogenesis. First, numerous studies showed that RANK contains six motifs that interact with various TRAF proteins in in vitro binding assays and/or inside cells in context of overexpression (10, 18–22). Then a systemic functional study of these putative TRAF binding motifs established that three of them (369PFQ373, 559PVQEET564, and 604PVQEQG609) redundantly play a functional role in osteoclastogenesis (23). Moreover, in accord with these data, using osteoclast precursors derived from TRAF2, TRAF5, and TRAF6 knock-out mice, several groups demonstrated that TRAF2, TRAF5, and TRAF6 are functionally involved in RANKL-induced osteoclastogenesis (45–48). A functional study indicates that 369PFQ373 mediates osteoclastogenesis by recruiting TRAF6 (24). Taken together, these data underscore the important involvement of TRAF-mediated signaling in RANK-induced osteoclastogenesis.

On the other hand, shortly after the unraveling of the essential role for the RANKL/RANK in osteoclastogenesis, it was already proposed that RANK may activate unidentified and unique signaling pathways that are essential for osteoclastogenesis (49). This early hypothesis was subsequently supported by some experimental evidence. The most convincing evidence came from the comparison of the capacity of RANKL and IL-1 in promoting osteoclastogenesis. It is well established that TRAF6 acts as a key downstream signaling molecule for both RANK and IL-1 receptor (50). Moreover, TRAF6 is involved in osteoclastogenesis (47, 48). In particular, a single TRAF6 binding motif is able to promote osteoclastogenesis (23, 24). However, administration of IL-1 to RANK−/− mice failed to induce any osteoclastogenesis in vivo (15), indicating that RANK activates TRAF6-independent novel signaling pathways.
pathways essential for osteoclastogenesis. Consistent with the in vivo finding, in vitro studies also demonstrated that IL-1 failed to stimulate osteoclastogenesis in vitro in the presence of M-CSF (27, 28).

The present study was undertaken to elucidate the hypothesized novel and unique RANK signaling pathways essential for osteoclastogenesis. Given that such a signaling pathway is most likely initiated by one or more motifs in the RANK cytoplasmic domain, we set out to unravel the novel signaling pathways by first identifying specific RANK cytoplasmic motifs that play an essential role in osteoclastogenesis. To this end we carried out a systematic structure/function study of the RANK cytoplasmic domain using a chimeric receptor approach that was well established in our previous study (23). Importantly, to carry out the study in an unbiased fashion, we initially generated 20 deletion mutants in which around 20 aa were deleted. This initial study revealed that two mutants failed to mediate osteoclastogenesis (Fig. 1). Notably, the segments deleted in the two mutants are located in a RANK cytoplasmic region that is highly conserved between human and mouse RANK (Fig. 2), further supporting that the deleted segments play a functional role in osteoclastogenesis. Subsequent studies employing additional internal deletion/point mutation mutants identified a specific 4-aa RANK motif essential for osteoclastogenesis (Fig. 4).

The novel RANK motif is unlikely to activate the known TRAF-dependent RANK signaling pathways for several reasons. First, the novel RANK motif (535IVVY538) does not share any homology with the TRAF binding consensus sequences (PXQT, EXGK, or VXVXXE) (26). Second, the numerous previous protein-protein interaction assays failed to identify this novel RANK motif as a TRAF-binding site (10, 18–22). More importantly, our previous study has identified all functional TRAF-binding sites (369PFQE373, 559PVQEP564, and 604PVQEG609) that play a role in osteoclastogenesis (23), and this novel RANK motif (535IVVY538) does not overlap with any of the three functional TRAF binding motifs (Fig. 2).

Nonetheless, although the novel RANK motif is not a TRAF binding domain, it may play the essential role in osteoclastogenesis by critically participating in the activation of TRAF-mediated signaling pathways (NF-κB, JNK, ERK, p38, NFATc1, and Akt). To address this possibility, we examined whether the mutation of the RANK motif abrogates the capacity of the RANK in activating these known TRAF-mediated signaling pathways. Our data demonstrated that the novel motif is not involved in the activation of the NF-κB, JNK, ERK, p38, and Akt pathways (Fig. 8, A–E). Furthermore, we also demonstrated that the novel RANK motif is dispensable for RANK-induced c-fos expression (Fig. 8F). Because RANKL also activates NFATc1 in TRAF6-dependent manner (51, 52), we also investigate whether the novel motif is involved in activating RANK-induced NFATc1 expression. However, our numerous attempts designed to compare the capacity of WT and PM3 in activating NFATc1 expression revealed that NFATc1 expression was fully activated before the addition of TNF-α (data not shown). This result indicates that although NFATc1 plays a critical role in osteoclastogenesis (51, 52), RANK-initiated NFATc1 expression may not be critical for osteoclastogenesis since NFATc1 can be activated by another mechanism. Consistently, it was shown that RANK-induced signal is only partially responsible for activation of NFATc1 expression in osteoclast precursors (51, 53) and immunoreceptor tyrosine-based activation motif-mediated costimulatory signals are also involved in induction of NFATc1 expression (53, 54). As such, we conclude that the novel motif is unlikely to play a crucial role in osteoclastogenesis by activating NFATc1 expression. In support of this notion, RANK-induced NFATc1 expression is dependent on TRAF6, and the novel RANK motif is not a TRAF-binding motif (discussed in detail in the previous paragraph).

Taken together, these data demonstrate that the novel RANK motif crucially regulates osteoclastogenesis by activating a novel and unique signaling pathway(s).

Further characterization of the novel motif revealed that this motif plays an essential role in osteoclastogenesis by committing macrophages to the osteoclast lineage. In support of the notion, the motif plays no role in osteoclast function or survival. These findings are consistent with the previous report that RANKL plays an essential role in committing macrophages to the osteoclast lineage (32). However, the precise molecular mechanism by which this novel RANK motif commits macrophages to the osteoclast lineage remains to be elucidated.

Based on our current study, we rule out the possibility that the inability of PM3 to promote osteoclastogenesis is caused by a change in three-dimensional structural integrity of the RANK cytoplasmic domain resulting from the mutation of the novel motif, since PM3 is fully capable of activating the various known RANK signaling pathways. As such, this novel RANK motif is likely to exert its effect on osteoclast lineage commitment by binding an unidentified signaling molecule to transmit downstream signaling pathways required for the commitment. We are currently using a two-hybrid screening system to identify the protein that specifically interacts with the motif.

The RANKL/RANK system not only plays an essential role in osteoclast formation and function but also is implicated in the pathogenesis of various bone diseases such as postmenopausal osteoporosis (55), bone loss in rheumatoid arthritis (RA) (56), and tumor-induced osteolysis (57). Consequently, since the discovery of the RANKL/RANK system, many studies have been focused on developing OPG and soluble RANK-Fc as therapeutic drugs to treat bone diseases (58, 59). However, both OPG and RANK-Fc have a potential drawback as therapeutic drugs, primarily due to the fact that their action lacks specificity. The RANKL/RANK system not only plays a pivotal role in osteoclast formation and function (10), but it is also a critical mediator of other biological processes such as the immune system (6, 60) and mammary gland development (7). Thus, use of either OPG or RANK-Fc to treat bone diseases will inevitably cause adverse effects on patient immune systems.

As a result, concomitantly, enormous efforts have also been undertaken by numerous research groups to investigate RANK signaling with aim of identifying intracellular pathways that can serve as more specific therapeutic targets for the various bone diseases. These previous signaling studies have established that RANK activates numerous signaling pathways such as NF-κB, JNK, ERK, p38, NFATc1, and Akt (8, 9, 25). However, since these pathways are also activated by a variety of other cytokines including other members of the TNFR family in distinct types of cells, they may not serve as appropriate therapeutic drug targets for specific targeting of the osteoclast. Given the observation that this novel RANK motif plays an essential role in osteoclastogenesis by activating the signaling pathways other than the six known signaling pathways, this study has raised the possibility that this motif and its downstream signaling pathways may serve as more specific and effective drug targets for treating bone diseases involving osteoclasts, such as postmenopausal osteoporosis, bone loss in rheumatoid arthritis, and cancer-induced osteolysis.

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Novel RANK Motif Essential for Osteoclastogenesis
