Functional Identification of Three Receptor Activator of NF-κB Cytoplasmic Motifs Mediating Osteoclast Differentiation and Function

Wei Liu‡‡, Duorong Xu‡‡, Hongmei Yang‡, Hui Xu‡, Zhenqi Shi‡, Xuemei Cao‡, Sunao Takeshita**†, Jianzhong Liu‡, Michael Teale‡, and Xu Feng‡ ‡‡

From the Departments of ‡Pathology and ‡Dermatology, University of Alabama at Birmingham, Birmingham, Alabama 35294, the ‡Department of Nephrology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong Province, China, and the **‡‡Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110.

Receptor activator of NF-κB ligand (RANKL) and its receptor activator of NF-κB (RANK) play pivotal roles in osteoclast differentiation and function. However, the structural determinants of the RANK that mediate osteoclast formation and function have not been definitively identified. To address this issue, we developed a chimeric receptor approach that permits a structure/function study of the RANK cytoplasmic domain in osteoclasts. Using this approach, we examined the role of six RANK putative tumor necrosis factor receptor-associated factor-binding motifs (PTM) (PTM1, ILLMTREEE398–403; PTM2, PSQPS849–533; PTM3, PFQEP369–373, PTM4, YVVSQTSQ637–642; PTM5, PVQET659–664; and PTM6, PVQEQG604–609) in osteoclast formation and function. Our data revealed that the RANK cytoplasmic domain possesses three functional motifs (PFQEP369–373, PVQET559–564, and PVQEQG604–609) capable of mediating osteoclast formation and function. Moreover, we demonstrated that these motifs play distinct roles in activating intracellular signaling. PFQEP369–373 initiates NF-κB, c-Jun N-terminal kinase, extracellular signal-regulated kinase, and p38 signaling pathways and PVQET559–564 activates NF-κB and p38 pathways in osteoclasts, whereas PVQEQG604–609 is only capable of activating NF-κB pathway. Significantly, the revelation of these functional RANK cytoplasmic motifs has not only laid a foundation for further delineating RANK signaling pathways in osteoclasts, but, more importantly, these RANK motifs themselves represent potential therapeutic targets for bone disorders such as osteoporosis.

Osteoclasts, the principal bone-resorbing cells, play a pivotal role in skeleton development and maintenance (1). Osteoclasts are derived from mononuclear precursors of monocyte/macrophage lineage upon stimulation of two key factors: monocyte/macrophage colony-stimulating factor (M-CSF)1 and receptor activator of nuclear factor κB (RANKL, also known as OPG ligand/osteoclast differentiation factor/TNF-related activation-induced cytokine) (1–3). RANKL was identified as a member of the TNF superfamily independently by several groups in the late 1990s (4–7). RANKL regulates both osteoclast formation and function by binding to its receptor RANK expressed on osteoclast precursors and mature osteoclasts (4, 8, 9). The essential role of both RANKL and RANK in the osteoclastogenic process has been well demonstrated by the findings that mice lacking the gene for either protein develop osteopetrosis caused by failure to form osteoclasts (10–12).

RANK was identified as a member of the TNF receptor family (6). Members of the TNF receptor family are characterized by a lack of intrinsic enzymatic activity, and thus they usually transduce intracellular signals by recruiting various adaptor proteins such as TNF receptor-associated factors (TRAFs) through the specific motifs in their cytoplasmic domains (13–15). Since the unraveling of the RANKL/RANK system, enormous efforts have been undertaken to elucidate RANK-initiated intracellular signaling. Particularly, many of the previous works have been focused on characterizing the receptor-proximal signaling events (8, 16–20), which represent the initial and critical component of intracellular signaling pathways initiated by membrane-bound receptors. Although these studies have mapped RANK cytoplasmic regions capable of interacting with TRAF proteins by various in vitro binding assays (8, 16–20), the physiological relevance of these data to osteoclast biology remains largely unexplored.

In the present study, we characterized the RANK-initiated signaling in osteoclast differentiation and function in physiological cellular background by identifying the specific RANK motifs regulating osteoclast formation and function. To this end, we developed a chimeric receptor approach that allows us to carry out a detailed structure/function study of RANK cytoplasmic domain in osteoclast differentiation and function. Using this approach, we have identified three functional motifs that regulate osteoclast differentiation and function. In addition, we show that these domains mediate osteoclast differentiation.

1 The abbreviations used are: M-CSF, monocyte/macrophage colony-stimulating factor; PTM, putative TRAF-binding motifs; BMM, bone marrow macrophages; OPG, osteoprotegerin; RANK, receptor activator of NF-κB; RANKL, RANK ligand; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNF receptor-associated factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; TRAP, tartrate-resistant acid phosphatase; TBS, Tris-buffered saline.
tiation and function by initiating the distinct signaling pathways. Our present work has established a direct functional link between specific RANK cytoplasmic motifs and a physiological process: osteoclast formation and function. Moreover, the identification of these functional RANK motifs has laid foundations for further delineating the downstream signaling pathways implicated in osteoclast formation and function.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—The chemicals were purchased from Sigma unless indicated otherwise. Synthetic oligonucleotides were purchased from Sigma Genosys (The Woodlands, TX). Bacterialidin was from EMD Biosciences, Inc. (San Diego, CA). Antibody against the external domain of mouse TNFR1 (for flow cytometry) (TNF-R1, sc-12746PE) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant mouse TNFs (416-TRNC-050) was from R & D Systems (Minneapolis, MN). The following antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA): antibodies against IsBo (antibody 9242), phospho-IsoBo (antibody 9241), p44/p42ERK (antibody 9102), phospho-p44/p42ERK (antibody 9101), JNK (antibody 9252), phospho-JNK (antibody 9251), p38 (antibody 9212), and phospho-p38 (antibody 9211).

Construction of TNF1/RANK Chimeric cDNA—Using the standard molecular cloning techniques, we engineered a chimeric cDNA comprising the mouse TNFR1 external domain (amino acids 1–210) linked in-frame to the transmembrane and cytoplasmic domains of mouse RANK (amino acids 210–625). cDNA fragment encoding the mouse TNFR1 external domain was amplified by reverse transcriptase-PCR using total RNA isolated from mouse bone marrow macrophages (BMMs) and a pair of primers containing XbaI sites. The TNFR1 cDNA fragment was then subcloned into pBluescript II SK+ cloning vector (Stratagene, La Jolla, CA) at the XbaI site, resulting in a plasmid named SK-TNF1-RANK. cDNA encoding the RANK transmembrane and cytoplasmic domains was also amplified by reverse transcriptase-PCR using total RNA from mouse BMMs with a forward primer containing the SpeI site and a reverse primer containing the BamHI site. The RANK cDNA fragment was then subcloned into SK-TNF1 between SpeI and BamHI sites, giving rise to a plasmid named SK-TNF1-RANK. The orientation and sequence of the chimeric cDNA was confirmed by sequencing.

Preparation of Retrovirus Encoding the Chimeric Receptors—The retrovirus vector pMX-puro (21) and the Plat-E packaging cells (22) were used. The chimeric cDNA (TNFR1/RANK) from SK-TNF1-RANK was subcloned into pMX-puro to generate plasmid construct named pMX-puro-TNF1-RANK. Plat-E cells were cultured in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum in the presence of 0.01 M β-mercaptoethanol. The selected cells were subsequently used for various studies.

In Vitro Osteoclastogenesis Assays and Bone Resorption Assay—Retrovirally infected BMMs were cultured in 24-well tissue culture plates (1 × 10⁵ cells/well) in α-minimal essential medium containing 10% heat-inactivated fetal bovine serum in the presence of 0.1 volume of culture supernatant of M-CSF-producing cells for 2 days as previously described (24). The cells were then infected with viruses for 24 h in the presence of 0.1 volume of culture supernatant of M-CSF-producing cells and 8 µg/ml polybrene. The cells were further cultured in the presence of M-CSF and 2 µg/ml puromycin for selection and expansion of transduced cells. The selected cells were subsequently used for various studies.

To perform bone resorption assays, osteoclasts were generated on whale dentin slices from infected or uninfected BMMs as described above. Dentin slices were harvested at day 9. The cells were removed from the dentin slices with 0.25 M ammonium hydroxide and mechan-
PCR amplification during the mutagenesis. The mutant chimeric cDNAs were then subcloned into pMX-puro plasmids as described above for virus preparation.

**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 before treatment with RANKL or TNFα for various times as indicated in individual experiments. The 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 (P-2850; Sigma) and 1× protease inhibitor mixture M-CSF (22 ng/ml) plus RANKL (100 ng/ml), or M-CSF (22 ng/ml) plus TNFα (10 ng/ml). After osteoclasts formed, the cultures were continued for 5 more days to allow the cells to resorb bone. The dentin slices were harvested and subjected to scanning electron microscopy analysis.

**Figure 2.** The chimeric receptor is capable of mediating osteoclast formation and function using TNFα as a surrogate. **A**, flow cytometric analysis showing the surface expression of the chimera on the infected BMMs. BMMs isolated from TNFR1 R2 knockout mice were infected with retrovirus encoding the chimeric receptor for 24 h. Infected cells were cultured in the presence of puromycin (2 μg/ml) for 2 or 4 days to select for the chimera expression-positive cells. The surface expression of the chimera was determined by flow cytometric analysis using TNFR1 antibody conjugated with phycoerythrin (PE). Uninfected BMMs were used as control. **B**, osteoclastogenesis assay with the chimeric receptor. TNFR1 R2 BMMs were either uninfected or infected with virus encoding chimeric receptor. Infected cells were then selected with puromycin for 2 days. Then uninfected BMMs or infected BMMs were treated with M-CSF alone (22 ng/ml), M-CSF (22 ng/ml) plus RANKL (100 ng/ml), or M-CSF (22 ng/ml) plus TNFα (10 ng/ml). Osteoclasts began to form at day 3, and the cultures were stained for TRAP activity at day 6. **C**, bone resorption assay. Uninfected or infected BMMs as described in B were plated on dentin slices, and the cultures were treated with M-CSF alone (22 ng/ml), M-CSF (22 ng/ml) plus RANKL (100 ng/ml), or M-CSF (22 ng/ml) plus TNFα (10 ng/ml). After osteoclasts formed, the cultures were continued for 5 more days to allow the cells to resorb bone. The dentin slices were harvested and subjected to scanning electron microscopy analysis.
40 μg of cell lysates were boiled in the presence of SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromphenol blue) for 5 min and loaded for electrophoresis on 10% SDS-PAGE. The proteins were transferred to nitrocellulose membranes (catalog number 162-0147) from Bio-Rad using a semi-dry blotter (Bio-Rad). The membranes were blocked in blocking solution (5% nonfat dry milk in TBS containing 0.1% Tween 20 (TBS-T)) for 1 h to prevent nonspecific binding and then washed three times with TBS-T. The membranes were incubated primary antibodies in TBS-T containing 5% bovine albumin (Sigma catalog number A-7030) overnight at 4 °C. Next day, the membranes were then washed three times with TBS-T and incubated with secondary antibody in TBS-T containing 5% nonfat dry milk for 1 h. The membranes were washed extensively and ECL detection assay was performed using a SuperSignal West Dura kit from Pierce.

Sequence Analysis—Sequence analysis was performed using the Genetic Computer Group (Madison, WI) sequence analysis software.

RESULTS

Construction of a Chimeric Receptor Capable of Mediating Osteoclast Formation and Function—To delineate functional motifs in the RANK cytoplasmic domain mediating osteoclast differentiation and function, we developed a chimeric receptor consisting of mouse TNFR1 external domain (amino acids 1–210) linked to the transmembrane and intracellular domains of mouse RANK (amino acids 210–625) (Fig. 1). Given that both TNFR1 and RANK belong to the same family of the cytokines and that they are both activated by trimerization (Fig. 1), we predicted that the TNFα-induced trimerization of the chimeric receptor would be sufficient to activate RANK intracellular signaling pathways required for the osteoclast formation and/or function (Fig. 1B).

To determine whether the chimeric receptor is capable of mediating osteoclast formation and function, we expressed the chimeric receptor in primary BMMs using a retroviral system (23, 28) (Fig. 2). Given that TNFα is also implicated in osteoclast formation and function, we used BMMs from TNFR1 and R2 double knockout mice (TNFR1−/−R2−/−) to eliminate any possibility of signaling through TNF receptors (29). BMMs derived from TNFR1−/−R2−/− mice were infected with the retrovirus encoding the chimeric receptor and the cells expressing the chimera were selected with 1 μg/ml puromycin for 2 or 4 days. Flow cytometric analysis with antibody against the ex-
ternal domain of TNFR1 demonstrated not only that the chimera was expressed on the cell surface but also that the surface expression levels of the chimera were increased with the selection time (Fig. 2A).

To determine whether the chimeric receptor is capable of mediating osteoclast differentiation, infected BMMs expressing the chimera were treated with M-CSF (22 ng/ml), M-CSF (22 ng/ml) plus RANKL (100 ng/ml), or M-CSF (22 ng/ml) plus TNFα (10 ng/ml) (Fig. 2B). Whereas infected BMMs treated with M-CSF alone remained in macrophage lineage, those treated with M-CSF and RANKL formed osteoclasts, indicating that the endogenous RANK in the infected cells is functional (Fig. 2B). Most importantly, when infected BMMs were treated with M-CSF and TNFα, they also formed osteoclasts, confirming that the chimeric receptor is working (Fig. 2B). As a negative control, uninfected BMMs failed to form osteoclasts in response to M-CSF and TNFα treatment (Fig. 2B). Notably, the infected cell culture treated with M-CSF alone also contained few TRAP-positive cells (Fig. 2B). This likely resulted from overexpression of the chimera in a few BMMs, because overexpression of TNFR family members can lead to the self-activation of their signaling pathways (30). Functionally, these TRAP-positive mononuclear cells may not be regarded as osteoclasts because the high power view of the culture indicates that they were still mononuclear. More importantly, as shown below in Fig. 2C, these TRAP-positive mononuclear cells failed to form any pits in the resorption assays. Thus, the formation of few TRAP-positive mononuclear cells in this culture will not undermine the potential of the chimera as a great tool to study RANK signaling. In Fig. 2B, we used TNFα at the concentration of 10 ng/ml. Our subsequent studies showed that lower TNFα concentrations (as low as 1 ng/ml) were still able to mediate osteoclast formation (data not shown).

To examine whether osteoclasts generated using the chimera strategy are capable of resorbing bone, we performed bone resorption assays (Fig. 2C). Osteoclasts generated using the chimera approach resorbed bone as efficiently as those formed using the endogenous RANK (Fig. 2C). Taken together, these data indicate that a chimera comprising TNFR1 external domain linked to the transmembrane and cytoplasmic domains of RANK is able to mediate osteoclast formation and function, using TNFα as a surrogate of RANKL. This chimera can serve as a useful tool for studying RANK signaling in osteoclast differentiation and function.

**Multiple RANK Cytoplasmic Motifs Are Involved in Osteoclast Formation and Function**—Next, we used the chimeric receptor approach to elucidate RANK cytoplasmic motifs that mediate osteoclast formation and function. Previously, several studies collectively suggested that the RANK cytoplasmic domain contains six PTMs that may be able to initiate RANK...
signaling: PTM1, ILLMTREE^{286–293} (17); PTM2, PSQPS^{349–353} (16–19); PTM3, PFQEP^{369–373} (17); PTM4, VYVSQTSQE^{537–545} (17); PTM5, PVQET^{559–564} (16–20); PTM6, and PVQE-QG^{604–609} (16–20). As shown in Fig. 3, we mutated these PTMs and investigated the role of these PTMs in osteoclast formation and function. The location and sequence of each PTM as well as the mutations introduced in these PTMs are highlighted in Fig. 3A. To minimize the potential effect of the point mutations on the three-dimensional structure of the RANK intracellular domain, each amino acid in a PTM was mutated to an amino acid with similar chemical characteristics (i.e., similar chemical structure, polarity, and charge). Fig. 3B shows the schematic structures of six mutant chimeric receptors constructed (named P1, P2, P3, P4, P5, and P6). These mutant chimeric constructs were then used to perform the osteoclastogenesis assay (Fig. 3C). Our data indicated that all six mutants exhibited no significant difference in their capacity to mediate osteoclast formation compared with the wild-type chimera (the one with no mutation in RANK cytoplasmic domain) (Fig. 3C). The assay was independently repeated three times, and the same result was obtained. These results raised two possibilities: (a) none of the six PTMs plays a functional role in osteoclast formation or (b) some of the PTMs are functionally redundant in mediating osteoclast formation.

To address the issue, we generated a mutant (P1–6) in which all six PTMs are mutated (Fig. 4A). We then prepared viruses encoding the P1–6 mutant and the wild-type chimera. Flow cytometric analysis showed that the P1–6 mutant and the wild-type chimeras were expressed at similar levels on cell surface of BMMs infected with these viruses (data not shown). As shown in Fig. 4B, when infected cells were treated with 22 ng/ml of M-CSF plus 5 ng/ml of TNFα, whereas the wild-type chimera is capable of mediating osteoclast formation, P1–6 failed to do so (Fig. 4B, top panels). These data indicate that some of the sites are functionally redundant. Interestingly, when 10 ng/ml TNFα was used for osteoclastogenesis assay, P1–6 resulted in a few TRAP positive cells (Fig. 4B, bottom panels). This may be due to the possibility that the introduced mutations are not

**FIG. 5. Osteoclastogenesis assay with mutant chimeric receptors with three PTMs mutated.** A, schematic diagram of two mutant chimeric receptors with three PTMs mutated (designated M1 and M2). B, osteoclastogenesis assay with M1 and M2. The assay was performed as described for Fig. 2B. Infected BMMs were treated with 22 ng/ml of M-CSF plus 5 ng/ml of TNFα. WT, wild type.
able to completely block the binding of downstream signaling molecules such as TRAF proteins. Taken together, these two sets of data indicate that some of the PTMs are indeed functionally redundant in mediating osteoclast formation.

Identification of Three Functional RANK Motifs Mediating Osteoclast Differentiation and Function—To identify the functional RANK cytoplasmic motifs, we first generated two mutant chimeric receptors: M1 and M2 (Fig. 5A). In M1, PTMs 1, 2, and 3 are mutated, whereas in M2, PTMs 4, 5, and 6 are mutated. When M1 and M2 were used to repeat the osteoclastogenesis assays, both mutants formed osteoclasts (Fig. 5B), suggesting that some of the three nonmutated PTMs in both M1 and M2 are functional. M1 showed a higher capacity to form osteoclasts than M2 (Fig. 5B), implying that more than one PTM among the three nonmutated PTMs in M1 (PTMs 4, 5, and 6) are probably capable of mediating osteoclast formation.

Based on the above data, we prepared six more mutants (W1, W2, W3, W4, W5, and W6) (Fig. 6A). In each of these mutants, only one PTM is not mutated. We reasoned that these mutants would allow us to identify the functional RANK motifs. BMNs infected with virus encoding these mutant chimeras were analyzed by flow cytometric analysis to confirm that all of the mutant chimeras were expressed at similar levels on cell surface (Fig. 6B). Then we used mutant chimeras W1–W6 to perform the osteoclastogenesis experiment with 22 ng/ml M-CSF and 5 ng/ml TNFα (Fig. 6C). Under these conditions, although W1, W2, and W4 failed to form any TRAP-positive cells, W3, W5, and W6 were capable of forming some TRAP-positive cells (Fig. 6C), indicating that PTM3, PTM5, and PTM6 are functional sites. We repeated the osteoclastogenesis assay with higher TNFα concentrations (10 ng/ml). As shown in Fig. 6D, whereas W1, W2, and W4 gave rise to a culture similar to P1–6 culture, W3, W5, and W6 formed much more and bigger osteoclasts than P1–6 mutant. These data demonstrated that PTM3, PTM5, and PTM6 are functional RANK motifs in mediating osteoclast formation. Moreover, both sets of the assays indicated that PTM5 and PTM6 are more potent than PTM3 in mediating osteoclast differentiation (Fig. 6, C and D).

To determine whether PTM3, PTM5, and PTM6 are able to mediate bone resorption, we performed bone resorption assays with BMNs infected with virus encoding the wild-type chimera, W1, W2, W3, W4, W5, W6, or P1–6 (Fig. 7). In this bone resorption assay, osteoclasts were generated in the presence of 22 ng/ml M-CSF and 5 ng/ml TNFα. As expected, no bone resorption pits were detected from the W1, W2, W4, and P1–6 because these mutants were not able to mediate osteoclast differentiation under the conditions (22 ng/ml M-CSF and 5 ng/ml TNFα) (Fig. 6C). Dentin slices from the wild-type chimera, W3, W5, and W6 cultures showed resorption activities (Fig. 7), revealing that the three RANK motifs are also capable of activating osteoclast bone resorption. Moreover, W3 gave rise to fewer bone resorption pits than W5 and W6, which is consistent with the osteoclastogenesis assay showing that W3 is less potent in modulating osteoclast differentiation than W5 and W6 (Fig. 6, C and D).

Identification of Signaling Pathways Activated by These Three Functional RANK Motifs—RANK mediates osteoclast formation and/or function by activating various intracellular signaling pathways including NF-κB (8, 31), JNK (8, 32), ERK
(8, 33), and p38 (33–35). Upon the elucidation of these three functional motifs, we next investigated whether the functional motifs are implicated in activation of these signaling pathways by performing Western analysis with antibodies against phosphorylated IkB, JNK, ERK, or p38 as previously described (33).

First, we infected TNFR1−/− R2−/− BMMs with virus encoding either the wild-type chimera or the mutant P1–6. Infected cells were then treated with TNFα for various times, and the activation of the signaling pathways was determined by Western analysis (Fig. 8). Although the wild-type chimera induced the phosphorylation of IkB with 5 min of treatment (Fig. 8A, lane 2), P1–6 mutant failed to do so (Fig. 8A, lane 5), indicating that some of the PTMs are implicated in the activation of the IkB/NF-κB pathway. In addition, the wild-type chimera also led to a high level of phosphorylation of JNK (Fig. 8B, lanes 2 and 3) and ERK (Fig. 8C, lanes 2 and 3) in response to TNFα treatment. In contrast, the TNFα-induced phosphorylation of JNK and ERK was profoundly reduced in the assays with P1–6 mutant (Fig. 8B and C, lanes 5 and 6), revealing that one or more of PTMs mutated in P1–6 plays a role in activating the JNK and ERK pathway. Finally, a similar result was also obtained for p38 phosphorylation (Fig. 8D). Taken together, these data indicate that the PTMs 3, 5, and 6 may regulate osteoclast formation/function by activating these signaling pathways.

Next, we examined which pathways each of the three functional RANK motifs activates in BMMs by repeating Western analysis with P1–6, W3, W5, and W6 mutants (Fig. 9). As shown in Fig. 9A, although P1–6 mutant failed to lead to IkB phosphorylation with 5 min of treatment (lanes 1 and 2), W3, W5, and W6 all induced IkB phosphorylation (lanes 4 and 5, lanes 7 and 8, and lanes 10 and 11, respectively), revealing that all three RANK motifs are able to activate IkB/NF-κB pathway.

Moreover, our data showed that W3 could also significantly activate all three MAPK pathways: JNK (Fig. 9B, lanes 4–6), ERK (Fig. 9C, lanes 4–6), and p38 (Fig. 9D, lanes 4–6). In contrast, W5 only induced p38 activation (Fig. 9D, lanes 7–9), and W6 did not activate any of the MAPK pathways (Fig. 9, B–D, lanes 10–12). In summary, PFQEP369–373 is involved in activation of IkB and all three MAPK pathways. PVQEQ559–564 activates IkB and p38 pathways. PVQEQ604–609 is only capable of activating IkB pathway (Fig. 9E).
DISCUSSION

Since the independent discovery of RANKL by two bone groups (4, 5) and two immunology groups (6, 7) in the late 1990s, RANKL has been shown to play pivotal roles in regulating various biological processes such as bone homeostasis (1, 5), immune function (6, 36), and mammary gland development (37). In addition, it has also been established that RANKL exerts its biological effects by binding to its receptor RANK, a member of the TNF receptor superfamily (6). However, the RANK-initiated intracellular signaling pathways in the various biological functions in response to RANKL ligation have not been fully defined.

Previously, many laboratories have investigated the interaction of RANK with the TRAF proteins and characterized the regions in RANK cytoplasmic domain that interact with various TRAFs (8, 16–20). These studies were mostly carried out either by in vitro binding assays or by using transformed cells irrelevant to osteoclasts, dendritic cells, or mammary gland epithelial cells. Therefore, the functional relevance of the data obtained from these studies to the three major biological processes has largely remained unknown. Moreover, it is controversial which TRAF proteins interact with RANK and which regions of the RANK interact with these TRAFs (8, 16–20).

Our present study focuses on identification of structural determinants in the RANK cytoplasmic domain that are involved in osteoclast formation and function. To obtain physio-
logical relevant data, we decided to carry out our structure-function study of RANK in physiological cellular background by using primary osteoclast precursor: primary BMMs. As reported here, this has been successfully achieved by a combination of our experience in efficient transduction of genes into primary BMMs using retrovirus technology with the creation of a chimeric receptor approach (23, 28) (Fig. 1). Moreover, the other strength of our current study is that we placed a special emphasis on identifying RANK cytoplasmic motifs that regulate cellular functions such as osteoclast formation and bone resorption. Significantly, we revealed that RANK contains three distinct motifs: PFQEP369–373, PVQEQ559–564, and PVQEQ604–609 (Fig. 9E), which are capable of mediating osteoclast formation and function. PFQEP369–373 has been previously shown to be a TRAF6-binding motif (38). A cell-permeable decapeptide derived from this motif blocked osteoclast formation involving RAW264.7 cell line (38), supporting indirectly that this motif might be functionally involved in osteoclast formation. Our present study provided direct evidence that PFQEP369–373 indeed plays a functional role not only in osteoclast formation but also in osteoclast bone resorption. Moreover, we revealed that RANK contains two other motifs (PVQEQ559–564 and PVQEQ604–609) that are able to promote osteoclast formation and function. PVQEQ559–564 and PVQEQ604–609 are more potent in promoting osteoclast formation than PFQEP369–373 (Fig. 6, C and D). Armstrong et al. (39) previously mapped RANK cytoplasmic regions capable of mediating osteoclast formation and function by reconstituting RANK−/− spleen macrophages with various RANK deletion mutants. Consistently, these two functional motifs are located in the functional RANK regions revealed by this group.

Upon the identification of the functional RANK motifs, we examined the capacity of these motifs to activate several known signaling pathways initiated by RANK. Interestingly, our signaling study showed that although all three motifs are able to activate IκB/NF-κB pathway, they differ in their ability to activate MAPK pathways (Fig. 9), indicating that these functional RANK motifs play distinct roles in initiating intracellular signaling pathways. In keeping with this finding, previous studies suggested that these functional motifs might transmit downstream signaling by recruiting different TRAF proteins. It has been shown that PFQEP369–373 is a TRAF6-binding motif (38). In addition, in vitro data suggested that PVQEQ559–564 and PVQEQ604–609 may bind TRAF proteins other than TRAF6 (20). However, it is still controversial which TRAF proteins specifically bind to PVQEQ559–564 and PVQEQ604–609, whereas Galibert et al. (20) showed that PVQEQ559–564 interacts with TRAF3 and PVQEQ604–609 is capable of binding of TRAF1, TRAF2, and TRAF5, another in vitro study demonstrated that neither TRAF1 nor TRAF3 interact with RANK (8). As such, research is underway in our laboratory to functionally identify TRAF proteins that specifically bind to these RANK motifs in transmitting intracellular signaling in osteoclast formation and function.

Intriguingly, our osteoclastogenesis assays demonstrated that the RANK motifs exhibited a distinct capacity in promoting osteoclast formation in the presence of M-CSF (Fig. 6, C and D). Both PVQEQ559–564 and PVQEQ604–609 are more potent than PFQEP369–373 in forming osteoclasts. Our signaling data revealed that these motifs differ in their ability to mediate signaling pathways (Fig. 9). In particular, they activate different MAPK pathways. However, it is unlikely that the difference in their capacity to form osteoclasts results from their ability to activate MAPK pathways, because all three MAPK pathways can be independently activated by M-CSF present in the osteoclastogenesis assay cultures (33). Therefore, these data rather imply that PVQEQ559–564 and PVQEQ604–609 may activate other as yet unidentified pathways to promote osteoclast formation. Future studies aimed at addressing this issue may provide more insights into the signaling mechanism underling the RANKL-induced osteoclast formation.

The identified functional RANK motifs have great potential to be further explored as therapeutic targets for bone diseases involving osteoclasts. The discovery of the RANKL/OPG regulatory axis has raised high expectation to develop OPG and soluble RANK-Fc as therapeutic drugs to treat bone diseases (40). However, both OPG and RANK-Fc have a possible drawback as therapeutic drugs, primarily because of the fact that their action lacks specificity. RANKL not only plays a pivotal role in osteoclast formation and differentiation (8), but it is also a critical mediator of other biological processes such as the immune system (36, 41) and mammary gland development (37). As such, a result of use of either OPG or RANK-Fc to treat bone diseases may cause potential adverse effects on patients’ immune systems. Thus, future studies of role of the three RANK motifs in other biological systems will help address whether these RANK motifs can serve as specific drug targets for preventing and treating bone diseases such as postmenopausal osteoporosis.

REFERENCES

1. Teitelbaum, S. L. (2000) Science 289, 1504–1508.
2. Suda, T., Takahashi, N., Udagawa, N., Jimi, E., Gillespie, M. T., and Martin, T. J. (1999) Endo. Rev. 20, 345–357.
3. Boyle, W. J., Simonet, W. S., and Lacey, D. L. (2003) Nature 423, 337–342.
4. Darnay, B. G., Ni, J., Moore, P. A., and Aggarwal, B. B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5357–5362.
5. Anderson, D. M., Maraskovsky, E., Billingsley, W. W., Dougall, W. C., Tometsko, M. E., Roos, D. R., Cosman, D., and Galibert, L. (1997) Nature 390, 175–179.
6. Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalichikov, S., Cayan, E., Bartlett, R. F., Frankel, W. N., Lee, S. Y., and Choi, Y. (1997) J. Biol. Chem. 272, 25190–25194.
7. Hsu, L., Lacey, D. L., Dunstan, C. R., Solovyev, I., Colombero, A., Timms, E., Van, G., Capparelli, C., Morony, S., Shiotohmatu, G., Bass, M. B., and Boyle, W. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3540–3545.
8. Burgess, T. L., Qian, Y., Kaufman, S., Ring, B. D., Van, G., Capparelli, C., Kelley, M., Hsu, H., Boyle, W. J., Dunstan, C. R., Hu, S., and Lacey, D. L. (1999) J. Cell Biol. 145, 527–538.
9. Kong, Y. Y., Yoshida, H., Huron, S., Van, T. L., Timms, E., Capparelli, C., Morony, S., Oliveira, d. S. A., Van, G., Ise, A., Kho, W., Wakeham, A., Dunstan, C. R., Lacey, D. L., Mak, T. W., Boyle, W. J., and Penninger, J. M. (1999) Nature 397, 315–323.
10. Dougall, W. C., Glaccum, M., Charrrier, K., Rohrbach, K., Brasel, K., De Smedt, T., Daro, E., Smith, J., Tometsko, M. E., Maliszewski, C. R., Armstrong, A., Shenv, B., Bain, S., Cosman, D., Anderson, D., Morrissey, P. J., Peschon, J. J., Schu, J., and Schu, J. (1999) Genes Dev. 13, 2432–2442.
11. Suda, T., Takahashi, N., Udagawa, N., Morony, S., Capparelli, C., Van, H.-L., McCabe, S., Huff, J., Shiotohmatu, G., Bass, M. B., and Boyle, W. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1566–1571.
12. Arch, R. H., and Thompson, C. B. (1999) Ann. Rev. Cell. Dev. Biol. 15, 113–140.
13. Darnay, B. G., and Aggarwal, B. B. (1999) Ann. Rheum. Dis. 58, (Suppl. 1) I–13.
14. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) Cell 104, 487–501.
15. Darnay, B. G., Daro, E., Ni, J., Moore, P. A., and Aggarwal, B. B. (1998) J. Biol. Chem. 273, 20539–20545.
16. Wang, B. R., Josin, R., Lee, S. Y., Volodyaskei, M., Steinman, R. M., and Choi, Y. W. (1998) J. Biol. Chem. 273, 28355–28359.
17. Kim, H. H., Lee, D. E., Shin, J. N., Lee, Y. S., Jeon, Y. M., Chung, C. H., Ni, J., Kwon, B. S., and Lee, Z. H. (1999) J. Biol. Chem. 274, 2724–2731.
18. Galibert, L., Tometsko, M. E., Anderson, D. M., Cosman, D., Dougall, W. C. (1998) J. Biol. Chem. 273, 34120–34127.
19. Onishi, M., Nosaka, T., Misawa, K., Mui, A. L., Gorman, D., McMahon, M., Miyajima, A., and Kitamura, T. (1998) Mol. Cell. Biol. 18, 3871–3879.
22. Morita, S., Kojima, T., and Kitamura, T. (2000) *Gene Ther.* 7, 1063–1066
23. Feng, X., Novack, D. V., Faccio, R., Ory, D. S., Aya, K., Boyer, M. I., McHugh, K. P., Ross, F. P., and Teitelbaum, S. L. (2001) *J. Clin. Invest.* 107, 1137–1144
24. Takeshita, S., Kaji, K., and Kudo, A. (2000) *J. Bone Miner. Res.* 15, 1477–1488
25. Lam, J., Takeshita, S., Barker, J. E., Kanagawa, O., Ross, F. P., and Teitelbaum, S. L. (2000) *J. Clin. Invest.* 106, 1481–1488
26. Greenfield, E. M., Alvarez, J. I., McLaurine, E. A., Oursler, M. J., Blair, H. C., Osdoby, P., Teitelbaum, S. L., and Ross, F. P. (1992) *Calcif. Tissue Int.* 51, 317–323
27. Unkeless, J. C. (1979) *J. Exp. Med.* 150, 580–596
28. Feng, X., Takeshita, S., Namba, N., Wei, S., Teitelbaum, S. L., and Ross, F. P. (2002) *Endocrinol.* 143, 4868–4874
29. Peschon, J. J., Torrance, D. S., Stocking, K. L., Glaccum, M. B., Otten, C., Willis, C. R., Charrier, K., Morrissey, P. J., Ware, C. B., and Mohler, K. M. (1998) *J. Immunol.* 160, 943–952
30. Inoue, J., Ishida, T., Tsukamoto, N., Kobayashi, N., Naito, A., Azuma, S., and Yamamoto, T. (2000) *Exp. Cell Res.* 254, 14–24
31. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999) *Molecular Cell* 4, 1041–1049
32. Jimi, E., Akiyama, S., Tsurukai, T., Okahashi, N., Kobayashi, K., Udagawa, N., Nishihara, T., Takahashi, N., and Suda, T. (1999) *J. Immunol.* 163, 434–442
33. Wei, S., Wang, M. W., Teitelbaum, S. L., and Ross, F. P. (2002) *J. Biol. Chem.* 277, 6622–6630
34. Matsumoto, M., Sudo, T., Saito, T., Osada, H., and Tsujimoto, M. (2000) *J. Biol. Chem.* 275, 31155–31161
35. Mansky, K. C., Sankar, U., Han, J., and Ostrowski, M. C. (2002) *J. Biol. Chem.* 277, 11077–11083
36. Wong, B. R., Josien, R., and Choi, Y. (1999) *J. Leukocyte Biol.* 65, 715–724
37. Fata, J. E., Kong, Y. Y., Li J., Sasaki, T., Irie-Sasaki, J., Moorehead, R. A., Elliott, R., Scully, S., Veurn, E. B., Khokha, R., and Penninger, J. M. (2000) *Cell* 103, 41–50
38. Ye, H., Arron, J. R., Lamothe, B., Cirilli, M., Kobayashi, T., Shevde, N. K., Segal, D., Daivenu, O. K., Vologodskaia, M., Yim, M., Du, K., Singh, S., Pike, J. W., Darnay, B. G., Choi, Y., and Wu, H. (2002) *Nature* 418, 444–447
39. Armstrong, A. P., Tometsko, M. E., Glaccum, M., Sutherland, C. L., Cosman, D., and Dougall, W. C. (2002) *J. Biol. Chem.* 277, 44347–44356
40. Doggrel, S. A. (2003) *Drugs Today* 39, 633–657
41. Wong, B. R., Josien, R., Lee, S. Y., Sauter, B., Li, H. L., Steinman, R. M., and Choi, Y. (1997) *J. Exp. Med.* 186, 2075–2080
