Activation of NF-\(\kappa\)B by RANK Requires Tumor Necrosis Factor Receptor-associated Factor (TRAF) 6 and NF-\(\kappa\)B-inducing Kinase

IDENTIFICATION OF A NOVEL TRAF6 INTERACTION MOTIF*

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Various members of the tumor necrosis factor (TNF) receptor superfamily activate nuclear factor \(\kappa\)B (NF-\(\kappa\)B) and the \(\kappa\)Jun N-terminal kinase (JNK) pathways through their interaction with TNF receptor-associated factors (TRAFs) and NF-\(\kappa\)B-inducing kinase (NIK). We have previously shown that the cytoplasmic domain of receptor activator of NF-\(\kappa\)B (RANK) interacts with TRAF2, TRAF5, and TRAF6 and that its overexpression activates NF-\(\kappa\)B and JNK pathways. Through a detailed mutational analysis of the cytoplasmic domain of RANK, we demonstrate that TRAF2 and TRAF5 bind to conserved TRAF binding motifs located in the C terminus at positions 565–568 and 606–611, respectively. In contrast, TRAF6 interacts with a novel motif located between residues 340 and 358 of RANK. Furthermore, transfection experiments with RANK and its deletion mutants in human embryonic 293 cells revealed that the TRAF6-binding region (340–358) was not the TRAF2 or TRAF5-binding region, but is necessary and sufficient for RANK-induced NF-\(\kappa\)B activation. Moreover, a kinase mutant of NIK (NIK-KM) inhibited RANK-induced NF-\(\kappa\)B activation. However, RANK-mediated JNK activation required a distal portion (427–603) of RANK containing the TRAF2-binding domain. Thus, our results indicate that RANK interacts with various TRAFs through distinct motifs and activates NF-\(\kappa\)B via a novel TRAF6 interaction motif, which then activates NIK, thus leading to NF-\(\kappa\)B activation, whereas RANK most likely activates JNK through a TRAF2-interacting region in RANK.

RANK* (for receptor activator of NF-\(\kappa\)B), a new member of the tumor necrosis factor (TNF) receptor superfamily, is a 616-amino acid receptor that includes a 383-amino acid intracellular domain with no significant homology to other members of this family (1). Although RANK is ubiquitously expressed in human tissues, its cell surface expression is limited to dendritic cells, the CD4+ T cell line MP-1, and foreskin fibroblasts (1, 2). Human RANK ligand (RANKL/TRANCE/OPGL/ODF), a type II transmembrane protein with an approximate molecular mass of 45 kDa, is expressed primarily on primary T cells, T cell lines, and lymphoid tissue (1, 3–5). Like other ligands of the TNF superfamily, RANKL has been demonstrated to activate nuclear factor \(\kappa\)B (NF-\(\kappa\)B) (1) and \(\kappa\)Jun-terminal kinase (JNK) (3). Furthermore, stimulation of dendritic cells with RANKL up-regulates the expression of the anti-apoptotic protein Bcl-X\(_L\), suggesting a potential role for RANK/RANKL in dendritic cell survival (2). Moreover, RANKL has been demonstrated to play an essential role in osteoclast differentiation and activation (4, 5).

Many of the TNF receptor superfamily members interact with a family of adaptor proteins referred to as TNF receptor-associated factors (TRAFs), which are characterized by a ring and zinc finger motif in their N termini and C-terminal domains that appear to be responsible for self- and non-self associations (6). Of the six known TRAF family members, only TRAF2, TRAF5, and TRAF6 activate NF-\(\kappa\)B and JNK (7), and only TRAF2 has been demonstrated to activate p38 kinase (8, 9). TRAF1, TRAF2, and TRAF5 interact with a characteristic TRAF binding motif, PXQXT, in the cytoplasmic domain of several members of the TNF receptor family (10–16). TRAF6 interacts with the cytoplasmic domain of RANK (16) and with CD40 via a distinct 16-amino acid region (residues 230–245) (11). Furthermore, TRAF6 interacts with interleukin-1 receptor-associated kinase 1 and 2 (IRAK1 and IRAK2) (17–19).

Besides TRAFs, the activation of NF-\(\kappa\)B is also mediated through a recently identified novel member of the mitogen-activated protein kinase kinase kinase family termed NF-\(\kappa\)B-inducing kinase (NIK) (20). NIK was originally identified as a TRAF2-interacting protein (20) and subsequently was found to interact with all TRAF molecules, except TRAF4 (7). When overexpressed in cultured cells, NIK, but not a kinase-inactive mutant (NIK-KM), activates NF-\(\kappa\)B (7, 15, 20) and JNK (15, 21). Furthermore, overexpression of NIK-KM inhibits NF-\(\kappa\)B activation by TNF, interleukin-1, CD27, human T-cell leukemia virus type 1 Tax, and Epstein-Barr virus-transforming protein latent infection membrane protein 1 (7, 15, 20, 22–24). Consequently, the activation of NF-\(\kappa\)B by NIK is mediated through its interaction with the I\(\kappa\)B\(_\alpha\) kinase (IKK\(_\alpha\) and IKK\(_\beta\)) complex (25–28), which results in the phosphorylation and degradation of I\(\kappa\)B\(_\alpha\).

Previous studies from our laboratory showed that the cytoplasmic domain of RANK interacts with TRAF2, TRAF5, and TRAF6 and that its overexpression activates NF-\(\kappa\)B and JNK pathways (16). However, it is not known whether these TRAFs bind to the same region of RANK or which TRAF or TRAFs are necessary for activation of NF-\(\kappa\)B and JNK. Similarly, it is not known whether NIK is involved in RANK-induced NF-\(\kappa\)B ac-
tivation. In addressing the role of various TRAFs and NIK in NF-κB and JNK activation mediated by RANK, we now demonstrate that RANK activates NF-κB by interacting with TRAF6 via a novel TRAF6 interaction motif and TRAF6 potentially activates NIK, leading to NF-κB activation, whereas RANK activates JNK through a TRAF2-interacting region in RANK.

**EXPERIMENTAL PROCEDURES**

**Reagents, Cell Lines, and Antibodies**—Human embryonic kidney 293 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in minimal essential medium supplemented with 10% fetal bovine serum and antibiotics. Monoclonal antibody to Myc (SC40) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-HA from Boehringer Mannheim (Indianapolis, IN); goat anti-rabbit IgG-conjugated horseradish peroxidase from Bio-Rad ( Hercules, CA); anti-FLAG (monoclonal antibody M2) from Eastman Kodak Co. (New Haven, CT); goat anti-mouse IgG-conjugated horseradish peroxidase from Transduction Laboratories (Lexington, KY); protein AG-Sepharose beads from Pierce (Rockford, IL); and the alkaline phosphatase fluorescent substrate 4-methylumbelliferyl phosphate (M3168) from Sigma.

**Expression Plasmids**—Expression plasmids encoding mouse FLAG-tagged TRAF5 and TRAF6 (15) were generously provided by H. Nakano (Juntendo University, Tokyo, Japan). FLAG-tagged TRAF molecules were PCR-amplified using FLAG-tagged NIK and NIK (KK429–430AA) (29). The resulting PCR fragment was digested with EcoRI and SalI sites, respectively, and cloned in-frame with GST in pGEX-KG (29). To generate deletion mutants of the cytoplasmic domain of RANK as glutathione S-transferase (GST) fusion proteins, specific 5’ and 3’ primers containing EcoRI and SalI sites, respectively, were used in PCR reactions with pSPORT3.0-TR6 (18). The resulting PCR products were digested with EcoRI/SalI and cloned in-frame with GST in pGEX-KG (29). To generate cytoplasmic deletion mutants of FLAG-tagged RANK, a parental plasmid containing the extracellular and transmembrane domains of RANK (residues 33–240) was first amplified by specific 5’ and 3’ primers containing HindIII and EcoRI, respectively, and cloned in-frame with the Flag epitope in pCMVFLAG1, resulting in pF-RANK241, which contains an in-frame stop codon 4 residues downstream of residue 240. All the RANK deletion mutants were prepared from the same PCR products utilized for subcloning into pGEX-KG and then subcloned into pF-RANK241 at the EcoRI/SalI site. Due to the subcloning at the EcoRI site, all FLAG-tagged RANK deletion mutants contained homologous amino acid substitutions (i.e. Ala94→Leu142→ Gly254−Ile282). These substitutions, however, did not effect the ability of RANK to activate signaling cascades (see “Results”). The sequence of all plasmids was verified by automated DNA sequencing. Expression and purification of GST-RANK and RANK deletion mutant fusion proteins were essentially as described (30).

**Transient Transfections and Western Blotting**—Human embryonic 293 cells (0.6 × 10⁶ cells/well on 6-well plates) were plated and transfected as described (16). The total amount of plasmid DNA was kept constant by addition of the control plasmid pCMVFLAG1. Cells and the conditioned supernatants were harvested 24–36 h after transfection. Lysates were prepared as described (16). For Western blot analysis, whole cell lysates (15–30 µg) or proteins from GST affinity precipitations were separated by 8.5% SDS-PAGE, electrophoresed onto nitrocellulose membranes (Bio-Rad), and incubated with the indicated antibodies. The membranes were then developed using the enhanced chemiluminescence (ECL) system (Amersham).

**GST-RANK Fusion Protein Affinity Binding Assays**—Equivalent amounts of each GST-RANK fusion protein attached to 20 µl of glutathione-agarose beads were mixed with lysates (50 µg) from 293 cells programmed to express the epitope-tagged TRAF protein in binding buffer (20 m Tris, pH 8, 150 mM NaCl, 1 mM dithiorethiol, 2 mM EDTA, and 0.1% Nonidet P-40) and allowed to rotate for 1 h at 4 °C. The beads were collected by centrifugation, washed three times in binding buffer once in low-salt buffer (20 m Tris, pH 8, 50 mM NaCl, and 1 mM dithiorethiol). Bound proteins were eluted with addition of SDS sample buffer and boiled. The eluted proteins were subjected to 7.5% SDS-PAGE and Western blot analysis was performed with either anti-Myc (TRAF2) or anti-FLAG (TRAF5 and TRAF6) as indicated in the figure legend.

**JNK Kinase Assays**—From transiently transfected 293 cells, lysates were prepared. Approximately 120 µg was then used for immunoprecipitation with anti-HA and protein AG-Sepharose beads for 1 h. Beads were collected by centrifugation, washed three times in lysis buffer, and then washed twice in low-salt buffer. JNK activity was analyzed on an immobilized GST-Jun (binder site) as a substrate as described previously (16). JNK activity was quantitated using PhosphorImager and Imagequant Software (Molecular Dynamics, Sunnyvale, CA). To verify equal transfection, lysates from the transiently transfected cells were subjected to Western blotting with anti-HA.

**NF-κB SEAP Reporter Assays**—To construct a synthetic NF-κB-containing promoter element, a PCR-based strategy was used. The upstream primer contained a fragment of (5′-GGGCGGCTTGGGGGACCTTTCGGGGGACCGTTCGGGACCTTTCGGGATTTTTCGGGACCTTTCGCTCCTGCGATCTGATCAGTGAGC-3′) containing four tandem copies of the NF-κB-binding site (GGGGACCTTTC) and 18 base pairs of a sequence complementary to the 5′ end of the SV40 early promoter sequence, and was flanked with an XhoI site. The downstream primer (5′-GCGGGCAGCTTTGCAAGGCTTGGGACCGTTCGGGATTTTTCGGGACCTTTCGCTCCTGCGATCTGATCAGTGAGC-3′) was complementary to the 3′ end of the SV40 promoter and was flanked with a HindIII site. PCR was performed using the SV40 promoter template. The resulting PCR fragment was digested with XhoI/HindIII and subcloned into a likewise digested SEAP-promoter plasmid to replace the SV40 minimal promoter element.

Human embryonic 293 cells were transiently transfected with pNF-κB-SEAP2 (0.5 µg) and the expression plasmids as indicated in the figure legend (24–36 h). Lysates were removed and assayed for SEAP activity essentially as described by the manufacturer (CLONTECH, Palo Alto, CA). In brief, medium (25 ml) was mixed with 30 µl of 5 times buffer (500 m Tris, pH 9, and 0.5% bovine serum albumin) in a total volume of 100 µl in a 96-well plate and incubated at 65 °C for 30 min. The plate was chilled on ice for 2 min. Then 50 µl of 1 mM 4-methylumbelliferyl phosphate was added to each well and incubated at 37 °C for 2 h. The activity of SEAP was assayed on a 96-well fluorescent plate reader (Fluorocount II, Lab Systems, Needham, Heights, MA) with excitation set at 360 nm and emission at 460 nm. The average (±S.D.) number of relative fluorescent light units for each transfection was then determined and reported as fold activation with respect to the vector-transfected cells. The NF-κB SEAP reporter gene in these assays was shown to be activated by TNFα by overexpression of TNFR2. Similar to the case in previously published reports (7, 9, 20), the specificity was established by this reporter system by the fact that TNF-induced NF-κB SEAP activity was inhibited by overexpression of either an IκBα mutant lacking Ser283/286, a kinase-inactive NIK, or a dominant negative TRAF2 mutant.²

**RESULTS**

In previous studies, we found that the intracellular domain (residues 234–616) of RANK contains three putative TRAF binding motifs of the sequence PXQXT: two located at the C terminus and one localized in the middle of the cytoplasmic domain (16). We also previously reported that TRAF2, TRAF5, and TRAF6 interact with RANK (16) and that overexpression of RANK in 293 cells activates the NF-κB and JNK pathways (1, 16). Therefore, in the present study, we sought to identify more specifically which regions of RANK are responsible for the activation of NF-κB and JNK and, furthermore, to define which TRAF molecules are responsible for these signaling pathways.

**Different Regions of RANK Are Responsible for Binding TRAF2, TRAF5, and TRAF6**—As we previously reported (16), RANK contains three putative TRAF binding motifs (Fig. 1A) and RANK interacts with TRAF2, TRAF5, and TRAF6. To identify which region of the cytoplasmic domain of RANK is necessary for binding TRAF2, TRAF5, and TRAF6, we constructed a series of deletion mutants of the cytoplasmic domain of RANK encompassing the various putative TRAF-binding domains (Fig. 1A). Each of these deletion mutants were fused in-frame with GST and purified by glutathione-agarose affinity chromatography. We examined the ability of each GST-RANK fusion protein to precipitate epitope-tagged TRAF2, TRAF5, and TRAF6 upon their overexpression in 293 cells (Fig. 1B). We observed strong interaction of TRAF2 and TRAF5 with GST-RANK fusion proteins containing residues 529–616. However,² B. G. Darnay and B. B. Aggarwal, unpublished observations.

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while TRAF2 was still capable of binding to GST-RANK fusion proteins lacking the last 13 amino acids, TRAF5 was not (Fig. 1B, top and middle). These data suggest that TRAF-binding domain III is responsible for TRAF5 interaction and that both TRAF II and TRAF III binding motifs are required for high-affinity binding of TRAF2, but the TRAF III binding motif is not essential for RANK's interaction with TRAF2. Unlike TRAF2 and TRAF5, TRAF6 did not interact with TRAF-binding domains II and III (Fig. 1B, bottom). This is consistent with a data indicating that TRAF6 does not bind to the PXQXT motif (11). Surprisingly, TRAF6 did bind to RANK between residues 326 and 427 (Fig. 1B, bottom). Conversely, GST-RANK deletion mutants that did not contain residues 326–427 did not bind TRAF6 (Fig. 1A and B). Inspection of the amino acid sequence between residues 326 and 427 revealed a putative TRAF6 binding motif (see below). Hence, the cytoplasmic domain of RANK appears to interact with TRAF2, TRAF5, and TRAF6 molecules using three distinct motifs.

**A Minimal Region of RANK (Residues 326–427) Activates NF-κB**—How interactions of different TRAFs with RANK affect RANK's ability to activate NF-κB and JNK is not known. To examine this, we constructed FLAG-tagged RANK deletion mutants (identical to those deletion mutants used to construct GST-RANK) in pCMVFLAG1 (Fig. 2A). Their expression was determined by transient transfection in 293 cells and Western blotting with anti-FLAG (Fig. 2B). As expected, all of the FLAG-tagged RANK deletion mutants were expressed similarly in 293 cells.
Next, we examined the ability of each RANK deletion mutant to activate a NF-κB-dependent SEAP reporter construct. Transient overexpression of RANK616 in 293 cells activated NF-κB-dependent reporter activity (Fig. 3A), which could be inhibited by co-transfection of an IκBα mutant lacking its N-terminal phosphorylation sites (data not shown). Deletion of the C-terminal region up to residue 427 (RANK427) had no effect on NF-κB-dependent reporter activity, but further deletion to residue 330 (RANK330) failed to activate NF-κB (Fig. 3A). Furthermore, when only the C-terminal region was fused to the transmembrane domain of RANK, NF-κB-dependent activity was either very weak (RANK429–616) or failed to respond (RANK529–616) (Fig. 3A), although each of the deletion mutants RANK429–616 and RANK529–616 interacted strongly with TRAF2 and TRAF5 (Fig. 2). Truncation of the TRAF5-binding region (RANK603 and RANK326–603) did not appear to affect NF-κB-dependent reporter activity. Together, these data indicate that residues 326–427 are responsible for activation of NF-κB. This was further confirmed by transfection of a deletion mutant, containing only residues 326–427 fused to the transmembrane region of RANK, which activated NF-κB-dependent reporter activity similar to that of RANK616 (Fig. 3A). The observations that some RANK deletion mutants (i.e. R530) activate NF-κB stronger than the full-length suggest that other factors may regulate RANK signaling such as cell surface receptor expression, other receptor-associated factors, and receptor processing. Nevertheless, taken together, these data suggest that the interaction of TRAF2 and TRAF5 with RANK is not required for RANK-induced NF-κB, but that the interaction of TRAF6 with RANK is necessary and sufficient for mediating NF-κB activation by RANK.

A Kinase-inactive NIK Inhibits NF-κB-dependent SEAP Activity Induced by RANK and RANK326–427—When transiently overexpressed in cultured cell lines, NIK, but not a kinase-inactive mutant (NIK-KM), activates NF-κB (7, 15, 20) (Fig. 3B), while NIK-KM inhibits TNF-induced NF-κB dependent reporter activity (7, 20) (data not shown). We thus examined the effect of NIK-KM on RANK616- and RANK326–427-
induced NF-κB reporter activity. Co-transfection of NIK-KM with RANK616 or RANK326–427 inhibited NF-κB-dependent reporter activity (Fig. 3B). Collectively, these data indicate that RANK activates NF-κB via residues 326–427, which interacts with TRAF6. Since TRAF6 has been demonstrated to interact with NIK (7), RANK most likely utilizes the TRAF6-NIK pathway for activation of NF-κB.

TRAF2-binding Domain of RANK Is Required for JNK Activation—Transient overexpression of RANK in 293 cells (16) or treatment of T cells with RANKL (3) has been demonstrated to activate the JNK pathway. We therefore examined the ability of each RANK deletion mutant to activate co-transfected HA-JNK1. RANK616 activated JNK strongly, RANK427 and RANK530 activated JNK marginally, while RANK330 failed to activate JNK (Fig. 4A). Similar to our results in the NF-κB-dependent reporter assay, the C-terminal region of RANK (residues 529–616) failed to activate JNK. Moreover, truncation of the TRAF5-binding domain, residues 604–616 (RANKE603), had no effect on JNK activation, which suggests that RANK’s interaction with TRAF5 is not required for JNK activation. The inability of these deletion mutants to activate JNK was not due to a lack of expression of transfected HA-JNK (Fig. 4B). Furthermore, unlike NF-κB activation by RANK, truncation of the TRAF2-binding domain (i.e. RANK326–427 and RANK326–530) reduced JNK activation by 3-fold when compared with RANKE616 (Fig. 4A). These data suggest that, unlike the TRAF6-binding domain of RANK, which is required for NF-κB activation, the TRAF2-binding domain is required but not sufficient for activation of JNK.

Identification of a Novel TRAF6 Binding Motif in RANK—Of all the members of the TNF receptor superfamily, only CD40 (11) and RANK (16) bind directly to TRAF6. Interestingly, the interleukin-1 receptor interacts indirectly with TRAF6 via its association with IRAK1 and IRAK2, which bind the adaptor protein MyD88 (17–19). Unlike TRAF1, TRAF2, and TRAF5, which interact with receptors through a common PXQXXT motif (10–16), no known binding motif has been described for TRAF6. However, deletion analysis of the cytoplasmic domain of CD40 has implicated a region between residues 230 and 245 of CD40 that interacts with TRAF6 (11). Inspection of this sequence in CD40, residues 326–427 of RANK, and the C terminus of IRAK1 and IRAK2 has revealed a putative TRAF6 binding motif (Fig. 5A). Alignment of these protein sequences suggests that TRAF6 interacts with a consensus sequence having the characteristic pattern, basic QXPXK acidic (Fig. 5A).

To further confirm that this putative TRAF6 interaction motif does indeed bind TRAF6, we constructed various deletion mutants between residues 326 and 427 (Fig. 5B). As stated
above, each of the deletion mutants was constructed as a GST fusion protein and as a FLAG-tagged receptor fused to the transmembrane and extracellular region of RANK. First we examined the ability of these GST-RANK fusion proteins to precipitate epitope-tagged TRAF6 upon its overexpression in 293 cells. In contrast to RANK330 which did not bind to TRAF6 (Fig. 1B), RANK358 interacted strongly with TRAF6 implicating residues between 330 and 358 for binding TRAF6 (Fig. 5C). Furthermore, we observed strong interaction of TRAF6 with all of the deletion mutants shown in Fig. 5B,
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except for RANK358–427, which failed to bind TRAF6 (Fig. 5C). Moreover, a GST-RANK fusion protein containing only residues 340–358 interacted with TRAF6 (Fig. 5C). Overall, these data support our observation that TRAF6 interacts with RANK via a novel motif located between residues 340 and 347.

Next, we examined whether this putative TRAF6 interaction motif is sufficient to activate a NF-κB-dependent SEAP reporter construct. Transient overexpression of RANK616, RANK326–427, and RANK358 activated NF-κB-dependent reporter activity, while RANK330 and RANK358–427 failed to activate NF-κB (Figs. 3A and 5D). These data suggest that residues located between 330 and 358 are critical for NF-κB activation by RANK, which was further confirmed by a RANK deletion mutant containing only residues 326–358 (Fig. 5D). Furthermore, a RANK deletion mutant containing only 19 residues (340–358) linked to the transmembrane and extracellular domain of RANK was sufficient to activate NF-κB-dependent reporter activity, which was not further increased by addition of residues 358–427 (i.e. RANK340–427) (Fig. 5D). The ability of these RANK deletion mutants to activate NF-κB is consistent with their ability to interact with TRAF6, supporting the notion that RANK activates NF-κB via TRAF6. Thus, as with the P6QX6 TRAF binding motif, this putative TRAF6 binding motif (Fig. 5A) may prove useful in identifying other proteins and receptors that could potentially interact with TRAF6.

**DISCUSSION**

Most members of the TNF receptor superfamily activate NF-κB and JNK via their association with TRAF molecules. With the exception of TRAF4, which has no known function, various members of this receptor family, most notably CD30, CD40, CD27, LTβ receptor, TNFR2, and RANK, interact directly with more than one TRAF family member (10, 12, 15, 16, 31–33). Although it is not fully understood how each of these TRAF molecules participates in signaling by these receptor family members, it appears that only TRAF2, TRAF5, and TRAF6 are functionally competent to activate downstream signaling pathways (7).

We have demonstrated that TRAF2, TRAF5, and TRAF6 interact with RANK via three distinct motifs in the cytoplasmic domain of RANK. Furthermore, our data suggest that NF-κB activation by RANK is dependent upon its interaction with TRAF6, while JNK activation by RANK is dependent in some way on TRAF2. This is consistent with the observation in mouse thymocytes that RANKL could activate JNK and that this activation could be inhibited by transgenic expression of a dominant negative TRAF2 (2). The significance of TRAF5 interaction with RANK is not clear. Moreover, on the basis of previous deletion studies with CD40 (11) and our deletion studies of RANK reported here, we have identified a novel TRAF6 binding motif (Fig. 5A) that is also present in two other TRAF6-interacting proteins, IRAK1 and IRAK2 (17–19). Whether the TRAF6-binding region in RANK is required for other biological signaling events in addition to NF-κB activation is unclear; however, it is likely that more than one signal could emanate from different regions of RANK and could cooperate to induce biological responses.

While our report was under review, a similar study was published indicating the interaction of various TRAF molecules with the mouse homolog of RANK (34). Consistent with our data, that study demonstrated that the C terminus of mouse RANK interacts with TRAF2 and TRAF5, but TRAF6 appears to interact near the middle and N terminus of the cytoplasmic domain of RANK. Furthermore, their data implicated the N terminus and middle regions of RANK for activation of NF-κB, although the C terminus of RANK could activate NF-κB, albeit weakly. In addition, these authors co-transfected dominant negative mutants of TRAF2, TRAF5, and TRAF6 with RANK to examine whether these TRAFs could block RANK-induced NF-κB activation. Similar to our observations, these authors were unable to completely inhibit NF-κB activation by coexpression of dominant negative versions of these TRAF molecules (34). Moreover, besides activation of NF-κB, transient expression of RANK also activated c-Jun and Elk-1-dependent transcriptional activity (34). These data would suggest that like CD30 (35), RANK may activate TRAF-independent signaling pathways or interact with unknown TRAF-like molecules to activate NF-κB and possibly other transcription factors.

Overall, we have demonstrated the ability of RANK to interact with various TRAF molecules through distinct motifs in the cytoplasmic domain of RANK. That more than one TRAF molecule interacts with RANK may suggest a cell-type- and TRAF-dependent signaling cascade initiated by RANK. To date, RANK activates at least three transcription factors, i.e. NF-κB, c-Jun, and Elk-1, which may be linked to the expression of anti-apoptotic genes in dendritic cells and the expression of genes involved in differentiation of osteoclasts. The identification of these genes may lead to the understanding of the function of RANKL/RANK in dendritic cells and osteoclasts.

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**Note Added in Proof**—While our paper was under review, a similar study was reported by Galibert et al. (Galibert, L., Tometsko, M. E., Anderson, D. M., Cosman, D., and Dougall, W. C. (1998) J. Biol. Chem. 273, 34120–34127) that demonstrated TRAF1, 2, 3, 5, and 6 interaction with RANK. Similar to our results, these authors observed interaction of TRAF1, 2, 3, and 5 with the C terminus of RANK whereas TRAF6 interacted with a membrane proximal region between residues 340 and 421 of RANK. Additionally, like our results, these authors demonstrated that deletion of residues 340–421 inhibited RANK-induced NF-κB activation, whereas JNK activation was only partially inhibited.

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