The Involvement of Multiple Tumor Necrosis Factor Receptor (TNFR)-associated Factors in the Signaling Mechanisms of Receptor Activator of NF-κB, a Member of the TNFR Superfamily*

(Received for publication, July 1, 1998, and in revised form, October 1, 1998)

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Receptor activator of NF-κB (RANK) is a recently identified member of the tumor necrosis factor receptor superfamily and is expressed on activated T cells and dendritic cells. Its cognate ligand (RANKL) plays significant roles in the activation of dendritic cell function and osteoclast differentiation. We demonstrate here the interaction of RANK with tumor necrosis factor receptor-associated factors (TRAFs) 1, 2, 3, 5, and 6 both in vitro and in cells. Mapping of the structural requirements for TRAF/RANK interaction revealed multiple TRAF binding sites clustered in two distinct domains in the RANK cytoplasmic tail. These TRAF binding domains were shown to be functionally important for the RANK-dependent induction of NF-κB and c-Jun NH2-terminal kinase activities. Site-directed mutagenesis demonstrated that these TRAF binding sites exhibited selective binding for different TRAF proteins. In particular, TRAF6 interacted with membrane-proximal determinants distinct from those binding TRAFs 1, 2, 3, and 5. When this membrane-proximal TRAF6 interaction domain was deleted, RANK-mediated NF-κB signaling was completely inhibited while c-Jun NH2-terminal kinase activation was partially inhibited. An NH2-terminal truncation mutant of TRAF6 inhibited RANKL-mediated NF-κB activation, but failed to affect constitutive signaling induced by receptor overexpression, revealing a selective role for TRAF6 in ligand-induced activation events.

RANK1 and RANK ligand (RANKL) are recently described cognate pair of the TNF receptor/ligand superfamilies (1). The receptor (RANK) cDNA was originally isolated from a human DC cDNA library and shows the highest homology (40% identity within the extracellular domain) with CD40 among TNFR family members. Among antigen-presenting cells, RANK surface expression appears to be specific to DC and can be significantly up-regulated by a DC activator, CD40 ligand. However, RANK protein expression is not DC-specific as RANK is also expressed on human peripheral blood T cells treated with phytohemagglutinin and IL-4 or transforming growth factor-β (1).

In contrast to the relatively specific protein expression, RANK mRNA is broadly expressed in a variety of tissues including skeletal muscle, thymus, liver, colon, adrenal gland, and small intestine. The discrepancy between mRNA and surface protein expression suggests complex post-transcriptional regulatory mechanisms for RANK expression. Cells may therefore express RANK after discrete activation or differentiation conditions.

The identification of the cognate ligand for RANK (RANKL) was performed by direct expression cloning from a mouse CD4+ thymoma cell line (1). The same ligand has also been identified by screening a T cell hybridoma cell line (termed TRANCE) (2) and as an osteoclast differentiation factor (3) whose activity can be inhibited by a soluble TNF receptor family member, osteoprotegerin (4). RANKL mRNA appears to have a more restricted tissue expression pattern than RANK and has only been detected in mouse thymus, lymph node, spleen (1, 2), bone marrow stroma, and trabecular bone (3). Specific lymphoid cells that express RANKL include both CD4+ and CD8+ T cells and B cell progenitors (1, 2). TCR stimulation of T cell hybrids leads to the rapid induction of RANKL/TRANCE mRNA (2).

Studies of the biological function of the RANK/RANKL interaction demonstrate that RANKL promotes the survival of transforming growth factor-β-treated T cells and increases the clustering and allo-stimulatory capacity of human DC (1). RANKL may promote DC survival by a BCL-XL-dependent mechanism (5). The recent characterization of RANKL as an essential factor for osteoclast differentiation and activation (3, 4) demonstrates additional activities of RANKL on myeloid lineages. Although the role of RANK in bone resorption/osteoclast differentiation has not been elucidated, RANK and its ligand appear to be important in the regulation of T cell/DC interactions and may also function in other important cellular differentiation processes.

Although the physiological functions of RANK and RANKL have been investigated, the mechanisms of RANK signal transduction have not been intensively studied. Multimerization of TNFR family members (as a result of ligand binding or receptor overexpression) is thought to lead to receptor activation and signal transduction, perhaps by revealing binding domains for enzymes or adaptor proteins. In recent years, substantial progress has been made to define the cytoplasmic proteins that function as adaptors (TRAFs 1–6, TRADD, FADD) or as serine/threonine kinases (RIP) and link TNF receptor stimulation with the induction of cell death, Jun kinase (JNK) or NF-κB activation pathways (reviewed in Ref. 6). RANK stimulation leads to activation of the nuclear transcription complex NF-κB in RANK-expressing human T cells and transfected 293 cells.

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1 The abbreviations used are: RANK, receptor activator of NF-κB; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNF receptor-associated factor; RANKL, RANK ligand; EMSA, electrophoretic mobility shift assay; DC, dendritic cell; GST, glutathione S-transferase; TBS, TRAF binding site; JNK, c-Jun NH2-terminal kinase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; IL, interleukin; MOPS, 4-morpholinepropanesulfonic acid.
(1) and JNK (2) in mouse thymocytes. However, the RANK cytoplasmic determinants or the cytoplasmic effector/adaptor proteins necessary for downstream signaling have not been described. While the 383-amino acid cytoplasmic domain of RANK is the largest of any known TNFR, it does not contain sequences suggestive of catalytic activity or significant homology with any known protein. The amino acid sequences of the human and mouse RANK cytoplasmic domains include multiple sections that show striking homology (64% amino acid identity and 78% similarity) between species (1), suggesting a conserved functional role for these structures. Since many members of the TNFR superfamily that do not contain a death domain (p80 TNFR, CD40, CD30, CD27, OX40, 4–1BB, LTβR, HVEM) interact with the cytoplasmic adaptor/effectors TRAF proteins (7–13), we designed an approach to experimentally define TRAF binding to RANK. In this study we have identified the structural and functional features of RANK required for both NF-κB and JNK-mediated signaling. Our analysis delineates multiple distinct and independent domains capable of binding TRAF proteins and transmitting downstream signals. In addition, TRAF6 appears to mediate RANK function through unique determinants independent of other TRAF proteins and plays a selective role in RANKL-induced signaling.

MATERIALS AND METHODS

Production and Purification of GST Fusion Proteins—Deletion constructs of RANK were generated by PCR amplification, and point mutants were generated by PCR using primers containing the appropriate nucleotide substitutions. The appropriate inserts were subcloned into the pGEX-4T (Amersham Pharmacia Biotech) vector at the BamHI and EcoRI sites. The correct sequence of each of the PCR-generated inserts was confirmed by DNA sequencing. A GST-human FAS cytoplasmic domain construct was used as a specificity control. GST fusion proteins were expressed and purified from E. coli and each of the zinc fingers (TRAF6-(289–522)) (19). A deletion (TRAF2-(87–501)) removed the first 86 amino acids (as described (21)). Oligonucleotides containing an NF-κB-binding site were annealed, radiolabeled with γ-32P-ATP and combined with 10 μg of nuclear extracts for 20 min at room temperature. Specificity of the reaction was confirmed by competition with 50-fold molar excess of non-labeled wild-type oligonucleotides or oligonucleotides containing a mutated NF-κB binding site. The protein-DNA complexes were resolved by 6% PAGE in 0.25× TBE buffer and visualized by autoradiography.

Protein Kinase Assays—For JNK assays, whole cell extracts were prepared from 293 cells transfected with full-length or cytoplasmic truncations of RANK or control vector 24 h after transfection as described (22). Oligonucleotides containing an NF-κB binding site were annealed, radiolabeled with γ-32P-ATP and combined with 10 μg of nuclear extracts for 20 min at room temperature. Specificity of the reaction was confirmed by competition with 50-fold molar excess of non-labeled wild-type oligonucleotides or oligonucleotides containing a mutated NF-κB binding site. The protein-DNA complexes were resolved by 6% PAGE in 0.25× TBE buffer and visualized by autoradiography.

RESULTS

Interaction of the RANK Cytoplasmic Domain with TRAFs 1, 2, 3, 5, and 6—In order to define whether TRAFs may bind to RANK, each of the known TRAFs was transcribed and translated in vitro in the presence of [35S]methionine/cysteine and coprecipitation assays were performed to determine the interaction with a GST fusion expressing the full-length RANK cytoplasmic domain. This domain (RANK amino acids 206–616) was able to interact with TRAFs 1, 2, 3, 5, and 6 in a specific manner (Fig. 1). We observed strong binding of RANK with TRAFs 1, 2, 3, and 6; weak interaction with TRAF5; and no binding with TRAF4. In contrast, none of the TRAFs interacted with a control GST protein (Fig. 1) or a GST-FAS cytoplasmic domain fusion protein (data not shown). To confirm that the in vitro interaction of TRAFs with RANK also occurred in cells, co-immunoprecipitation experiments were performed in 293 cells cotransfected with RANK and epitope-tagged TRAFs and revealed that full-length RANK associated with the same repertoire of TRAFs (TRAFs 1, 2, 3, 5, and 6) but not TRAF4 (data not shown).

To identify the binding sites in the RANK cytoplasmic domain, four individual COOH-terminal deletions were constructed and the in vitro binding assays were repeated. In contrast to the full-length RANK cytoplasmic domain, a deletion construct that lacks the COOH-terminal 72 amino acids (RANK Δ544) was unable to interact with TRAFs 1, 2, 3, and 5 (Fig. 1), and TRAF6 binding was reduced by approximately 60% (19) to the manufacturer's instructions (Promega) using an EG&G/Berthold luminometer. Relative luciferase activities were normalized to the β-galactosidase activity.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from 293 cells transfected with full-length or cytoplasmic truncations of RANK or control vector 24 h after transfection as described (21). Oligonucleotides containing an NF-κB binding site were annealed, radiolabeled with γ-32P-ATP and combined with 10 μg of nuclear extracts for 20 min at room temperature. Specificity of the reaction was confirmed by competition with 50-fold molar excess of non-labeled wild-type oligonucleotides or oligonucleotides containing a mutated NF-κB binding site. The protein-DNA complexes were resolved by 6% PAGE in 0.25× TBE buffer and visualized by autoradiography.

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Protein Kinase Assays—For JNK assays, whole cell extracts were prepared from 293 cells 24 h after transfection. Cells were lysed in a buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerolphosphate, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, and the protease inhibitors leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride. Clarified lysates were immunoprecipitated with 1 μg each of anti-JNK (FL) and anti-JNK (C17) (both from Santa Cruz Biotechnology, Inc. Santa Cruz, CA). The immune complexes were washed three times in lysis buffer, two times with wash buffer (500 mM LiCl, 100 mM Tris, pH 7.5, 0.1% Triton X-100, 1 mM dithiothreitol) and three times in assay buffer (20 mM MOPS, pH 7.0, 2 mM EGTA, 10 mM MgCl2, 1 mM dithiothreitol, 0.1% Triton X-100). JNK activity was determined by an immune-complex assay using 1 μg of GST-c-Jun-(1–169) (Upstate Biotechnology Inc., Lake Placid, NY) and 5 μCi of [32P]ATP as substrate in 40 μl of assay buffer at 30 °C for 20 min. Reaction products were resolved on 4–20% SDS-PAGE and visualized by autoradiography.
revealed multiple regions with limited homology with experimentally defined TRAF binding sites (TBS) in other TNFR family members. We defined two potential RANK TBS in this region (residues 569–574 (P-V-Q-E-E-T) and residues 607–611 (P-V-Q-E-Q)) due to their similarity to the P-X-Q-X-T TBS found in CD40 and CD30 (22) as well as the HVEM TBS.

**FIG. 1.** Interaction of TRAFs with the RANK cytoplasmic region. A, schematic representation of RANK cytoplasmic mutant constructs. The amino acid positions of the cytoplasmic region of RANK are shown for the full-length RANK beginning at residue 206. The stippled box from amino acid 213 to 233 represents the transmembrane region, and the shaded boxes marked with Roman numerals I, II, and III represent putative TRAF binding sites as discussed under “Results.” The COOH-terminal deletions (RANK Δ constructs) are shown, including the position of the COOH-terminal amino acid. The constructs containing point mutations are described with the amino acid substitutions above the putative TRAF binding sites. The RANK Δ340–421 mutation is an internal in-frame deletion of residues 340–420 including the TRAF binding site I. These mutants were expressed either in bacteria as GST fusion proteins with the RANK sequence beginning at position 206 or in mammalian cells within the context of the remaining NH2-terminal RANK sequence. B, TRAF binding to RANK was measured by incubating purified GST fusion proteins containing the RANK cytoplasmic domain (and COOH-terminal RANK deletions) separately with TRAF proteins 1–6 translated in vitro with [35S]methionine/cysteine. Bound complexes were precipitated with glutathione-agarose as described under “Materials and Methods,” resolved by SDS-PAGE, and visualized by fluorography. A fraction of the labeled input protein (2%) has also been directly run on the gel. As a specificity control, GST beads were also incubated with labeled TRAFs. Each of the GST fusion proteins were used at the same concentration, and equal loading of lanes was confirmed by visualization with Coomassie Brilliant Blue R-250 staining.
To determine if these residues in RANK were important for TRAF binding, we substituted certain amino acids within these putative TBS with alanine and assayed TRAF binding. Mutagenesis of amino acids 609–610 (Q-E) to alanine within the full-length RANK cytoplasmic domain significantly abolished TRAF1, TRAF2, and TRAF5 binding (Fig. 2), similar to that seen with the GST-RANK Δ544 deletion. Binding of TRAF3 and TRAF6 was unaffected. Substitution of residues 571–573 (Q-E-E) with alanine resulted in a loss of TRAF3 binding without reducing the binding of any other TRAF. These results identify two separate regions critical for binding TRAFs 1, 2, 3, and 5. By combining mutations of these two sites (571–573 and 609–610), loss of TRAFs 1, 2, and 3 binding was more pronounced (Fig. 2), suggesting that these two sites cooperate in binding. None of the mutations examined, either alone or in combination, reduced TRAF6 binding, indicating that TRAF6 recognizes distinct TBS in RANK.

RANK Stimulation Induces the Activation of NF-κB—We have previously reported that activation of RANK either by receptor overexpression or by RANKL treatment leads to the activation of NF-κB complexes (1). In order to study the role of RANK cytoplasmic sequences and other cellular protein effectors in RANK signaling, we established a transient transfection/NF-κB-responsive reporter system. We first examined reporter activity resulting from increased RANK expression in the 293 cells. NF-κB-dependent reporter activity increased in a RANK dose-dependent manner until optimal (30-fold) induction was achieved with 6.4 ng of RANK DNA transfected (Fig. 3A). These data illustrate that ectopic overexpression of RANK can lead to ligand-independent NF-κB signaling, similar to that seen with other TNFR family members, p80 TNFR and CD40 (7, 23).

RANKL-dependent signaling was next examined by the transfection/reporter system using a suboptimal RANK DNA concentration (0.4 ng). NF-κB-responsive luciferase reporter plasmid (pIL-8/REP) and pDC304/β-galactosidase. Cells were treated for 18 h with 500 ng/ml soluble recombinant human RANKL-leucine zipper (rhRANKL) (see “Materials and Methods”). Luciferase activity was measured as described under “Materials and Methods.” In order to determine the activity of transmembrane ligand expression on RANK-mediated reporter activity, cells were also cotransfected with expression vectors for the full-length RANKL. Transfection of the transmembrane ligand had no significant affect on reporter activity in the absence of transfected receptors (not shown).
that these responses are due to NF-κB activation.

*The Induction of NF-κB Is Mediated by Two Separate RANK Cytoplasmic Domains*—The interaction of TRAFs with multiple RANK cytoplasmic domains and the ability of some TRAFs to mediate NF-κB activation (7, 24) suggests that the deletion of TBS may abrogate NF-κB-dependent signaling. To address this possibility, COOH-terminal cytoplasmic deletions of RANK were expressed in 293 cells and reporter activity was measured. Equivalent expression levels of each RANK construct were confirmed by immunoprecipitation of metabolically labeled proteins and by flow cytometry (data not shown). Transfection of 293 cells with the RANK deletion construct lacking the COOH-terminal 72 amino acids (RANK Δ544) resulted in reduced NF-κB reporter activity in the absence of RANKL activation (Fig. 4A). However, RANKL treatment of RANK Δ544-expressing cells induced NF-κB activation to levels similar to that seen with full-length RANK. Further deletion of COOH-terminal sequences had minimal effects on the constitutive and RANKL-mediated reporter activity until the removal of amino acids 339–422 (construct RANK Δ339), which completely abrogated both constitutive signaling and responsiveness to RANKL. The deletion of RANK cytoplasmic determinants had similar effects on NF-κB activation as determined by direct EMSA assays (Fig. 4B). Taken together, these data suggest that RANK contains two domains (amino acids 339–422 and 544–616) within its cytoplasmic tail important for NF-κB signaling.

These two RANK functional regions correspond with the two domains that affect binding of TRAFs 1, 2, 3, 5, and 6 (amino acids 544–616) and TRAF6 (amino acids 339–422). Moreover, the deletional analysis of RANK domains important for NF-κB signaling expose differences between RANK signal transduction as a result of receptor overexpression and RANKL treatment. Constitutive signaling resulting from RANK overexpression was significantly affected by the loss of the COOH-terminal 72 amino acids (RANK Δ544). However, RANKL-mediated signaling was only affected by the deletion of amino acids 339–422 correlating to the loss of direct TRAF6 binding in vitro.

**Activation of NF-κB by RANK/RANKL Is Mediated by TRAFs**—In the absence of receptor expression, TRAFs 2, 5, and 6 activated NF-κB (data not shown) as has been reported previously (7, 24). To examine the functional involvement of these TRAFs in RANK-mediated NF-κB activation, 293 cells were co-transfected with RANK and expression vectors encoding NH2-terminal truncations of TRAF2, TRAF5, and TRAF6, each of which has been demonstrated to suppress NF-κB signaling in a dominant negative manner (7, 18, 24). Co-expression of the zinc ring-deleted forms of TRAFs 2, 5, or 6 each inhibited the RANKL-inducible reporter activity in a concentration-dependent fashion (Fig. 5). However, only NH2-truncated TRAF2 and TRAF5 suppressed constitutive signaling from the full-length RANK. The NH2-truncated TRAF6 selectively inhibited RANKL-induced signaling, but not constitutive signaling, revealing a specific role for TRAF6 in RANKL-induced signaling.

**Activation of JNK by RANK**—To determine whether human RANK receptor will lead to activation of JNK, protein kinase assays were performed in 293 cells overexpressing RANK. Transfection of RANK significantly induced JNK activity to levels exceeding that seen with TNFα treatment or overexpression of p60 TNFR (Fig. 6). Truncation of the COOH-terminal 72 amino acids of RANK abrogated the majority of JNK activity. The residual JNK activity was completely inhibited after the truncation of amino acids 339–422 in the RANK cytoplasmic domain (RANK Δ339). These results demonstrate that two distinct RANK cytoplasmic domains (residues 544–616 and 339–422) play functional roles in JNK activation, similar to the domains necessary for NF-κB activation described above (Figs. 4 and 5).

**Essential Role of the TRAF6 Binding Site in NF-κB, but Not JNK, Signaling by RANK**—The experiments utilizing overexpression of COOH-terminal RANK truncation mutants demonstrate that TBS II and III contribute to JNK and NF-κB activation. However, treatment of these RANK mutants with exogenously added RANKL revealed that the region which binds TRAF6 in vitro (residues 340–421) plays the most critical role in NF-κB activation. To test the role of this domain directly, we constructed an in-frame deletion (RANK Δ340–...
which removes the TRAF6 binding site and leaves intact the downstream domains capable of interacting with TRAFs 1, 2, 3, and 5 (TBS II and III). As shown in Fig. 7A, there was no detectable NF-κB activation resulting from either overexpression or RANKL activation of RANK Δ340–421 as compared with full-length RANK. This lack of response was very similar to that of the RANK Δ339 mutant, which has been shown to be incapable of binding any TRAF (Fig. 1). This result confirms the critical requirement for TRAF6 binding to this region for NF-κB activation and illustrates that the downstream TBS II and III cannot function independently to mediate NF-κB activation. Deletion of RANK residues 340–421 reduced JNK activity relative to that seen with full-length RANK (Fig. 7B); however, in contrast to the NF-κB results, this internal deletion still retained some activation of JNK above the vector control. This reduced, yet detectable, JNK activity was also

FIG. 5. Involvement of TRAF2, TRAF5, and TRAF6 in RANKL- and overexpression-induced NF-κB activation. 293 cells were transiently co-transfected with the full-length RANK and the NF-κB-responsive IL-8 luciferase reporter plasmid. In addition, expression vectors for the indicated dominant negative TRAF2, TRAF5, or TRAF6 constructs were also co-transfected in increasing concentrations (50–250 ng/transfection). The amounts of DNA were equalized by dsDNA transfection by the addition of empty vector pDC304. Cells were treated with 500 ng/ml recombinant human RANKL-leucine zipper (rhRANKL) where indicated, and luciferase activities were measured 16–18 h after treatment. As a specificity control for the dominant negative activity of the TRAFs, transfection of cells with up to 250 ng of pDC304/LACZ did not affect RANK- or RANKL-dependent signaling (not shown). Relative luciferase activities were normalized to β-galactosidase expression levels and are reported as mean values ± S.E. from triplicate transfections. The data are representative of three experiments.

FIG. 6. Activation of JNK by RANK. 293 cells were transiently transfected with empty vector (pDC304) control, p60 TNFR expression construct, full-length RANK, or the indicated COOH-terminal RANK deletion expression vectors. After 24 h, cells were treated with TNF-α (50 ng/ml) for 20 min or left untreated. Cell lysates were immunoprecipitated with anti-JNK antibodies and the JNK activity was determined by an immune-complex kinase assay using GST-c-Jun(1–169) as a substrate. The products of the kinase assay were resolved by SDS-PAGE and visualized by autoradiography. The position of phosphorylated GST-c-Jun is indicated by the arrow.

FIG. 7. An internal deletion of the TRAF6 binding site completely inhibits RANK-mediated NF-κB activation but only partially inhibits JNK activation. A, human 293 cells were transiently transfected with the expression vector DNA and relative luciferase activities were measured as described under "Materials and Methods" and Fig. 3. The RANK Δ340/421 mutation is an internal in-frame deletion of residues 340–420 including the TRAF6 binding site. B, human 293 cells were transiently transfected with 2.5 μg of each plasmid and JNK activity was measured as described under "Materials and Methods" 24 h after transfection. C, to examine RANKL-induced JNK activation, 293 cells were transiently transfected with 50 ng of each expression vector and JNK activity was measured using GST-c-Jun(1–169) as a substrate 24 h later. This concentration of full-length RANK expression vector did not activate JNK in the absence of added RANKL. Where indicated, cells were treated with 1 μg/ml soluble rhRANKL for 20 min prior to cell lysis. For each experiment, immunoprecipitation of35S-labeled RANK demonstrated equivalent expression of each protein construct (data not shown). The data shown are representative of three independent transfections.
exhibited after RANKL activation of the RANK Δ340–421 construct (Fig. 7C). Therefore, this region (340–421), which we have shown to be capable of binding TRAF6 directly, is required for NF-κB and contributes to JNK activation by RANK.

**DISCUSSION**

The TNFR superfamily consists of type I membrane proteins that can mediate diverse effects including apoptosis, differentiation, and survival upon ligand-mediated receptor activation. To date, each TNFR family member without a death domain has been shown to interact with cytoplasmic TRAF adapter proteins either directly or indirectly. However, signaling pathways affected by different TNFR members are unique and frequently non-redundant. The specificity of RANK signaling through TRAFs may then be determined by multiple factors: 1) the cellular abundance and localization of the TRAF proteins; 2) the relative affinity of TRAFs for cytoplasmic TRAF-binding proteins including other TRAFs, I-TRAP/TANK, TRIP, C-IAPs, and A20 (reviewed in Ref. 6); and 3) the relative affinity of these adaptor/effecter proteins for the RANK cytoplasmic domain. We have shown that five of the six known TRAF can directly bind RANK cytoplasmic determinants, constituting the largest complement of TRAF proteins known to bind any TNFR family member and suggesting that RANK may utilize a versatile cast of effector molecules to transmit signals.

RANK contains two independent TRAF binding regions: amino acids 544–616, which affects binding of multiple TRAFs (TRAFs 1, 2, 3, 5, and 6); and 340–421, which binds TRAF6 only. We engineered specific point mutants of the putative RANK TBS in order to reveal critical RANK features for TRAF binding in vitro. Alanine substitutions within positions 571–573 uncovered selective binding determinants for TRAF3 at this site. Point mutations at positions 609–610 significantly affected TRAFs 1, 2, and 5 binding to RANK without affecting TRAF3 or TRAF6 binding. The critical Q-E element of these two TBS in RANK is similar to TRAF binding elements found in CD30 (22) and OX40 (12). However, receptor-specific selectivity and affinity of TRAF binding appears to be influenced by sequences surrounding this core region.

Deletion analysis clearly shows that TRAF6 directly binds to RANK within amino acids 340–421 (Fig. 1). Removal of amino acids 544–616 resulted in a 50% loss of TRAF6 binding intensity, but direct binding of TRAF6 to this latter region of RANK was not unambiguously determined. Since alanine substitutions of TBS within this COOH-terminal 72 amino acids did not reduce TRAF6 binding, either TRAF6 binds to two disparate sites (within positions 340–421 and 544–616) independently of other TRAF proteins or some structure within the COOH-terminal tail can positively affect TRAF6 binding to the upstream (340–421) region. Current experiments are under way to distinguish these possibilities.

We have demonstrated that RANK triggering of both the NF-κB and JNK pathways requires cytoplasmic elements necessary for TRAF binding. The deletion of the COOH-terminal 72 residues (RANK Δ544), which decreases binding of TRAFs 1, 2, 3, 5, and 6, resulted in a major reduction of RANK overexpression-induced NF-κB and JNK activity (Figs. 4 and 6). Any residual NF-κB or JNK activity was eliminated when the TRAF6 binding region (amino acids 340–421) was removed. RANKL treatment of RANK Δ544-expressing cells induced NF-κB activation to levels equivalent to full-length RANK (Fig. 4A), suggesting that the ability to directly bind TRAF6, in the absence of direct binding of other TRAFs, allows optimal RANK signaling.

Although we identified a region with limited homology to other TBS at residues 353–357 (site I; P-S-Q-P-T) within the upstream region important for RANK signal transduction, we were unable to identify the specific binding site for TRAF6 by site-directed mutagenesis (data not shown). Therefore, to directly test the hypothesis that TRAF6 binding is pivotal for RANK-mediated NF-κB and JNK activation, we constructed an in-frame deletion of amino acids 340–421 (RANK Δ340–421). The deletion of this region resulted in complete inhibition of NF-κB activation, while JNK activation was inhibited to a lesser degree. Thus, two separate regions of RANK (340–421 and 544–616) can independently activate JNK by virtue of the appropriate TRAF binding to each of these domains. These data also suggest that for activation of JNK, TRAF6 is functionally redundant with other TRAFs (TRAF2 and TRAF5) binding downstream. Since residues 340–421 are necessary for NF-κB signaling by RANK in this model cell system, TRAF6 binding and coupling to the appropriate downstream effector proteins plays a critical role in this pathway. Similar to TRAF2 and TRAF5, TRAF6 has been shown to act upstream of both NF-κB and JNK pathways (24); however, these data suggest that, after RANK activation, TRAF6 may couple to distinct downstream effector proteins from those activated by TRAF2 or TRAF5.

The ability of TRAF6 to functionally complement NF-κB signaling in RANK is reminiscent of its role in CD40 signaling (8). Similar to RANK, CD40 binds TRAF6 via a distinct domain independently of other TRAF interactions and can mediate NF-κB signaling in the absence of direct TRAF2 or TRAF5 binding. The N-terminal deletion of TRAF6 only affects RANK-induced reporter activity, while the NH2-terminal deletions of TRAFs 2 and 5 affected signals resulting from both RANKL treatment and RANK overexpression (Fig. 5). This illustrates another unique feature of TRAF6 in RANK signaling, and also suggests that there may be qualitative differences in TNFR family signaling resulting from the normal interaction of ligand and receptor versus the artificial signal emanating from overexpressed receptor. This latter observation has implications for the signaling mechanisms of the Epstein-Barr virus latent membrane protein 1 (LMP1), which mimics a constitutively active receptor by aggregating in the plasma membrane (25) and utilizes TRAF proteins for the activation of NF-κB (26–29) and other pathways (30).

Although it is not clear whether RANK may utilize TRAF-independent signaling mechanisms during activation or differentiation of RANK-expressing cells, we show here that the functional consequence of TRAF binding to RANK appears to be the downstream regulation of multiple gene activation pathways. The resulting activation of nuclear transcription factors NF-κB and AP-1 play an unequivocal role to trigger inflammatory, immune, and acute phase gene expression. While these studies expose a particular role for TRAF6 in RANK signal transduction, the exact localization of the TRAF6 binding sites will help to further elucidate the role of TRAF6 binding in RANKL-induced signaling events. The examination of RANK constructs incapable of binding each of the different TRAFs in the context of cellular activation (e.g. DC) or differentiation will be important to evaluate TRAF-dependent and independent RANK signaling mechanisms.

**Acknowledgments**—We thank Kathy Maggiora for cell transfection help; Dirk Kooistra for the pDC904 TRAF6–(289–522) construct; Jeanette Bertles, Chang-Pin Huang, Marty Timour and Gordana Sapina for DNA sequencing; Drs. Doug Williams and Timothy Bird for critically reviewing the manuscript; Mari Hall and Gary Carlton for graphics assistance; and Anne Aumell for editorial assistance.

**Addendum**—While this paper was under review, Darnay et al. (31) also described the association of RANK with TRAFs 2, 3, 5, and 6. In contrast to our observations here, these authors did not detect TRAF6 binding to RANK cytoplasmic elements upstream of residue 530. This discrepancy may be explained by the use of RANK purified from trans-
fected cells (31), which may already have bound cytoplasmic proteins capable of competing with exogenously added TRAFs. Our data reported in the current study, using bacterially expressed RANK cytoplasmic domain and in vitro translated TRAFs, clearly show the direct association of TRAF6 with RANK residues 340–421. In addition, Darnay et al. (31) did not observe a role for residues upstream of RANK 530 in NF-κB activation, while we demonstrate here a critical role of residues 340–421 in NF-κB activation in response to receptor overexpression and RANKL-mediated activation using two separate assay systems. The reasons for this discrepancy are not clear and may be due to subtle differences in the experimental systems used.

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J. Biol. Chem. 1998, 273:34120-34127.
doi: 10.1074/jbc.273.51.34120

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