Characterization of the Intracellular Domain of Receptor Activator of NF-κB (RANK)

INTERACTION WITH TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTORS AND ACTIVATION OF NF-κB AND c-JUN N-TERMINAL KINASE*

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Various members of the tumor necrosis factor (TNF) receptor superfamily interact directly with signaling molecules of the TNF receptor-associated factor (TRAF) family to activate nuclear factor κB (NF-κB) and the c-Jun N-terminal kinase (JNK) pathway. The receptor activator of NF-κB (RANK), a recently described TNF receptor family member, and its ligand, RANKL, promote survival of dendritic cells and differentiation of osteoclasts. RANK contains 383 amino acids in its intracellular domain (residues 234–616), which contain three putative TRAF-binding domains (termed I, II, and III). In this study, we set out to identify the region of RANK needed for interaction with TRAF molecules and for stimulation of NF-κB and JNK activity. We constructed epitope-tagged RANK (F-RANK616) and three C-terminal truncations, F-RANK330, F-RANK427, and F-RANK530, lacking 85, 188, and 285 amino acids, respectively. From this deletion analysis, we determined that TRAF2, TRAF5, and TRAF6 interact with RANK at its C-terminal 85-amino acid tail; the binding affinity appeared to be in the order of TRAF2 > TRAF5 > TRAF6. Furthermore, overexpression of RANK stimulated JNK and NF-κB activation. When the C-terminal tail, which is necessary for TRAF binding, was deleted, the truncated RANK receptor was still capable of stimulating JNK activity but not NF-κB, suggesting that interaction with TRAFs is necessary for NF-κB activation but not necessary for activation of the JNK pathway.

To date, over 20 members of the tumor necrosis factor (TNF) ligand and receptor superfamilies have been identified. Most of these receptors activate signaling cascades involving the activation of nuclear factor κB (NF-κB), protein kinases (MAPK/JNK/p38), and apoptosis through engagement of various adaptor proteins (1–3). Activation of apoptosis is typically transmitted through death domain-containing receptors (4). Additionally, many TNFR family members activate NF-κB and JNK pathways via interaction with various TRAF family members (1, 3, 5–12). The TRAF family consists of six distinct proteins, each containing a ring and zinc finger motif in their N termini and C-terminal domains that appear to be responsible for self-association and protein interaction. TRAF1, TRAF2, and TRAF3 bind to distinct motifs within CD40, CD30, ATAR/HVEM, and p80 TNFR (6–8, 13). The PXQX(T/S) motif is characteristic for binding TRAF1, TRAF2, and TRAF5 (6, 7, 14). Moreover, TRAF6 interacts with CD40 via a 16-amino acid region (residues 230–245) (7). Of the TRAF molecules, only TRAF2, TRAF5, and TRAF6 have been demonstrated to mediate signaling of NF-κB and JNK (3, 5, 10, 11).

RANK (for receptor activator of NF-κB), a recently described novel TNFR family member, bears high similarity in its extracellular domain to CD40 (15). It consists of a 616-amino acid transmembrane receptor, of which 383 amino acids reside in the intracellular domain. The intracellular domain does not show any homology to any of the known TNFR family members. RANK mRNA is ubiquitously expressed in human tissues, but cell surface RANK is expressed only on dendritic cells, the CD4+ T cell line MP-1, and foreskin fibroblasts (15). CD40L greatly enhances expression of RANK on mature dendritic cells (15), suggesting a potential role for RANK in dendritic cell function.

The human and mouse ligands for RANK (RANKL) share 85% identity (15). This ligand consists of 317 residues and is a type II transmembrane protein, whose expression is restricted to primary T cells, T cell lines, and lymphoid tissue (15). Furthermore, RANKL was cloned independently by three groups as an osteoclast differentiation factor (16), as an apoptosis-regulatory gene (TRANCE, for TNF-related activation-induced cytokine) (17), and as a ligand for the soluble TNFR family member osteoprotegerin (18). Overexpression of RANK and RANKL has been demonstrated to activate NF-κB (15). RANKL was also shown to stimulate JNK activity in mouse thymocytes and T cell hybridomas, but not B cells (17), and was partially inhibited in thymocytes from dominant negative TRAF2 transgenic mice (19). Additionally, RANKL appears to enhance T cell growth and dendritic cell survival by up-regulation of Bel-7 (15, 17).

To date, there is no report to indicate the region of the RANK receptor necessary for activation of JNK and NF-κB. Thus, we constructed various C-terminal truncations of RANK and transiently expressed them in human cultured cell lines to characterize their ability to interact with various TRAF family members and to activate JNK and NF-κB. From this deletion analysis, we have identified specific regions of RANK that...
interact with TRAF2, TRAF5, and TRAF6 and that stimulate JNK and NF-κB activation.

EXPERIMENTAL PROCEDURES

Reagents, Cell Lines, and Antibodies—HeLa, an epithelial carcinoma cell line, and 293, a human embryonic kidney cell line, were obtained from the American Type Culture Collection (Rockville, MD) and cultured in minimal essential medium supplemented with 10% fetal bovine serum and antibiotics. Affinity-purified rabbit anti-TRAF2 (SC-876, C-20) and anti-JNK1 (SC-474, C-17) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG-conjugated horseradish peroxidase was obtained from Bio-Rad. Anti-FLAG (monoclonal antibody M2) and anti-FLAG (M2)-conjugated agarose were obtained from Eastman Kodak Co. (New Haven, CT). Goat anti-mouse IgG-conjugated horseradish peroxidase was obtained from Transduction Laboratories (Lexington, KY). Protein A/G-Sepharose was obtained from Pierce.

Expression Plasmids—The complete cDNA for RANK (pSPORT3.0-TR8) was identified through a homology search of an expressed sequence tag cDNA database (Human Genome Sciences, Inc., Rockville, MD) obtained from a primary dendritic cell cDNA library for proteins containing the cysteine-rich repeat characteristic of TNFR family members. C-terminal cDNA is identical to hRANK (15). To generate FLAG-tagged RANK616, the 5′-primer CTAAGAAGGACTCTGAGGATCC and the 3′-primer GACCTGAGCTCACTGAGCTGTTGGC were used in a PCR reaction with pSPORT3.0-TR8 to generate a PCR product that would encode residues 33–616 (lacking the signal sequence), which was cloned into the HindIII/SalI site to generate the expression vector pCMVFGLAG1 (Eastman Kodak Co.). RANK deletion mutants were generated by PCR using the above 5′ primer and the 3′ primers (for RANK330: TCTTACCGTCGACTCAAGCCTTGCC; for RANK530: GCAGGTGCAGCTGAGTCACTGAGCTGTTGGC; for RANK427: AACCAGGTGCAGCTGAGTCACTGAGCTGTTGGC; and for RANK330: GCAGGTGCAGCTGAGTCACTGAGCTGTTGGC) to give rise to pBS-FLAGTR8, pBS-FLAGTR6, pBS-FLAGTR5, and pBS-FLAGTR3, respectively. Initial transient transfections of 293 cells with 0.5–1 μg of each of these expression plasmids resulted in a strong nuclear signal, indicating efficient expression of the transfected proteins. For coimmunoprecipitations, transfected cells were harvested 36–40 h after transfection.

In Vitro Translation of 35S-Labeled TRAFs—Expression vectors encoding the indicated full-length TRAF cDNAs were transcribed and translated with a coupled transcription/translation system as described in the manufacturer’s instructions (Promega, Madison, WI). Translation products were analyzed by SDS-PAGE, transferred to nitrocellulose membranes, and detected with an autoradiograph.

RESULTS AND DISCUSSION

A human cDNA (TR8, for TNF receptor-like 8) encoding a TNFR-related protein was identified through a homology search of an expressed sequence tag cDNA database. The full-length cDNA encodes a protein of 616 amino acid residues. The extracellular domain (residues 1–208) contains a signal sequence and the conserved cysteine-rich repeats characteristic of the TNFR family (21). The intracellular domain (residues 234–616) is the largest of all the TNFR family members to date and contains 85, 188, and 285 amino acids, respectively. This domain is needed for signaling, we constructed a FLAG epitope-tagged version of RANK in the plasmid pCMVFGLAG1. The mature polyprotein encodes residues 33–616 (F-RANK616) with a FLAG epitope tag at its N terminus (Fig. 1A). To identify which region of the cytoplasmic domain is needed for signaling, we constructed three C-terminal deletions, designated F-RANK530, 427, and 330 (Fig. 1A) and lacking 85, 188, and 285 amino acids, respectively.

Most of the TNFR family members interact directly with various members of the TRAF family of signaling proteins. In some of these receptors, a consensus TRAP-binding motif (PX-QX/T/S) is required to bind to TRAF2, TRAF3, and TRAF5 (7, 13, 14, 22, 23). Inspection of the intracellular domain of RANK suggests three potential TRAF-binding domains, two at the C terminus (TRAFII and III) and one in the middle of the intracellular domain.

![Deletion Analysis of RANK](image-url)
Molecular mass standards (in kDa) are indicated at clonal antibody as described under “Experimental Procedures.” After 24 h, cell lysates were prepared and subjected to SDS-PAGE and Western blotting with an anti-FLAG monoclonal antibody as described under “Experimental Procedures.” Transient overexpression of F-RANK616 in 293 cells activated JNK (Fig. 4B). Furthermore, F-RANK530 and -427 deletion mutants, which lack 85 and 188 residues from the C terminus, respectively, could still activate JNK (Fig. 4B). However, C-terminal truncation of 285 residues (which leaves approximately 98 amino acids intact) could not activate JNK (Fig. 4B). From at least three independent transfection experiments, we found that F-RANK616, -530, and -427 could increase JNK activity between 4- and 10-fold, whereas F-RANK330 increased activity by no more than 1.5-fold relative to vector-transfected cells. These data suggest that F-RANK530 and F-RANK427 may stimulate JNK activation without binding directly to TRAFs. Because F-RANK330 had no significant effect on JNK activation, we tentatively localized a JNK activation domain between residues 330 and 427 within the cytoplasmic domain of RANK.

TRAF2, TRAF5, and TRAF6 Interact with the C Terminus of RANK—Because most TNFR family members utilize TRAFs as signaling components and RANK contains putative TRAF-binding domains, we examined the ability of RANK to interact with various TRAFs. We transiently transfected HeLa and 293 cells with vectors directing expression of F-RANK616 and F-RANK deletion mutants. After 24–36 h, cell lysates were prepared, and epitope-tagged receptors were immunoprecipitated with anti-FLAG-conjugated agarose. Coprecipitation of endogenous TRAF2 was detected by Western blotting with anti-TRAF2 polyclonal antibodies. When expressed in HeLa (Fig. 3A, top) and 293 cells (Fig. 3A, bottom), only F-RANK616 and none of the F-RANK deletion mutants precipitated endogenous TRAF2. Membranes were also probed with anti-FLAG to ensure the precipitation of epitope-tagged receptors (data not shown).

To examine whether other TRAFs could interact with RANK, we transiently transfected 293 cells with F-RANK expression vectors. After 36 h, cell lysates were prepared and in vitro translated 35S-labeled TRAF2, TRAF5, and TRAF6 were added to each of the lysates. The epitope-tagged receptors were immunoprecipitated with anti-FLAG-conjugated agarose, and bound proteins were eluted in SDS-sample buffer and subjected to SDS-PAGE. The bound 35S-labeled TRAFs were detected by exposure of the dried SDS-PAGE gel to x-ray film. Like endogenous TRAF2, 35S-labeled TRAF2 coprecipitated only with F-RANK616 and not with the deletion mutants (Fig. 3B, top). Similarly, 35S-labeled TRAF5 (Fig. 3B, middle) and TRAF6 (Fig. 3B, bottom) coprecipitated with F-RANK616 and not with the deletion mutants. Quantitation of 35S-labeled TRAF2, TRAF5, and TRAF6 bound to F-RANK616 showed 145-, 11-, and 5-fold increases, respectively, in binding relative to vector-transfected cells. Thus, we have shown that TRAF2, TRAF5, and TRAF6 interacted with RANK at its C-terminal 85 residues.

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RANK Deletion Mutants Lacking TRAF Binding Domains (II and III) Activate JNK—TRAF2, TRAF5, and TRAF6 are involved in JNK activation (3) by various members of the TNFR family and the interleukin-1 receptor (5) (i.e., TRAF6). We tested whether RANK and the various C-terminal deletion mutants were capable of activating JNK. When overexpressed in cultured cell lines, most TNFR family members activate signal transduction pathways in the absence of ligand (2). Thus, we transiently transfected 293 cells with increasing amounts of F-RANK expression vectors. Cell lysates were prepared 36 h after transfection and analyzed for receptor expression by Western blotting with anti-FLAG antibodies (Fig. 4A). Furthermore, the cell lysates were assayed for JNK activation by immune complex kinase assays using GST-Jun-(1–79) as a substrate. Transient overexpression of F-RANK616 in 293 cells activated JNK (Fig. 4B). Furthermore, F-RANK530 and -427 deletion mutants, which lack 85 and 188 residues from the C terminus, respectively, could still activate JNK (Fig. 4B). However, C-terminal truncation of 285 residues (which leaves approximately 98 amino acids intact) could not activate JNK (Fig. 4B). From at least three independent transfection experiments, we found that F-RANK616, -530, and -427 could increase JNK activity between 4- and 10-fold, whereas F-RANK330 increased activity by no more than 1.5-fold relative to vector-transfected cells. These data suggest that F-RANK530 and F-RANK427 may stimulate JNK activation without binding directly to TRAFs. Because F-RANK330 had no significant effect on JNK activation, we tentatively localized a JNK activation domain between residues 330 and 427 within the cytoplasmic domain of RANK.

The C Terminus of RANK Is Necessary for NF-κB Activation—According to gel mobility shift assays, overexpression of RANK in 293 cells activates NF-κB (15). To explore whether RANK deletion mutants activate NF-κB, we transiently transfected 293 cells with F-RANK616 and the F-RANK deletion mutants. Western blotting with anti-FLAG antibodies indicated expression of the epitope-tagged receptors (Fig. 5A). Analysis of NF-κB by a gel mobility shift assay indicated that only F-RANK616 activated NF-κB (Fig. 5B). None of the F-RANK deletions were capable of activating NF-κB in three independent transient transfection experiments, even though from the same transfections F-RANK530 and -427 could activate JNK.

Our data are consistent with a previous report (15) indicating that transient overexpression of RANK in 293 cells induces NF-κB. We further demonstrated by deletion of the C-terminal 85 residues that this domain is necessary for TRAF interaction and most likely NF-κB activation as well. Whether the interaction between RANK and TRAFs is responsible for NF-κB activation remains to be determined. Our data are in agreement with reports that show that TRAF2, TRAF5, and TRAF6 participate in NF-κB activation by other TNFR family members (3).

Stimulation of mouse thymocytes or T-cells, but not B-cells, by RANKL/TRANCE induces JNK activation (17), which could be inhibited in thymocytes from transgenic mice expressing a dominant negative form of TRAF2 (19). Our deletion analysis of RANK provided evidence that RANK lacking the TRAF binding domain could still stimulate JNK activity. Furthermore, our deletion analysis implies that RANK residues between 330 and 427 are too different to allow valid comparisons. Indeed, it is pos-

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2 B. G. Darnay and B. B. Aggarwal, unpublished observations.
Deletion Analysis of RANK

possible that RANK can activate the JNK pathway in both a TRAF-dependent and -independent fashion. Moreover, it is possible that other unidentified adaptor proteins and TRAF-like molecules are responsible for signaling by RANK.

In summary, RANK encodes the largest cytoplasmic domain (383 amino acids) of any TNFR family member identified thus far. For the first time, we provide evidence that TRAF2, TRAF5, and TRAF6 bind to the C-terminal 85 amino acids; however, TRAF2 appeared to bind better than TRAF5 and TRAF6. Furthermore, we demonstrated that deletion of the

FIG. 3. Coprecipitation of TRAF2, TRAF5, and TRAF6 with F-RANK616. A, human HeLa (top) or embryonic kidney 293 (bottom) cells were transiently transfected with the indicated expression vectors as described under “Experimental Procedures.” After 24 h, whole cell lysates were prepared, and epitope-tagged receptors were immunoprecipitated with anti-FLAG-conjugated agarose and washed, and bound proteins were eluted with SDS-sample buffer. Samples were subjected to SDS-PAGE, and coprecipitating TRAF2 was detected by Western blotting with anti-TRA2 polyclonal antibodies. B, human 293 cells were transiently transfected with the indicated expression vectors as described under “Experimental Procedures,” and after 36 h cell lysates were prepared. In vitro translated 35S-TRA2 (top), 35S-TRA5 (middle), or 35S-TRA6 (bottom) were added to the lysates, and the epitope-tagged receptors were immunoprecipitated as described in A. Samples were subjected to SDS-PAGE, and the dried gel was exposed to x-ray film for 24 h to detect bound 35S-TRA2, 35S-TRA5, and 35S-TRA6. IP, immunoprecipitate.

FIG. 4. Overexpression of F-RANK616, -530, and -427 stimulate JNK activity. Human 293 cells were transiently transfected with 0.5, 1.5, and 3.0 μg of the indicated expression vectors as described under “Experimental Procedures.” After 36 h, cell lysates were prepared and subjected to Western blotting with anti-FLAG (A) and immunoprecipitated with anti-JNK1 antibodies. The activity of coprecipitating JNK1 was measured by an immune complex kinase assay (B) using exogenous GST-Jun-(1–79) as described under “Experimental Procedures.” Phosphorylation of GST-Jun-(1–79) was quantitated on a PhosphorImager, and -fold activation was measured relative to the vector-transfected cells. Data are representative of three independent transfection experiments.

FIG. 5. The C terminus of RANK is necessary for NF-κB activation. Human 293 cells were transiently transfected with 0.5 and 2 μg of the indicated expression vectors as described under “Experimental Procedures.” After 36 h, cell lysates were prepared and subjected to Western blotting with anti-FLAG (A), and nuclear extracts were subjected to an EMSA (B) as described under “Experimental Procedures.” NF-κB binding was quantitated on a PhosphorImager, and -fold activation was measured relative to the vector-transfected cells. Data are representative of three independent transfection experiments.
TRAF interaction motif at the C terminus did not diminish RANK's stimulation of JNK activity, suggesting that RANK could activate JNK in a TRAF-independent manner. However, deletion of the C-terminal 85 residues results in loss of NF-κB activation. Thus, we have demonstrated that TRAF family members interact with the novel TNFR family member RANK and may participate in RANK signal transduction.

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