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The TRAF Family of Signal Transducers Mediates NF-κB Activation by the TRANCE Receptor*

(Received for publication, July 27, 1998, and in revised form, September 6, 1998)

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Tumor necrosis factor (TNF)-related activation-induced cytokine (TRANCE), a member of the TNF family expressed on activated T-cells, bone marrow stromal cells, and osteoblasts, regulates the function of dendritic cells (DC) and osteoclasts. The TRANCE receptor (TRANCE-R), recently identified as receptor activator of NF-κB (RANK), activates NF-κB, a transcription factor critical in the differentiation and activation of those cells. In this report we identify the TNF receptor-associated factor (TRAF) family of signal transducers as important components of TRANCE-R-mediated NF-κB activation. Coimmunoprecipitation experiments suggested potential interactions between the cytoplasmic tail of TRANCE-R with TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6. Dominant negative forms of TRAF2, TRAF5, and TRAF6 and an endogenous inhibitor of TRAF2, TRAF-interacting protein (TRIP), substantially inhibited TRANCE-R-mediated NF-κB activation, suggesting a role of TRAFs in regulating DC and osteoclast function. Overexpression of combinations of TRAF dominant negative proteins revealed competition between TRAF proteins for the TRANCE-R and the possibility of a TRAF-independent NF-κB pathway. Analysis of TRANCE-R deletion mutants suggested that the TRAF2 and TRAF5 interaction sites were restricted to the C-terminal 93 amino acids (C-region). TRAF6 also complexed to the C-region in addition to several regions N-terminal to the TRAF2 and TRAF5 association sites. Furthermore, transfection experiments with TRANCE-R deletion mutants revealed that multiple regions of the TRANCE-R can mediate NF-κB activation.

TRANCE,1 also called RANKL (1), osteoclast differentiation

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1 The abbreviations used are: TRANCE, TNF-related activation-induced cytokine; TNF, tumor necrosis factor; TRANCE-R, TRANCE receptor; TNFR, TNF receptor; DC, dendritic cell; OCL, osteoclast; IL, interleukin; TRAF, TNF receptor-associated factor; NF-κB, nuclear factor-κB; JNK, c-Jun N-terminal kinase; BA, amino acids; OPG, osteoprotegerin; ORF, open reading frame; PCR, polymerase chain reaction; HA, hemagglutinin; MASP, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; hTRADD, human TNF receptor-associated death domain; TRIP, TNF receptor-interacting protein; MEKK, MEK kinase; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

2 B. R. Wong, R. Josien, S. Y. Lee, M. Vologodskaya, R. M. Steinman, and Y. Choi, unpublished observation.
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inducing effects (19), suggesting that TRANCE is part of a complex cytokine network that coordinates an array of biological processes.

The NF-κB family of transcription factors plays an important role in DC and osteoclast function. Dendritic cell development is inhibited in RelB-deficient mice (20) and in bone marrow cultures infected with adenovirus harboring the IκB repressor (21). NF-κB1 (p50) and NF-κB2 (p52) double knockout mice develop osteoporosis because of a defect in osteoclast differentiation (22). In addition, IL-1 enhances OCL survival by activating NF-κB (23). Therefore, discovering the mechanisms leading to NF-κB activation from the TRANCE-R will aid in our understanding of the molecular events involved in DC and osteoclast function. Our results demonstrate that TRANCE-R associates with TRAF2, TRAF5, and TRAF6 at distinct regions of the cytoplasmic tail to initiate NF-κB activation. Therefore, TRANCE may direct DC and OCL differentiation and activation through the TRANCE-R by stimulating NF-κB via TRAFs.

MATERIALS AND METHODS

Constructs—TRAF5.DN (aa 236–559) and TRAF6.DN (289–522) open reading frames (ORFs) were amplified by PCR and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) containing a 3′-hemagglutinin (HA) epitope (product encoding TRAF2.DN (241–501) was cloned into pcDNA3.1 containing a 3′-ID4 tag. The HA-TRAF1, TRAF2, HA-TRIP, and HA-hTRADD expression vectors were previously described (24). pBABE-TRAF3 was kindly provided by Dr. Genhong Cheng (UCLA, Los Angeles, CA). FLAG-TRAF5, FLAG-TRAF6, and FLAG-hHi-67 (hHi) expression vectors were constructed by cloning the ORF derived by PCR into the pFLAG-CMV-2 vector (pFLAG-2; Kodak, Rochester, NY). The various TRANCE-R cytoplasmic tails (aa 235–625, 235–559, 235–358, 532–625, and 354–536) were obtained by reverse transcription-PCR of murine thymus RNA. The cytoplasmic tails were fused to the 3′ end of GST in pEBG (GST-TRcyt and GST-TRcyt mutants) or the 3′ end of the TRANCE-R extracellular/transmembrane domain (TR-E) in pFLAG-CMV1 (pFLAG-1; Kodak). The c-Jun (pFA-Jun) and Elk-1 (pFA-E1k) pathway-specific transactivators, the GAL4 UAS-containing luciferase reporter (pGFR-Luc) plasmid, and the MEKK (pPC-MEKKK) and MEK1 (pPC-MEKK1) expression vectors were purchased from Stratagene (La Jolla, CA). The NF-κB reporter vector (κB)-interferon-luciferase and pCMV-β-gal plasmids (Invitrogen) were described previously (26). All constructs were made from mouse ORFs unless indicated. ORFs obtained by PCR were confirmed by DNA sequencing.

Transfections and Reporter Assays—293T cells were grown under standard conditions (Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 37 °C, 5% CO2). One day prior to transfection cells were split into 6-well dishes (4 × 105/well) with 0.1% gelatin for luciferase assays or in 10-cm dishes (26). Measurements of luciferase were normalized to β-galactosidase activity. Luciferase values were normalized to β-galactosidase activity, and the results are displayed as a fold induction over vector alone. A representative result of three independent experiments is shown. Error bars denote standard deviations between samples performed in triplicate.

was examined in 293T cells to determine whether an overexpression system in tumor cell lines could accurately model TRANCE-R signaling. Activation of the ETS domain-containing transcription factor Elk-1 was also examined because it is a substrate for the mitogen-activated protein kinases (MAPK): JNK, p38, and extracellular signal-regulated kinase (ERK), which are often activated by TNFR family members. In addition, Elk-1 regulates the expression of c-Fos, a transcription factor important for osteoclast differentiation (27). An epitope-tagged murine TRANCE-R expression vector was cotransfected with luciferase reporter constructs that monitor either c-Jun, Elk-1, or NF-κB transcriptional activity. Fig. 1 demonstrates that overexpression of TRANCE-R induced c-Jun (→3-fold; Fig. 1A), Elk-1 (→5-fold; Fig. 1B), and NF-κB (→100-fold; Fig. 1C) when compared with cells transfected with vector alone. As expected a mutant TRANCE-R lacking the cytoplasmic tail (TR-E) failed to activate c-Jun, Elk-1, or NF-κB. MEKK, MEK1, and TRAF2 expression provided positive controls for c-Jun, Elk-1, and NF-κB activation, respectively. A dose-response curve showed that 50 ng of TRANCE-R expression vector was sufficient for achieving maximal NF-κB activation (data not shown).

The TRANCE-R Cytoplasmic Tail Associates with TRAF Adaptor Proteins—Immunoprecipitation experiments were performed to test the association of TRANCE-R with all the known TRAF proteins except for TRAF4, which was shown to be a nuclear protein (28). The association of TRAF1, TRAF3, TRAF5, and TRAF6 with the cytoplasmic tail of TRANCE-R fused to GST (GST-TRcyt) was observed when coexpressed in 293T cells (data not shown). However, TRAF2 could not be analyzed by coexpression because it possessed high levels of expression of GST-TRcyt for unknown reasons (data not shown). Therefore, lysates from 293T cells overexpressing GST or GST-TRcyt were mixed with lysates from 293T cells overexpressing either TRAF1, TRAF2, TRAF3, TRAF5, TRAF6, human TRADD (hTRADD), or hHi-67 (hHi). GST-TRcyt-interacting protein complexes were isolated with glutathione-Sepharose
TRANCE Receptor Activates NF-κB via TRAF Adaptor Proteins

Dominant Negative Forms of TRAF2, TRAF5, and TRAF6

TRAF2, TRAF5, and TRAF6 are detected with the input lysates. The GST-TRcyt-interacting protein complexes were precipitated with glutathione-Sepharose beads, washed extensively in lysis buffer, and analyzed by SDS-PAGE/Western blot analysis. Ten percent of the input lysates were also analyzed to confirm the expression of the putative interacting proteins (Input 0.1). TRAF1, TRAF3, and hTRADD were detected at the α-HA monoclonal antibody (12CA5). TRAF2 was detected with N-terminal α-TRAF2 polyclonal antibodies. TRAF5, TRAF6, and hKi were detected with an α-FLAG monoclonal antibody (M2). All associations were confirmed at least three times in independent experiments.

TRAF1 recruits cellular inhibitors of apoptosis to the receptor complex (29), and overexpression of TRAF1 inhibits T-cell receptor-mediated cell death of CD8+ T-cells (30). Therefore, TRAF1 may partially mediate TRANCE-induced DC survival. TRAF2, TRAF5, and TRAF6, in addition to NF-κB activation, are responsible for JNK induction leading to Jun/Fos activator protein-1 (AP-1) transactivational activity. TRAF6 also stimulates ERK (31), which phosphorylates and activates a distinct set of transcription factors including c-Myc, Elk-1, C/EBP, Tal-1, and ATF-2. MAPK-induced transcriptional activity may integrate with the NF-κB pathway to mediate the various effects of TRANCE on DC and osteoclasts.

**FIG. 2.** TRANCE-R associates with the TRAF family of adaptor proteins. Lysates of 293T cells overexpressing the cytoplasmic tail of TRANCE-R (aa 235–625) fused to GST (GST-TRcyt) were mixed with lysates from 293T cells expressing HA-TRAF1, TRAF2, HA-TRAF3 (TRAF3), FLAG-TRAF5 (TRAF5), FLAG-TRAF6 (TRAF6), HA-hTRADD (hTRADD), or FLAG-hKi-67 (hKi) as described under “Materials and Methods.” The GST-TRcyt-interacting protein complexes were precipitated with glutathione-Sepharose beads, washed extensively in lysis buffer, and analyzed by SDS-PAGE/Western blot analysis. Ten percent of the input lysates were also analyzed to confirm the expression of the putative interacting proteins (Input 0.1). TRAF1, TRAF3, and hTRADD were detected with the α-HA monoclonal antibody (12CA5). TRAF2 was detected with N-terminal α-TRAF2 polyclonal antibodies. TRAF5, TRAF6, and hKi were detected with an α-FLAG monoclonal antibody (M2). All associations were confirmed at least three times in independent experiments.

**FIG. 3.** Dominant negative TRAF2, TRAF5, and TRAF6 proteins and TRIP inhibit TRANCE-R-mediated NF-κB activation. pFLAG-1/TRANSE-R (TR; 50 ng) was cotransfected with increasing amounts of expression vectors encoding murine TRAF2.DN, TRAF5.DN, TRAF6.DN, TRIP, or human Ki-67 (hKi). After transfection (24–36 h), the cells were harvested, and NF-κB-induced luciferase activity was assessed as described in Fig. 1. The average ± fold induction observed in three independent experiments performed in duplicate is shown. Error bars denote the standard error of the mean (S.E.) of the three experiments.

TRAF5.DN, or TRAF6.DN resulted in a dose-dependent inhibition of NF-κB activation (Fig. 3). However, NF-κB induction was incompletely blocked despite a 20-fold excess of any of the vectors encoding TRAF.DN proteins. TRIP, a TRAF2-interacting protein that inhibits TRAF2-dependent NF-κB activation (24), also decreased NF-κB activation by the TRANCE-R in a dose-dependent manner. In contrast, overexpression of an irrelevant protein, human autoimmune antigen Ki-67 (hKi), failed to inhibit TRANCE-R-mediated NF-κB activation, thus indicating the specificity of TRAF.DN and TRIP proteins. Therefore, NF-κB activation induced by the TRANCE-R signaling is, in part, mediated by TRAF2, TRAF5, and TRAF6 and can be negatively regulated by TRIP.

Functional and Biochemical Mapping of the TRANCE-R Cytoplasmic Tail—Deletion mutants of the TRANCE-R cytoplasmic tail (Fig. 4A) were fused with GST (GST-TR) or with the FLAG-tagged extracellular transmembrane domain of TRANCE-R (TR-E). The design of TRANCE-R cytoplasmic tail deletions was based on PXXQ(E/T)S or VXXT/SXXE TRAF-binding sites determined in other TNFR family members (26, 33). The cytoplasmic tail was arbitrarily divided into a membrane-proximal N-terminal region (N-region; aa 235–358), a middle region (M-region; aa 359–531), and a C-terminal region (C-region; aa 532–625). Associations between GST-TR mutants and TRAF2, TRAF5, and TRAF6 were examined. Lysates from 293T cells overexpressing TRAF proteins were mixed with lysates containing the different GST-TR fusion proteins, and GST-TR/TRAF complexes were precipitated with glutathione-Sepharose and analyzed by Western blot. These membranes were also stained with Coomassie brilliant blue to confirm the presence of the GST-TR proteins. TRAF2 associated most strongly with GST-TR-235–625, weakly with -354–536, but not with -235–358 (Fig. 4B). These results suggest that amino acid residues 532–559 contain the major TRAF2-interacting site. Similar reasoning suggested that residues 559–603 of the C-region were required for TRAF5 binding (Fig. 4B). Therefore, both TRAF2 and TRAF5 associate with the TRANCE-R at distinct but juxtaposed sites within the C-region. TRAF6 associated with the N-region and M-region containing mutants but less efficiently to the C-region (Fig. 4B). Thus TRAF6 can associate with...
multiple sites N-terminal to the C-region.

The various transmembrane-anchored cytoplasmic tails were tested for their ability to activate NF-κB. Western blot analysis using the α-FLAG antibody demonstrated comparable levels of expression produced by the various TR-E-cytoplasmic tail constructs (Fig. 4C). Fig. 4D shows that mutants TR-E\(^{235–603}\) and TR-E\(^{235–559}\), which lack the C-terminal 22 and 66 aa, respectively, stimulated similar levels of NF-κB activity compared with the wild-type TRANCE-R (TR-E\(^{235–625}\)). In addition, TR-E\(^{235–358}\), the mutant encompassing the N-terminal region (N-region), and TR-E\(^{354–536}\), the mutant delimiting the middle region (M-region), also induced relatively high levels of NF-κB activity. Unexpectedly, the C-region mutant, TR-E\(^{532–625}\), consistently elicited relatively low, yet substantial, NF-κB activity (10–20-fold), whereas the N-region and M-region, which primarily interacted with TRAF6, generated significantly higher levels of NF-κB activity. These results suggest that TRAF6, in addition to TRAF2 and TRAF5, is an important NF-κB-inducing element in the TRANCE-R signaling complex.

**Competition between TRAF Members for the TRANCE-R Signaling Complex**—Combinations of TRAF.DN proteins were co-expressed with TRANCE-R in an attempt to enhance the NF-κB inhibition caused by individual dominant negatives. Expression of TRAF2.DN or TRAF6.DN in combination with TRAF5.DN did not cause a further decrease in TRANCE-R-mediated NF-κB activity compared with any TRAF.DN alone (Fig. 5). However, TRAF2.DN in combination with TRAF6.DN reduced NF-κB activity by an additional ~50% of the activity observed with TRAF2.DN or TRAF6.DN alone. Addition of TRAF2, TRAF5, and TRAF6 dominant negative proteins together did not inhibit NF-κB further than that observed with TRAF2.DN plus TRAF6.DN. Conceivably, TRAF2 may compete with TRAF5 by concealing residues important for its association with the TRANCE-R. TRAF6 may also hinder the association of TRAF5 with the TRANCE-R in a similar manner, although the proximity between the TRAF5 and TRAF6 asso-

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**Fig. 4.** Structure-function analysis of the TRANCE-R. A, diagram of deletion mutants of the TRANCE-R cytoplasmic tail (TRCyT). N and C denote the N and C termini, respectively. The amino acid sequence of potential TRAF-interacting motifs and their locations relative to the various TRANCE-R mutants (thin lines) are shown. The N- regions (aa 235–358), M-regions (aa 359–531), and C-regions (aa 532–625) are indicated with thick lines. B, association of TRAF2, TRAF5, and TRAF6 with GST-TRCyT mutants. Lysates from 293T cells transfected with plasmids expressing GST-TRCyT (aa 235–625) and GST- TRCyT mutants were combined with lysates from 293T cells transfected with TRAF2, FLAG-TRAF5 (TRAF5), or FLAG-TRAF6 (TRAF6). Protein complexes were precipitated with glutathione-Sepharose and analyzed by SDS-PAGE/Western blot analysis. 10% of the lysates from 293T cells overexpressing TRAF proteins (Input 0.1) were analyzed to confirm their expression and compare relative binding efficiencies. After Western analysis, the blots were stained with Coomassie brilliant blue (CBB) to verify the expression of wild-type or mutant GST-TR expression. The bands corresponding to the various GST-TR proteins are labeled with asterisks (*). Results from experiments that failed to express high levels of wild-type or mutant GST-TRs in any sample were discarded. Similar results were obtained from three independent experiments. C, Western blot analysis (α-FLAG Ab) of 293T cells transfected with constructs (0.5 μg) encoding the TRANCE-R extracellular/transmembrane domain (TR-E) fused to the various cytoplasmic tails as described in Fig. 1. Bands corresponding to the various TR-E fusion proteins are labeled with asterisks (*). D, NF-κB-dependent luciferase activity measured from 293T cells 24–36 h after transfection with the various TR-E fusion constructs (0.5 μg). A representative result of five independent experiments is shown. Error bars denote standard deviations of conditions performed in triplicate.
cation sites was not resolved. Residual NF-kB activity (~10–20-fold induction) could not be inhibited despite coexpression of TRAF2.DN, TRAF5.DN, and TRAF6.DN together. Thus similarly to CD30 (34, 35), TRANCE-R may initiate TRAF-independent pathways or interact with unknown TRAFs to activate NF-kB.

In this report we demonstrate that TRAF adaptor proteins can associate with the cytoplasmic tail of TRANCE-R and mediate NF-kB activation. During the review of this article a study was published showing associations between the human TRANCE-R and TRAFs (36). However, in that study, the C-terminal 86 residues (aa 530–616) were shown to be essential for NF-kB activation and TRAF interaction whereas our data define other regions capable of those functions. It is possible that the human receptor has distinct properties compared with the mouse receptor used in this study. More likely, however, the discrepancies reflect the sensitivities of the different methods employed to study protein interactions or NF-kB activation. TRAFs may be responsible for some of the effects of TRANCE on DC and osteoclasts. Perhaps TRAFs via NF-kB and/or MAPKs are linked to the expression of anti-apoptotic genes such as bcl-xL or genes involved in differentiation and activation. The importance of TRAFs in TRANCE-R signaling in DC or OCL will be further explored with TRAF-deficient mutant mice or by overexpressing TRAF.DN proteins in those cells.

Acknowledgments—We thank Joe Arron, Nacksung Kim, Michael Klein, John MacMicking, and Su Tsao for comments and suggestions regarding this manuscript. We also thank Angela Santana for excellent technical help.

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*J. Biol. Chem.* 1998, 273:28355-28359.
doi: 10.1074/jbc.273.43.28355

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