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TRAF5, an Activator of NF-κB and Putative Signal Transducer for the Lymphotoxin-β Receptor*

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Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) are signal transducers for several members of the TNF receptor superfamily. We have identified a novel member of the TRAF family by degenerate oligonucleotide polymerase chain reaction amplification that contains a zinc RING finger and zinc finger motifs, a coiled-coil region, and a C-terminal "TRAF" homology domain. *In vitro* translated TRAF5 binds to the cytoplasmic region of the lymphotoxin-β receptor (LT-βR) but not to several other related receptors including CD40, both TNF receptors, Fas, and nerve growth factor receptor. TRAF5 and LT-βR coimmunoprecipitate when overexpressed in COS7 cells. TRAF5 mRNA expression is found in all visceral organs and overlaps with LT-βR. These features distinguish TRAF5 from the other members of the TRAF family. The transcription factor NF-κB is activated in HEK293 cells by overexpression of full-length TRAF5 but not a truncated form lacking the zinc binding region. Furthermore, overexpression of LT-βR in HEK293 cells also results in activation of NF-κB, which is partially inhibited by the truncated TRAF5 mutant. These results show TRAF5 is functionally similar to TRAF2 in that both mediate activation NF-κB and implicate TRAF5 as a signal transducer for LT-βR.

The tumor necrosis factor (TNF)[1] family of cytokines initiates multiple cellular responses, including cell death and growth, that regulate inflammatory and immune defenses (1, 2). TNF and other members of this superfamily, such as lymphotoxin-αβ complex (3) and CD40 ligand (4), are trimers that are thought to initiate signal transduction by clustering specific cell surface receptors (5, 6). The corresponding family of receptors that binds TNF-related cytokines consists of single transmembrane-spanning glycoproteins related by their cysteine-rich, extracellular domains (7). The lack of an enzymatic domain in the cytoplasmic region of these receptors suggests that signaling is achieved through receptor-associated proteins. Two distinct families of proteins, the death domain homologs and the TNF receptor-associated factors (TRAF), have emerged as candidate signaling proteins for this receptor superfamily (8, 9). Rothe *et al.* (10) identified two proteins, designated TRAF1 and -2, that associate with the 75–80-kDa TNF receptor (TNFR80; type 2) but not with Fas or 55–60-kDa TNFR (TNFR60; type 1). TRAF3 (also known as CRAF1, CD40bp, CAP1, or LAP1) was identified by association with CD40 (11–13) or binding to the dominant oncogene of Epstein-Barr virus protein, LMP-1 (14). Recently, a fourth member, CART1, was identified (referred to as TRAF4) in breast cancer, and the protein is found in the nucleus (15). TRAFs are composed of an N-terminal cysteine/histidine-rich region containing zinc RING and/or zinc finger motifs, a coiled-coil (leucine zipper) motif, and a homology region in their C terminus that defines the TRAF family (10). The TRAF domain of TRAF2 and -3 is sufficient for self-association and receptor binding. TRAF1 and -2 form homo- and heteromeric complexes with each other, and TRAF2, not TRAF1, binds directly to TNFR80 and CD40 (10, 16). TRAF3 binds CD40, LT-βR, and TNFR80, revealing a functional similarity between these receptors not appreciated from sequence analysis (14). The activation of NF-κB, a member of the Rel family of transcription factors involved in the activation of genes involved in inflammatory and immune responses (17, 18), by TNFR80 and CD40 is dependent upon the zinc RING finger of TRAF2 (16), a function not associated with TRAF1 or -3. However, TRAF3 is involved in expression of CD23 in B cells (12), whereas a receptor binding function or signaling activity for TRAF4 has not been reported. Although evidence is accumulating, together these results strongly suggest a fundamental role for TRAFs in signaling cellular responses by receptors in the TNFR superfamily.

The sequence divergence in the cytoplasmic domains among members of the TNFR family suggests that additional TRAFs may exist that signal pleiotropic functions initiated by these receptors. We have used degenerative oligonucleotides corresponding to a TRAF domain homology to amplify TRAF-related sequences. This approach has yielded a novel member of the TRAF family that principally interacts with the LT-βR (19) and activates NF-κB, suggesting its designation as TRAF5.

MATERIALS AND METHODS

Molecular Cloning of Murine TRAF5—Total RNA from murine B cell lymphoma, A20.2J, was used to prepare cDNA with reverse transcriptase of Moloney murine leukemia virus (Superscript, Life Technologies, Inc.) according to the manufacturer's protocol. The cDNA was amplified by PCR utilizing degenerative oligonucleotide primers corresponding to highly conserved amino acids in the TRAF domains of TRAF1, -2, and -3. The sense and antisense primers corresponded to amino acid sequences YLNGDG and DD(T/A)(I/M)F(I/L), respectively. The PCR was performed under the following conditions: 1 min at 94 °C; 2 min at 45 °C; and 1 min at 72 °C for 40 cycles. Amplified DNA was subcloned and sequenced, and a novel TRAF-related sequence was used to screen a cDNA library from the murine monocytic cell line J774A.1 (Uni-ZAP, Stratagene) using a ^{32}P-labeled probe (Rediprime kit; Amersham Corp.). Sixteen clones were isolated, and the DNA sequence of several overlapping clones was determined on both strands using a series of oligonucleotide primers. The 2222-base pair sequence con-

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[1] The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNF receptor-associated factor; LT, lymphotoxin; LT-βR, LT-β receptor; NGFR, nerve growth factor receptor; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; HA, hemagglutinin; TRADD, TNFR1-associated death domain protein.

tained a single open reading frame of 1674 nucleotides preceded by a 322-nucleotide 5′-untranslated region with an in-frame upstream stop codon from the initiation site. The nucleotide sequence has been submitted to the DDBJ/GenBank/EMBL Data Bank (accession number D78141) and referred to as TRAF5.

Glutathione S-Transferase (GST) Fusion Protein Expression and Binding Assay—The source and construction of the cytoplasmic domains of several of the TNF-related receptors as GST fusion proteins have been described (13, 14). NGFR-GST was kindly provided by Dr. Dale Bredesen (Burnham Institute, formerly the La Jolla Cancer Research Foundation, La Jolla, CA). Fusion proteins were expressed and purified as described (20, 21). TRAF5 was synthesized *in vitro* with ^{35}S-labeled methionine using a coupled transcription and translation system (TNT, Promega). Binding of ^{35}S-TRAF5 to GST fusion proteins was performed as described previously (14).

Expression Vectors—Influenza virus hemagglutinin (HA)-tagged TRAF5 and a truncated TRAF5-(233–558) were generated by inserting the PCR product of full-length TRAF5 or truncated TRAF5-(233–558) into the *Eco*RI site of pHAKITneo, in which HA epitope tag (YPYDVPDYA) was placed downstream of the SRα promoter in pMKITneo (22) (kindly provided by Dr. Kazuo Maruyama, Department of Public Health, Tokyo Medical and Dental University). LT-βR expression vector (23) was generated by subcloning into pcDNA1 (Invitrogen).

Transfection, Immunoprecipitation, and Western Blot Analysis—COS7 cells (2×10^6) were transiently transfected with 2.5 μg of pHAKIT-TRAF5 and 2.5 μg of LT-βR-pcDNA1 by the DEAE-dextran method as described previously (24). After 2 days in culture, cells were lysed in 1.0 ml of 0.1% Nonidet P-40 lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin) for 30 min on ice. Immunoprecipitation and Western blot were performed as described previously (22). Anti-HA monoclonal antibody was purchased from Boehringer Mannheim. Goat anti-LT-βR was produced by immunization with purified human LT-βR-Fc fusion protein (19) as described previously for related proteins (25).

NF-κB Activation—Human embryonic kidney 293 cells (2×10^6) were transfected by the calcium phosphate method as described previously (16) with 2.5 μg of the LT-βR, TRAF5, or empty vector DNA to bring the total DNA to 7.5 μg. Nuclear extracts were prepared from transfected cells after 2 days in culture, and DNA binding interactions were performed by electrophoretic mobility shift assay as described previously with a slight modification (26). NF-κB oligonucleotide containing two tandemly arranged NF-κB sites from the HIV-1 enhancer (5′-ATCAGGGACTTTCCGCTGGGGACTT-3′ and 5′-CGGAAAGTCCCCAGCGGAAAAGTCCC-3′) and a random oligonucleotide with the sequences 5′-AGGATGGGAGTGTGATATATCCTTGAT-3′ and 5′-ATCAAGGATATATCACACTCCCATCCT-3′ were used in this assay. The composition of the activated NF-κB complex was examined by supershift analysis with antibodies that recognize specific NF-κB subunits (Santa Cruz Biotechnology, Inc.).

RESULTS AND DISCUSSION

A novel cDNA was obtained from the murine monocytic cell line J774A.1 by amplification of expressed sequences using degenerate oligonucleotide primers corresponding to the conserved amino acids in the TRAF domain of TRAF1, -2, and -3. This cDNA contains 2222 nucleotides with an open reading frame of 558 amino acids (predicted molecular mass of 64 kDa) (Fig. 1*A*). Several structural homologies were recognized in the amino acid sequence including a N-terminal cysteine- and histidine-rich region consistent with a zinc RING finger and five zinc fingers found in TRAF2 and -3 (10–14). The zinc binding region is followed by a coiled-coil (leucine zipper) region starting at Ile-264 that contains seven heptad repeats similar to TRAF3 (11–14). Also like TRAF3 a second coiled-coil-like region (starting at Leu-342) is found in TRAF5 that is similar, but not identical, to the coiled-coil region in TRAF1 and -2. This region is also referred to as the N-TRAF domain (amino-proximal region of the TRAF domain (10)). The C-terminal region (C-TRAF domain) defines this cDNA as a TRAF family member (10) with the highest homology to TRAF3 (65%) and TRAF2 (56%) (Fig. 1*B*). These structural characteristics suggest this cDNA encodes a novel member of the TRAF family and is referred to as TRAF5.

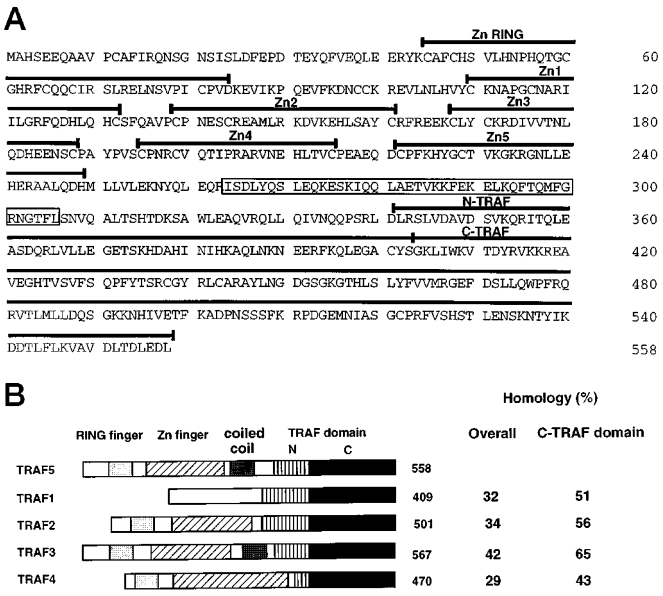

FIG. 1. **Primary structure of murine TRAF5.** *A*, the amino acid sequence of mouse TRAF5 deduced from cDNA sequence. *B*, structural domains of the TRAF family. Amino acids that form a zinc RING finger, zinc fingers, N-TRAF, and C-TRAF domain are indicated by *bold bars*, respectively. A coiled-coil region is shown by *boxes*. The percentages of homology at the amino acid level to TRAF5 were calculated by Lipman-Pearson's method (Genetyx). In TRAF5 the domains correspond to: a zinc RING (residues 45–84), zinc fingers (110–249), a coiled-coil (264–306), N-TRAF (342–403), and C-TRAF (404–558).

The structural similarity of TRAF5 with TRAF2 and -3 predicts that TRAF5 should bind directly to the cytoplasmic domain of one or more member(s) of the TNFR family. Receptor binding function was tested by incubating *in vitro* translated ^{35}S-labeled TRAF5 with several different TNF receptor-GST fusion proteins immobilized on glutathione beads. In this assay only LT-βR-GST bound TRAF5 (Fig. 2*A*). CD40, TNFR80 and TNFR60, Fas, and NGFR-GST fusion proteins failed to bind TRAF5 even though the amounts of these receptors used in the binding assay were substantially higher than for LT-βR-GST (data not shown). Both CD40- and TNFR80-GST preparations used here were active in binding *in vitro* translated TRAF3 (14); TNFR60-GST binds to TRADD and Fas-GST binds to Fas-associated phosphatase 1 (FAP-1) and Fas/APO-1-associated death domain protein (FADD) (27) demonstrating the functional integrity of these reagents (data not shown). These results indicate that TRAF5 interacts relatively specifically with LT-βR, although this conclusion is tentative until other TNF-related receptors have been tested. Unfortunately, the lack of obvious sequence homology in the cytoplasmic domains prevents a prediction of which other receptor(s) might bind TRAF5.

Northern blot analysis with a full-length cDNA probe demonstrated a 2.2-kilobase transcript for TRAF5 in all major visceral organs of the mouse with the most prominent expression in the lung and spleen (data not shown). Although mRNA for TRAFs are low abundance, this pattern of tissue distribution is similar to TRAF2 and -3, which are also broadly expressed (10, 14). TRAF5 mRNA is expressed in both lymphocytic and monocytic cell lines. By contrast, LT-βR mRNA is expressed in all major visceral organs of adult mouse, including lung and spleen, and also in thymic epithelial, monocytic, but not lymphocytic cell lines (28). The overlapping pattern of expression of TRAF5 and LT-βR is consistent with the idea that TRAF5 may be a signaling protein for the LT-βR. However, the disparate expression pattern in T lymphocytes suggests that TRAF5 may associate with other receptors.

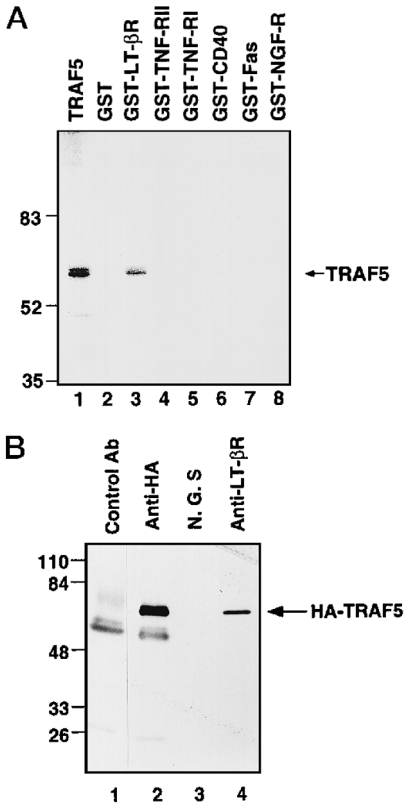

FIG. 2. **TRAF5 associates with LT-βR *in vitro* and *in vivo*.** *A*, association of *in vitro* translated TRAF5 with LT-βR-GST. TRAF5 cDNA was translated *in vitro* with [^{35}S]methionine, and 5 μl of the product (*lane 1*) was added to binding buffer and mixed with 10 μl of glutathione beads containing GST (*lane 2*) or individual TNFR-related-GST fusion proteins (*lanes 3–8*). The bound fraction was analyzed by SDS-polyacrylamide gel electrophoresis (10% gel) and phosphoimage. The fraction bound to LT-βR-GST is 56% of the total offered. *B*, association of TRAF5 and LT-βR in COS7 cells. Expression vectors containing HA-tagged TRAF5 (HA-TRAF5) or LT-βR were cotransfected into COS7 cells. After 2 days in culture cells, lysates were immunoprecipitated with normal mouse Ig (control antibody (*Ab*), *lane 1*), anti-HA monoclonal antibody (*lane 2*), normal goat serum (*N.G.S*, *lane 3*), or goat anti-LT-βR serum (*lane 4*). The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis, and the presence of HA epitope was detected by Western blot with anti-HA antibody. The molecular size standards (in kilodaltons) are indicated at the *left*. The *arrow* indicates the position of HA-TRAF5; the *lower band* is mouse Ig heavy chain.

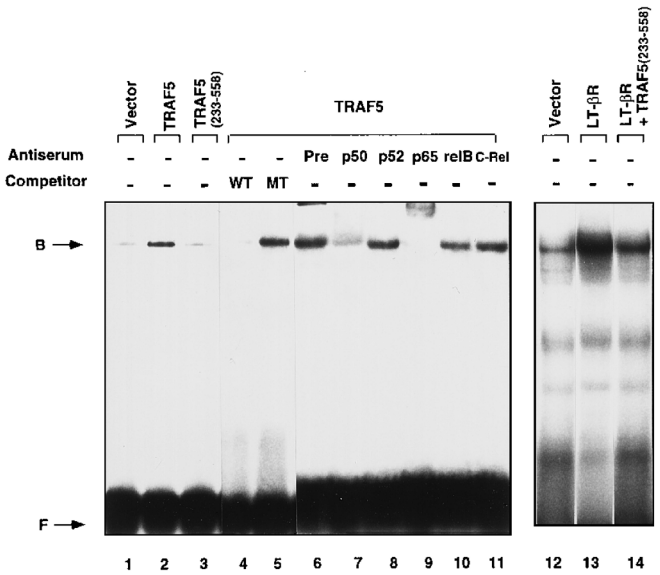

FIG. 3. **Overexpression of TRAF5 or LT-βR induces NF-κB activation.** HEK293 cells (2×10^6) were transfected with empty vector (*lane 1* or *12*) or expression vectors for a full-length TRAF5 (*lanes 2* and *4–11*), truncated TRAF5-(233–558) (*lane 3*), LT-βR (*lane 13*), or mutant TRAF5-(233–558) and LT-βR (*lane 14*). Nuclear extracts were prepared after 2 days, and 4 μg of the nuclear extract was incubated with a ^{32}P-labeled double-stranded κB oligonucleotide. Unlabeled competitor oligonucleotide containing either κB oligonucleotide (*WT*) (*lane 4*) or random oligonucleotide (*MT*) (*lane 5*) was added at a 50-fold molar excess. For supershift assays, reaction mixtures were incubated with 1 μl of either preimmune serum (*lane 6*), anti-p50 (*lane 7*), anti-p52 (*lane 8*), anti-p65 (*lane 9*), anti-relB (*lane 10*), or anti-C-Rel (*lane 11*) antiserum. *B* and *F* indicate the position of the bound or free fraction, respectively. *WT* and *MT* indicate wild type oligonucleotide or random oligonucleotide, respectively.

To determine whether TRAF5 associates with LT-βR *in vivo*, COS7 cells were cotransfected with TRAF5, tagged with HA epitope (HA-TRAF5) and LT-βR cDNAs. Cell lysates were prepared and subjected to immunoprecipitation with anti-HA, anti-LT-βR, or their respective control antibodies. The immunoprecipitates were analyzed by Western blot using anti-HA antibody (Fig. 2*B*). A band at 64 kDa consistent with the predicted molecular mass of HA-TRAF5 was efficiently co-immunoprecipitated with anti-HA (*lane 2*) or anti-LT-βR (*lane 4*) but not with nonspecific antibodies (*lanes 1* and *3*). This result indicates that TRAF5 and LT-βR form a complex when overexpressed in COS7 cells and suggests that TRAF5 is a component of the signal transduction complex for the LT-βR.

Overexpression of TRAF2, but not TRAF1 or -3, in HEK293 cells has been shown to directly activate the transcription factor, NF-κB (16). To determine whether TRAF5 mediates NF-κB activation, HEK293 cells were transfected with cDNAs encoding TRAF5, a truncated TRAF5 lacking the zinc binding region (233–558) or LT-βR. Deletion analysis of TRAF5 has shown that both the TRAF and coiled-coil domains are required for association with LT-βR (data not shown). Thus a truncated region (233–558) still has the ability to interact with LT-βR. Activation of NF-κB was assessed by gel shift assay using ^{32}P-labeled κB oligonucleotide (Fig. 3). Overexpression of the full-length TRAF5, but not truncated TRAF5-(233–558) or empty vector, induced a specific shift in migration of the labeled κB probe identifying activation of NF-κB (*lanes 1–5*). In addition, antibodies to NF-κB subunits p65 and p50 induced a supershift in the migration of the labeled probe indicating that overexpression of TRAF5 specifically activates the p65-p50 complex (*lanes 6–11*). These results reveal a strong functional similarity between TRAF5 and TRAF2. Both TRAFs activate p65-p50 NF-κB complex, which is dependent upon the zinc binding region (more specifically the RING finger in TRAF2 (16)). TRAF5 does not bind TNFR80 or CD40, a distinguishing feature from TRAF2, which suggests TRAF5 may be a specific signaling protein for the LT-βR. Overexpression of LT-βR cDNA alone also induced NF-κB activation (Fig. 3, *lane 13*). However, cotransfection of truncated TRAF5-(233–558) partially inhibited the activation of NF-κB by LT-βR cDNA (*lane 14*). The incomplete dominant negative effect of TRAF5-(233–558) indicates a compensatory molecule exists for LT-βR-mediated activation of NF-κB, perhaps TRAF2 or another protein(s). This issue is complicated in light of recent studies showing that TRAF2, and to a lesser extent TRAF3, interact with TRADD (29). TRADD is a death domain protein mediating the activation of NF-κB by TNFR60 (30). One possible scenario accounting for the incomplete effect of TRAF5-(233–558) mutant is that the LT-βR-TRAF3 complex could recruit TRADD, which in turn activates NF-κB independently from TRAF5. These results suggest multiple signaling pathways are utilized by LT-βR to elicit cellular responses.

That different TRAFs bind to the same receptor (*e.g.* TRAF3 and -5 binding to LT-βR or TRAF2 binding to CD40 and

TNFR80) raises the question of how specificity is controlled during signal transduction. Interactions among different receptors with a common signaling protein may explain why cell death or NF-κB activation is a common response signaled by several members of this receptor superfamily. However, the unique phenotypes associated with genetic mutations in several of the ligands or receptors indicate that signaling *in vivo* is highly specific. A pertinent example is failure of peripheral lymphoid organs to develop in mice genetically deficient in the LT-α gene (31, 32), a finding that implicates the LT-$\alpha 1\beta 2$ and LT-βR as a developmental regulatory system (19, 31). Recently, a detailed analysis of animals deficient in LT-α or TNFR60, but not TNFR80, revealed a failure of splenic germinal centers to form after immunization (33). The defect in germinal center formation in the LT-α and TNFR60 knockout mice is apparently due to distinct processes, which reinforces the exquisite specificity of the signaling pathways *in vivo*. Whether the specificity in signaling these complex processes is controlled at the level of receptor-associated proteins, or perhaps by downstream events, is a major goal of research in this area. In this regard, the identification of TRAF5 provides another step to achieve this goal.

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