Identification of TRAF6, a Novel Tumor Necrosis Factor Receptor-associated Factor Protein That Mediates Signaling from an Amino-terminal Domain of the CD40 Cytoplasmic Region*

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Takaoami Ishida, Sei-ichi Mizushima,†, Sakura Azuma, Norihiro Kobayashi, Tadashi Tojo, Kimie Suzuki, Shigemi Aizawa,‡, Yoshiki Watanabe,§ George Mosialos,¶, Elliott Kieff,¶, Tadashi Yamamoto, and Jun-ichiho Inoue**

From the Department of Oncology and the §Department of Pathology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan, the †Biosciences Research Laboratory, Mochida Pharmaceutical Co. LTD, 1-1-1 Kamiya, Kita-ku, Tokyo 115, Japan, and the ¶Infectious Disease Division, Brigham & Women’s Hospital, Boston, Massachusetts 02115

CD40 signalings play crucial roles in B-cell function. To identify molecules which transduce CD40 signalings, we have utilized the yeast two-hybrid system to clone cDNAs encoding proteins that bind the cytoplasmic tail of CD40. A cDNA encoding a putative signal transducer, designated TRAF6, has been molecularly cloned. TRAF6 has a tumor necrosis factor receptor (TNFR)-associated factor (TRAF) domain in its carboxyl terminus and has a RING finger domain, a cluster of zinc fingers and a coiled-coil domain, which are also present in other TRAF family proteins. TRAF6 does not associate with the cytoplasmic tails of TNFR2, CD30, lymphotoxin-β receptor, and LMP1 of Epstein-Barr virus. Deletion analysis showed that residues 246–269 of CD40 which are required for its association with TRAF2, TRAF3, and TRAF5 are dispensable for its interaction with TRAF6, whereas residues 230–245 were required. Overexpression of TRAF6 activates transcription factor NFκB, and its TRAF-C domain suppresses NFκB activation triggered by CD40 lacking residues 246–277. These results suggest that TRAF6 could mediate the CD40 signal that is transduced by the amino-terminal domain (230–245) of the CD40 cytoplasmic region and appears to be independent of other known TRAF family proteins.

CD40 is a member of the tumor necrosis factor receptor (TNFR) superfamily, which includes TNFR1 and -2 (1, 2), Fas (3), lymphotoxin-β receptor (4), CD30 (5), OX40 (6), and the low affinity nerve growth factor receptor (7), all of which share a ligand-binding domain composed of tandemly repeated cysteine-rich modules. CD40 is expressed in late B cells in bone marrow, mature B cells, and certain accessory cells, including bone marrow-derived dendritic cells and follicular dendritic cells (8–10), and is a receptor for CD40 ligand (CD40L) present on activated CD4+ T cells (11). Signals through CD40 rescue B cells from apoptosis induced by cross-linking of the surface immunoglobulin M complex (12) and also induce B cells to differentiate and to undergo Ig isotype switching (13, 14).

CD40 signalings were reported to include modulation of the activity of non-receptor-type tyrosine kinases such as Lyn, Fyn, and Syk, activation of phosphatidylinositol-3-kinase, phosphorylation of phospholipase Cγ2 (15–17), activation of the Rel/NFκB transcription factors (18), and induction of the Bel-xL, Cdk4, and Cdk6 proteins (19). However, the mechanisms of signal transduction from CD40 are uncertain. Biochemical purification of receptor-associated proteins or the recently developed cDNA cloning system that uses yeast genetic selection led to the discovery of two groups of signal transducer molecules that are utilized by members of the TNFR superfamily. Members of the first group are proteins with a highly conserved domain known as the TRAF domain and include TRAF1, TRAF2, TRAF3, and TRAF6, also known as CD40bp, LAP-1, or CRAF (21–23). TRAF proteins have been implicated in signal transduction from TNFR2 and CD40. We (24) and Nakano et al. (25) have recently cloned a cDNA encoding TRAF5 which mediates signals emanating from CD40 and the lymphotoxin-β receptor. The second group includes proteins with a death domain involved in Fas and TNFR1 signaling such as FADD (26) also known as MORT1 (27) or RIP (28), and TRADD (29). Among various CD40 signaling events, the Rel/NFκB activation was demonstrated to be mediated by TRAF2 (30), TRAF5 (24, 25), and RIP (31). To further characterize the initial stage of signaling by CD40, we have used the yeast two-hybrid system to identify cDNAs encoding proteins that interact with the cytoplasmic tail of CD40. Here we report the identification of a novel member of the TRAF family, designated TRAF6, that binds to the amino-terminal region of the CD40 cytoplasmic tail, which is distinct from the binding domain for TRAF2, TRAF3, and TRAF5.

MATERIALS AND METHODS

Yeast Two-hybrid System—A DNA fragment encoding the cytoplasmic tail of mouse CD40 (amino acids 216–306) was cloned into the yeast LexA DNA-binding domain vector pBTM116. The resulting plasmid, pBTM40cyt, was used as bait in a two-hybrid screening of a murine C57 Black Kaplan cDNA library fused to the activation domain of Gal4 in the pHACT plasmid (Clontech). Seventy-two out of the 2 × 10⁶ transformants screened grew in the absence of histidine and had detectable...
Total RNA from WEHI-231 and EL-4 cell lines was prepared, and the total RNA was converted to cDNA with random hexanucleotide primers (pd(N)6, Pharmacia Biotech Inc.). The PCR primers were designed to correspond to the 5′ region of the C40–65 cDNA (probe D, see Fig. 2B) and the 65–69 (probe A) and 3′ region (probe B) of the C40–65 cDNA (probe A) and the 65–69 (probe B). The PCR products were separated on a 1.5% agarose gel and transferred to nylon membrane (Hybond N, Amersham). The filter was hybridized with 32P-labeled oligo probe c (see panel B). The unique restriction sites of the cDNA fragments were cleaved, and cDNA was subjected to nucleotide sequencing. A mouse multiple clone library was prepared from mouse spleen cDNA at 65°C as described (19). The filter was hybridized with 32P-labeled TRAF6 cDNA (65–69) or with β-actin cDNA (24) followed by centrifugation. The amount of DNA transfected was always adjusted to 5 μg with a control expression vector, pME18S-FLAG. Forty hours after transfection, cell extracts were prepared by freeze-thawing followed by centrifugation. β-Galactosidase activity was used to standardize transfection efficiency, and CAT assays were performed for 1 h at 37°C as described (32).

RESULTS AND DISCUSSION

From 2 × 10⁶ clones of a murine C57 Black Kaplan T cell lymphoma cDNA library, 10 independent clones were isolated that met all specific criteria for binding to the cytoplasmic tail of mouse CD40 in yeast. Nucleotide sequencing of all cDNA fragments revealed that one of them (clone C40–65) encoded a peptide which has a TRAF domain in its carboxyl-terminal region (Fig. 1A). Since it is the sixth member of the TRAF family of proteins, we termed this protein “TRAF6.” Using the C40–65 cDNA as a probe, a cDNA encompassing the entire coding region was obtained from a cDNA library prepared from the murine EL-4 T cell line. The longest cDNA clone (clone 65–69) contained 2312 base pairs (bp) including a potential poly(A) tail (Fig. 2B). An in-frame stop codon 69 nucleotides upstream from the first methionine indicates that the clone 65–69 encodes the full-length TRAF6 protein. However, Northern blotting of poly(A)+ RNA from various murine tissues and cell lines revealed that the TRAF6 mRNA is 5.5 kb (Fig. 2A and C). The C40–65 cDNA starts at nucleotide 829 of the 65–69 cDNA and has an extra 2868 bp of 3′-untranslated region (Fig. 2B). To further investigate the relation between the 65–69 and the C40–65 cDNAs, Northern blotting was performed using the 5′-region of the 65–69 cDNA (probe A) and the 3′-region of the C40–65 (probe B) as probes (Fig. 2B). Both probes hybridized to a 5.5-kb mRNA (Fig. 2C). Furthermore, the C40–65 cDNA was not artificially generated by ligating

**Fig. 2. The TRAF6 mRNA analysis.** A, Northern blot analysis of the TRAF6 mRNA in mouse tissues. The filter was hybridized with 32P-labeled TRAF6 cDNA (65–69) (upper) or with β-actin cDNA (bottom). B, structure of various TRAF6 cDNAs. Three thin lines indicate the region of the cDNA fragments. The bold lines indicate the region of the probes used for library screening (probe D) and the Northern blotting analysis of cell line RNAs (probes A and B). Arrows indicate the position of the PCR primers (a, b) and oligonucleotide probe (c) used for the Southern blotting analysis of the PCR products. C, Northern blot analysis of the TRAF6 mRNA from WEHI-231 and EL-4 cell lines. The left panel (lanes 1 and 2) and the right panel (lanes 3 and 4) were hybridized with probe A and probe B, respectively. D, PCR analysis of the TRAF6 mRNA. PCR was performed without templates (lanes 2 and 6), with spleen cDNA (lanes 3 and 7) or C40–65 cDNA (lanes 4 and 8). The left panel shows the ethidium bromide stain of the PCR products, and the right panel shows the result of the Southern blot analysis of the same agarose gel. The filter was hybridized with 32P-labeled oligo probe c (see panel B).
two independent cDNAs during the preparation of the cDNA library, because a TRAF6 cDNA obtained from spleen mRNA contains an extra 3'-untranslated region identical with that of the C40–65 cDNA (Fig. 2D). Thus, these short cDNAs could be generated by the minor poly(A) addition site at 2291 or misannealing of oligo(dT) primer for cDNA synthesis. The length of combined TRAF6 cDNAs is 5277 bp, which is consistent with the size of the TRAF6 mRNA (Fig. 2A).

The TRAF6 cDNA can encode a protein of 530 amino acids (Fig. 1B) with a calculated molecular weight of 60,083. The carboxyl terminus of the encoded protein is homologous to that of TRAF family protein for approximately 150 amino acids, called TRAF-C domain (20). Although the TRAF-C domain of TRAF6 is most homologous to that of TRAF2 (35.9% identity), identities among other TRAF family proteins range from 41.7% to 66.2%. In addition to the TRAF-C domain, TRAF6 has three potential domains including a coiled-coil domain, a cluster of five zinc fingers, and a RING finger, which are also present in TNF receptor type 2, CD30, and lymphotoxin-β receptor (Fig. 2B).

To characterize the CD40-TRAF6 interaction, we carried out in vivo binding assays using GST pull down experiments. Cell extracts were prepared from 293T cells cotransfected with FLAG-tagged TRAF6 or TRAF2, and GST-tagged CD40 or its mutants. Whereas the interaction of TRAF2 (middle), TRAF3 (24), or TRAF5 with CD40 is mediated by residues 246–269 of CD40, the interaction of TRAF6 with CD40 does not fully require residues 245–269 and is mediated by 230–245 (lanes 2–5, top). The interaction of TRAF6 with CD40 was little affected when Thr254 of CD40 was converted to Ala (TA mutant, our amino acid numbering includes the signal peptide) (lane 6, top), an alteration known to impair CD40 signaling linked to growth inhibition (33), whereas this mutation abolished the interaction of CD40 with TRAF6 (lane 6, middle), TRAF3 (21), and TRAF5 (24). However, we could not rule out the possibility that TRAF6 might also recognize residues 245–269, because the amount of TRAF6 bound to GST246 or GST-TRAF6 was about 65% of that bound to GST246 or GST-WT. These results suggest that TRAF6 could mediate CD40 signaling events that are distinct from those mediated by TRAF2, TRAF3, or TRAF5. Since it is possible that the tertiary structure of CD40 cytoplasmic region in GST fusion protein is different from that in natural membrane-bound CD40, our conclusion described above should be interpreted in consideration of this point.

We next analyzed the interaction of TRAF6 with other members of the TNF receptor superfamily by in vitro binding assay. TRAF6 did not bind to any other receptor examined including TNF receptor type 2, CD30, and lymphotoxin-β receptor (Fig. 3C, lanes 3–5), and to LMP-1 of Epstein-Barr virus (lane 6) to which TRAF3 binds (23).

One of the signals emanating from CD40 is the activation of the transcription factor NFκB. It has been demonstrated that TRAF2 and TRAF5, but not TRAF3, TRAF4, or TRAF6, mediate signals linked to NFκB activation (26). To examine the possible role of TRAF6 in CD40-mediated NFκB activation, transient transfection experiments were performed to determine whether TRAF6 expression might lead to activation of transcription from an NFκB-site-dependent reporter gene. The NFκB-site-dependent reporter construct ([κB]6TK-CAT) (32) was cotransfected with a TRAF6 expression vector (pME-FLAG-TRAF6) into human Jurkat T cells. To confirm the specificity of transcription, the same reporter construct carrying mutant κB sites ([κB]m6TK-CAT) was transfected. In Jurkat cells, TRAF6 as
well as TRAF2 activates αB-site-dependent transcription in a dose-dependent manner (Fig. 4A). Cheng and Baltimore (34) demonstrated the existence of two non-overlapping regions in the cytoplasmic tail of CD40, each of which is sufficient for NFκB activation. One region, called TIMct, consists of 17 amino acids (residues 250–266) and is required and sufficient for the association of CD40 with TRAF2 and TRAF3. The other is a more amino-terminal region which partly overlaps with the TRAF6 binding region. We have also shown here that residues 230–245 of CD40 required for NFκB activation (19) coincide with the region required for the binding of TRAF6, suggesting that activation of NFκB by CD40 signaling could be partly mediated by TRAF6. To further elucidate the role of TRAF6 in CD40-mediated NFκB activation, we asked whether the TRAF-C domain of TRAF6 acts as a transdominant negative mutant to suppress NFκB activation induced by CD40ΔΔ246 mutant in which the binding site for TRAF2 was removed. Expression of the TRAF-C domain of TRAF6 resulted in the suppression of NFκB activity, while expression of the TRAF-C domain of TRAF2 had little effect (Fig. 4B). Thus, it is possible that NFκB activation through TIMct could be mediated by either TRAF2 or TRAF5 or both, whereas NFκB activation from a more amino-terminal region is mediated by TRAF6.

The TNF receptor family of proteins, including Fas, CD40, CD30, and the lymphotixin-β receptor, have been shown to associate with downstream signal transducer molecules carrying either a TRAF or a death domain (20–29). TRAF2 can interact with the death domain containing molecule TRADD (35), indicating that cross-talk between TRAF proteins and death domain proteins could mediate diverse signaling processes emanating from the TNF receptor family members. Accumulating evidence indicated that three members of the TRAF family proteins including TRAF2, TRAF3, and TRAF5 can directly associate with CD40 (21–24, 30). We have now identified and characterized a new member of CD40-associated factors, designated as TRAF6. We have previously shown that CD40 residues 246–266, which contains the TIMct domain (34), were required for blocking apoptosis of WEHI 231 cells induced by surface IgM signaling (19). 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