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TIFAB inhibits TIFA, TRAF-interacting protein with a forkhead-associated domain

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Abstract

Tumor necrosis factor receptor-associated factor 6 (TRAF6) transduces signals that lead to activation of NFκB and AP-1, which is essential for cell differentiation and establishment of the immune and inflammatory systems. TRAF-interacting protein with a forkhead-associated domain (TIFA) was identified as a TRAF6-binding protein that could link IRAK-1 to TRAF6 and then activate TRAF6 upon stimulation. We report identification of a TIFA-related protein, TIFAB, that inhibits TIFA-mediated activation of NFκB. TIFAB does not associate with members of the TRAF family but does bind TIFA. We analyzed the effect of TIFAB expression on the TRAF6/TIFA interaction by immunoprecipitation of TRAF6 and found that TIFA coprecipitated with TRAF6 was not changed. However, when we analyzed this interaction by immunoprecipitation of TIFA, we found that TIFAB significantly increased the amount of TRAF6 coprecipitated with TIFA. These findings suggest that TIFAB inhibits the TIFA-mediated TRAF6 activation possibly by inducing a conformational change in TIFA.

Keywords: NFκB; TRAF; TIFA; Negative regulator; IL-1; TLR; IRAK-1

The tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family proteins are cytoplasmic proteins that mediate cytokine signaling emanating from members of the TNFR superfamily and the Toll/interleukin-1 receptor (IL-1R) family [1,2]. To date, six members of the TRAF family have been described. TRAF2, TRAF5, and TRAF6 activate transcription factors NFκB through IκB kinase (IKK) and AP-1 through mitogen-activated protein kinases (MAPKs) including Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK). TRAF6 is the most divergent member of the TRAF family and is the only TRAF that is involved in signaling from members of the Toll/IL-1R family by interacting with IL-1R-associated kinase-1 (IRAK-1) [3,4]. IL-1 signaling is completely blocked in the absence of TRAF6 [5–7]. We previously reported that TRAF6-deficient ($TRAF6^{-/-}$) mice exhibit severe osteopetrosis and lack lymph nodes due to defective signaling from RANK upon binding of osteoclast differentiation factor/RANK ligand (ODF/RANKL) (also known as OPGL and TRANCE) [6–8]. Furthermore, $TRAF6^{-/-}$ mice display hypohidrotic ectodermal dysplasia, which is a congenic disorder of formation of skin appendices including hair follicles and sweat glands [9]. Thus, TRAF6 plays essential roles in differentiation of specific cell types and development of the immune and inflammatory systems. Although TRAF6-mediated signal transduction is necessary for proper development, the molecular mechanism by which TRAF6 exerts its biological effects remains unknown. To address this question, we have screened for proteins that bind and regulate TRAF6. TRAF-interacting protein with a forkhead-associated (FHA) domain (TIFA), which is also known as T2BP, has been identified as such

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a protein [10,11]. When overexpressed, TIFA activates NFκB and JNK. Furthermore, introduction of a mutation into TIFA that abolishes binding of TIFA to TRAF6 leads to loss of ability of TIFA to activate NFκB and JNK, indicating that interaction with TRAF6 is essential for the activity of TIFA.

FHA domains are conserved sequences of 60–100 amino acids found mainly in eukaryotic nuclear proteins [12]. Some of the FHA domain-containing proteins bind directly to phosphoserine/phosphothreonine residues via the FHA domain in much the same way that SH2 domains interact with phosphotyrosine residues [13,14]. TIFA carrying mutations in the FHA domain that are known to abolish FHA domain binding to phosphopeptide (G50ES66A mutant) cannot activate NFκB and JNK, suggesting that TIFA may be regulated by an unidentified phosphoprotein. Furthermore, analysis of endogenous proteins indicated that TIFA associates with TRAF6 constitutively, whereas it associates with IRAK-1 in an IL-1 stimulation-dependent manner. Therefore, TIFA is likely to link IRAK-1 to TRAF6 upon stimulation as a signal-dependent activator of TRAF6 [11]. In the present study, we performed homology searches of human and mouse genome databases and identified a gene that encodes a protein with structural similarity to TIFA. This TIFA-related protein, designated TIFAB, associates with TIFA and blocks TIFA-mediated activation of NFκB.

Materials and methods

Identification of an open reading frame in the human TIFAB cDNA. To identify the first nucleotide of the human TIFAB open reading frame (ORF), a DNA fragment containing the 5′-untranslated region was amplified by polymerase chain reaction (PCR) from a human spleen cDNA library in λ ZapLox (Invitrogen). The first PCR was performed with the phage-specific primer 1F (5′-AAGAACGTGGA CTCCAACG-3′) and the TIFAB-specific primer TB-3 (5′-ATGTG CCTTCTTCTACGCGA-3′) and LA *Taq* polymerase (Takara Bio). After 30 cycles of amplification at 55 °C, a nested second PCR was performed to increase the specificity and efficiency of the amplification reaction with the internal primers 4F (5′-GTCCATTCGCCATTCA GG-3′) and TB-2 (5′-CAGAGGGCTGGTATCATGCT-3′). The amplified fragments were cloned into pGEM-T Easy (Promega) and nucleotide sequences were determined.

Northern blotting. Total RNA was prepared from various tissues of a 4-week-old male ICR mouse as described [15]. Total RNA (20 μg) was separated by 1% formaldehyde denaturing agarose gel and transferred to nylon membrane (Hybond N, Amersham–Pharmacia). The filter was incubated with [^{32}P]-labeled full length mouse TIFAB cDNA probe and washed with 0.5× SSC/0.1% (wt/vol) SDS at 65 °C for 60 min.

Immunoprecipitation and Western blotting. HEK293T cells (1×10^6) in 90-mm dishes were cotransfected with the indicated combinations of pME-FLAG-TIFA, pME-Myc-TIFA, pME-FLAG-TIFAB, pME-Myc-TIFAB, and pME-Myc-TRAF6. Thirty-six hours after transfection, cells were lysed in TNE buffer as described previously [7] and subjected to immunoprecipitation by addition of anti-FLAG monoclonal antibody (M2) (Sigma) and protein G–Sepharose (Amersham–Pharmacia Biotech). The resulting immunoprecipitates were separated on 10% or 12.5% polyacrylamide/SDS gels, and each protein was visualized by Western blotting with either anti-FLAG or anti-Myc (9E10) (Santa Cruz) monoclonal antibody. In some experiments, TRAF6 was detected using anti-TRAF6 polyclonal antibody (H-274) (Santa Cruz). Cell lysates were also used to analyze the level of expression of each protein.

Luciferase reporter assay. HEK293T cells (2×10^5) in 6-well dishes were transfected with 5 ng of 3× κB-luc, 50 ng β-actin-β-gal, and the indicated amounts of various expression plasmids. Thirty-six hours after transfection, luciferase activity was measured with the PicaGene Luciferase Assay System (Toyo Ink) and β-galactosidase activity was used to standardize transfection efficiency.

Results and discussion

Identification of TIFAB in human and mouse

To identify TIFA-related proteins, we screened in silico for sequences homologous to the FHA domain of mouse TIFA [11]. A cDNA clone, AK036643, was identified and predicted to encode a polypeptide containing an FHA domain that is 60% similar (30% identical) to that of mouse TIFA (Fig. 1A). AK036643 contains a complete ORF that encodes 147 amino acids from the first methionine. The putative protein has the same architecture as that of TIFA [11]; the single FHA domain lies in the middle of the protein flanked by the N-terminal (N-domain) and C-terminal (C-domain) regions. The N-domain and C-domain of this protein have 44% and 55% similarities, respectively, to the corresponding domains of TIFA. Therefore, we designated this protein TIFAB. We then searched for a human homologue of mouse TIFAB by screening in silico with the predicted amino acid sequence of mouse TIFAB. Although a cDNA clone (AK131088) was identified, this cDNA does not contain a termination codon upstream of a putative coding region. To determine the first methionine of human TIFAB, we carried out PCR-based amplification of the 5′-ends of cDNAs. Two independent cDNAs isolated from a human spleen cDNA library contained an in-frame termination codon upstream of the predicted first methionine of human TIFAB (Fig. 1B). The human TIFAB protein is 80% identical to mouse TIFAB but contains 14 additional amino acids at the C-terminal end. The human and mouse TIFAB genes mapped to chromosomes 5q31.2 and 13, respectively. The entire coding region is contained in a single exon, as is the case for the TIFA gene (data not shown). TIFAB mRNA is expressed at high levels in spleen and at moderate levels in lung, thymus, and small intestine (Fig. 2). This pattern of expression is very similar to that of TIFA [11], suggesting that TIFA and TIFAB may be involved in similar signal transduction pathways.

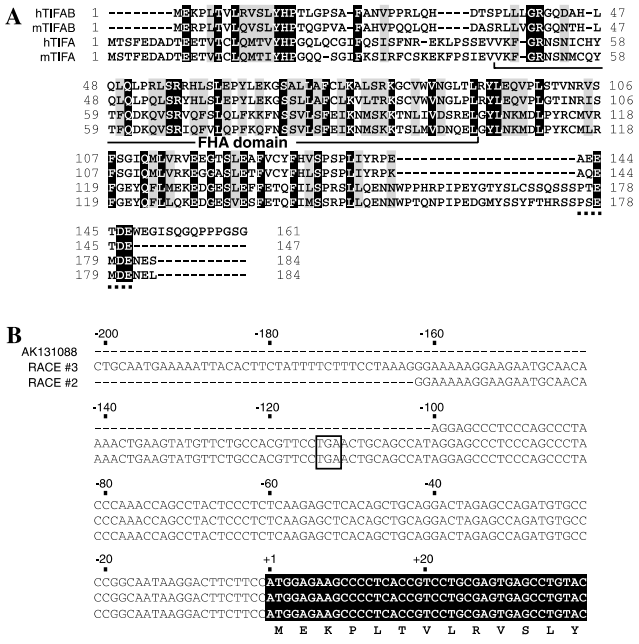

Fig. 1. Structure of human and mouse TIFAB. (A) Comparison of the amino acid sequences of the human and mouse TIFA family proteins. Amino acid sequences of the TIFAB and TIFA from human (h) and mouse (m) were aligned with the ClustalX software program. Residues identical or similar in all four proteins are indicated in black or gray boxes, respectively. A dotted line indicates the TRAF6-binding sites in human and mouse TIFA. The FHA domain is indicated with solid lines. The DDBJ/EMBL/GenBank Accession Nos. are: AK036643 (mouse TIFAB), AB062110 (human TIFA), AB062111 (mouse TIFA). The nucleotide sequence of human TIFAB cDNA has been submitted to DDBJ/EMBL/GenBank (Accession No. AB161513). (B) Structure of the 5′-region of the human TIFAB cDNA. Nucleotide sequences of the 5′-regions of AK131088 and two independently amplified cDNA fragments (RACE#3 and #2) are shown. Coding regions are marked in black, and in-frame stop codons upstream of the first methionine are indicated by a box. The nucleotide sequence of RACE#2 has been submitted to DDBJ/EMBL/GenBank (Accession No. AB161513).

TIFAB does not activate NFκB but blocks NFκB activation induced by TIFA

When TIFA is overexpressed, it activates NFκB (Fig. 3A) [11], and the C-terminal TRAF6-binding motif, PxExxAcidic/Aromatic (PSEMDE in mouse TIFA), is required for this activation. In fact, E178A mutant of TIFA, in which Glu-178 is replaced with Ala, is unable to activate NFκB in HEK293T cells endogenously expressing TRAF6 [11]. To determine if TIFAB activates NFκB, an NFκB-driven luciferase reporter plasmid was cotransfected with a mouse TIFAB expression plasmid in HEK293T cells. Even though the TIFAB protein was expressed efficiently and the TRAF6-binding motif is partially conserved in TIFAB, TIFAB did not activate NFκB (Fig. 3A). We then hypothesized that TIFAB might inhibit activation of NFκB induced by TIFA. To check this hypothesis, TIFA-mediated activation of NFκB was monitored in

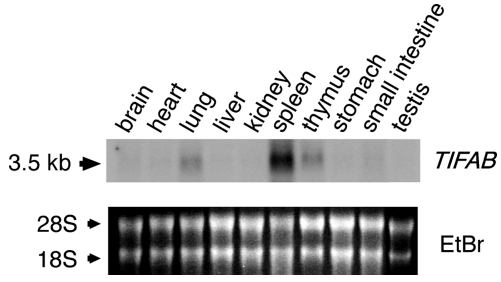

Fig. 2. Northern blotting analysis of *TIFAB* mRNA in mouse tissue. Total RNAs (20 g) extracted from various mouse tissues were separated for Northern blotting as described under Materials and methods. Ethidium bromide (EtBr) staining of rRNAs is shown as a loading control.

the absence or the presence of increasing amounts of TIFAB. TIFAB blocked TIFA-induced NFκB activation in a dose-dependent manner without affecting TIFA levels (Fig. 3B). A similar inhibitory effect of TIFAB was also observed with human TIFA and TIFAB (data not shown).

Formation of a complex containing TIFAB, TIFA, and TRAF6

To understand the molecular mechanism by which TIFAB inhibits TIFA-mediated activation of NFκB, the interaction of TIFAB with TIFA and TRAF family proteins was analyzed. When Myc-TIFA and FLAG-TIFAB were coexpressed and immunoprecipitation was performed with anti-FLAG antibody, Myc-TIFA was coprecipitated with FLAG-TIFAB, indicating that TIFAB was associated with TIFA (Fig. 4A). We then examined whether TIFAB associates with TRAF6 or other members of the TRAF family. FLAG-TIFAB or FLAG-TIFA was coexpressed with Myc-TRAF6, and cell lysates were immunoprecipitated with anti-Myc antibody. TIFAB was not coprecipitated with TRAF6, whereas TIFA was associated with TRAF6 (Fig. 4B). TIFA was not associated with other members of the TRAF family (data not shown). Binding of TIFA to TRAF6 is required for TIFA-mediated activation of NFκB and JNK [11]. In addition, TRAF6 transduces signals when oligomerized [16,17]. Therefore, we hypothesized that TIFA may form a homo-oligomer and facilitate formation of TRAF6 oligomers. Various TIFA deletion mutants that cannot form homo-oligomers did not activate NFκB. However, the G50ES66A mutant of TIFA, which contains two amino acid substitutions in its FHA domain bound to TRAF6 and formed homo-oligomer but did not activate NFκB [11]. Gel filtration analysis of recombinant TIFA protein and the inactive TIFA mutant revealed that TIFA forms a trimer and the TIFA G50ES66A mutant forms pentamers or hexamers [11]. These results suggest that formation of the correct TIFA oligomer, which appears to be dependent on the

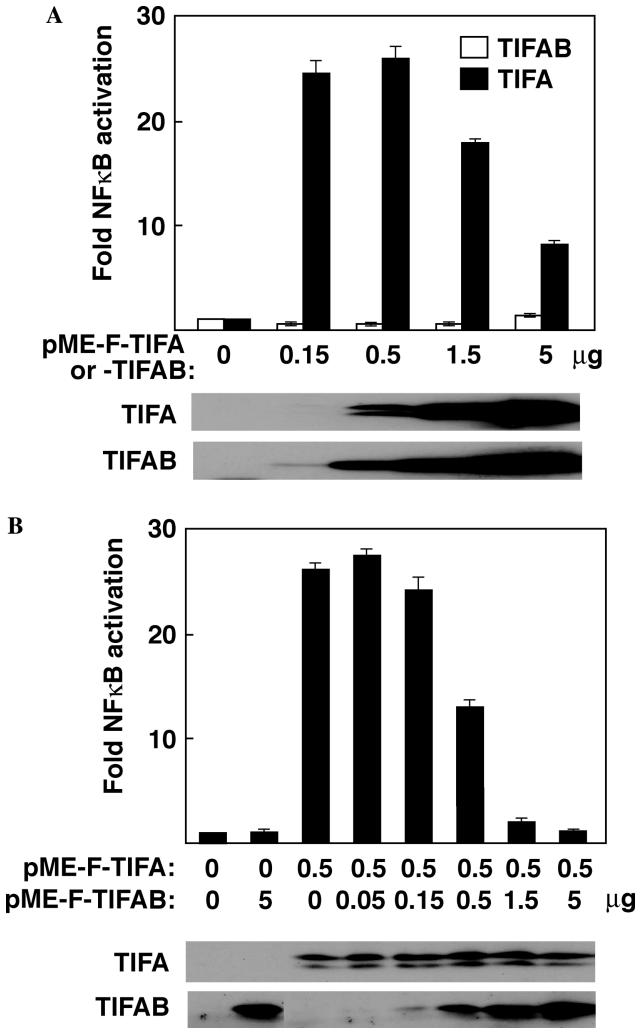

Fig. 3. TIFAB blocks NFκB activation induced by TIFA. (A) TIFAB does not activate NFκB. HEK293T cells were transfected with 5 ng of 3× κB-luc, 50 ng β-actin-β-gal, and the indicated amounts of pME-FLAG-TIFAB (open bars) or pME-FLAG-TIFA (closed bars). Thirty-six hours after transfection, luciferase assays were performed. Results are means ± SD of triplicate experiments and are representative of two independent experiments. Expression of FLAG-TIFAB and that of FLAG-TIFA were measured by Western blotting with anti-FLAG antibody. (B) TIFA-mediated activation of NFκB is inhibited by TIFAB. Experiments were performed as described in (A) except that NFκB activation induced by constant amount of TIFA was measured in the absence or presence of increasing amounts of TIFAB. Expression of FLAG-TIFAB and that of FLAG-TIFA were measured by Western blotting with anti-FLAG antibody.

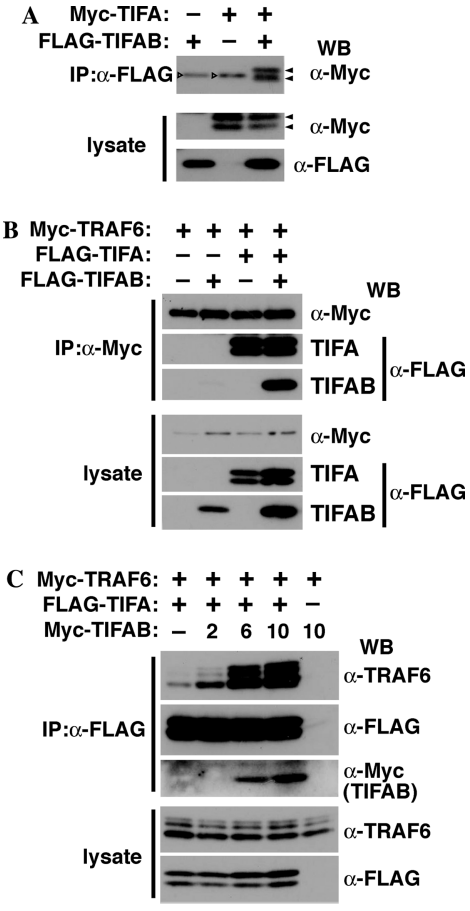

Fig. 4. Formation of a complex containing TIFAB, TIFA, and TRAF6. (A) TIFAB binds to TIFA. HEK293T cells were transfected with 5 μg pME-Myc-TIFA and/or 5 μg pME-FLAG-TIFAB. Thirty-six hours after transfection, cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibody or directly used for Western blotting (WB). Closed arrowheads indicate Myc-TIFA. Open arrowheads indicate background bands derived from immunoglobulin light chain. (B) TIFAB associates with TRAF6 via TIFA. HEK293T cells were transfected with 2 μg pME-Myc-TRAF6 alone or together with 2 μg pME-FLAG-TIFA and/or 10 μg pME-FLAG-TIFAB. Thirty-six hours after transfection, cell lysates were subjected to immunoprecipitation with anti-Myc antibody or used directly for Western blotting. (C) Association of TIFAB with TIFA results in increased association of TRAF6 with TIFA. HEK293T cells were transfected with 2 μg pME-Myc-TRAF6 together with 2 μg pME-FLAG-TIFA and/or increasing amounts of pME-Myc-TIFAB. Thirty-six hours after transfection, cell lysates were subjected to immunoprecipitation with anti-FLAG antibody or used directly for Western blotting.

structure of TIFA, may be critical for TRAF6 activation. Thus, there are two possible mechanisms by which TIFAB inhibits TIFA-mediated activation of NFκB. First, binding of TIFAB to TIFA may cause TIFA to dissociate from TRAF6 and TIFA. Alternatively, binding of TIFAB to TIFA may cause a conformational change in TIFA without affecting the interaction between TIFA and TRAF6. To distinguish these possibilities, we examined formation of complexes containing TIFAB, TIFA, and TRAF6. When both FLAG-TIFA and FLAG-TIFAB were coexpressed with Myc-TRAF6, TIFAB was coprecipitated with TRAF6, indicating that TIFAB was indirectly associated with TRAF6 via TIFA (Fig. 4B). To further characterize the TIFAB/TIFA/TRAF6 complex, TRAF6 coprecipitated with TIFA was analyzed in the absence or presence of increasing amounts of TIFAB by immunoprecipitation of TIFA with anti-FLAG antibody. The amount of TRAF6 coprecipitated with TIFA was dramatically increased in the presence of TIFAB in a dose-dependent

manner (Fig. 4C). However, the amount of TIFA coprecipitated with TRAF6 analyzed by immunoprecipitation of TRAF6 with anti-Myc antibody was not changed in the absence or presence of TIFAB (Fig. 4B), suggesting that the TRAF6/TIFA interaction is stable irrespective of TIFAB. This discrepancy appears to indicate that a conformational change in TIFA occurs. The FLAG-epitope of TIFA may not be fully accessible to the anti-FLAG antibody possibly due to steric hindrance caused by TRAF6 associated with TIFA, and binding of TIFAB to TIFA could change the conformation of TIFA and thus that of TRAF6, allowing the anti-FLAG antibody to bind efficiently to the epitope present in TIFA. Because the amount of TRAF6-associated TIFA could be significantly lower than that of free TIFA, the amount of TIFA immunoprecipitated by anti-FLAG antibody appeared to be constant. Taken together, these findings suggest that TIFAB inhibits TIFA-mediated activation of NFκB through induction of conformational changes in TIFA and TRAF6.

TIFA is thought to link IRAK-1 to TRAF6 upon stimulation [11]. Thus, TIFA may play a role in signaling from IL-1R and some members of the TLR family. Furthermore, TIFA associates with TRAF6 constitutively, suggesting that TIFA is involved in other TRAF6 signaling critical for cell differentiation. Thus, TIFAB could be an important regulator of cell differentiation and development of immune and inflammatory systems.

Acknowledgments

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