THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 16, Issue of April 21, pp. 12102–12107, 2000

Printed in U.S.A.

The *Drosophila* Tumor Necrosis Factor Receptor-associated Factor-1 (DTRAF1) Interacts with Pelle and Regulates NFκB Activity*

(Received for publication, November 22, 1999, and in revised form, January 20, 2000)

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A member of the tumor necrosis factor (TNF) receptor-associated factor (TRAF) family was identified in *Drosophila*. DTRAF1 contains 7 zinc finger domains followed by a TRAF domain, similar to mammalian TRAFs and other members of the family identified in data bases from *Caenorhabditis elegans*, *Arabidopsis*, and *Dictyostelium*. Analysis of DTRAF1 binding to different members of the human TNF receptor family showed that this protein can interact through its TRAF domain with the p75 neurotrophin receptor and weakly with the lymphotoxin-β receptor. DTRAF1 can also self-associate and binds to human TRAF1, TRAF2, and TRAF4. Interestingly, DTRAF1 interacts with human cIAP-1 and cIAP-2 but not with *Drosophila* DIAP-1 and -2. By itself, DTRAF1 did not induce significant NFκB activation when overexpressed in mammalian cells, although it specifically increased NFκB induction by TRAF6. In contrast, TRAF2-mediated NFκB induction was partially inhibited by DTRAF1. Mutants of DTRAF1 lacking the N-terminal region inhibited NFκB induction by either TRAF2 or TRAF6. DTRAF1 specifically associated with the regulatory N-terminal domain of Pelle, a *Drosophila* homolog of the human kinase interleukin-1 receptor-associated kinase (IRAK). Interestingly, though Pelle and DTRAF1 individually were unable to induce NFκB in a human cell line, co-expression of Pelle and DTRAF1 resulted in significant NFκB activity. Interactions of DTRAF1 with human TRAF-, TNF receptor-, and IAP-family proteins imply strong evolutionary conservation of TRAF protein structure and function throughout Metazoan evolution.

Members of the tumor necrosis factor receptor (TNFR)[1] superfamily can elicit a wide spectrum of cellular responses, including regulation of cell proliferation, apoptosis, and differentiation (1, 2). Several of these functions are mediated by a family of intracellular TNFR-binding proteins, the TNF receptor-associated factors (TRAFs) (1, 3). In humans and mice, six members of the TRAF family (TRAF1 through TRAF6) have been identified. All of these proteins share as a defining characteristic a conserved stretch of amino acids near their C termini termed the TRAF domain. The TRAF domain is required for binding of these signal-transducing adaptor proteins to specific members of the TNF family of receptors (3). Intrinsic redundancies may exist among TRAF family members. For instance, different TRAFs can bind to the same TNFR-family receptor. Also, different TRAFs can activate similar transduction pathways. In this regard, TRAF2, TRAF5, and TRAF6 have been shown to activate NFκB and c-Jun NH_2-terminal kinase when overexpressed in cells, whereas TRAF1, TRAF3, and TRAF4 do not (4–9). Despite the apparent redundancy, specific roles for each of the TRAF-family proteins in the signal transduction pathways regulated by particular TNF-family receptors are beginning to emerge from gene knock-out studies in mice, strongly suggesting cell context dependence (10–13).

Another family of receptor proteins utilizing TRAF proteins as signal transduction mediators is the interleukin-1 receptor/Toll family. The interleukin-1 receptor/Toll family of receptors is involved in host defenses against microbial pathogens (14, 15). Among the TRAFs, only TRAF6 has been shown to participate in signal transduction processes mediated by the interleukin-1 receptor/Toll family of receptors (8, 16).

In *Drosophila*, the Toll signaling pathway regulates dorsoventral polarity in the developing embryo (14), promotes larval hematopoiesis (17), and is essential for innate and adaptive immunity (14, 15). Two proteins known as Tube and Pelle mediate signaling by the cytoplasmic tail of Toll. Tube is a unique protein (18), whereas Pelle is a serine/threonine protein kinase homologous to the mammalian kinase IRAK (19). Both proteins contain in their N-terminal region a death domain that is required for interaction between Pelle and Tube (20–22). Upon ligand binding to Toll, Pelle becomes activated and phosphorylates itself, Toll, Tube, and several other substrates. This process culminates in the phosphorylation and subsequent degradation of Cactus, the fly homologue of mammalian IκB, thus allowing Dorsal, the *Drosophila* counterpart of mammalian NFκB, to translocate to the nucleus and regulate gene expression (14, 20).

In this report we have identified a *Drosophila* TRAF protein. We show that this TRAF-family protein is able to bind some mammalian TNFR-family receptors and TRAFs and that it can modulate the NFκB pathway in human cells, suggesting strong structural and functional conservation of TRAFs throughout Metazoan evolution. Also, we show that *Drosophila* TRAF (DTRAF-1) is a likely component of the Toll/Spatzle/Pelle signaling complex, based on its ability to interact with Pelle and to regulate Pelle-mediated NFκB activation.

* This work was supported in part by National Institutes of Health Grants CA-69381 and GM50545 and also by Zaiya Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TNFR, tumor necrosis factor receptor; TRAF, TNF receptor-associated factor; PCR, polymerase chain reaction; LTβR, lymphotoxin-β receptor; NGFR, p75 neurotrophin receptor; GST, glutathione *S*-transferase; IAP, inhibitor of apoptosis; IRAK, interleukin-1 receptor-associated kinase; DR4, death receptor 4.

MATERIALS AND METHODS

Identification and Isolation of cDNAs Encoding DTRAF—A *Drosophila* TRAF homologue (DTRAF1) was identified by sequence comparison searches of the public data bases using the Blast program (NCBI-NIH) and using as template the cDNA encoding the TRAF domain of hTRAF4. Multiple sequence alignments were made using the clustalW program. Exon and splice-site predictions were performed using the gene finder program from Baylor College of Medicine.

DTRAF1 cDNAs were isolated from a *Drosophila* cDNA embryo (0–4 h) pNB40 library by PCR (23). The primers used for the amplification of the full-length DTRAF1 cDNA consisted of a primer containing the SP6 polymerase recognition sequence located upstream of the cloning site in the pNB40 plasmid and the reverse primer 5′-GTACTCGAGCTTAGACGGCCACTATCTTGCT-3′. The PCR fragment was subcloned into pcR-BluntII topo plasmid (Invitrogen). Further PCR amplification for subcloning into the mammalian expression vector pcDNA-3-myc (Invitrogen) was performed using the primer-pair 5′-CCAGGATCCATTATGGGCTCCTTGG-3′ and 5′-ATATCTCGAGTTAGACGGCCACTATCTTGCTGAGATCCAC-3′. The DTRAF1 N-terminal deletion mutants DTRAF1 (Δ1–124) and DTRAF1 (Δ1–226) were also amplified by PCR using the forward primers 5′-ATAGAATTCTCGGCTGACACACTG-3′ and 5′-CCAAGGGATCCATCAACTACACAGGC-3′, respectively, and the reverse primer described above. After heating at 94 °C for 2 min PCR was performed using 35 cycles of 94 °C for 15 s, 58 °C for 20 s, and 72 °C for 100 s, followed by a final cycle of 72 °C for 5 min.

Cells—293T cells were obtained from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle's high glucose medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Hyclone; Logan, UT), 1 mM glutamine, and antibiotics.

Plasmids—Plasmids containing cDNAs encompassing the complete open reading frames of hTRAF1 (pSG5-TRAF1), hTRAF2 (pcDNA3-HA-TRAF2), hTRAF3 (pbluscriptKS-TRAF3b), hTRAF4 (pcDNA3-HA-TRAF4), hTRAF5 (pcDNA3-Flag-TRAF5), and hTRAF6 (pcDNA3-myc-TRAF6) have been previously described (6, 9, 24–28). pGEX-TRAF2(263–501), pGEX-CD40(ct), pGEX-Fas(ct), pGEX-LT-βR(ct), pGEX-DR4(ct), and pGEX-NGFR(ct) have been previously described (9, 24, 29–31). The promoter-containing reporter gene plasmid pUC13–4xNFκB-luc (containing four tandem human immunodeficiency virus-NFκB response elements and the minimal fos promoter) and pCMV-β-galactosidase have been previously described (32, 33).

Yeast Two-hybrid Assay—Yeast two-hybrid assays were performed as described (34) using the EGY48 strain *Saccharomyces cerevisiae* (MATa, trp1, ura3, his, leu::plexApo6-leu2). Cells were grown in either yeast extract/peptone/dextrose medium with 1% yeast extract, 2% polypeptone, and 2% glucose or in Burkholder minimal medium fortified with appropriate amino acids as described previously (35). Transformations were performed using a LiCl method with 1 μg of plasmid DNA and 100 μg of denatured salmon sperm carrier DNA. The DNA binding domain plasmids, pEG202 or pGilda, and the activation domain plasmid, pJG4–5, containing cDNAs encoding Bax, Ras, the cytoplasmic domain of Fas, the cytoplasmic domain of $Toll_{(829–1097)}$, Tube, $Pelle_{(1–501)}$, and the deletion mutants $Pelle_{(1–209)}$ and $Pelle_{(219–501)}$ were previously described (24, 36). The fragment of DTRAF1 cDNA encoding amino acids 125 to 387, DTRAF1 (Δ1–124), was amplified by PCR as described above and subcloned into pGilda, which produces fusion proteins with an N-terminal *LexA* DNA binding domain.

Production and Purification of Recombinant Drosophila TRAF Domain—For GST fusion protein production, pGEX plasmids were transformed into competent XL-1 blue bacteria cells and grown in LB medium. GST-protein production was induced at an A_{600} = 1.0 with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 25 °C. Cells were then recovered and resuspended in phosphate-buffered saline containing 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml lysozyme and lysed by sonication. The GST-DTRAF1 (Δ1–124) protein was purified from bacterial lysates by affinity chromatography using glutathione-Sepharose (Amersham Pharmacia Biotech). The resins were then washed with phosphate-buffered saline containing 1 mM dithiothreitol until the $A_{280\ nm}$ reached <0.01.

Protein Binding Experiments—In vitro GST-protein binding assays were performed as described (9, 35, 37, 38). Briefly, [^{35}S]methionine-labeled TRAF proteins were produced by *in vitro* translation using the transcription/translation-coupled reticulocyte system following the manufacturer's instructions (Promega Inc.). Equal amounts of each labeled protein (2–6 μl of lysate) were diluted with 250 μl of binding buffer (142 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes, pH 7.4, 0.2% Nonidet P-40, 0.5 mM dithiothreitol, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride and a mixture of other protease inhibitors (Roche Molecular Biochemicals)) and incubated with the GST-protein resins (0.5 μg of protein immobilized on 10 μl of glutathione-Sepharose) at 4 °C for 2 h. The resins were then extensively washed with binding buffer, and the GST-protein binding complexes were eluted with buffer containing 50 mM Tris-HCl, pH 8, 1 mM dithiothreitol, and 100 mM glutathione, followed by analysis by SDS-polyacrylamide gel electrophoresis and fluorography.

Reporter Gene Assays—For NFκB reporter gene assays, 293T cells were transfected using a calcium phosphate method and a total of 12 μg of plasmid DNA (including 0.5 μg of pUC13–4xNFκB-luc plasmid and 1 μg of pCMV-β-galactosidase plasmid) at ≈50% confluency in 6-well plates in duplicate. After 36 h, cells were lysed with 0.5 ml of Promega lysis buffer. The luciferase activity of 10 μl of each cell lysate was determined using the luciferase assay system from Promega following the manufacturer's protocol and measured using a luminometer (EG&G Berthold). Luciferase activity was normalized relative to β-galactosidase activity (32).

RESULTS AND DISCUSSION

Identification of a TRAF Protein from Drosophila Embryos—A *Drosophila* gene encoding a protein homologous to the mammalian TRAF family was identified by searching the public data bases. The full-length *Drosophila* TRAF (DTRAF) cDNA was isolated from a cDNA library prepared from 0–4-h *Drosophila* embryos (23). This DTRAF cDNA was contained within a unique exon located in the *Drosophila* chromosome 2L (24F1–24F2) and encoded a predicted protein of 387 amino acids in length. The AUG that initiates this open reading frame is present within a favorable context for translation (39). DTRAF contains seven zinc finger domains in its N-terminal region, followed by a TRAF domain located near the C terminus of the molecule. Thus, the domain topology of DTRAF is similar to other previously identified mammalian TRAF proteins (Fig. 1). In contrast to many TRAFs, however, DTRAF does not contain a RING finger domain, and no evidence of a coiled-coil region upstream of the TRAF domain was found. Recently, a fly TRAF homologue was isolated based on its ability to interact with the Ste20 kinase Msn in a yeast two-hybrid screen (40). This *Drosophila* TRAF appears to be the same protein as ours, except that it contains an additional 99 amino acids located at the N terminus of the protein, suggesting that our protein and DTRAF1 arise from a common gene.

Using computer data base searches, we have also identified TRAF proteins in *C. elegans* and in *Arabidopsis* (Fig. 2). The *C. elegans* TRAF contains a RING finger domain and five zinc fingers domains at the N terminus of the protein in addition to the TRAF domain at the C terminus. The *Arabidopsis* TRAF protein described here is an example of a family of hypothetical proteins containing TRAF domains found in *Arabidopsis thaliana*. Contrary to the other known TRAF proteins, the TRAF domain is located in the N terminus of this protein. The *Arabidopsis* TRAF protein does not contain either RING or zinc finger domains. A schematic representation of these new TRAF proteins is presented in Fig. 2 along with sequence alignments of the TRAF domains of the human and nonmammalian TRAF proteins. The predicted amino acid sequence of DTRAF1 is most similar to TRAF4 (47% identity) among the TRAFs (Fig. 2*A*), both when considering only the homology within the TRAF domain and when evaluating the entire molecule. The predicted amino acid sequence *C. elegans* TRAF shares similar extents of similarity with TRAF3, TRAF4, and TRAF5 (36 to 37% identity). *Arabidopsis* TRAF was most similar to TRAF3 (25% identity). A phylogenetic-tree analysis illustrating the theoretical evolutionary distances of these TRAF proteins is shown in Fig. 2*C*.

The sequences corresponding to the TRAF domains of DTRAF1, *C. elegans* TRAF, *Arabidopsis* TRAF, and *Dictyostelium* TRAF (41) were threaded onto the recently published x-ray crystal structure of human TRAF2 (42, 43). As shown in

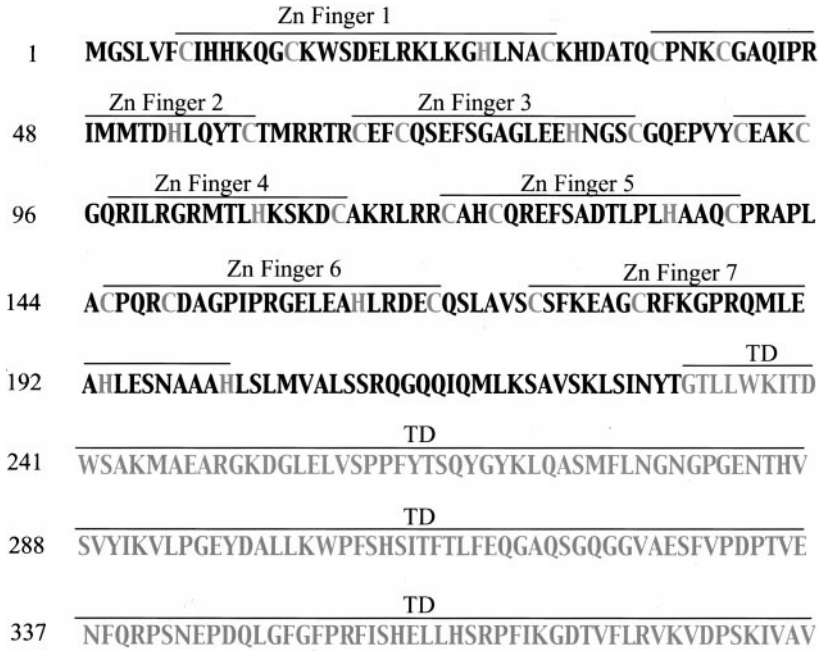

FIG. 1. **Amino acid sequence of DTRAF1.** The predicted amino acid sequence of DTRAF1 is presented. The residues composing the seven zinc finger domains and the TRAF domain are *underlined*.

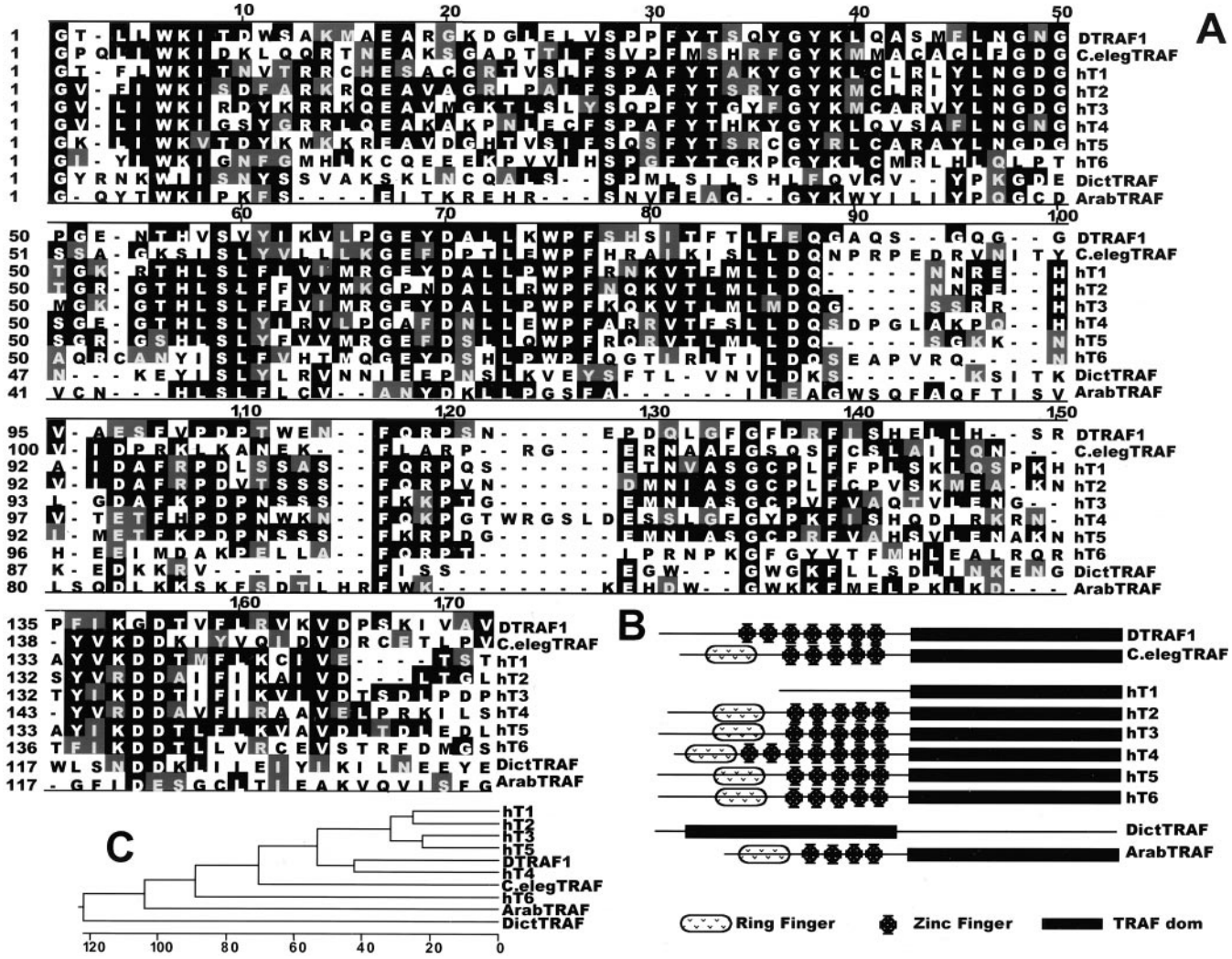

FIG. 2. **Comparison of DTRAF1 with other TRAFs.** *A*, amino acid sequences and alignments have presented for the TRAF domains of the six human TRAF proteins, DTRAF1, *C. elegans* TRAF, *Arabidopsis* TRAF, and *Dictyostelium* (DG17) TRAF (41). *Black boxes* indicate shared residues between family members. *Gray boxes* indicate conservative amino acid replacements. Alignments were made using the clustalW program. *B*, schematic representation of the different protein domains of the nonmammalian TRAF proteins and the human TRAFs. *C*, schematic representation of the relative phylogenetic distances of the TRAF domains.

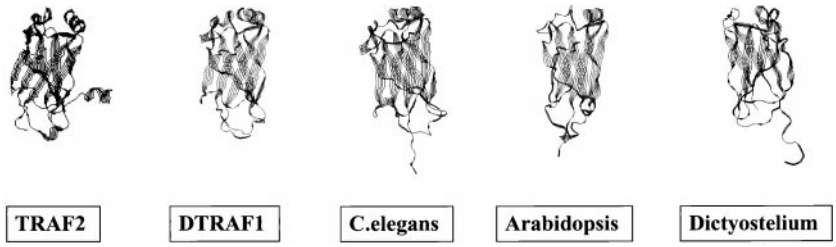

FIG. 3. **Models of TRAF domains.** Computer-generated models of TRAF domains are presented for human TRAF2, DTRAF1, *C. elegans*, *Arabidopsis*, and *Dictyostelium*.

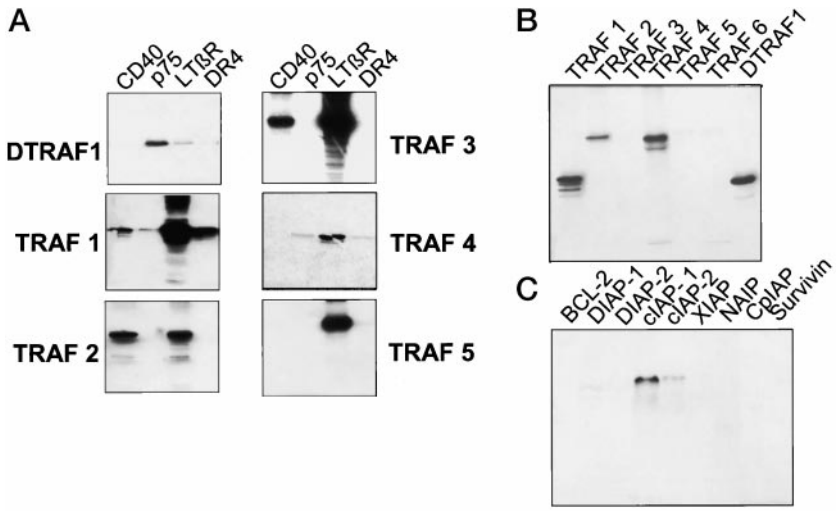

FIG. 4. **Analysis of DTRAF interactions with TNFR, TRAF, and IAP-family proteins.** *In vitro* protein binding assays were performed. Various proteins were *in vitro* translated in the presence of L-[^{35}S]methionine in reticulocyte lysates, and then equal volumes (10 μl) were incubated with GST fusion proteins (1 μg) immobilized on glutathione-Sepharose. Control GST and other GST control proteins were included in all assays, although results are not always presented. After washing, bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. *A*, the interaction of vitro translated ^{35}S-labeled DTRAF1 (Δ1–124) with GST fusion proteins containing the cytoplasmic domains of CD40, p75NGFR, LTβR, and DR4 was tested. *B*, the interactions of *in vitro* translated ^{35}S-labeled human TRAFs and DTRAF1 (Δ1–124) with a GST fusion protein containing the DTRAF1 fragment 125–387 were tested. *C*, interactions of ^{35}S-labeled IAP-family proteins with a GST fusion protein containing the DTRAF1 fragment 125–387 were tested.

Fig. 3, all of these TRAFs from lower organisms adopted the same overall fold as the TRAF domain of human TRAF2.

Analysis of the Binding Properties of DTRAF1—To investigate whether DTRAF1 can interact with the cytosolic domains of various members of the TNFR family, GST fusion proteins containing the cytoplasmic domains of CD40, p75NGFR, LTβR, and DR4 were tested for their ability to bind an *in vitro* translated ^{35}S-labeled DTRAF1 (Δ1–124) fragment that included the TRAF domain (Fig. 4*A*). DTRAF1 (Δ1–124) specifically bound to the cytosolic tail (ct) of p75NGFR and more weakly to LTβR(ct). This pattern of binding is similar to that of TRAF4, which also binds specifically to these two members of the TNFR family *in vitro* (26). This observation is consistent with the more extensive sequence similarity of DTRAF1 with TRAF4.

Next, we studied the ability of the GST-DTRAF1 (Δ1–124) fusion protein to interact with the six known human TRAFs and to itself (Fig. 4*B*). DTRAF1 bound to TRAF1, TRAF2, and TRAF4, whereas no binding was detected to TRAF3, TRAF5, or TRAF6. Also, DTRAF was able to self-associate, similar to other TRAF family proteins (42–45).

Another type of protein that has been shown to interact with the TRAF domain of TRAF2 is IAP-family proteins, specifically cIAP-1 and cIAP-2 (44, 46). IAP family proteins are involved in regulating cell death by direct inhibition of caspases (47). Two members of this family have been identified in *Drosophila* (48). Using the same approach described above, we investigated the ability of the TRAF domain of DTRAF1 to interact with all known human members of the IAP family as well as baculovirus CpIAP and the two *Drosophila* IAPs, DIAP-1 and DIAP-2 (Fig. 4*C*). DTRAF1 (Δ1–124) interacted with human cIAP-1 and weakly with cIAP-2. In contrast, none of the other IAPs tested, including DIAP-1 and DIAP-2, bound to DTRAF1. Thus, DTRAF1 may not be a regulator of IAPs in *Drosophila*, although it evidently shares structural features in common with TRAF2 that permit binding to cIAP-1 and cIAP-2.

DTRAF1 Regulates NFκB Activity in a Mammalian Cell Line—Mammalian TRAF2, TRAF5, and TRAF6 induce NF-kB activation when overexpressed in cells. To investigate if DTRAF1 has a role in regulating NFκB activity, we overexpressed DTRAF1 and the deletion mutants DTRAF1 (Δ1–124) and DTRAF1 (Δ1–226) in human epithelial 293T cells, together with a NFκB-responsive reporter plasmid. None of these proteins induced significant NFκB activity in this cell line (Fig. 5*A*). However, when DTRAF1 was co-transfected with TRAF6, a clear increase in NFκB activity was detected as compared with the levels of NFκB activity generated by TRAF6 alone. In contrast, co-transfection of TRAF6 with the DTRAF1 deletion mutants dramatically reduced TRAF6-mediated NFκB (Fig. 5*A*). Interestingly, co-expression of TRAF2 with either full-length DTRAF1 or the deletion mutants resulted in an inhibition of the TRAF2-mediated NFκB induction (Fig. 5*A*). The levels of expression of these proteins in 293T cells after transfection were examined by immunoblotting, revealing that DTRAF(Δ1–124) did not alter the levels of TRAF2 or TRAF6 (Fig. 5*B*). Since DTRAF1 binds TRAF2 but not TRAF6 *in vitro*, these data suggest that full-length DTRAF1 may interfere with TRAF2 function through direct interaction. In contrast, the effects of DTRAF1 on TRAF6 are presumably indirect, perhaps reflecting an interaction of DTRAF1 with signal transducing proteins downstream of TRAF6.

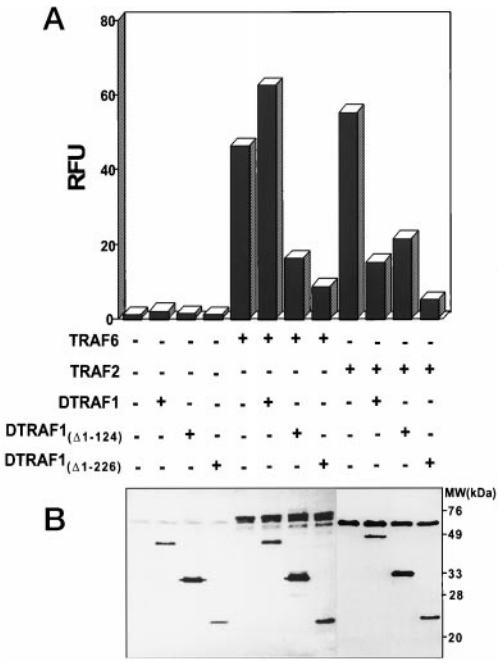

FIG. 5. **DTRAF can regulate the NFκB activity induced by TRAF2 and TRAF6 in a mammalian cell line.** *A*, 293T cells were transfected in 6-well dishes with ≈10 μg of pcDNA3 control plasmid, 3.5 μg of either pcDNA3-myc-hTRAF6 or pcDNA3-hTRAF2, or 7 μg of either pcDNA3-myc-DTRAF1, DTRAF1 (Δ1–124), and DTRAF1 (Δ1–226) together with 0.5 μg of pUC13–4xNFκB-luc plasmid and 1 μg of pCMV-β-galactosidase plasmid, as indicated. Relative NFκB activity was assessed using 10 μl of each of the cell lysates prepared 36 h after transfection by luciferase assays, with normalization for β-galactosidase activity. Three independent experiments were performed. The results are presented as relative fluorescence units (*RFU*) (representative data). *B*, protein expression levels of TRAF6, TRAF 2, and myc-tagged DTRAF1, DTRAF1 (Δ1–124), and DTRAF1 (Δ1–226) were assessed by analyzing 5 μl of each cell lysate by immunoblotting using anti-TRAF6, anti-TRAF2, or anti-myc antibodies, followed by ECL-based detection.

TABLE I
Specific interaction of DTRAF1 with Pelle

Yeast strain EGY48 was transformed with yeast expression plasmids encoding various proteins expressed as fusions with either an N-terminal *LexA* DNA binding domain (left column) or B42 transactivation domain (right column). Transformants were scored for activation of *LEU2* and *lacZ* reporter genes under the control of *LexA* operators, as described (34, 52). Plasmid combinations that resulted in growth on leucine-deficient media within 4 days were scored as positive (+). β-Galactosidase activity of each colony was tested by filter assay and scored as blue (+) or white (−) after 60 min. The DTRAF1 plasmid used here encodes residues 125–387.

Lex A	B42	−Leu	β-Gal
DTRAF1	$Pelle_{(1–501)}$	+	+
DTRAF1	Tube	−	−
DTRAF1	Bax	−	−
DTRAF1	Ras	−	−
DTRAF1	$Pelle_{(1–209)}$	+	+
DTRAF1	$Pelle_{(210–501)}$	−	−
Fas	$Pelle_{(1–209)}$	−	−
$Pelle_{(1–209)}$	DTRAF1	+	+
$Pelle_{(1–209)}$	Tube	+	+
$Pelle_{(1–209)}$	Bax	−	−
$Pelle_{(210–501)}$	DTRAF1	−	−
Toll	DTRAF1	−	−
Fas	DTRAF1	−	−
Bax	DTRAF1	−	−

DTRAF1 Binds to and Regulates Pelle—Although TRAF2, TRAF5, and TRAF6 have been shown to activate NFκB via the kinases NFκB-inducing kinase and receptor interacting protein (49–51), TRAF6 has also been shown to regulate NFκB through the kinase IRAK (8). IRAK is the human counterpart

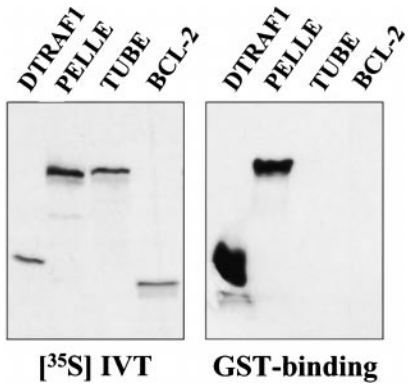

FIG. 6. **DTRAF interacts with Pelle *in vitro*.** *In vitro* binding analyses were performed using GST-DTRAF1 (Δ1–124) fusion protein and [35S]methionine-labeled-DTRAF1 (Δ1–124), Pelle, Tube, and Bcl-2. The *in vitro* translated (*IVT*) proteins are shown in the *left panel*. The analysis of the GST-bound proteins is shown in the *right panel*.

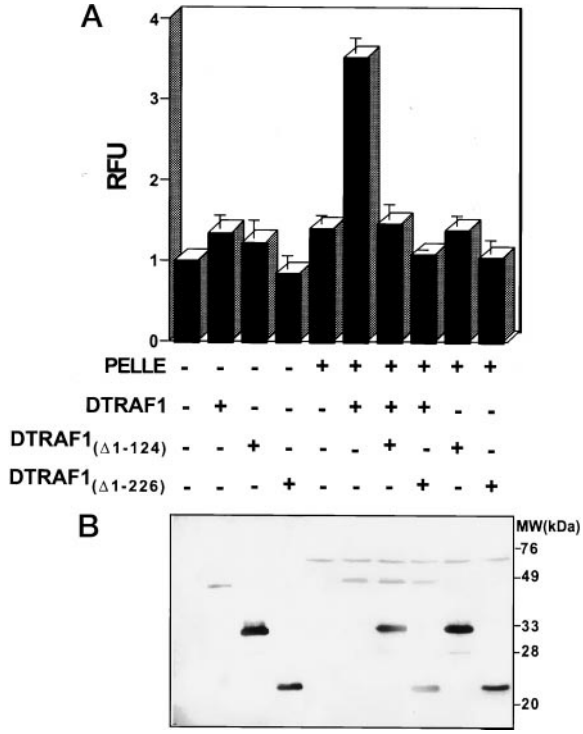

FIG. 7. **DTRAF increases the NFκB activity induced by Pelle in a mammalian cell line.** 293T cells were transfected in 6-well dishes with ≈10 μg of pcDNA3 control plasmid, 2.5 μg of pcDNA3-myc-Pelle or pcDNA3-myc-DTRAF1, and 2.5 or 5 μg of pcDNA3-myc DTRAF1 (Δ1–124) and DTRAF1 (Δ1–226), together with 0.5 μg of pUC13–4xNFκB-luc plasmid and 1 μg of pCMV-β-galactosidase plasmid, as indicated. Relative NFκB activity was assessed using 10 μl of each of cell lysates prepared 36 h after transfection by luciferase assays, with normalization for β-galactosidase activity. The results are presented as fold activation relative to control ($n = 3$). Statistical significance was assessed by unpaired, independent t test. *B*, levels of myc-tagged Pelle, DTRAF1, DTRAF1 (Δ1–124), and DTRAF1 (Δ1–226) were assessed by SDS-polyacrylamide gel electrophoresis/immunoblot assay using 5 μl of cell lysate derived from transfected 293T cells and employing anti-myc antibodies for immunodetection.

of the *Drosophila* kinase Pelle and mediates signal transduction by the Toll/interleukin 1 receptor family. In *Drosophila*, the transmembrane receptor signaling by Toll is mediated by two proteins, Tube and Pelle, the latter representing the *Drosophila* counterpart of mammalian IRAK kinase (36). To determine if the TRAF domain of DTRAF1 can interact with components of the *Drosophila* Toll signal transduction pathway, we performed yeast two-hybrid assays (Table I). These two-hybrid

experiments revealed a strong interaction of DTRAF1 (Δ1–124) with Pelle but not with Tube or Toll. The interaction of DTRAF1 (Δ1–124) with Pelle was mediated by the N-terminal regulatory domain of Pelle, which contains a death Domain (21), and not by the C-terminal protein kinase domain (Table I), similar to the interaction previously described for Pelle and Tube (Table I) (21, 36). The interaction of DTRAF1 with Pelle was further demonstrated by *in vitro* binding assays (Fig. 6). *In vitro* translated Pelle specifically bound to a GST-DTRAF1 (Δ1–124) fusion protein, whereas Tube did not.

Next, we explored whether DTRAF1 can regulate NFκB induction by Pelle. When expressed in HEK293T cells, neither Pelle nor any of the DTRAF1 constructs induced an increase in NFκB activity (Fig. 7). However, when Pelle and DTRAF1 were co-transfected, a clear increase in NFκB activity was detected. This induction of NFκB was not observed when Pelle was co-transfected with DTRAF1 (Δ1–124) or DTRAF1 (Δ1–236), suggesting an important role for the zinc finger domains of DTRAF1 in this co-stimulatory effect. Moreover, both DTRAF1 (Δ1–124) and DTRAF1 (Δ1–236) were able to abrogate the induction of NFκB activity mediated by co-transfection of Pelle and DTRAF1, suggesting that these two DTRAF1 deletion mutants function in a dominant negative fashion (Fig. 7). The induction of NFκB activity in 293T cells by the combination of DTRAF1 and Pelle was specific in that reporter genes containing other promoters (*e.g.* p53-responsive; retinoid-responsive; androgen-responsive) were not affected (not shown).

Altogether these results demonstrate that DTRAF1 is able to regulate NFκB activation in collaboration with Pelle, suggesting that DTRAF1 is a component of the Toll pathway in *Drosophila*. This pathway has been implicated in the regulation of the dorsal-ventral polarization of developing embryos. However, microinjection of DTRAF1 (Δ1–124) mRNA into *Drosophila* embryos failed to affect the normal dorsal-ventral patterning (not shown), suggesting that DTRAF1 may not be required for this developmental process. This observation, together with the recent characterization of another member of the TRAF family in *Drosophila* (40) implies that flies may contain other TRAF-family genes that create redundancy in the pathways available for NFκB induction, similar to the situation with mammalian TRAFs (9). A role for DTRAF1 in innate immune responses to pathogens in flies, however, remains to be explored.

Conclusions—In summary, we have identified cDNA sequences encoding TRAF domain proteins in *Drosophila*, *C. elegans*, *Arabidopsis*, and *Dictyostelium*. The *Drosophila* TRAF homologue exhibits a variety of properties commonly observed in TRAF-family proteins of mammals including an ability to (*a*) associate with other TRAFs, (*b*) bind TNFR- and IAP-family proteins, (*c*) interact with IRAK-like kinases, and (*d*) modulate NF-kB activity. These findings suggest strong evolutionary conservation of the structure and function of vertebrate and invertebrate TRAFs. The finding of TRAF-like proteins in mammalians, invertebrates, plants, and slime mold suggests that TRAF domains represent an ancient component of the innate immune response.

Acknowledgments—We thank C. Ware, D. Bredesen, and E. Alnemri for plasmids, C. Fux, S. Fuess, and S. Johnson for technical assistance, and R. Cornell for manuscript preparation.

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