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# T3JAM, a novel protein that specifically interacts with TRAF3 and promotes the activation of JNK[1]

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**Abstract Previous studies suggest that localization of tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family members is important for regulating their signal transduction. During a screen for TRAF3-associated proteins that potentially alter TRAF3 subcellular localization and enable signal transduction, we identified a novel protein, T3JAM (TRAF3-interacting Jun N-terminal kinase (JNK)-activating modulator). This protein associates specifically with TRAF3 but not other TRAF family members. Coexpression of T3JAM with TRAF3 recruits TRAF3 to the detergent-insoluble fraction. More importantly, T3JAM and TRAF3 synergistically activate JNK but not nuclear factor (NF)-κB. Our studies indicate that T3JAM may function as an adapter molecule that specifically regulates TRAF3-mediated JNK activation.**


*Key words:* Tumor necrosis factor receptor-associated factor 3; Tumor necrosis factor receptor; Jun N-terminal kinase; Nuclear factor κB; Subcellular localization; Signal transduction

## 1. Introduction

Tumor necrosis factor receptor-associated factors (TRAFs), are a family of adapters that mediate the activation of signaling pathways downstream of tumor necrosis factor receptors (TNFRs) [1]. Among six known TRAF family members, TRAF3 has been shown to interact with many TNFR superfamily members including CD40, LT-βR and BR3 [2–5]. Studies have shown that TRAF3 may negatively regulate Jun N-terminal kinase (JNK) and nuclear factor (NF)-κB signaling [5–7]. However, TRAF3 also promotes NF-κB activation in airway epithelial cells and may be able to activate p38 and promote LT-βR-mediated death signaling [6,8,9]. These findings suggest the presence of an intricate regulatory mechanism that modulates the functional role of TRAF3.

When overexpressed, TRAF2, 5 and 6 activate the JNK and NF-κB signaling pathways, possibly in membrane-localized complexes [10,11]. TRAF3, on the other hand, is cytosolic and is unable to activate JNK or NF-κB when overexpressed, suggesting that recruitment to membrane-bound complexes may be important for the activation of these pathways. We have shown that artificial localization of TRAF3 to the membrane enables TRAF3 to activate JNK but not NF-κB [10]. In the cell, this may be mediated through interactions between TRAF3 and other proteins.

Although multiple TRAF3-interacting proteins, including p62 nucleoporin, the microtubule network-associated molecule MIP-T3 and Act1, have been identified [12–14], the functional relevance of these interactions in TRAF3-mediated signaling is unclear. Here we report discovery of a novel protein, T3JAM (TRAF3-interacting JNK-activating modulator). T3JAM specifically interacts with TRAF3 and alters the subcellular localization of TRAF3 while promoting the specific activation of JNK signaling. To our knowledge this is the first report describing a TRAF3-interacting protein that regulates the specific activation of the JNK pathway.

## 2. Materials and methods

### 2.1. Yeast two-hybrid screening

Yeast two-hybrid screening was performed as previously described [15]. Full-length coding sequence for TRAF3 was used as bait to screen a lambda phage mouse T cell library, kindly provided by Dr. S. Elledge [16]. DNA was prepared from 40 positive clones and transformed into *Escherichia coli* strain Topp10 and examined by restriction analysis and sequencing.

### 2.2. Antibody production

A clone coding for amino acids 300–513 of T3JAM was subcloned into the *Bam*HI/*Xho*I sites of pGEX1λT and transformed into the Topp10 strain of *E. coli*. Recombinant glutathione S-transferase (GST)-T3JAM 300–513 was purified using standard GST purification methods and the purified protein was sent to ProSci Incorporated for antibody production in rabbits.

### 2.3. Plasmid construction

To obtain a full-length clone of T3JAM, we used the fragment from the yeast two-hybrid screen (coding for amino acids 300–513) to probe a murine T cell library using standard methods. Both strands of the full-length T3JAM clone were sequenced, and the sequence deposited into GenBank under accession number AY383616. Full-length and truncated forms of T3JAM were amplified by polymerase chain reaction (PCR) and subcloned into the *Bam*HI/*Xho*I sites of pCMVmyc. To make an in-frame GST fusion protein, T3JAM 300–513 was subcloned into the *Bam*HI/*Sal*I sites of pEBG. The TRAF3/TRAF5 do-

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[1] Nucleotide sequence data reported are available in the DDB/EMBL/GenBank databases under accession number AY383616.
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main-swapping plasmids and pEBB-HA-TRAF2, 3, 5 and 6 plasmids were constructed as previously described [10,11].

### 2.4. mRNA quantification

RNA was isolated by standard guanidium isothiocyanate methods. The cDNA template for quantitative real-time PCR analysis was then synthesized and PCR was performed using the iCycler thermocycler (Bio-Rad) as previously described [17,18]. Levels of the ribosomal protein L32 were used to normalize expression levels of T3JAM in different tissues. The primers used to amplify T3JAM are as follows: T3JAM forward, ggagacatttcagctccaagtca; T3JAM reverse, tgtagcaatcaccaccatcagc.

### 2.5. Fractionation, immunoprecipitation and Western blotting

For fractionation studies, cell transfection, lysis and fractionation were performed as described previously [10]. Equivalent amounts of cytoplasmic and pellet samples were prepared for Western blot analysis of TRAF distribution. For immunoprecipitation experiments, cells were harvested and lysed in a Triton lysis buffer consisting of 20 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA), 1% Triton X-100 and 10% glycerol plus a protease inhibitor cocktail. An equal proportion of each sample was removed prior to immunoprecipitation to analyze expression levels of transfected constructs. Immunoprecipitation was performed using rabbit anti-GST-T3JAM antibody (ProSci Incorporated), and samples were analyzed by Western blot.

For analysis of endogenous murine T3JAM expression levels, bone marrow, heart, kidney, liver, spleen and thymus were solubilized in radioimmunoprecipitation assay (RIPA) buffer by sonication, and 30 μg of protein from each organ was analyzed by Western blot using rabbit anti-GST-T3JAM 300–513 antibodies.

Antibodies used in this manuscript include rabbit anti-GST-T3JAM 300–513 antibodies (ProSci Incorporated), the 12CA5 mouse anti-HA antibody, the 9E10 mouse anti-myc antibody and mouse anti-GST antibodies (Santa Cruz Biotechnologies).

### 2.6. In vitro kinase assay and gel shift analysis

JNK activity was measured as previously described [10]. Nuclear extract isolation and electrophoretic mobility shift assays were performed as described previously for NF-κB [19].

## 3. Results

### 3.1. Identification of T3JAM, a novel TRAF3-interacting protein

To search for proteins that can recruit TRAF3 to the membrane fraction and facilitate TRAF3-mediated signaling, we conducted a yeast two-hybrid screen using full-length murine TRAF3 as bait. From this screen many known TRAF3-interacting proteins were identified, including TANK and p62 nucleoporin. We also obtained numerous overlapping clones representing a novel gene that we called T3JAM. To isolate the full-length T3JAM sequence, we screened a murine T cell cDNA library with the longest fragment from the yeast two-hybrid screen (encoding 214 amino acids, T3JAM 300–513) and recovered an insert of 2094 bases containing a single open reading frame coding for a protein of 513 amino acids (Fig. 1A).

The University of California, Santa Cruz Genome Bioinformatics Database (http://genome.ucsc.edu/) localized the T3JAM sequence to chromosome 1 in the mouse and its genomic structure is predicted to contain 14 exons. T3JAM was

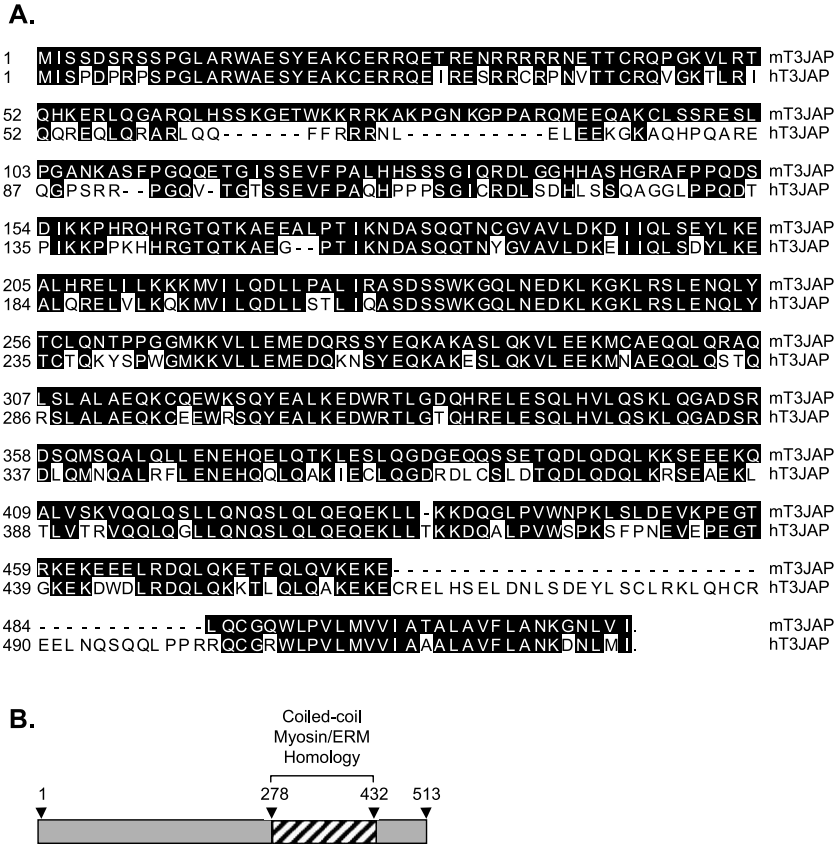


Fig. 1. T3JAM protein structural features. An alignment of the human and murine amino acid sequences of T3JAP are shown in A. Residues identical to murine sequence are highlighted in black, missing residues are indicated by dashes and stop codons by periods. B: Graphical representation of murine T3JAM protein structural features. Gray represents sequence with no significant homology to known conserved protein motifs and the putative coiled-coil domain is indicated by diagonal lines.

found to have a single human homolog (represented by DNA sequence from PAC 434O14; GenBank accession numbers AL049667, AK022798) on chromosome 1q32.3.−41. The human gene codes for a product of 531 amino acids in length. Murine and human T3JAM are 82% identical at the nucleotide level, and have 66% amino acid identity and 73% amino acid similarity (Fig. 1A). While the N-terminal half of T3JAM contains no known conserved protein motifs, the C-terminal half of T3JAM was found to contain a putative coiled-coil domain homologous to the myosin tail heptad repeat and the ezrin/radixin/moesin (ERM) family domain (Fig. 1B). Both of these domains mediate protein–protein interactions, suggesting a possible mechanism for T3JAM interaction with TRAF3 [20,21].

### 3.2. T3JAM is expressed in a tissue-specific manner

To determine the tissue expression pattern of T3JAM in the mouse we used quantitative real-time PCR and Western blotting techniques. We found that T3JAM was preferentially expressed in the bone marrow, spleen and thymus and not present in the heart, kidney or liver (Fig. 2A and B). Interestingly, in addition to the band representing full-length T3JAM protein (62 kDa), we observed bands of other sizes indicating that T3JAM may be modified posttranslationally or its transcript may be alternatively spliced.

### 3.3. T3JAM interacts specifically with TRAF3

To determine if T3JAM specifically interacts with TRAF3 we coexpressed GST-tagged T3JAM with various HA-tagged TRAF molecules and performed coimmunoprecipitation experiments. In fractionation experiments, we found that T3JAM is relatively detergent-insoluble and truncation mutants showed that the N-terminal 300 amino acids of T3JAM are responsible for localizing of T3JAM to the detergent-insoluble fraction (data not shown). Because of this, it

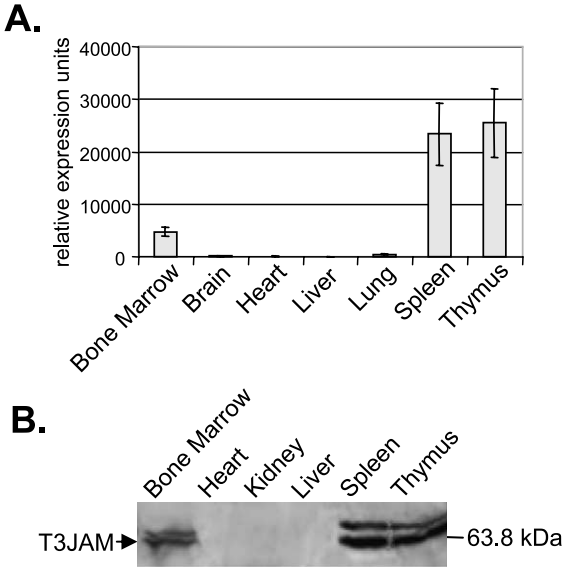


Fig. 2. Tissue-specific expression of T3JAM. A: Total RNA was extracted from the indicated organs of C57B/6 mice and used to make cDNA template. T3JAM expression was then assayed by quantitative real-time PCR. Samples were run in triplicate and data are represented as relative expression units. B: RIPA extracts (30 μg) from indicated organs were analyzed by Western blot using anti-GST/T3JAM 300–513.

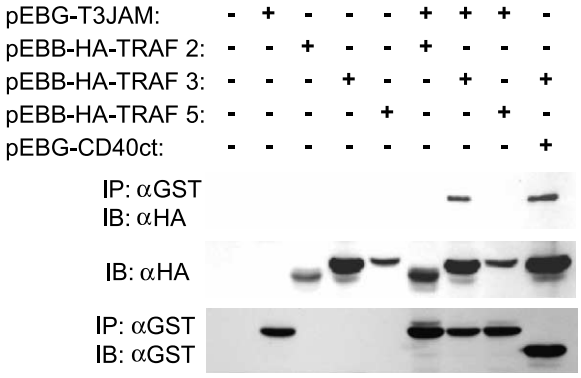


Fig. 3. T3JAM specifically associates with TRAF3. Indicated constructs were transfected into 293T cells using the calcium phosphate method. GST-tagged T3JAM 300–513 was immunoprecipitated using rabbit polyclonal anti-GST antibodies and interaction with HA-tagged TRAF2, 3, or 5 was assayed via Western blotting techniques using the 12CA5 antibody. The data shown are representative of four independent experiments.

was necessary to use a soluble truncation mutant of T3JAM coding for amino acids 300–513 (T3JAM 300–513) for all immunoprecipitation studies. As a positive control, TRAF3 was cotransfected with a construct coding for a GST-tagged version of the CD40 cytoplasmic tail (pEBG-CD40ct). Fig. 3 demonstrates that TRAF3 strongly interacts with both the CD40 cytoplasmic tail and with T3JAM. In contrast, neither TRAF2 nor TRAF5 were able to associate with T3JAM. In

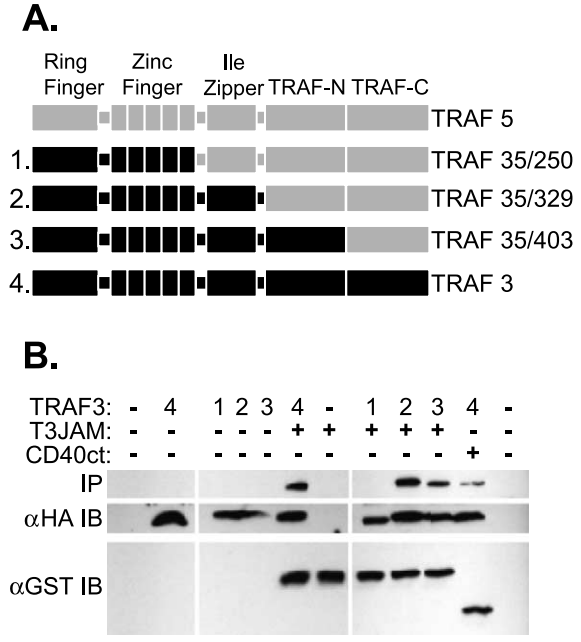


Fig. 4. Mapping of TRAF3 and T3JAM interaction domains. Coimmunoprecipitation experiments were carried out using HA-tagged TRAF3/TRAF5 domain-swapping constructs depicted in A. Indicated constructs were coexpressed with GST-tagged T3JAM 300–513 in 293T cells and immune complexes were precipitated (IP) using rabbit anti-GST-T3JAM antibodies, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted (IB) with either 12CA5 mouse anti-HA or mouse anti-GST antibodies (B). The data shown are representative of two independent experiments.

addition, we found that T3JAM was unable to interact with TRAF6 (data not shown).

### 3.4. The coiled-coil domain of T3JAM mediates interaction with the isoleucine zipper domain of TRAF3

Our yeast two-hybrid results showed that the C-terminal coiled-coil-containing region of T3JAM (amino acids 300–513) is sufficient to mediate interaction with TRAF3. We performed coimmunoprecipitation experiments with T3JAM 300–513 and various mutants of TRAF3 to determine which region of TRAF3 mediated interaction with T3JAM. Since truncation mutants may adversely affect the structural integrity or solubility of TRAF3, we used domain-swapping constructs in which various regions of TRAF3 were replaced with the homologous regions of TRAF5 [11]. As described above, TRAF5 is unable to interact with T3JAM and thus any interaction between T3JAM and one of these chimeric molecules would presumably involve the portion of the mutant contributed by TRAF3.

Constructs encoding either HA-tagged full-length TRAF3 or domain-swapping mutants in which various domains of TRAF3 were replaced with those of TRAF5 (Fig. 4A) were cotransfected with GST-tagged T3JAM 300–513. As seen in Fig. 4B, full-length TRAF3 robustly interacts with T3JAM. However, the construct that contains only the N-terminal zinc-binding domain of TRAF3 (TRAF 35/250) is unable to interact with T3JAM. Addition of the TRAF3 sequence coding for the isoleucine zipper (TRAF 35/329) allows for strong association with T3JAM. Moreover, the protein containing all TRAF3 sequences except the TRAF-C domain (TRAF 35/403) also interacts with T3JAM. These results indicate that the isoleucine zipper domain of TRAF3 mediates interaction with the coiled-coil domain of T3JAM.

### 3.5. T3JAM recruits TRAF3 to the detergent-insoluble fraction

We had previously found that TRAF3 was the most soluble member of the TRAF family, and its low presence in the insoluble fraction correlated with a poor ability to activate the JNK [10]. As T3JAM is an insoluble, TRAF3-interacting protein, we hypothesized that it may be able to recruit TRAF3 to the insoluble fraction. In Fig. 5 we examined the solubility profiles of T3JAM and TRAF3 either alone or in combination and found that T3JAM did indeed recruit a significant amount of TRAF3 protein to the pellet fraction.

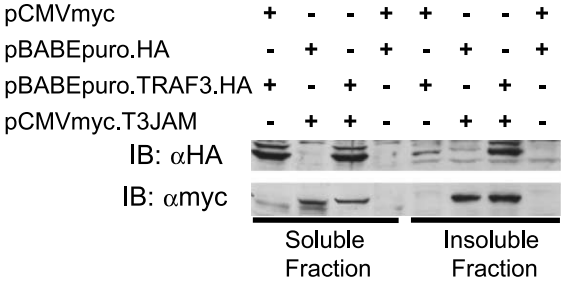


Fig. 5. T3JAM alters TRAF3 localization to the insoluble fraction of cell lysates. 293T cells were transfected with the indicated plasmids. Cells were harvested as indicated in Section 2 and an equal volume of each fraction was analyzed using Western blotting techniques. Blots were probed with either the 12CA5 anti-HA antibody or the 9E10 anti-myc antibody. The data shown are representative of three independent experiments.

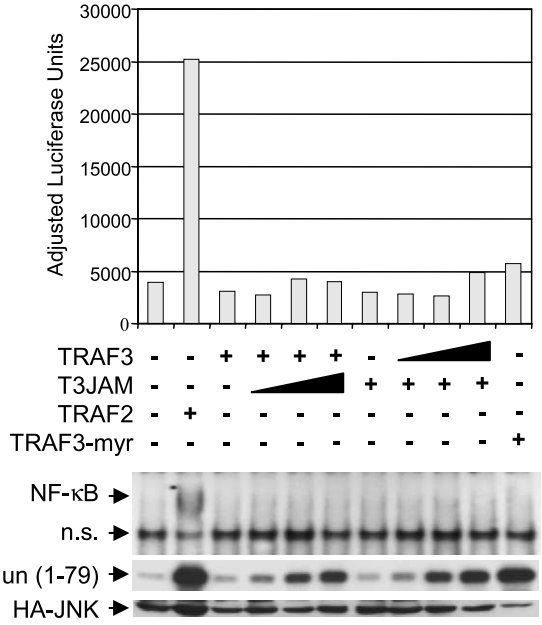


Fig. 6. T3JAM and TRAF3 cooperate to activate JNK signaling. Indicated constructs were transfected into 293T cells using the calcium phosphate technique. Nuclear and cytoplasmic fractions were isolated and used to test for NF-κB or JNK activation. NF-κB activity was tested by luciferase reporter assay (top panel) and electrophoretic mobility shift assay (EMSA) (top middle panel). JNK activity was examined by in vitro kinase assay from the same cell extracts (bottom middle panel). Equivalent expression levels of HA-JNK were determined by Western blotting using the 12CA5 antibody (bottom panel). The data shown are representative of three independent experiments.

While T3JAM consistently recruited TRAF3 to the insoluble fraction in numerous experiments, we found that the degree of recruitment was dependent upon the ratio of expression levels of TRAF3 and T3JAM (data not shown). Thus, the stoichiometry of TRAF3 and T3JAM may be an important factor in the regulation of TRAF3.

### 3.6. Coexpression of T3JAM and TRAF3 causes the activation of JNK but not NF-κB

We had previously observed a link between TRAF insolubility and JNK activation [10]. In addition, preliminary functional studies indicated that overexpressed T3JAM on its own led to weak JNK activation and not of the type 1 NF-κB pathway (data not shown). These observations led us to examine the effect of combined TRAF3 and T3JAM expression on JNK activation. As shown in Fig. 6, overexpression of either TRAF3 or T3JAM alone in 293T cells does not lead to significant JNK activation. However, coexpression of T3JAM and TRAF3 strongly activates JNK, suggesting that these two molecules have a synergistic effect.

In the same cellular extracts, we simultaneously examined the activation of the type 1 NF-κB pathway through a luciferase reporter assay as well as through electrophoretic mobility shift assays using an NF-κB-specific oligonucleotide probe (Fig. 6). In both cases, T3JAM/TRAF3 coexpression appears to have no effect on the type 1 NF-κB pathway. These studies demonstrate that the synergistic effect between T3JAM and TRAF3 is specific for the activation of JNK and not NF-κB. Thus, T3JAM may be a specific regulator for TRAF3-mediated JNK activation.

## 4. Discussion

While TRAF2, 5 and 6 all potently activate signal transduction downstream of TNFRs, the role of TRAF3 in promoting signaling events has been enigmatic. In this study, we have identified a novel TRAF3-interacting protein that specifically interacts with the isoleucine zipper of TRAF3 via its coiled-coil domain. This interaction appears to recruit TRAF3 to an as yet uncharacterized detergent-insoluble complex where T3JAM cooperates with TRAF3 to specifically activate the JNK pathway. This scenario is consistent with our previous results which showed that forced localization of TRAF3 to the cell membrane allowed for activation of the JNK pathway but not the NF-κB pathway [10]. In addition, TRAF3 has recently been shown to associate with a number of detergent-insoluble subcellular microdomains including the cytoskeleton and lipid rafts [13,22,23]. Thus, localization of TRAF3 may be an important determinant in its ability to activate specific signaling pathways. Because T3JAM is able to modulate the localization of TRAF3, it may be one of the critical factors that determine which signal transduction pathway is activated by TRAF3. Future studies on the nature of the insoluble complex to which T3JAM recruits TRAF3 and its role as a JNK-specific signaling complex should provide further insight into the mechanisms that regulate TRAF3-mediated signal transduction.

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