TRAF3 FORMS HETEROTRIMERS WITH TRAF2 AND MODULATES ITS ABILITY TO MEDIATE NF-κB ACTIVATION

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FRET experiments utilizing confocal microscopy or flow cytometry assessed homo- and hetero- trimeric association of human TRAFs in living cells. Following transfection of Hela cells with plasmids expressing CFP- or YFP- TRAF fusion proteins, constitutive homotypic association of TRAFs -2, -3 and -5 was observed, as well as heterotypic association of TRAF1-TRAF2 and TRAF3-TRAF5. A novel heterotypic association between TRAFs –2 and –3 was detected and confirmed by immunoprecipitation in Ramos B cells that constitutively express both TRAFs –2 and –3. Experiments employing deletion mutants of TRAF2 and TRAF3 revealed that this heterotypic interaction minimally involved the TRAF-C domain of TRAF3 as well as the TRAF-N domain and Zn fingers 4 and 5 of TRAF2. A novel flow cytometric FRET analysis utilizing a two step approach to achieve linked FRET from CFP to YFP to HcRed established that TRAFs –2 and –3 constitutively form homo- and hetero- trimers. The functional importance of TRAF2-TRAF3 heterotrimerization was demonstrated by the finding that TRAF3 inhibited spontaneous NF-kB, but not AP-1, activation induced by TRAF2. Ligation of CD40 on Ramos B cells by recombinant CD154 caused TRAF2 and TRAF3 to dissociate, whereas overexpression of TRAF3 in Ramos B cells inhibited CD154-induced TRAF2-mediated activation of NF-kB. Together, these results reveal a novel association between TRAF2 and TRAF3 that is mediated by unique portions of each protein and that specifically regulates activation of NF-kB, but not AP-1.
INTRODUCTION

A family of TNF-receptor associated factors (TRAFs 1-7) functions as adaptor molecules for TNF-receptor superfamily members by associating with the intracellular domain of these proteins and subsequently mediating downstream signaling events such as NF-κB and AP-1 (1, 2). Biochemical approaches have revealed that TRAFs form homotypic multimers (3-6) as well as certain heterotypic multimers, such as those between TRAF1 and TRAF2 and between TRAF3 and TRAF5 (7-10).

Previous reports have demonstrated that TRAFs –2 and –3 play an important role in cellular activation and differentiation following engagement of a variety of TNF-receptor superfamily members such as CD40/TNFRSF5 (1), OX40/TNFRSF4 (11), LTβR (12-14), XEDAR (15), BCMA/TNFRSF17 (16) and Fn14/TWEAKR/TNFRSF12A (17, 18). The functional significance of TRAFs –2 and –3 in immune responses mediated by one or more of these TNF-receptor superfamily molecules was revealed by experiments with mice that were genetically altered in expression of TRAFs –2 or –3. Experiments using mice transgenic for only the TRAF domain of TRAF2 (aa245-501; TRAF2.DN; 19) or mice genetically deficient in TRAF3 expression (20) revealed a role for both adaptor proteins in T cell-dependent humoral immune responses. Of note, mice expressing a dominant negative (DN) form of TRAF2 exhibited an expanded B cell compartment that was evidenced by splenomegalaly and lymphadenopathy (21), whereas TRAF3-/- mice exhibited decreased numbers of B cell precursors (20). Examination of signaling mechanisms mediated by TRAFs –2 or –3 revealed that both adaptor proteins induce activation of the mitogen activated protein kinase (MAPK) jun N-terminal kinase (JNK) as well as playing a role in the regulation of
NF-κB activation (21-23). Importantly, a number of reports have indicated that TRAF3 inhibits NF-κB activation induced by TRAF2 following engagement of TNF-receptor superfamily members such as CD40/TNFRSF5 (29) and OX40/TNFRSF4 (30, 31), but the precise mechanism of this observation has not been delineated. However, overexpression of wild-type (WT) TRAF3 has been shown to inhibit TRAF2-induced NF-κB activation (30, 31). Furthermore, proteolysis of TRAF3 by a pepstatinA inhibitable mechanism enhanced CD40-mediated NF-κB activation (32), whereas TRAF2-induced degradation of TRAF3 enhanced NF-κB activation (33). By contrast, expression of an alternatively spliced form of TRAF3 has been shown to activate NF-κB (34, 35). These findings suggest that a complex role for TRAF2 and TRAF3 in the regulation of NF-κB, but the precise molecular mechanism has not been delineated. Importantly, no direct physical interaction between TRAF2 and TRAF3 has been documented to date.

The purpose of the current study was to examine whether functional inhibition of TRAF2-induced NF-κB activation was mediated by a direct interaction between TRAFs –2 and –3. Experiments utilizing one- and two- step FRET performed by confocal microscopy or flow cytometry clearly demonstrated that TRAF3 directly associates with TRAF2 and inhibits TRAF2-induced NF-κB, but not AP-1, activation.
MATERIALS AND METHODS

Plasmids

Plasmids that encode CFP- or YFP- fused to TRAFs 3 or 5 have been described previously (36). Where indicated, the fragments containing TRAF3 were cloned into the appropriate sites of HcRed-C1 to prepare HcRed-TRAF3. Plasmids containing human cDNAs encompassing the complete open reading frames of hTRAF1 and hTRAF6 have been previously described (37, 38). To prepare CFP or YFP fusion protein constructs, the relevant PCR fragment was amplified using a pair of oligonucleotides (TRAF1-EcoR1-top 5’- TCG AAT TCT ATG GCC TCC ACC AGC TCA GGC AGC-3’ and TRAF1-BamHI-bottom 5’-ATT GGA TCC CTA AGT GCT GGT CTC CAC AAT GC-3’; TRAF6-Bgl II top 5’-CTC AGA TCT CGA ATG AGT CTG CTA AAC TGT GAA-3’ and TRAF6-HindIII-bottom 5’-TCG AAG CT TCT GCT AT A CCA GCT CAT CAG TA-3’) and then cloned into the appropriate sites of CFP-C1 or YFP-C1 vectors (Clontech, San Diego, CA). Plasmids for human TRAF2 were directly amplified from a thymus cDNA library, according to the manufacturer’s instructions, using a pair of oligonucleotides (TRAF2-Bgl II-top 5’-CTC GGA TCC ATG GCT GCA GCT AGC GTG-3’ and TRAF2-Hind III-bottom 5’-AA CAA GCT TAG TTA GAG CCC TGT CAG GTC-3’). Afterward, these fragments were cut with appropriate restriction enzymes and cloned into their compatible sites in CFP-C1, YFP-C1 and HcRed-C1 (Clontech) to prepare CFP-TRAF2, YFP-TRAF2 (38) and HcRed, respectively. The same PCR fragments for all TRAFs were cloned into His-tagged pcDNA3 (Invitrogen Life Technologies, Grand Island, NY) to prepare His-TRAF2, His-TRAF3, His-TRAF5.
and His-TRAF6 plasmids. A plasmid that encodes a FRET-negative control (CFP-TRAF2TRAF-YFP) has been previously described (36). All constructs were confirmed by DNA sequencing. The NF-κB and AP-1 luciferase reporter plasmids were purchased from Stratagene (La Jolla, CA).

Cell Culture and Plasmid Transfection

Hela cells were obtained from the ATCC and cultured in DMEM high glucose medium supplemented with 10% FBS, 1mM glutamine and antibiotics. Transient transfection was done with the LipoFectamine Plus reagent into log-phase growing cells (Invitrogen). Routinely, transfected cells were cultured overnight and then analyzed by confocal microscopy and flow cytometry. Any samples involving TRAF6 transfection were incubated in the presence of 30µM lactacystin (Calbiochem, La Jolla, CA) or 25µM MG132 (Calbiochem, La Jolla, CA) and were processed for the assays indicated 3 hours post-transfection. The EBV-negative B cell line, Ramos/R2G6, was cultured as described previously (24).

Immunoprecipitation and Immunoblotting

Sixteen hours post-transfection, cells were lysed in hyper-salt on ice. Cell extracts were examined by immunoblotting with antibodies specific for GFP (Roche, Indianapolis, IN) or individual TRAF molecules (TRAFs –1, -3, -5 and –6, Santa Cruz Biotechnologies, Santa Cruz, CA; TRAF2, BD Pharmingen, LaJolla, CA). Immunoprecipitation was performed with Ramos cells to determine whether there are endogenous TRAF2/TRAF3 complexes. Briefly, affinity-purified rabbit anti-TRAF2
(Santa Cruz Biotechnologies) or anti-rabbit IgG1 was crosslinked to protein A sepharose (Roche) beads with dimethylpimelimidate (Pierce). Twenty-five µg of protein from whole cell extracts from Ramos B cells were mixed with beads and incubated overnight at 4°C in buffer BC-100 (20mM HEPES, pH 7.3; 20% glycerol; 100mM KCl; 4mM DTT; 0.2mM EDTA; 0.5mM PMSF). The beads were then washed four times with 20mM HEPES (pH 7.9), 250 mM NaCl, 0.05% Triton X-100 and subjected to electrophoresis and immunoblot analysis.

FRET Detection by Flow Cytometry

All cytometric data were collected using a FACS DiVa (Digital Vantage SE; Becton Dickinson, San Jose, CA). The optical configuration for FRET measurement (FRET1) between CFP and YFP has been described previously (36, 38). Briefly, the argon-ion 488nm laser line at 150mW and the krypton-ion UV 407 nm laser line at 50mW were employed to excite the YFP and CFP, respectively. YFP signals were collected using a 546/10nm bandpass filter in the primary laser pathway (laser 1). CFP signals were collected using a 460/20 nm bandpass filter in the third laser pathway (laser 3). FRET1 signals directly emitted from YFP during CFP→YFP FRET were collected using 546/10nm bandpass filter in the third laser pathway (UV1-FL7). To study FRET (FRET2) signals between YFP and HcRed, a 630/22 nm bandpass filter (FL8) was used to detect emission signals from HcRed in the primary laser pathway (laser 1), whereas HcRed was directly excited by the 568 nm line emitted from the spectrum laser at 50mW and its emission was monitored by the signals detected in the second pathway (laser 2) using a 610 LP filter (FL6). The detector in the UV1-FL7 position of the UV-laser
pathway was also used to collect either two-step FRET signals emitted from HcRed during CFP→YFP→HcRed FRET using a 630/22 nm bandpass filter. All data were analyzed using CellQuest software (Becton Dickinson).

**FRET Detection by Laser-Scanning Confocal Microscopy**

The method employed has been described previously (37, 38). Briefly, at sixteen hours post-transfection, cells were fixed in 4% paraformaldehyde in PBS and mounted on silicon-coated slides. Fixation does not change fluorescence protein localization and cellular morphology (37, 38; data not shown). Any samples involving TRAF6 transfection were cultured with 30µM lacatacystin and fixed 3h post-transfection. Hela cells transfected with plasmids expressing CFP- and YFP- fusion proteins were examined routinely using a 100x objective. Confocal microscopic images were obtained with the Carl Zeiss laser scanning microscope with LSM 510 software. An excitation wavelength of 458nm and an emission wavelength of 480 to 500 nm were used for CFP, whereas an excitation wavelength of 514nm and an emission wavelength of 515 to 545 nm were used for YFP. FRET was assessed and quantitated using an acceptor photobleaching method that was developed for laser-scanning confocal microscopy (37, 38). The method assessed the extent of FRET by measuring the donor fluorescence before (Da) and after (D) photobleaching of the acceptor. The amount of energy transfer detected by confocal microscopy (FRETc) was calculated as the ratio of donor fluorescence in the presence or absence of acceptor: FRETc = D/Da.

The ratio of D/Da equals or is less than 1.0 in the absence of FRET. If D/Da is >1.0, FRET is considered to have occurred (39). The magnitude of the D/Da ratio > 1.0 is
proportional to the proximity of the fluorophore. The ratio of D/D_a was compared to the null hypothesis value of 1.0 by one group t-tests.

*Reporter Gene Assays and Flow Cytometry*

For NF-κB and AP-1 reporter gene assays, Hela cells were transfected using the LipoFectamine Plus reagent and a total of 4.5 µg of DNA (including 0.5 µg of the NF-κB- or the AP-1 luciferase reporter constructs) and 2 µg of pEYFP plasmid to monitor transfection efficiency and 2 µg of plasmid expressing YFP- or His- tagged TRAFs) at about 60% confluence in 6-well plates in triplicate. For experiments to examine whether an interaction between TRAF2 and TRAF3 affected TRAF2-mediated NF-κB or AP-1 activation, Hela cells were transfected with 0.5 µg of the NF-κB- or AP-1 luciferase reporter plasmid and 2 or 5 µg of plasmids expressing YFP, YFP-TRAF2 or YFP-TRAF3, or 2 µg of YFP-TRAF2 plus 2 or 5 µg of YFP-TRAF3. Sixteen hours post-transfection, cells were lysed with 300 µl of Promega lysis buffer. The luciferase activity of 10 µl of each lysate was measured using the Luciferase assay kit from Promega. Luciferase activity was normalized relative to YFP levels.
RESULTS

Verification of the Functional Integrity of TRAF Fusion Proteins

The plasmids expressing fluorescent fusion proteins of each TRAF were prepared and transiently expressed in Hela cells. Immunoblots were carried out with antibodies specific for the fluorescent tag as well as the specific TRAF proteins to document correct expression of each TRAF fusion protein (Fig. 1, A-E). Each of the constructs was expressed and detected at the expected molecular mass. For each of the fusion proteins, except TRAF2, smaller molecular weight fragments were also detected. This was most marked for TRAF6, that was ubiquitinated within 3 hours and completely degraded by 16 hours post-transfection. Even when the cells were cultured in the presence of an inhibitor of the proteasome, lactacystin, only a small amount of TRAF6 was expressed at 16 hours post-transfection. It is notable that Hela cells expressed detectable levels of only two of the TRAF family members constitutively, TRAFs –2 and –5.

Because of the relatively large size (27kD) of the fluorescent CFP or YFP tag fused to each TRAF, it was important to document that the TRAF fusion proteins were functionally intact. To accomplish this, plasmids were prepared that expressed TRAF proteins fused to a small His-tag. As can been seen in Figure 1F, overexpression of His- or fluorescent- fusion proteins of TRAFs –2 or –6 induced activation of NF-κB equivalently.
**FRET Reveals TRAF-domain Mediated Self-Association of TRAFs –2, -3 and –5 in Living Cells**

By confocal microscopy, TRAF fusion proteins were primarily expressed as aggregates in the cytosol (Fig. 2A, data not shown). Importantly, CFP- and YFP- tagged TRAFs localized comparably in cells. Homotypic association of TRAFs –2, -3 and –5, but not TRAF6, was detected in the cytosol of transfected cells by confocal FRET (Fig. 2B, C; data not shown). Flow cytometric FRET also demonstrated homotypic association of TRAFs –2, -3 and –5 as well as weak, but reproducible homotypic association of TRAF6 (Fig. 2D). The ability of flow cytometry to detect homomers of TRAF6 is likely related to the enhanced sensitivity of flow cytometric FRET compared with confocal microscopic FRET (36-38). Absence of FRET was observed in cells transfected with a plasmid expressing CFP-TRAF2TRAF-YFP in which a structurally restricted linker of nearly 100 angstroms (TRAF domain from TRAF2) was inserted between CFP and YFP (Fig. 2D, row 4). Importantly, self association of TRAFs was mediated by their respective TRAF domains as comparable FRET was detected between full-length YFP-TRAFs and CFP fusion proteins of the respective TRAF domains (Fig. 3). The FRET signal for self-association of full-length TRAFs or TRAF domains of each TRAF were not statistically significant implying that the TRAF domains account for all homotypic associations detected between these family members.
FRET Reveals TRAF1-TRAF2, TRAF3-TRAF5 and TRAF2-TRAF3 Heterotypic Interactions in Living Cells

Heterotypic interactions between TRAF1 and TRAF2 as well as TRAF3 and TRAF5 have been previously reported (7-10). To determine whether TRAF1-TRAF2 and TRAF3-TRAF5 heterotrimers could be detected in living cells, FRET was assessed in Hela cells co-transfected with plasmids expressing CFP-TRAF1 and YFP-TRAF2 or CFP-TRAF3 and YFP-TRAF5. Along with its uniform distribution in the cytoplasm, CFP-TRAF1 co-localized with YFP-TRAF2 in punctate regions (data not shown). This close association was accompanied by positive FRET (Fig. 4). Similar results were noted for interactions between TRAFs –3 and –5 (Fig. 4).

To determine whether TRAF2 interacted with other TRAFs, Hela cells were co-transfected with a plasmid expressing CFP-TRAF2, along with plasmids expressing either YFP-tagged TRAFs –3, –5 or –6. Figure 5 reveals that TRAF2 colocalized with TRAF3 and that FRET could be detected between TRAF2 and TRAF3 tagged with CFP and YFP, respectively (data not shown). By contrast, there were no direct interactions between TRAFs –2 and –5 although in some spreading cells they appeared to colocalize (Fig. 5A). FRET between TRAFs –2 and –6 was difficult to detect by confocal microscopy. Even when analyzed after three hours, colocalization of TRAF2 and TRAF6 was not found reproducibly. Moreover, when colocalization was found, FRET was not routinely detected between TRAFs –2 and –6. Even by the more sensitive approach of flow cytometric FRET, physical interactions between TRAF2 and TRAF6 could not be reproducibly detected. The direct interaction between full-length TRAF2 and TRAF3 detected by confocal microscopy was confirmed by flow cytometry (Fig. 5B).
TRAF2-TRAF3 Heterotypic Interactions are Mediated by the TRAF-C domain of TRAF3 and TRAF-N, ZnF4 and ZnF5 regions of TRAF2

To identify the sequence elements that direct TRAF2 to interact with TRAF3, a series of deletion mutants of TRAF2 were fused to YFP and each was tested for an interaction with CFP-TRAF3. As noted previously, markedly positive FRET signals were detected between full-length CFP-TRAF3 and YFP-TRAF2 (Fig. 6). Of interest, the intensity of FRET for TRAF2-TRAF3 heterotypic interactions was not significantly different from FRET observed for either the TRAF2 or TRAF3 homotypic interactions. Removal of the TRAF-C of TRAF2 did not affect FRET, whereas further deletion of the TRAF-N domain and the ZnFs resulted in complete loss of FRET. Expression of either ZnF1 to ZnF5 or the TRAF-domain alone of TRAF2 resulted in no FRET with CFP-TRAF3. However, ZnF1 to ZnF5 and TRAF-N of TRAF2 or the RING and ZnFs of TRAF2 were sufficient to interact with TRAF3, although these interactions were less efficient than with full-length TRAF2. Furthermore, removal of ZnF4 and ZnF5 from the RING and ZnF mutant of TRAF2 eliminated interaction with TRAF3. Taken together, the data indicate that the TRAF-N domain, ZnF4 and ZnF5 of TRAF2 are sufficient to interact with TRAF3.

A series of plasmids expressing C-terminal deletion mutants of TRAF3 fused with CFP were used to examine the motifs of TRAF3 involved in heterotypic interaction with YFP-TRAF2. Deletion of TRAF-C of TRAF3 abolished the ability of TRAF3 to interact with TRAF2 (Fig. 7), although TRAF3 lacking its TRAF-C domain co-localized with TRAF2. In contrast, a mutant of TRAF3 lacking the entire TRAF domain (TRAF-C and TRAF-N) and consisting of only the RING and ZnFs did associate with TRAF2.
(1.15±0.10, n=11), although to a lesser degree than the full-length TRAF3. Further deletion of ZnFs 2-5 abolished the interaction of this TRAF3 mutant with TRAF2. Moreover, a TRAF3 mutant of the ZnFs and TRAF-N in the presence or absence of the TRAF-C domain also associated with TRAF2. These observations suggest that the heterotypic interaction of TRAF3 with TRAF2 may be mediated by a number of complicated interactions of different regions of TRAF3 with TRAF2.

TRAF3 Specifically Inhibits TRAF2-induced NF-κB, but not AP-1, Activation

The functional impact and specificity of TRAF2-TRAF3 heterotypic interactions on cellular signaling was examined in Hela cells following transfection with an NF-κB or AP-1 reporter construct and plasmids expressing TRAFs –2 and –3. TRAF3 specifically inhibited TRAF2-induced activation of NF-κB, but not AP-1 (Fig. 8). Notably, a truncation mutant of TRAF3 containing only the ring finger and ZnF1 (TRAF3RZF1), that did not interact with TRAF2, had no effect on TRAF2-mediated NF-κB activation. As an additional control, the impact of TRAF3 on TRAF6-mediated NF-κB was also examined. No interaction between TRAF3 and TRAF6 could be detected by flow cytometric FRET. Moreover, TRAF6 induced activation of NF-κB was not inhibited but was substantially enhanced by TRAF3 (Fig. 8).

Two-Step Triple FRET Detects TRAF2-TRAF3 Heterotrimers in Living Cells

A CFP→YFP→HcRed two step linked FRET flow cytometric technique has been developed to examine interactions of three separate proteins in living cells. This method is based upon the establishment of FRET1 between CFP and YFP and FRET2 between
YFP and HcRed, as well as undetectable FRET between CFP and HcRed. Using this approach, we examined whether homo- and hetero- trimerization between TRAFs –2 and –3 occurred in living cells. As can be seen from Fig. 10, the linked CFP→YFP→HcRed FRET (Two Step FRET) was able to detect homotrimers of TRAF2, homotrimers of TRAF3 and TRAF2-TRAF3 heterotrimers in cells co-transfected with CFP-TRAF2, YFP-TRAF2 and HcRed-TRAF2 (Fig. 10, row 5), or CFP-TRAF3, YFP-TRAF3 and HcRed-TRAF3 (Fig. 9, row 4) and CFP-TRAF3, YFP-TRAF2 and HcRed-TRAF3 (Fig. 9, row 6), respectively.

*Heterotypic Interactions Between TRAF2-TRAF3 Occur Constitutively in Ramos B Cells, Whereas Ligation of CD40 by Recombinant CD154 Induces Dissociation of TRAF2 and TRAF3.*

To determine whether TRAF2-TRAF3 interactions occur in cells constitutively expressing both TRAF2 and TRAF3, experiments were carried out in Ramos B cells that express both TRAFs. As can be seen in Fig. 10A, immunoprecipitation of TRAF2 in Ramos B cells resulted in co-precipitation of TRAF3. It is notable that ligation of CD40 on Ramos B cells induced both a decrease in the relative abundance of TRAF3 relative to TRAF2 and dissociation of TRAF2 and TRAF3. Both of these events required an amount of recombinant CD154 (0.1 ug per $10^5$ cells) that engaged approximately 40 % of surface CD40. Although engagement of CD40 caused a decline in the abundance of TRAF3 relative to TRAF2, this could not explain the apparent dissociation of TRAF2 and TRAF3 induced by recombinant CD154. Thus, CD40 engagement caused a maximal decline of TRAF3 abundance 4.3 fold, whereas detected association of TRAF2 and
TRAF3 decreased by a maximum of 26.7 fold. Although residual TRAF3 could be detected following stimulation with recombinant CD154 that engaged approximately 100% of surface CD40 (10 µg per 10^5 cells), minimal TRAF2-TRAF3 interaction was observed. Finally, although signaling through CD40 caused dissociation of TRAF2 and TRAF3, overexpression of TRAF3 inhibited CD154-induced TRAF2-mediated activation of NF-κB (Figure 10B).
DISCUSSION

The current data indicate that TRAF2 and TRAF3 form heterotrimers and suggest that functional inhibition of TRAF2-induced NF-κB activation may be mediated by a direct interaction between TRAFs –2 and –3. Experiments utilizing one- and two-step FRET performed by confocal microscopy or flow cytometry clearly demonstrated that TRAF3 forms heterotrimers with TRAF2 in living cells in a complex manner that minimally involves the TRAF-C domain of TRAF3 as well as the TRAF-N domain and Zn fingers 4 and 5 of TRAF2. Immunoprecipitation experiments in Ramos B cells that express both proteins constitutively confirm that formation of TRAF2-TRAF3 multimers is a physiologic event not related only to overexpression. In addition, the current results confirm previous suggestions that homotypic interactions between TRAF2 and TRAF3 might develop by demonstrating that these complexes exist endogenously in living cells by confocal microscopic and flow cytometric FRET, including a novel two-step triple FRET approach that directly documented the existence of homotypic trimeric multimers of TRAF2 and TRAF3. Furthermore, the present study confirms and extends previously published reports that TRAF3 inhibits TRAF2-induced NF-κB activation (29-31) by demonstrating that this inhibition is specific since TRAF3 has no effect on TRAF2-induced activation of AP-1. Moreover, TRAF3 did not inhibit NF-κB activation induced by TRAF6. These findings provide new insight into the role of TRAFs –2 and –3 in the NF-κB and AP-1 signaling pathways mediated by TNF-receptor superfamily members by demonstrating the existence and functional role of TRAF2-TRAF3 multimers in living cells.
TRAF3 specifically interferes with TRAF2-induced activation of NF-κB, but not activation of AP-1 (Fig. 9). Previous reports have demonstrated that TRAF3 inhibits NF-κB activation induced by TRAF2 following engagement of TNF-receptor superfamily members (29-31). Moreover, overexpression of wild-type (WT) TRAF3 has been shown to inhibit TRAF2-induced NF-κB activation (31). Furthermore, proteolysis of TRAF3 by a pepstatinA inhibitable mechanism enhanced CD40-mediated NF-κB activation (32) and TRAF2-induced degradation of TRAF3 enhances NF-κB activation (33). These findings are all consistent with the current observation that TRAF3 specifically inhibits TRAF2-induced NF-κB activation.

The mechanism by which TRAF3 specifically inhibits TRAF2-induced NF-κB but not JNK activation has not been previously delineated. Of note, other molecules such as Schnurri3/KRC/ZAS2 (41-43) and CYLD (44, 45) have been shown to inhibit TRAF2-induced activation of both signaling pathways, although the mechanism of inhibition is quite different. The Zn-finger protein, KRC/ZAS2, directly binds the TRAF-C domain of TRAF2 and inhibits binding of TRAF2 to TNF-receptor family members such as TNFR1. CYLD is a ubiquitin (Ub) C-terminal hydrolase (44, 45) that inhibits TRAF2-induced activation of JNK and NF-κB by preventing K63-Ub of TRAF2 that is necessary for activation of the upstream kinases involved in these signaling pathways (46, 47). By contrast, CSN3 has been shown to inhibit TRAF2-induced NF-κB, but not JNK, activation by directly interfering with IKK complex association with TRAF2 (48). The current data suggest that the formation of a direct physical interaction with TRAF3 may also serve to limit the capacity of TRAF2 to activate NF-κB specifically, but to have no effect on TRAF2-induced activation of AP-1.
TRAF3 may specifically inhibit TRAF2-induced NF-κB, but not JNK, activation by alteration of TRAF membrane localization. Recent reports emphasize this possibility since membrane localization of TRAFs has been shown to influence the downstream signaling pathways activated. For example, whereas TRAF2 sequestration in the cytoplasm has been shown to mediate NF-κB but not JNK activation, TRAF2 localization in insoluble RAFT membrane fractions has been shown to mediate activation of JNK but not NF-κB (40, 49). Moreover, the N-terminal RING finger of TRAF2 has been shown to be required for spontaneous as well as ligand-induced RAFT localization of TRAF2 (49). Importantly, recruitment of TRAF3 to RAFT fractions results in activation of JNK, but not NF-κB (25, 28).

Although TRAF2-TRAF3 heterotrimers may direct TRAF2 to RAFTs and thus alter its signaling capability, we could find no gross alteration in the distribution of TRAF2 when TRAF3 was coexpressed. It remains possible, however, that subtle redistribution of TRAF2 as a result of binding to TRAF3 may have occurred. It is of interest that caspase-3 cleaves TRAF3 to two fragments, one lacking the RING and zinc fingers and the other consisting of only the TRAF domains (50). Whereas full length TRAF3 is found primarily in the cytoplasm, caspase-3 cleaved TRAF3 localizes to membrane RAFT fractions. Importantly, the current data demonstrate that a TRAF3 molecule consisting of only the RING and Zn fingers can associate with TRAF2 as measured by FRET (Fig. 7). It is of interest to hypothesize that caspase-3 cleaved TRAF3 (RING and Zn fingers only) may associate with TRAF2 and keep TRAF2 in membrane RAFT fractions. In this manner, caspase-3 cleaved TRAF3 in association
with TRAF2 may deplete TRAF2 from the cytoplasm and thus inhibit TRAF2-induced NF-κB.

Experiments performed in a B cell line that was genetically modified to lack TRAF2 demonstrated that TRAF3 is degraded by TRAF2 in a manner that is independent of new protein synthesis (33). Earlier studies had demonstrated that the TRAF domain of TRAF2 was not sufficient to mediate the degradation of TRAF3 induced by full-length TRAF2 (26, 51). Of note, the current experiments demonstrated that the TRAF domain of TRAF2 is not sufficient to mediate association with TRAF3 (Fig. 6), making this an unlikely explanation for the results.

Some reports have observed that TRAF2 spontaneously associates with TNF-receptor superfamily members such as RANK and CD40 in membrane RAFT fractions by a mechanism that requires the N-terminal RING and Zn fingers of TRAF2 (49, 52). Following receptor engagement in situations where TRAF1 is absent, such as genetically deficient mice or cell lines such as Hela that are TRAF1-negative, RAFT-localized TRAF2 underwent K48-ubiquitination by E3 ubiquitin (Ub) ligases such as Siah2 and was degraded by the proteosome (53, 54). If TRAF1 was expressed, TRAF2 was recycled to the soluble membrane fraction and was not degraded by the proteosome (49). It is interesting to hypothesize that whereas TRAF1 associates with TRAF2 and recycles TRAF2 from membrane RAFTs to the cytoplasm, TRAF3 may associate with TRAF2 and force TRAF2 to associate with membrane RAFTs or prevent RAFT-localized TRAF2 from reentering the cytoplasm. It remains to be tested whether TRAF2-TRAF3 multimers stabilize expression of both TRAFs and make them resistant to either
degradation by each other or by K^{48}-Ub mediated mechanisms, but this is an intriguing possibility.

The full-length TRAF2 molecule interacts with full-length TRAF3 to form heterotrimers, demonstrated here by a novel two step linked FRET flow cytometric approach. Importantly, although previous reports have suggested interactions between various TRAFs (1), this is the first demonstration that TRAF2 and TRAF3 directly interact and that heterotrimers of TRAFs 2 and 3 spontaneously form in living cells. The development of two step linked FRET permitted this to be documented definitively. Of note, multimerization was minimally mediated by the TRAF-C domain of TRAF3 as well as the TRAF-N domain and Zn fingers 4 and 5 of TRAF2. Previous studies have shown that deletion of the TRAF-C domain prevented homo-multimerization of all TRAFs (6). In addition, previous reports have demonstrated that TRAF2-TRAF1 multimers are mediated by the TRAF-C domains of both proteins, whereas TRAF3-TRAF5 multimers are independent of TRAF-C domains (7-10). In this regard, TRAF2-TRAF3 interactions are somewhat different in that they are dependent only on the TRAF-C domain of TRAF3, but not TRAF2. Of note, overexpression of TRAF3 has been shown to co-simulate TRAF5-induced NF-κB activation (8), whereas the current data are consistent with the reports that TRAF3 inhibits TRAF2-induced activation of NF-κB. Finally, the ability of TRAF2 to induce downstream signaling cascades has been shown to be dependent upon its ability to multimerize (6). The current data show that multimerization of TRAF2 with TRAF3 limits the ability of TRAF2 to induce NF-κB activation and therefore is consistent with the conclusion that TRAF2 homotrimerization is required for effective activation of NF-κB, but not AP-1.
Previous reports have investigated the domains of TRAF2 that contribute to activation of NF-κB and JNK. Mutational analysis of TRAF2 revealed that the N-terminal region containing the RING and Zn fingers is required for its ability to activate NF-κB and JNK (55). By contrast, the addition of seven additional amino acids in the RING domain of TRAF2 by alternative splicing abrogated the ability of TRAF2 to induce activation of NF-κB but had no effect on TRAF2-induced activation of JNK (56, 57). Deletion of either the RING domain or the most N-terminal Zn finger (ZnF1) abrogated the ability of TRAF2 to induce NF-κB or JNK activation. Surprisingly, deletion of ZnF2 had no effect on TRAF2-induced NF-κB or JNK activation and mutation of ZnF3 doubled the level of TRAF2-induced NF-κB but had no effect on JNK, suggesting that ZnF3 of TRAF2 regulates the ability of TRAF2 to activate NF-κB but has no role in activation of JNK (6). Importantly, it should be noted that the current data demonstrate that the domains of TRAF2 that play a direct role in signal transduction (RING, ZnF1, ZnF3; 6, 55) and TRAF3 association (TRAF-N, ZnF4, ZnF5; Fig. 6) are unique. Similarly, the domains of TRAF3 that have been shown to induce JNK (RING and ZnF; 24-28, 57) are different from the domain of TRAF3 that mediates its interaction with TRAF2 (TRAF-C; Fig. 7). Although TRAF2-TRAF3 heterotrimer formation is mediated by regions of the TRAF2 molecule that are not directly involved in initiation of signaling cascades, formation of TRAF2-TRAF3 heterotrimers may alter binding or activation of signaling molecules that have been shown to activate NF-κB, but not AP-1, following their association with TRAF2.

It is notable that signaling through CD40 induced a decrease in the abundance of TRAF3 relative to TRAF2 as well as dissociation of constitutively-expressed TRAF2 and
TRAF3 in Ramos B cells. Whether this is related to the previously reported TRAF2-induced proteolysis of TRAF3 (32, 33) is currently unknown. However, CD154-stimulated proteolysis of TRAF3 is unlikely to be the complete explanation for the CD154-induced dissociation of TRAF2 and TRAF3 even after maximal engagement of CD40 since residual TRAF3 could be detected and minimal TRAF2-TRAF3 interaction was noted. Finally, it is notable that overexpression of TRAF3 could still inhibit CD40-induced TRAF2-mediated activation of NF-κB. These results imply that there may be multiple mechanisms by which TRAF3 can regulate the signaling capacity of TRAF2. In resting cells, the formation of heterotrimers of TRAF2 and TRAF3 may be the major mechanisms that inhibits the constitutive activation of NF-κB by TRAF2.

In summary, the current data demonstrate that TRAF3 specifically inhibits TRAF2-induced NF-κB, but not AP-1, activation. TRAFs –2 and –3 form heterotrimers in living cells that can be documented by immunoprecipitation, confocal microscopic or flow cytometric CFP→YFP FRET as well as a new two step linked flow cytometric CFP→YFP→HcRed FRET technique. The interactions between TRAFs –2 and –3 are complicated, but minimally involved the TRAF-C domain of TRAF3 as well as TRAF-N, ZnF4 and ZnF5 of TRAF2. Together, these observations indicate that TRAF3 and TRAF2 form functional heterotrimers in living cells that have the capacity to modulate TRAF-mediated signal transduction, specifically.
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FIGURE LEGENDS

FIGURE 1. Fluorescently-tagged or HA-tagged TRAFs are expressed in Hela cells and equivalently affect NF-κB activation. Hela cells were transfected with 500 ng of each plasmid as indicated, cultured overnight, lysed in hypersalt buffer for 30 minutes on ice and then the supernatant was assessed for GFP and TRAF expression by immunoblotting. All cells were harvested and assayed 16 hours after transfection, except for cells transfected with YFP-TRAF6 that were harvested 3 hours and 16 hours after transfection with and without the presence of 30 µM lactacystin post-transfection. Immunoblotting for the specific TRAF and GFP was carried out with lysates of cells transfected with TRAF1 (A), TRAF2 (B), TRAF3 (C), TRAF5 (D) and TRAF6 (E). The open star indicates the molecular weight of the TRAF -CFP or -YFP construct and the closed star indicates the molecular weight of the endogenous TRAF. (F) Hela cells were transfected with 2 µg of plasmids encoding YFP, YFP-TRAF2, YFP-TRAF3, YFP-TRAF5 or YFP-TRAF6, along with 500 ng of an NF-κB-driven luciferase reporter. Sixteen hours post-transfection, the cells were processed for luciferase activity.

FIGURE 2. FRET reveals self-association of TRAFs -2, -3 and -5. (A) Colocalization of TRAF2 or TRAF3 by confocal microscopy. Hela cells were cotransfected with either CFP-TRAF2 and YFP-TRAF2 or CFP-TRAF3 and YFP-TRAF3. Sixteen hours later, the cells were fixed in 4% paraformaldehyde in PBS and then mounted for confocal analysis. CFP-TRAF2 co-localizes with YFP-TRAF2 (top row) and CFP-TRAF3 colocalizes with YFP-TRAF3 (lower panel). (B) Homotypic interactions of TRAF2 (I) or TRAF3 (II)
demonstrated by confocal microscopic FRET. The pseudo-color images of CFP and YFP are shown immediately before and after acceptor YFP photobleaching. (C) Analysis of homotypic TRAF interactions by confocal microscopy. A (D/D_{0}) ratio >1 indicates that FRET has occurred. Data from multiple analysis (n) are shown with the statistical significance (p value). (D) Flow cytometric FRET demonstrates prominent self-association of TRAFs -2, -3 and -5, and modest self-asociation of TRAF6. The region 2 (R2) included the transfected cells expressing both YFP and CFP was gated and the cells in R2 were analyzed for FRET. Only few events were found in R2 with control transfected cells (1) and those cells transfected with CFP (2) or YFP (3) alone. The cells transfected with a FRET-negative control plasmid (CFP-TRAF2TRAF-YFP) showed negative FRET with an MFI of 3.5 (4). In contrast, the cells co-transfected with CFP-tagged and YFP-tagged TRAF2, 3 and 5 showed strong positive FRET signals (5-7). In contrast, homotypic interaction of CFP-TRAF6 and YFP-TRAF6 (8) was considerably weaker, even in the presence of lactacystin. Cells expressing CFP-TRAF6 and YFP-TRAF6 were assessed for FRET 3 hours after transfection. Other cells were assessed 16 hours after transfection.

**FIGURE 3.** The TRAF domain (TD) mediates homotypic association of TRAF 2, 3 and 5. Hela cells were cotransfected with either YFP-TRAF2 and CFP-TRAF2TRAF or YFP-TRAF3 and CFP-TRAF3TRAF or YFP-TRAF5 and CFP-TRAF5TRAF and analyzed by confocal microscopic FRET. Data from multiple analysis (n) are shown with the statistical significance (p value).
**FIGURE 4.** Confocal microscopic FRET reveals TRAF1-TRAF2 and TRAF3-TRAF5 heterotypic interactions. Hela cells were cotransfected with either YFP-TRAF2 and CFP-TRAF1 or CFP-TRAF3 and analyzed by confocal microscopic FRET. Data from multiple analysis (n) are shown with the statistical significance (p value).

**FIGURE 5.** Confocal microscopic FRET reveals TRAF2-TRAF3, but not TRAF2-TRAF5 or TRAF2-TRAF6, heterotypic interactions. FRET between TRAF2, but not between TRAFs -2 and -5 or TRAFs -2 and -6 from multiple experiments is shown in A. Association between full-length TRAF2 and TRAF3 was confirmed by flow cytometric FRET (B).

**FIGURE 6.** TRAF2-TRAF3 heterotypic interactions are mediated by the TRAF-N, ZnF4 and ZnF5 regions of TRAF2. Hela cells were co-transfected with successive TRAF2 C-terminal deletion or N-terminal truncation mutants coupled with YFP along with CFP-TRAF3. The structure of the various TRAF2 mutants as well as the degree of confocal microscopic FRET and the extent and nature of co-localization are summarized.

**FIGURE 7.** TRAF2-TRAF3 heterotypic interactions are mediated by the TRAF-C domain of TRAF3. Hela cells were transfected with successive TRAF3 C-terminal deletion or N-terminal truncation mutants coupled with CFP along with YFP-TRAF2. The structure of the various TRAF3 mutants as well as the degree of confocal microscopic FRET and the extent and nature of co-localization are summarized.
FIGURE 8. **TRAF3 inhibits TRAF2-induced NF-κB, but not AP-1 activation, and does not inhibit TRAF6-induced activation of NF-κB.** (A) Hela cells were cotransfected with 0.5 µg of an NF-κB luciferase reporter construct along with either 4 or 7 µg of pEYFP vector as control, or with 2 or 5 µg of YFP-TRAF2 (I, II) or with 2 or 5 µg of YFP-TRAF3 (I, III), or with 2 µg of YFP-TRAF2 (A) plus 2 or 5 µg of YFP-TRAF3RZF1 (II). The blank pEYFP vectors were used to balance the amount of plasmid transfected in each sample. Sixteen hours after transfection, lysates of the cells were prepared and assayed for luciferase activity. Relative luciferase activity was normalized by transfection efficiency, relative protein expression level and protein amount. The data presented are the mean and standard deviation for three independent experiments. (B) TRAF3 neither interacts with TRAF6 nor inhibits TRAF6-mediated NF-κB activation. I. Cotransfection of YFP-TRAF3, YFP-TRAF6 and the NF-κB luciferase reporter construct was carried out as described in A. The data are the mean and standard deviation of three independent experiments. II. Hela cells were co-transfected with 2 µg each of CFP-TRAF3 and YFP or YFP-TRAF3 or YFP-TRAF6. Three hours after transfection, FRET was assessed by flow cytometry.

FIGURE 9. **Two-step triple flow cytometric FRET detects TRAF2-TRAF3 heterotrimers in living cells.** (A) Flow cytometric profiles showing simultaneous measurement of both FRET1 (CFP->YFP, R1) and FRET2 (YFP->HcRed, R2). FRET was not detected in the cells co-expressing CFP/YFP-TRAF3/HcRed (1), whereas FRET1 was detected in the cells co-expressing CFP-TRAF3/YFP-TRAF3/HcRed (2), FRET2 was detected in the cells co-expressing CFP/YFP-TRAF3/HcRed-TRAF3 (3), and both FRET1 and FRET2
were detected in those co-expressing CFP-TRAF3/YFP-TRAF3/HcRed-TRAF3 (4), CFP-TRAF2/YFP-TRAF2/HcRed-TRAF2 (5) or CFP-TRAF3/YFP-TRAF2/HcRed-TRAF3 (6). The MFIs of FRET1 and FRET2 are shown for each panel as well as the percentage of cells transfected. (B) Flow cytometric profiles showing homo- and heterotrimerization of TRAF2 and TRAF3 evidenced by a positive linked 2-step FRET (CFP->YFP->HcRed, R3). The same samples from Figure 10A were reanalyzed with a 633/20 band-pass filter positioned in the UV 3rd laser pathway (FL7) to measure the linked 2-step-FRET signal emitted from HcRed fluorophore that was sequentially excited by CFP->YFP FRET. No two-step-FRET signals were detected in the cells co-expressing CFP/YFP-TRAF3/HcRed (1), in those co-expressing CFP-TRAF3/YFP-TRAF3/HcRed (2) or CFP/YFP-TRAF3/HcRed-TRAF3 (3). In contrast, linked 2-step-FRET was detected in cells co-expressing CFP-TRAF3/YFP-TRAF3/HcRed-TRAF3 (4), CFP-TRAF2/YFP-TRAF2/HcRed-TRAF2 (5), or CFP-TRAF3/YFP-TRAF2/HcRed-TRAF3 (6). Mean fluorescence intensities of the linked two-step FRET and FRET2 as well as the transfection efficiency are shown in each FRET panel.

**FIGURE 10.** The impact of CD40 engagement on heterotypic interactions between TRAF 2 and TRAF 3 in Ramos B cells. (A) CD40 ligation by recombinant CD154 leads to dissociation of TRAF2-TRAF3 complexes. Ramos B cells were stimulated with the indicated amounts (in ug per 100,000 cells) of recombinant CD154 for 15 minutes at 37 °C. Afterward, protein extracts were prepared and immunoprecipitated with rabbit antibody against human TRAF2, washed twice with 250 mM NaCl and then the precipitated complexes were separated on a protein PAGE gel. After being transferred
to nitrocellulose membranes, immunoblotting for TRAF2 and TRAF3 was carried out using specific monoclonal antibodies. The relative densities of the TRAF2 and TRAF3 bands were calculated using the BioRad Molecular Imager, and the ratio between the densities of TRAF2 and TRAF3 is shown. (B) Expression of TRAF3 in Ramos B cells inhibited CD154-induced NF-κB activation. Ramos B-cells were co-transfected with 0.5 µg of an NF-κB luciferase reporter plasmid along with either 3 µg of His-tagged control vector, or with 1 µg of YFP-TRAF2 and 2 µg of control vector, or with 1 µg of YFP-TRAF2 and either 1 or 2 µg of CFP-TRAF3. Cells were stimulated with the indicated amounts of CD154 (0.005 and 0.1 µg of CD154 per 100,000 cells) for 3 hours at 37 °C, lysed directly in luciferase lysis buffer, and then assessed for luciferase activity.
FIGURE 1
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FIGURE 2A,B,C
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FIGURE 2D
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FIGURE 3
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FIGURE 4
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TRAF1/ TRAF2

1.12 ± 0.07
(p<0.001, n=15)

TRAF3/ TRAF5

1.15 ± 0.05
(p<0.001, n=15)
FIGURE 5A,B
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FIGURE 6
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FIGURE 7
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+++ dots 1.40 + 0.08 (n=18, p<0.001)

- 1.00 + 0.02 (n=18, NS)

R             Z1  Z2  Z3  Z4  Z5       TRAF-N                                TRAF-Ch
FRET with YFP-TRAF2

+++, dots 0.99 + 0.03 (n=18, NS)

CFP-TRAF3

∆ TRAF-C +++ dots 1.15 + 0.10 (n=11, p<0.01)

CFP-TRAF3RZF1

R            Z1   Z2  Z3  Z4  Z5 ++, dots 1.15 + 0.10 (n=11, p<0.01)

CFP-TRAF3RZF1-3

CFP-TRAF3ZF4-5 TRAF-N

+++, dots 1.13 + 0.08 (n=29, p<0.001)

+++, dots 1.13 + 0.08 (n=29, p<0.001)

+/-, uniform 1.01 + 0.05 (n=12, NS)

-    0.96 + 0.02 (n=10, NS)

+/- 1.00 + 0.03 (n=19, NS)

Z2  Z3   Z4  Z5      TRAF-N                                 TRAF-C

-    0.93 + 0.07 (n=12, NS)

-    0.98 + 0.05 (n=27, NS)
A) i) NF-κB activity (RLU)

- Control: 4
- TRAF2: 7
- TRAF3: 2
- TRAF3RZF1: 0

ii) NF-κB activity (RLU)

- Control: 4
- TRAF2: 2
- TRAF3: 2
- TRAF3RZF1: 0

iii) AP-1 activity (RLU)

- Control: 4
- TRAF2: 2
- TRAF3: 2

B) i) NF-κB activity (RLU)

- Control: 4
- TRAF6: 7
- TRAF3: 2

ii) FRET Intensity vs. CFP Intensity

- 1. CFP-TRAF3 + YFP
- 2. CFP-TRAF3 + YFP-TRAF6
- 3. CFP-TRAF3 + YFP-TRAF3

iii) CFP Intensity vs. YFP Intensity

- 1. CFP-TRAF3 + YFP
- 2. CFP-TRAF3 + YFP-TRAF6
- 3. CFP-TRAF3 + YFP-TRAF3
FIGURE 9
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FIGURE 10
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TRAF3 forms heterotrimers with TRAF2 and modulates its ability to mediate NF-kB activation
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