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Research Article

TRAF4 functions as an intermediate of GITR-induced NF-κB activation

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Abstract. Members of the tumor necrosis factor receptor (TNFR) family regulate the activation, differentiation, and function of many cell types, including cells of the immune system. TNFR-associated factors (TRAFs) function as adapter molecules controlling signaling pathways triggered by TNFR family members, such as activation of nuclear factor κB (NF-κB). Despite intensive research, the function of TRAF4 in signaling pathways triggered by TNFR-related proteins remains enigmatic. Intriguingly, our functional studies indicated that TRAF4 augments NF-κB activation triggered by glucocorticoid-induced TNFR (GITR), a receptor expressed on T cells, B cells, and macrophages. Further analyses revealed that TRAF4-mediated NF-κB activation downstream of GITR depends on a previously mapped TRAF-binding site in the cytoplasmic domain of the receptor and is inhibited by the cytoplasmic protein A20. GITR is thought to inhibit the suppressive function of regulatory T cells (T_{reg} cells) and to promote activation of T cells. Taken together, our studies provide the first indications that TRAF4 elaborates GITR signaling and suggest that TRAF4 can modulate the suppressive functions of T_{reg} cells.

Key words. TNFR-associated factor (TRAF); glucocorticoid-induced TNFR (GITR); signal transduction; nuclear factor κB (NF-κB).

Tumor necrosis factor receptor (TNFR)-related proteins lack inherent enzymatic activity associated with their cytoplasmic domains. Upon ligand engagement, signaling complexes composed of adapter proteins assemble at the cytoplasmic domain of TNFRs to transmit extracellular signals [1, 2]. TNFR-associated factors (TRAFs) are one such family of adapter proteins that interact directly or indirectly with TNFRs to regulate signaling events, such as activation of nuclear factor κB (NF-κB) and c-Jun N-terminal kinase (JNK) [3, 4]. A conserved TRAF domain in their C termini defines TRAF family members and mediates homo- and heteromeric protein-protein interactions [5, 6]. In their N termini, TRAFs contain stretches of zinc finger motifs and, with the exception of TRAF1, RING finger motifs that are essential for TRAF-mediated signaling [5, 7].

Little is known about the role of TRAF4 in mammalian signaling pathways. TRAF4 shares most sequence similarity with *Drosophila* TRAF1, an adapter molecule critical for JNK activation and eye development in the fruit fly [8]. Originally identified as a protein localized in the nuclei of breast carcinoma cells, TRAF4 has also been detected in the cytoplasm [9–11]. Consistent with its cytoplasmic localization, TRAF4 has been observed in over-expression systems and in vitro binding assays to interact with the p75 neural growth factor receptor and the lymphotoxin-β receptor [10, 12]. Paradoxically, TRAF4 has been implicated in promoting apoptotic pathways mediated by p53, yet has been observed to inhibit Fas-mediated cell death [13, 14]. Expression of TRAF4 in T

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cells is dependent on stimulators of the NF-κB pathway [11]. TRAF4 deficiency in mice results in developmental defects of the upper respiratory tract and axial skeleton as well as incomplete closure of the neural tube [15, 16]. Although TRAF4-deficient mice exhibit an appreciable phenotype, the receptor-mediated pathways regulated by TRAF4 are not readily apparent.

Glucocorticoid-induced TNFR (GITR), a member of the TNFR superfamily, has been implicated in inhibiting the suppressive function of CD^{4+}/CD^{25+} regulatory T cells (T_{reg} cells) that control immune effector cells [17–19]. Furthermore, GITR functions as a co-stimulatory receptor on various T cell subsets [20–22]. Development of GITR-deficient mice is grossly normal with no obvious defects in naïve T cells [23]. However, GITR-deficient T cells exhibit an exaggerated activation phenotype when stimulated with CD3-specific antibodies. Taken together, these studies suggest that GITR plays a crucial role in regulating immune responses to self and foreign antigens.

While the immunomodulatory role of GITR is beginning to be appreciated, mechanisms of signal transduction triggered by the receptor are not well characterized. All three subsets of mitogen-activated protein kinases (MAPKs) and NF-κB are induced by GITR in activated T cells (unpublished data). Functional studies of GITR suggest that TRAFs regulate signaling pathways downstream of the receptor [24, 25]. Intriguingly, expression of TRAF2, a prototypical positive regulator of TNFR signaling, inhibits NF-κB activation induced by GITR, which argues that TRAF molecules beside TRAF2 may positively regulate GITR-induced NF-κB activation (unpublished data). Consistent with this hypothesis, mutation of TRAF interaction sites in the cytoplasmic domain of GITR eliminated the capacity of the receptor to activate NF-κB. The data presented here extend our analysis of molecular mechanisms of signal transduction mediated by GITR and provide novel insights into the role of TRAF4 in NF-κB activation triggered by the TNFR-related protein.

Material and methods

Cell line, transfection, and luciferase assay

For all transfection experiments, human embryonic kidney (HEK) 293 cells (ATCC) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 10 mM HEPES, 100 mM non-essential amino acids (NEAA), 100 U/ml penicillin, and 100 μg/ml streptomycin. One million HEK293 cells were transfected with a constant amount of DNA using plasmids indicated in the figure legends and Effectene reagent (Qiagen) according to the manufacturer's protocol. Twenty-four hours post-transfection, cells were lysed in reporter lysis buffer (Promega) and luciferase assays were performed as described previously [26]. The induction of NF-κB activity was calculated by dividing the relative luciferase units from the reporter plasmid containing two canonical NF-κB sites with units from the reporter plasmid with the minimal promoter.

Plasmids

GITR-L and GITR were cloned into pcDNA3.1 (Invitrogen). Corresponding alanine substitution mutations of pcDNA3.1[GITR] were generated using the QuikChange mutagenesis system (Stratagene). FLAG-A20 (a kind gift of Dr. R. Ulevitch) and TRAF4 were subcloned into pcDNA3.

Results

NF-κB activation triggered by GITR is enhanced by TRAF4

Our recent studies have shown that TRAFs elaborate signaling pathways triggered by GITR distinct from other TNFR-related proteins (unpublished data). Despite its similarity in primary structure to other TRAFs, the exact involvement of TRAF4 in TNFR-mediated signaling pathways remains unclear. The cellular localization of TRAF4 argues that the adapter protein may function as a proximal signaling intermediate of GITR or may elaborate further distal pathways that have originated at the plasma membrane. To determine if TRAF4 regulates GITR signal transduction, GITR and GITR ligand (GITR-L) were co-expressed in HEK293 cells in the presence or absence of TRAF4, and NF-κB activity was measured by a luciferase reporter assay. Consistent with previous reports, GITR interacting with GITR-L activates NF-κB, whereas expression of either the receptor or the ligand by themselves was insufficient (fig. 1 and data not shown). When TRAF4 was co-expressed with GITR and GITR-L, receptor-induced NF-κB activation was significantly increased. Expression of TRAF4 in the absence of GITR-induced signaling did not affect NF-κB activity, indicating the specificity of the finding. This result suggests that TRAF4 plays a role in GITR-triggered signal transduction pathways.

TRAF-binding sites in GITR are critical for TRAF4-mediated augmentation of receptor-mediated NF-κB activation

A single TRAF-binding site, which includes acidic residues 202/203 and 211–213 in the cytoplasmic domain of GITR, has been mapped previously and is required for receptor-induced NF-κB activation (unpublished data). In contrast, mutation of acidic residues 219/220 did not adversely affect TRAF interaction or the ability of the receptor to activate NF-κB. To test if TRAF4-mediated effects depend on TRAF recruitment to GITR, HEK293 cells were transfected with TRAF4 as

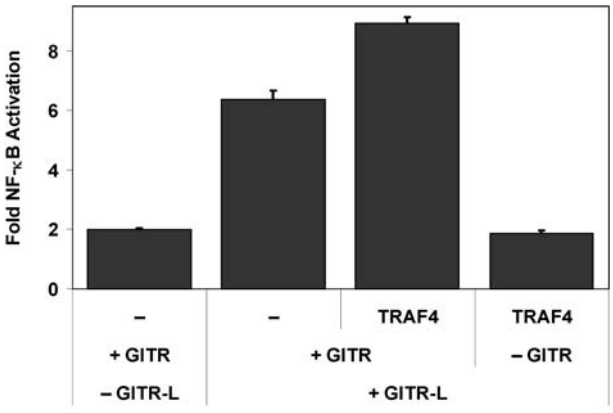

Figure 1. TRAF4 positively regulates GITR-induced NF-κB activation. HEK293 cells were transfected with the indicated expression constructs and luciferase reporter constructs containing two canonical NF-κB sites in the promoter. Twenty-four hours post-transfection, luciferase assays were performed on cell lysates as described in Materials and methods. The experiment shown is representative of three independent experiments, where error bars indicate standard deviations of triplicate samples.

well as GITR-L and full-length GITR or mutants of the receptor deficient or sufficient to interact with TRAFs. Similar to the augmentation of signaling originating from the intact receptor, TRAF4 amplified NF-κB activation triggered by the GITR mutant (219EE>2A) that was capable of interacting with TRAFs (fig. 2 and data not shown). In contrast, expression of TRAF4 did not significantly increase NF-κB activation induced by GITR mutants (202ED>2A and 211EEE>3A) that were defective in TRAF interaction. These studies revealed the relevance of TRAF-binding sites for the ability of TRAF4 to augment GITR-induced NF-κB activation, consistent with TRAF4 elaborating proximal steps of GITR signal transduction.

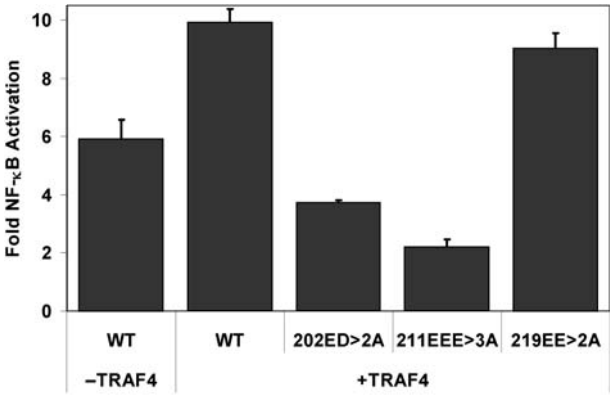

Figure 2. Mutation of TRAF interaction sites results in loss of TRAF4 augmentation of GITR-induced NF-κB activation. HEK293 cells were co-transfected with GITR-L and TRAF4 as well as the indicated wildtype (WT) and mutant GITR receptors. Depicted is a representative NF-κB-dependent luciferase assay of three independent experiments.

A20 inhibits TRAF4 enhancement of GITR-induced NF-κB activation

One mechanism of down-regulating signaling pathways triggered by TNFR-related proteins involves NF-κB-dependent synthesis of A20, a TRAF-interacting adapter protein that inhibits both NF-κB activation and apoptotic pathways triggered by TNFRs [27–29]. Similar to previous observations, expression of A20 inhibited NF-κB activation induced by GITR-GITR-L interaction in HEK293 cells (fig. 3). When expressed simultaneously, A20 eliminated the ability of TRAF4 to augment GITR-triggered NF-κB activation. This observation indicates that TRAF4 functions in GITR-induced signal transduction pathways leading to NF-κB activation, similar to TRAF2, TRAF5, and TRAF6 downstream of other TNFR-related proteins.

TRAF4 abrogates TRAF2 inhibition of NF-κB activation triggered by GITR

Previous functional studies of GITR signal transduction suggested a novel inhibitory function of TRAF2 in NF-κB activation triggered by this TNFR-related protein (unpublished data). To examine the functional interplay between TRAF2 and TRAF4 in the context of GITR signaling, NF-κB activation was monitored in HEK293 expressing GITR and GITR-L with the distinct TRAF molecules. HEK293 cells did not express amounts of TRAF2 or TRAF4 that were detectable by Western blot (data not shown). Downstream signaling effects triggered by TNFR-related proteins have been shown to be sensitive to the ratios of adapter components [30–32]. Therefore, the expression of TRAF2 was kept constant, while the amount of TRAF4 was increased to distinguish specific TRAF4 effects on TRAF2 inhibition of GITR signaling. Consistent with TRAF4 functioning in parallel with TRAF2, we observed that expressing increasing amounts of TRAF4 antagonized and overcame the inhibitory

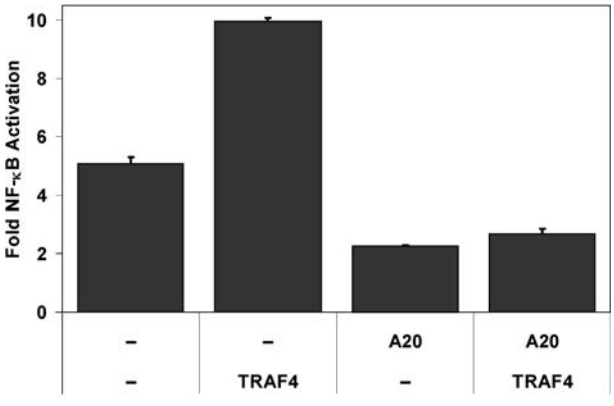

Figure 3. TRAF4 enhancement of GITR-induced NF-κB activation is diminished by A20. GITR, GITR-L, and the indicated proteins were expressed in HEK293 cells. The experiment shown is representative of three independent experiments.

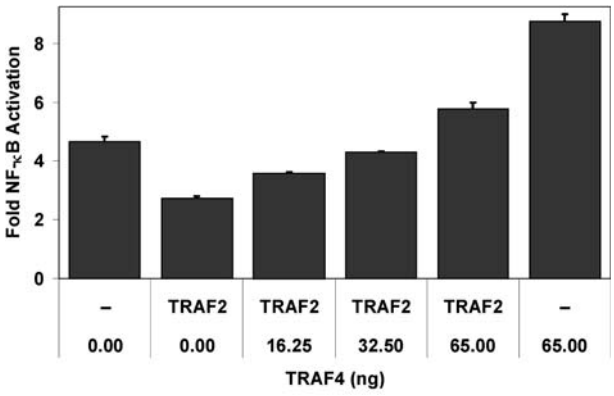

Figure 4. Cross-talk between TRAF2 and TRAF4-mediated signaling triggered by GITR. HEK293 cells were transfected with 65 ng each of GITR, GITR-L, and TRAF2 as well as increasing amounts of TRAF4 expression constructs as indicated. Similar trends were observed in repeated experiments.

effect of TRAF2 on GITR-mediated NF-κB activation (fig. 4). This result further argues that TRAF4 exerts its effects at a proximal stage of GITR signal transduction.

Discussion

Signal transduction pathways triggered by TNFR-related proteins rely upon the assembly and disassembly of multi-protein complexes to induce downstream events, such as activation of NF-κB and MAPKs [1, 2, 7]. TRAFs function as critical adapter molecules regulating the composition of signaling complexes induced by TNFRs and post-translational modifications, such as ubiquitination, of signaling intermediates [5, 33, 34]. Although evolutionarily conserved and extensively studied, the function of TRAF4 beyond regulating tracheal development and closure of the neural tube remains elusive. Because previous work indicated that GITR induces TRAF-mediated signaling, we hypothesized that TRAF4 may serve as a molecular mediator of GITR signaling.

Our functional studies revealed that TRAF4 promotes NF-κB activation triggered by GITR. TRAF4-mediated NF-κB activation downstream of GITR is dependent on an intact TRAF-binding site in the cytoplasmic domain of GITR. Furthermore, co-expression of A20 – an adapter protein, which is upregulated in response to NF-κB activation and can inhibit TRAF-mediated NF-κB activation – interfered with TRAF4-mediated NF-κB activation triggered by GITR. Taken together, these observations revealed that TRAF4 amplifies NF-κB activation triggered by GITR at the level of other TRAFs, which implies that TRAF4 acts as a convergent platform and is consistent with the adapter function of the TRAF family in proximal events of GITR-induced signaling. The mechanism underlying the interplay between TRAF4 and other TRAFs is intriguing and will be the subject of future studies. TRAFs are known to hetero-oligomerize through interactions at the coiled-coil region of the TRAF domain [35]. Whether TRAF4 and other TRAFs are components within the same complexes assembled in response to GITR signaling and regulatory mechanisms affected by these potential interactions remains to be determined.

TRAF4 is expressed in most tissues throughout development, especially in epithelial and neural tissues as well as in breast carcinoma, and thus overlaps with several members of the TNFR family [9, 10, 36, 37]. As GITR utilizes TRAF4 as a signaling intermediate, it is tempting to speculate that TRAF4 modulates signaling triggered by other TNFR-related proteins expressed in these tissues. Furthermore, TRAF4 expression, like that of A20, is induced in response to NF-κB activation, which suggests that A20 may counterbalance the effects of TRAF4 during the maintenance phase of TNFR-mediated signaling [11, 38, 39].

Previous studies indicated that GITR activates the classical NF-κB pathway as well as the three MAPK subfamilies: p38, ERK, and JNK (unpublished data). Through its interaction with $p47^{phox}$, the adapter subunit of NAD(P)H oxidase, TRAF4 is involved in the oxidative activation of JNK mediated by TNF-α and HIV-1 Tat [40]. Furthermore, TRAF4 expression facilitates TNF-α-induced S6 kinase activity [14]. Signaling through GITR has been shown to counteract the suppressive function of T_{reg} cells, leading to organ-specific autoimmune disease [18, 19, 41]. GITR also co-stimulates the proliferation and cytokine production of T cells [20–22, 42]. These other results and the data presented here suggest that TRAF4 and its divergent signaling function(s) may be attractive targets for modulating GITR signaling in therapeutic strategies targeting the inflammatory immune response and autoimmunity.

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