

NF- κ B activator Act1 associates with IL-1/Toll pathway adaptor molecule TRAF6

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Abstract NF- κ B activator 1 (Act1), also called CIKS, is a recently identified protein with NF- κ B and AP-1 activation activities through its association with the I κ B kinase complex. We identified and confirmed that Act1 interacts with tumor necrosis factor receptor-associated factor 6 (TRAF6); notably, Act1 binds to TRAF6 only among TRAF family proteins. The amino-terminal half of Act1 is required for its interaction with the TRAF domain. Act1-mediated NF- κ B activation was inhibited by a dominant-negative mutant of TRAF6 in a dose-dependent manner, and IL-1-induced NF- κ B activation was inhibited by a high level of Act1 expression. Our results suggest that Act1 is involved in IL-1/Toll-mediated signaling through TRAF6.

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Key words: Act1; CIKS; TRAF6; Two-hybrid; Protein–protein interaction; NF- κ B

1. Introduction

Interleukin-1 (IL-1) plays an important role in inflammatory responses by activating transcription factors, such as nuclear factor kappa B (NF- κ B), AP-1, and ATF [1–3]. The IL-1-induced signaling pathway for NF- κ B activation has been studied extensively, and a plausible working model has been proposed [4–6]. When IL-1 binds to its receptor (IL-1R), a receptor complex comprising IL-1R and its coreceptor (IL-1RAcP) is formed [7]. The cytoplasmic proteins MyD88 (protein encoded in myeloid differentiation gene 88) [8] and Tollip (Toll-interacting protein) [9] are both recruited to the receptor complex, resulting in recruitment of IL-1 receptor-associated kinase 1 (IRAK1) [10]. IRAK1 is activated by autophosphorylation, disassociates from the complex, and interacts with tumor necrosis factor receptor-associated factor 6 (TRAF6) [11], a key adapter molecule in the pathway. The IRAK1–

TRAF6 complex activates the TAB1/TAK1–NIK (transforming growth factor- β activated kinase 1 (binding) protein–NF- κ B-inducing kinase) pathway [5,12], leading to activation of the I κ B kinase (IKK) complex that is essential to activate NF- κ B [13]. Because the molecular mechanisms downstream of IRAK1 in the IL-1-induced signaling pathway are shared by those of the Toll signaling pathway, the pathway is also called the IL-1/Toll signaling pathway [6]. Activated TAK1 is also implicated in the activation of SAPK/JNK (c-Jun N-terminal kinase), a member of the MAPK kinase kinase (MAPKKK) family. It is also proposed that IL-1-induced activation of JNK may diverge from NF- κ B activation at the level of TRAF6 [14].

NF- κ B activator 1 (Act1), also called CIKS (connection to IKK and SAPK/JNK), has previously been identified as an NF- κ B and JNK-activating protein by two independent groups [15,16]. Act1 does not have any enzymatic domain; instead it contains a helix-loop-helix at the amino terminus and a coiled-coil motif at the carboxyl terminus. Direct interaction of Act1 with the IKK complex leads to NF- κ B activation. This mechanism is supported also by the result that a kinase-deficient dominant-negative mutant of NIK does not affect Act1-mediated NF- κ B activation. Despite clear evidence of the signaling pathway downstream of Act1 that leads to activation of NF- κ B, the signal that modulates Act1 activity has not been clarified. Recently, Qian et al. [17] reported that Act1 is recruited to the CD40 receptor in epithelial cells upon their stimulation with CD40 ligand, suggesting that Act1 plays a role in CD40-mediated signaling. However, these authors also described their seemingly inconsistent result that Act1 interacts with TRAF3, as TRAF3 seems to play a negative role in Act1-dependent CD40-mediated NF- κ B activation [17].

Here we report the identification and confirmation of Act1 interaction with TRAF6, an adapter molecule essential for NF- κ B activation in the IL-1/Toll signaling pathway. We also propose a working model for the signal-transduction mechanism of Act1 that is consistent with our present data.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney cell line HEK293 was cultured in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 U/ml penicillin, and 200 μ g/ml streptomycin. CHO-K1 cells were maintained in F-12 Nutrient Mixture (Ham's F-12), both supplemented with 10% FBS and antibiotics. Both cells were purchased from the RIKEN cell bank Japan.

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Abbreviations: Act1, NF- κ B activator 1; NF- κ B, nuclear factor kappa B; CIKS, connection to IKK and SAPK/JNK; JNK, c-Jun N-terminal kinase; TRAF6, tumor necrosis factor receptor-associated factor 6; IKK, I κ B kinase; IL-1, interleukin-1; MyD88, protein encoded in myeloid differentiation gene 88; Tollip, Toll-interacting protein; IRAK1, interleukin-1 receptor-associated kinase 1; TAB1, transforming growth factor- β activated kinase 1 binding protein; TAK1, transforming growth factor- β activated kinase 1; NIK, NF- κ B-inducing kinase

2.2. Mammalian two-hybrid assay

The detailed method has been published in [18]. Briefly, the assay samples were prepared by two-step polymerase chain reaction (PCR); we constructed the PCR products to express fusion proteins with the Gal4 DNA-binding or herpes virus VP16 transcriptional activation domains. We transfected the PCR products together with 20 ng of the reporter plasmid pG5luc (Promega, CA, USA) into 2.2×10^4 CHO-K1 cells by using the transfection reagent LF2000 (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. After 20 h of incubation, the luciferase reporter activity was measured with the Steady-Glo Luciferase Assay System (Promega).

2.3. Co-immunoprecipitation analysis

The detailed method has been published in [19]. The protein-coding sequences of Act1 and TRAF6 cDNAs were amplified by PCR and subcloned into the expression vectors pCMV-HA and pCMV-Myc (Clontech, CA, USA), respectively. We then transfected 1×10^6 HEK293 cells with 2.5 µg of the expression vectors for HA-tagged Act1 (HA-Act1) and Myc-tagged TRAF6 (Myc-TRAF6) by using LF2000. After 24 h, cells were harvested and lysed by TNE buffer [19]. After centrifugation at $10000 \times g$ for 15 min, the supernatants were isolated and immunoprecipitated with 5 µg anti-HA antibody (Santa Cruz, CA, USA). The co-precipitated Myc-TRAF6 was detected by Western blot analysis. The supernatants described above were also subjected to direct Western blot analysis to confirm the expression of HA-ACT1 and Myc-TRAF6.

2.4. Glutathione S-transferase pull-down assays

In vitro transcription and translation of TRAF6 was performed with the TNT T7 Quick Master Mix kit (Promega) in the presence of [35 S]methionine, according to the manufacturer's protocol. Glutathione S-transferase (GST) or the GST-Act1 fusion protein was expressed in *Escherichia coli* and purified. An aliquot of GST-Act1 or GST, bound to glutathione-Sepharose beads (Amersham, Buckinghamshire, UK), was incubated with 5 µl of in vitro translated TRAF6 in 500 µl NETN buffer (100 mM NaCl, 1 mM EDTA [pH 8.0], 20 mM Tris-HCl [pH 8.0], 0.2% NP40) for 6 h. Beads were washed five times with 1 ml NETN buffer, resuspended in Laemmli sample buffer, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before autoradiography.

2.5. Reporter gene analysis

The expression vectors for Act1 and a dominant-negative mutant of TRAF6 that lacks amino acid residues 1–226 (dnTRAF6) were each transfected with 10 ng of the reporter vector pNFκB-Luc (Clontech) into 5×10^4 HEK293 cells per well of a 96-well plate by using LF2000. After 24 h, cells were treated or untreated with 10 ng/ml IL-1 (R&D systems, MN, USA) for 6 h. The luciferase activity of the reporter gene was measured as described previously.

3. Results

3.1. Interaction of Act1 with TRAF6

We previously reported the development of a novel assay system for the systematic analysis of protein–protein interactions, which involves rapid, PCR-mediated sample preparation and a high-throughput assay system based on the mammalian two-hybrid method [18]. Using our previously developed system, we systematically assayed approximately 6000 cDNAs derived from mouse full-length enriched cDNA libraries. We detected strong luciferase reporter activity corresponding to an interaction between Act1 and TRAF6 when TRAF6 was used as a prey sample (Fig. 1A). To confirm the interaction, we carried out a co-immunoprecipitation experiment (Fig. 1B). We transfected the expression vectors for HA-Act1 and Myc-TRAF6 into HEK293 cells and subjected the cell extracts to immunoprecipitation using an anti-HA antibody. Western blot analysis using an anti-Myc antibody showed that Myc-TRAF6 was specifically co-immunoprecipitated with HA-Act1 (top panel, Fig. 1B). We confirmed the

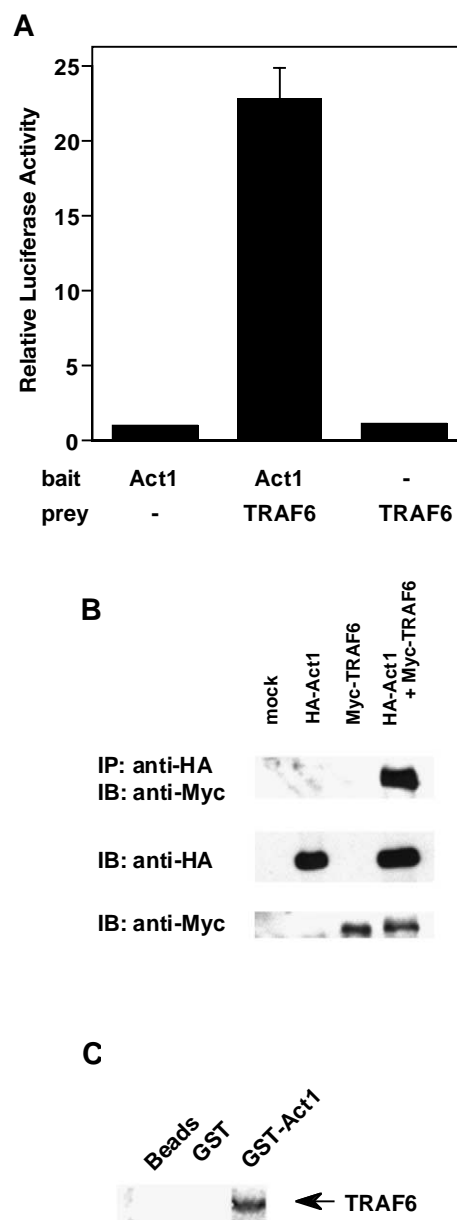


Fig. 1. Identification and confirmation of the interaction between Act1 and TRAF6. A: Identification of Act1 interaction with TRAF6. The relative luciferase activity of the reporter gene was measured when TRAF6 (prey) and Act1 (bait) were co-transfected with the reporter vector pG5luc into CHO-K1 cells. Mean values for transfections of either bait- or prey-samples were calculated as baselines. The nucleotide sequence for mouse Act1 cDNA has been deposited in the DDBJ/EMBL/GenBank database (accession no. AK031556). B: Co-immunoprecipitation of Act1 and TRAF6. The expression vectors encoding HA-Act1 and Myc-TRAF6 were transfected into HEK293 cells as indicated at the top of the panel. Cell extracts were immunoprecipitated by using an anti-HA antibody. The co-precipitated Myc-TRAF6 was detected by Western blot analysis using an anti-Myc antibody (top row). The extracts were also subjected to Western blot analysis to confirm the expression of HA-Act1 (middle row) and Myc-TRAF6 (bottom row). The antibodies used for immunoprecipitation (IP) and immunoblotting (IB) are indicated to the left of the panel. C: In vitro binding of Act1 to TRAF6. The GST-Act1 fusion protein, immobilized on glutathione-Sepharose beads, was incubated with in vitro translated [35 S]-labeled TRAF6. After the beads were washed, bound TRAF6 was analyzed by SDS-PAGE and autoradiography. We did not detect co-precipitated TRAF6 in incubations of glutathione-Sepharose beads only (Beads) or GST immobilized on beads (GST).

result by using *in vitro* GST pull-down assays and showed that [35 S]-labeled TRAF6 is specifically co-precipitated with GST-Act1, indicating that Act1 directly interacts with TRAF6 (Fig. 1C). A correlation of expression profiles between Act1 and TRAF6 is essential for the interaction to be biologically significant, and Northern blot analysis from sev-

eral groups indicates that both Act1 and TRAF6 are ubiquitously expressed in almost all adult tissues [11,16].

3.2. Act1 specifically interacts with TRAF6 through the TRAF domain

Because TRAF6 is an adapter protein with several well-

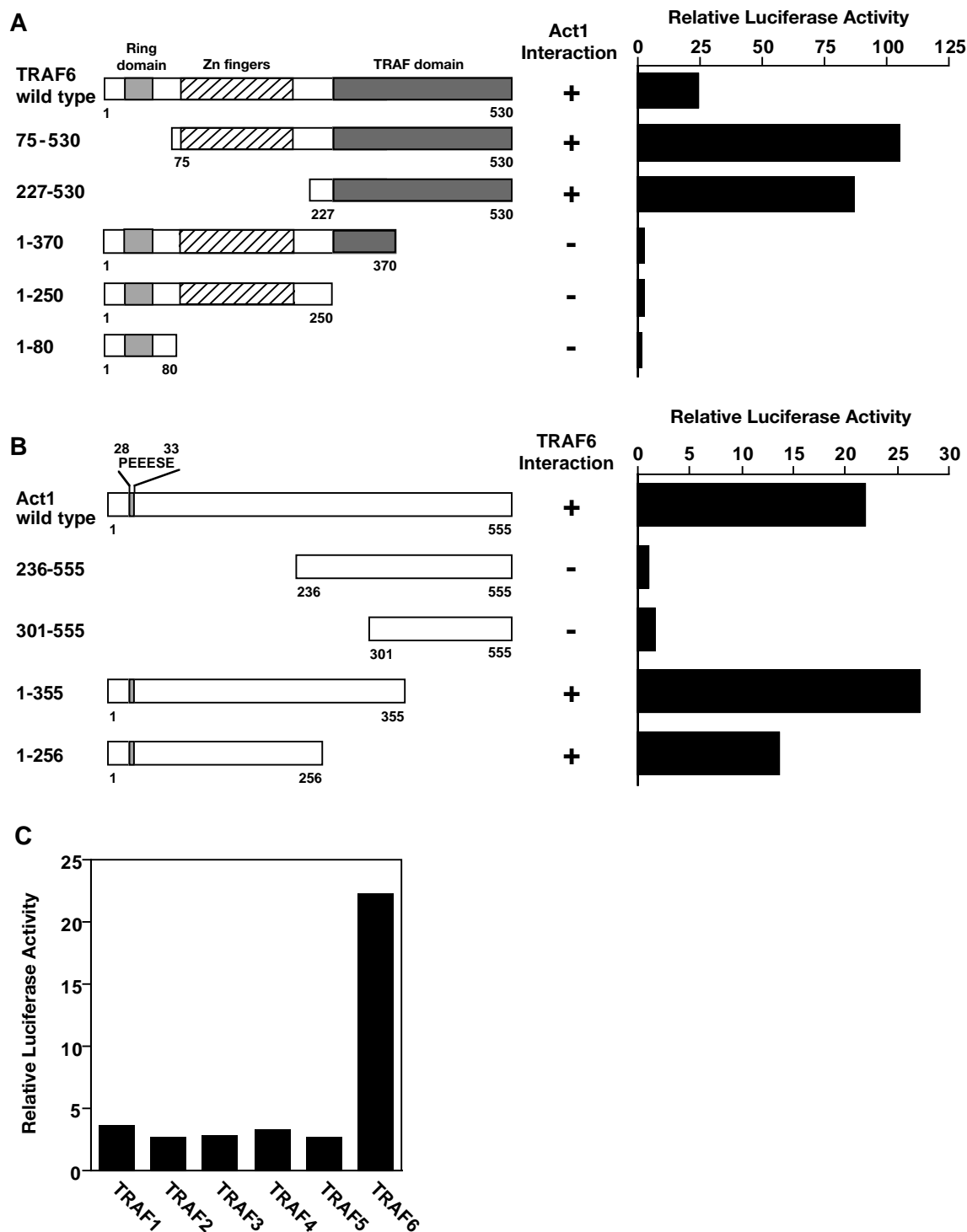


Fig. 2. Mapping of Act1-TRAF6 interaction domains. The interaction was examined by using the mammalian two-hybrid assay. The relative luciferase activity of the reporter gene was measured. A: Schematic representation of TRAF6, its deletion mutants and the interactions with wild-type Act1. B: Schematic representation of Act1, its deletion mutants, and the interaction with wild-type TRAF6. C: Interaction specificity of Act1 with TRAF family proteins.

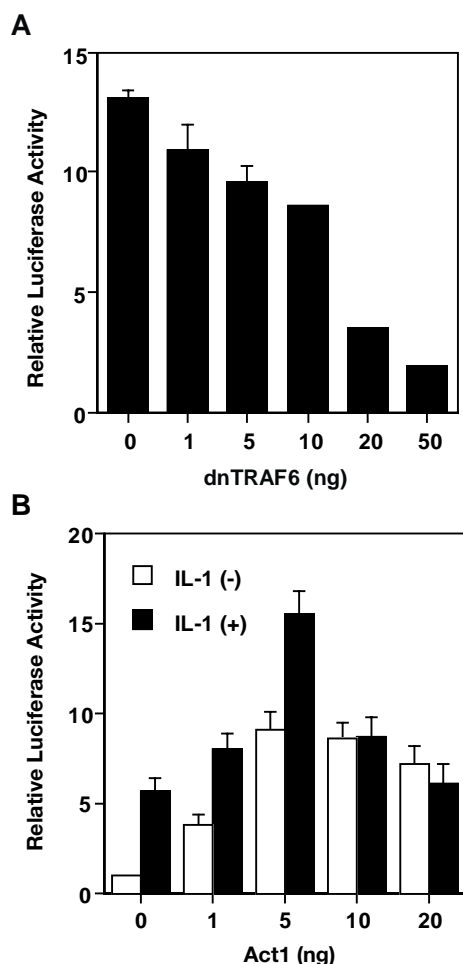


Fig. 3. The influence of dnTRAF6 or IL-1 on the activation of NF- κ B by Act1. HEK293 cells in 96-well plates were transiently transfected with the various expression vectors described and the reporter vector pNF κ B-Luc, followed by measurement of the reporter luciferase activity. A: We co-transfected the expression vectors for Act1 (10 ng/well) and dnTRAF6. B: Cells transfected with the expression vector for Act1 were treated (solid bars) or untreated (open bars) with 10 ng/ml IL-1 for 6 h.

known motifs, including the RING domain, a Zn finger motif, and the TRAF domain (Fig. 2A), we evaluated which motif is responsible for interaction with Act1. We assessed the interaction of Act1 with wild-type TRAF6 or its deletion mutants by using the mammalian two-hybrid method (Fig. 2A). Our results showed that the minimal Act1-binding region includes the carboxyl-terminal half of TRAF6, which is known as the TRAF domain. We performed similar experiments to map the region of Act1 responsible for interaction with TRAF6 (Fig. 2B). The carboxyl-terminal deletion mutants, Act1(1–355) and Act1(1–256), could interact with TRAF6, whereas the amino-terminal deletion mutants, Act1(236–555) and Act1(301–555), could not. This result indicates that Act1 associates with TRAF6 through its amino-terminal half. Recently, the crystal structure of TRAF6 complexed with TRAF6-binding peptides from CD40 and RANK revealed that the TRAF6-binding motif has a consensus motif, PXEXXZ (Z, aromatic/acidic residue) [20]. Our results are consistent with this report because this consensus motif is present in the amino-terminal region of Act1 (PEEESE at amino acid residues 28–33 in Fig. 2B).

The TRAF family proteins, TRAFs 1–6, are characterized by the conserved TRAF domains in their carboxyl-terminal regions [21,22]. We examined the specificity of Act1's interaction with TRAF family proteins. We detected prominent luciferase activity only corresponding to the interaction between Act1 and TRAF6, although marginal luciferase activities occurred with the interactions between Act1 and TRAFs 1–5 (Fig. 2C).

3.3. Reporter gene analysis using the expression vector for Act1

Overexpression of Act1 activates NF- κ B, and this activation is mediated by association of the amino-terminal half of Act1 with IKK γ [15,16]. To evaluate whether the interaction between Act1 and TRAF6 leads to NF- κ B activation, we transiently co-expressed HA-Act1 and Myc-dnTRAF6(227–530), a dominant-negative mutant of TRAF6 [11], in HEK293 cells. The overexpression of dnTRAF6(227–530) inhibited Act1-mediated NF- κ B activation in a dose-dependent manner (Fig. 3A). Because dnTRAF6 itself cannot activate NF- κ B but has the TRAF domain necessary to interact

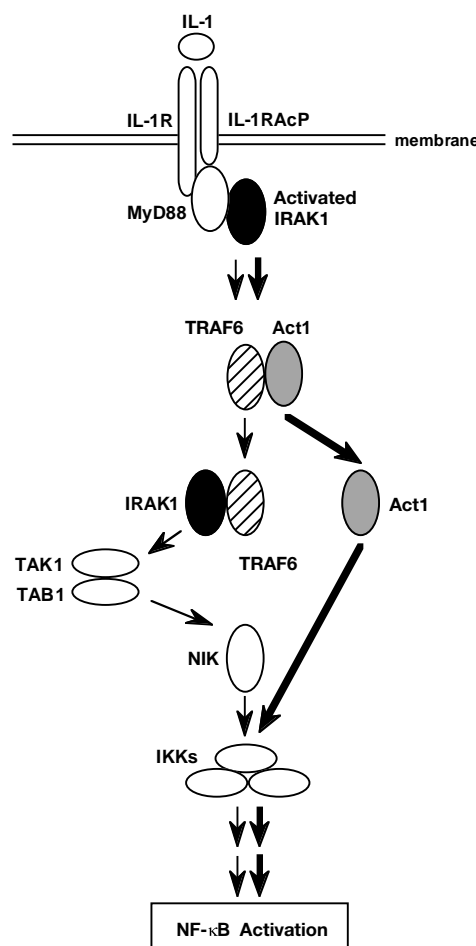


Fig. 4. A working model for the Act1 signaling pathway. The IL-1 signaling pathway, shown by thin arrows, has been well characterized and involves MyD88, IRAK1, TRAF6, TAK1, TAB1, NIK, and IKKs [6,29]. In addition, two independent groups have reported that interaction of Act1 with IKK γ directly activates the IKK complex [15,16]. We propose in the model (thick arrows) that TRAF6 functions as an anchor for Act1, retaining it in unstimulated cells, and that activated IRAK1 associates with TRAF6, resulting in the release of Act1. Thus, Act1 directly binds to and activates IKKs.

with Act1, our result suggests that the overexpressed dnTRAF6 sequesters Act1 so that Act1 cannot associate with IKK γ to activate NF- κ B.

TRAF6 transduces IL-1-induced signals to downstream molecules such as TAB1 and TAK1, resulting in NF- κ B activation [12]. To explore the relevance of the Act1–TRAF6 interaction in IL-1-induced NF- κ B activation, we transfected the expression vector for HA-Act1 into HEK293 cells and treated them with IL-1 (Fig. 3B). We observed that Act1-induced NF- κ B activation became maximal when the vector concentration was ≥ 5 ng/well, and IL-1 treatment had an additive effect for NF- κ B activation at concentrations ≤ 5 ng/well. Interestingly, the additive effect disappeared when we transfected more than 10 ng/well of Act1 expression vector into the cells (Fig. 3B). Our results suggest that a high level of Act1 expression interferes with IL-1-induced NF- κ B activation, most likely by interaction with TRAF6.

4. Discussion

Here we describe the identification and confirmation of the specific interaction of Act1 with TRAF6. Our results are inconsistent with a recent report by Qian et al., in which Act1 co-immunoprecipitated TRAF3 strongly and TRAFs 1 and 5 weakly but not TRAFs 2, 4, and 6 [17]. One of the possible explanations for the inconsistency is that those authors used human Act1 cDNA corresponding to a minor transcript that is alternatively spliced, whereas we used mouse Act1 cDNA corresponding to the human Act1 major transcript [23]. The Act1 minor transcript possesses an extra exon encoding an additional nine amino acids in the amino terminus of the protein, which may affect TRAF-binding specificity of Act1. Further, the marginal reporter activities we observed associated with the interactions between Act1 and TRAFs 1–5 (Fig. 2C) may reflect the interactions that Qian et al. detected in immunoprecipitation experiments.

The interaction between Act1 and TRAF6 seems to need neither post-translational modification nor any signaling triggers, because we detected the interaction by using *in vitro* translated proteins (Fig. 1C). Therefore, it is conceivable that Act1 could associate with TRAF6 in unstimulated cells, and this situation might explain why Act1 has not previously been identified as a co-purifying component of the IKK complex despite the strong Act1–IKK γ interaction detected by two independent groups [15,16]. Together with our finding that overexpression of dnTRAF6 inhibited Act1-induced activation of NF- κ B (Fig. 3A), these results suggest that Act1 cannot interact with and activate IKK γ unless Act1 dissociates from TRAF6. The interaction with IKK γ occurs through the amino-terminal half of Act1 [15], and TRAF6 interacts with Act1 in the same region. We further showed that IL-1 treatment lacked an additive effect on Act1-mediated NF- κ B activation when the expression vector for Act1 was transfected at high levels (Fig. 4B). In addition, the TRAF domain of TRAF6 mediates the interaction with Act1 (Fig. 2A); activated IRAK1 binds to TRAF6 in the same region [11]. We also confirmed that TRAF6 interacts with IRAK1 through the TRAF domain (data not shown). These results suggest that excess Act1 sequesters TRAF6 so that activated IRAK1 cannot interact with TRAF6 to make the IRAK1–TRAF6 complex required for the transduction of the NF- κ B activation signaling to the TAB1/TAK1–NIK pathway.

According to our experimental results, we propose a working model that accounts for the upstream signaling pathways of Act1, in which Act1 functions in cooperation with TRAF6 to cause NF- κ B activation in the IL-1/Toll signaling pathway (Fig. 4). We hypothesize that Act1 associates with TRAF6 in unstimulated cells. Ligand-induced activation of the IL-1R complex (and the Toll-like receptor complex) leads to the release of activated IRAK1 to interact with and thereby transduce the signal to TRAF6. This signal causes the release of Act1 from TRAF6. The IRAK1–TRAF6 complex then transduces the signal to the TAB1/TAK1–NIK pathway, resulting in activation of the IKK complex. Concurrently, the released Act1 directly binds to IKKs to activate the IKK complex. Therefore, activation of the IKK complex in both the IRAK1–TRAF6 complex and Act1 pathways results in NF- κ B activation, and this duality may be useful for the effective signal transduction.

The TRAF domain serves as an adapter module and is involved in interactions with various signal molecules. In addition to several molecules that are essential for TNF signaling pathways [24], many other molecules, including TRIP (TRAF-interacting protein), T2BP (TRAF2 binding protein), and A20, interact with the TRAF domain of TRAF2 [19,25,26]. In case of TRAF6, TTRAP (TRAF and TNF-R associated protein) and ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) have also been reported to interact with the TRAF domain [27,28]. Although these TRAF-domain-binding proteins affect NF- κ B and AP-1 activation of the TNF and IL-1/Toll signaling pathways, their precise mechanisms are unclear. Our working model for Act1 signaling pathways suggests that these TRAF-domain-binding proteins may act similarly to Act1 in that the TRAF proteins exchange their binding partners during ligand-induced activation. Further analysis is necessary to evaluate our working model.

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