Interaction of the TNFR-receptor associated factor TRAF1 with I-kappa B kinase 2 (IKK2, IKK-beta, IKBKB) and TRAF2 indicating a dose dependent regulatory function of TRAF1 for NF-kappa B signaling

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IKK2 is one of the most crucial signaling kinases for activation of the transcription factor NF-kappa B. Since many NF-kappa B activating pathways converge at the level of IKK2, we searched for interaction partners of this kinase using the C-terminal part (aa 466-756) as bait in a yeast two-hybrid system. We identified the N-terminal part (aa 1-228) of the TNF-receptor associated factor TRAF1 as putative interaction partner, which was subsequently confirmed in mammalian cells by coimmunoprecipitation experiments. However, this interaction seemed weaker than the interaction between TRAF1 and TRAF2, an important activating adapter molecule of NF-kappa B signaling indicating that relative levels of IKK2, TRAF1 and TRAF2 might be important for the final biological readout. Reporter gene and kinase assays using ectopic expression of TRAF1 indicated that it can have both activating and inhibiting functions for IKK2 and NF-kappa B. Co-expression of fluorescently tagged TRAF1 and TRAF2 at different ratios implied that TRAF1 can affect clustering and presumably the activating function of TRAF2 in a dose dependent manner.

INTRODUCTION

The NF-kappa B family of transcription factors is essential for a great variety of biological processes such as inflammation, cell survival, regulation of apoptosis, proliferation and cell differentiation. There are two major signaling pathways leading to NF-kappa B: the classical or canonical pathway originating at TNFα-, IL-1 or Toll-like receptors and the alternative pathway initiated for instance at CD40 [1]. Both pathways converge at the level of the IκB kinase (IKK) complex, which consists basically of two related kinases (IKK1 and IKK2) and an essential adapter (termed NEMO for NF-kappa B essential modulator). The IκB kinases can then phosphorylate inhibitors of NF-kappa B on two adjacent serine residues, marking them for polyubiquitination, which results in their degradation by 26S proteasomes and release of active NF-kappa B. The classical activation pathway signals primarily to IKK2, whereas the alternative pathway triggers predominantly IKK1 activity [1, 2]. Nevertheless, these two kinases influence each other [3, 4] and interact with a variety of additional signaling molecules [1]. It is currently still not clear, which interactions can occur simultaneously and whether certain molecular associations are mutually exclusive or influence each other and as a consequence also the NF-kappa B signaling cascade. In the last few years, it became increasingly clear that ubiquitination processes exert important functions in the activation of the IKK complex [2]. These ubiquitinations are triggered by TRAF molecules (mainly TRAF2, TRAF5 and TRAF6), which contain RING domains that have E3 ligase activity catalyzing non-degradative K63-linked polyubiquitination. In contrast to K48-linked polyubiquitin, K63-linked polyubiquitin chains do not lead to proteasomal degradation but rather serve as an association and signaling platform for certain ubiquitin binding proteins, such as TAB1 and TAB2 in combination with the kinase TAK1 [5]. K63-linked polyubiquitination thereby results in binding and activation of TAK1, which then activates IKK2. TRAF1 is the only TRAF-adapter molecule lacking a RING domain and therefore does not act as a ubiquitinase [6, 7]. Of note, TRAF molecules form homo- or heterotrimeric complexes. It has been suggested that the composition of heterotrimers is important for signaling function. Interestingly, both positive and negative regulatory effects of TRAF1 on NF-kappa B signaling have been reported. In cell culture systems overexpression of TRAF1 resulted either in inhibition [8] or in augmentation [9] of NF-kappa B activity. Similar conflicting data have been obtained in knockout mouse models. T-cells from TRAF1-deficient mice showed enhanced NF-kappa B activity and increased IKK2 activity [10], whereas dendritic cells from TRAF1-deficient mice showed attenuated NF-kappa B signaling in a different study [11]. The effect of TRAF1 on signaling is further complicated by the fact that it is a substrate of caspases and therefore cleaved in the course of apoptosis. This leads to a release of the TRAF-domain, which then acts as an inhibitor of NF-kappa B signaling [12].
In this study we provide evidence for a specific interaction of TRAF1 with IKK2 and we demonstrate that this molecular association is weaker than TRAF1 / TRAF2 interaction. Based on this observation and the findings that ectopic expression of TRAF1 can have both inhibitory and stimulatory effects on IKK2 and NF-kappa B activity, we propose a model, in which relative levels of TRAF1, TRAF2 and IKK2 are important for regulating the signaling activity of IKK2.

METHODS

Yeast two-hybrid screening was performed with the C-terminal part of IKK2 (amino acids 466 – 756) essentially as described [13] using a library from activated leukocytes. After identification of TRAF1 as potential binding partner, all the TRAF molecules (TRAF1-TRAF6; kindly provided by David Sassoon, [14]) were tested in the yeast two-hybrid system for interaction with the IKK2 bait. **Mammalian two-hybrid assays** were performed with the Matchmaker™ system provided by Clontech according to the instructions of the manufacturer with the exception that the pFR-Luc vector from Stratagene was used in combination with a **luciferase reporter gene assay** as described in [15].
Coimmunoprecipitations were carried out as specified in [16] using transfected HeLa cells and **kinase assays** were done as depicted in [13].

RESULTS AND DISCUSSION

Our aim was to identify interaction partners of IKK2 as a key enzyme for NF-kappa B activation. To that end, we performed a yeast two-hybrid screening with the C-terminal part of IKK2 as bait. This part contains a helix-loop-helix domain and a leucine zipper as potential protein interaction domains. Among various signaling molecules, we also identified an N-terminal fragment of TRAF1 (amino acids 1 – 228) as putative binding partner. Next, we tested, whether other members of the TRAF family are capable of interacting with IKK2 using yeast two-hybrid constructs for all TRAFs. In this system, only TRAF1 interacted with the IKK2-bait (Fig.1A). Testing IKK1 as bait in combination with all the TRAF molecules (TRAF1 – TRAF6) did not reveal any significant interaction (data not shown). The binding of IKK2 and TRAF1 could be verified in a mammalian two-hybrid reporter assay, demonstrating that the interaction was not an artifact of the yeast system (Fig. 1B). Furthermore, we could clearly demonstrate the interaction between full length IKK2 and full-length TRAF1 in human cells by applying co-immunoprecipitation experiments (Fig. 1C). Similar experiments with the N-terminal part (aa 1 – 228) of TRAF1 verified the results of the yeast two hybrid system that the N-terminal domain lacking the TRAF domain is sufficient for the interaction with IKK2 (data not shown). However, it has to be noted that also the TRAF-domain seems to be capable of interacting with the IKK-complex [12]. In contrast to the co-precipitation of TRAF1 with IKK2, we could not detect any co-immunoprecipitation of TRAF2 with IKK2. Moreover, co-expression of TRAF2 reduced the amount of TRAF1 that was coprecipitated with IKK2 indicating that

TRAF2 might compete with IKK2 for TRAF1 binding (Fig. 1C). This possibility was further tested by co-immunoprecipitation of TRAF1 and TRAF2, which revealed that these two molecules interact with each other at a rather high NaCl concentration of 500 mM (Fig. 1D). At this salt concentration, we could not detect any significant interaction between TRAF1 and IKK2 (data not shown) indicating that TRAF1/TRAF2 binding may be stronger than the association between TRAF1 and IKK2. As a consequence, it has to be expected that TRAF1 would rather bind TRAF2 as long as the later is not saturated. Specific interaction between IKK2 and TRAF1 but not other TRAF family members is supported by the fact that IKK2 interacts with the N-terminal part of TRAF1, which differs from all the other TRAF family members (Fig. 1e). The C-terminal interaction domain of IKK2 does not include the kinase domain, suggesting that it may still be accessible for substrates.

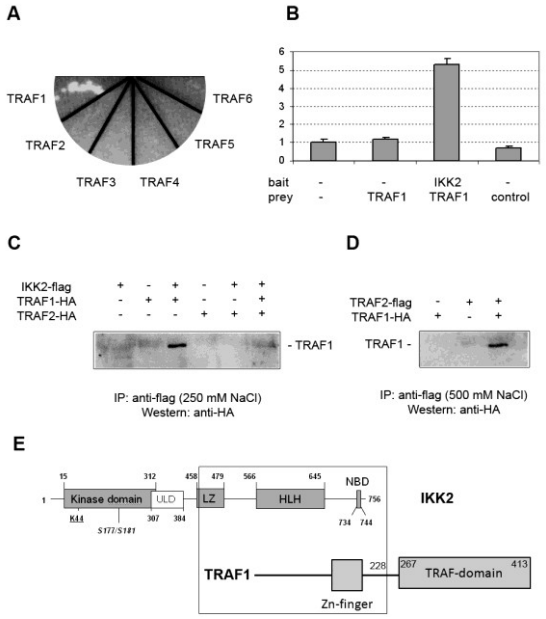

Fig. 1: IKK2 interacts specifically with TRAF1. **A)** Yeast two hybrid assay: The C-terminal domain of IKK2 interacts specifically with TRAF1 but not TRAF2 – TRAF6. **B)** Verification of the interaction in mammalian cells using a mammalian two-hybrid system. A Luciferase reporter assay was done with empty (-) bait or prey constructs, IKK2, TRAF1 or control as indicated. **C)** Coimmuno-precipitation of IKK2 and TRAF1 after transfection of HeLa cells with flag- or HA-tagged expression constructs as indicated and immunoprecipitation (IP) in presence of 250 mM NaCl. **D)** Coimmuno-precipitation of TRAF1 and TRAF2 in presence of 500 mM NaCl. **E)** Schematic illustration of the interaction domains as depicted by the overlapping rectangle. Amino acids 466-756 of IKK2 including a leucine zipper (LZ), a helix-loop-helix domain (HLH) and the NEMO binding domain (NBD) interact with amino acids 1 – 228 of TRAF1 (containing a Zn-finger).

Testing the effect of TRAF1 expression in NF-kappa B reporter gene assays revealed at first sight conflicting results. While TRAF1 clearly inhibited IKK2-mediated NF-kappa B activation in some experiments (Fig. 2A), some expression constructs also led to stimulation of NF-kappa B activity in other experiments (Fig. 2A right). This variable effect was also observed in kinase assays, where TRAF1 resulted either in a slight inhibition of IKK2 activity (Fig. 2B, upper part), or in a significant stimulation of the activity (Fig. 2B, lower part). Interestingly, we could observe that another IKK2-interacting protein (Rpn5) also led to a stimulation of IKK2 activity in the later case. A possible explanation for this phenomenon is that IKK2 interacting proteins might trigger clustering of IKK2 molecules and subsequent self-activation by auto-phosphorylation. According to this hypothesis the level of the IKK2 interacting molecules relative to the amount of IKK2 might be important for exerting either a stimulatory or an inhibitory effect. This model can also be applied to other interaction partners of TRAF1. Since TRAF2 is an important positively acting signaling molecule interacting with TRAF1, we also tested the effect of TRAF1 on TRAF2. Upon ectopic expression as a fluorescent fusion protein in cells, TRAF2 forms distinct clusters in the cytosol. This is most likely reflecting the inherent propensity of TRAF2 to trigger the oligomerization of other signaling molecules or kinases by self-interaction via the TRAF-domain. Co-expression of a CFP-tagged TRAF1 with YFP-tagged TRAF2 resulted in a dose dependent disaggregation of the TRAF2 clusters, revealing that TRAF1 has the capability of influencing the oligomerization of TRAF2 (Fig. 3) important for its signaling function.

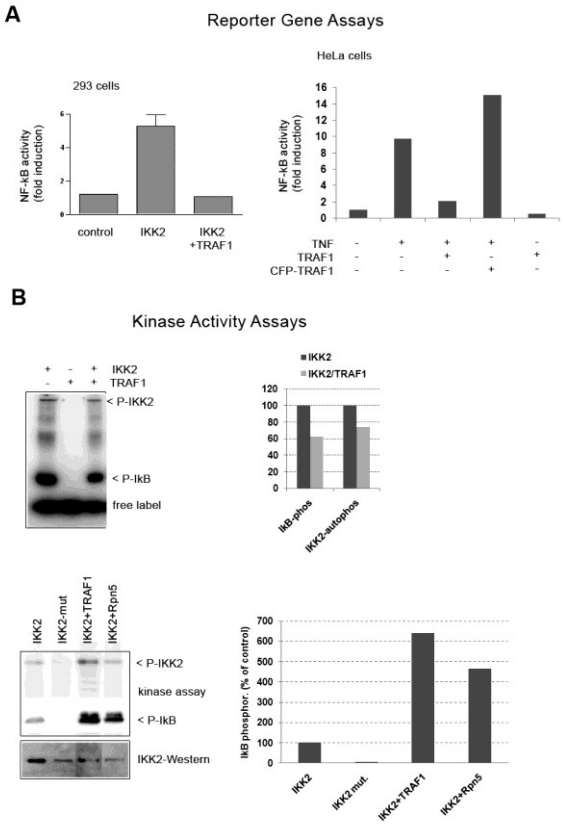

Fig. 2: TRAF1 can exert both inhibitory and stimulatory effects on IKK2 and NF-kappa B activity. A) Effect of TRAF1 in NF-kappa B reporter gene assays. Left panel: 293 cells were transfected with a NF-kappa B luciferase reporter and IKK2 in absence or presence of a TRAF1 expression construct. Right panel: HeLa cells were transfected with the NF-kappa B reporter and two different TRAF1 constructs as indicated followed by treatment with TNFα (50 ng/ml for 6 h). **B)** Effect of TRAF1 on IKK2 activity. Upper left panel: In vitro kinase assay using IKK2 immunoprecipitated from 293 cells transfected with IKK2 alone or in combination with TRAF1. Upper right: quantification of phosphorylated IκBα and auto-phosphorylated IKK2. Lower left panel: Kinase assay and Western Blot of IKK2 or mutant IKK2 (IKK2-mut) in combination with TRAF1 or the IKK2 interacting protein Rpn5. Lower right: Quantification of IκBα phosphorylation.

Of note, TRAF1 is transcriptionally upregulated by NF-kappa B [7, 9], and degradation of TRAF2 is triggered by the NF-kappa B signaling pathway [6, 7]. The combination of these two effects is expected to shift the

balance of the TRAF1/TRAF2 ratio and might therefore lead to a shift from TRAF1/TRAF2 complexes to TRAF1/IKK2 complexes. This has potential consequences for IKK2 and NF-kappa B activity dependent on the cellular context and the relative levels of TRAF1 and IKK2. Since TRAF1 does not contain a RING domain for K63-linked polyubiquitination, it might be expected that it is not an activator of the NF-kappa B pathway by itself, but rather an inhibitor. However, since TRAF1 binds other TRAF molecules which are activators of NF-kappa B signaling, it may also function as an activator in conjunction with these proteins at a certain stoichiometry. Moreover, binding of TRAF1 to IKK2 may also directly influence IKK2 activity by affecting proximity-induced auto-phosphorylation and self-activation of IKK2. Taken together it seems likely that TRAF1 exerts variable regulatory functions in NF-kappa B signaling dependent on the presence and relative levels of other signaling molecules and the cellular context.

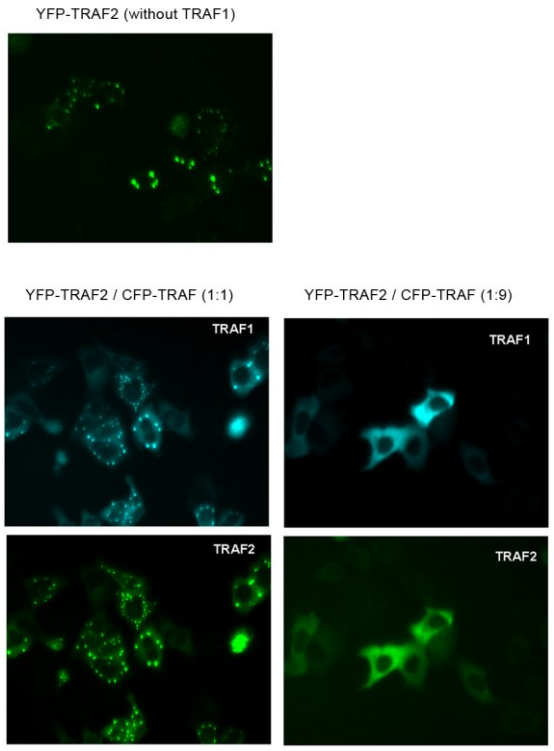

Fig. 3: TRAF1 influences the clustering tendency of TRAF2 in a dose dependent manner. YFP-tagged TRAF2 was expressed either alone or in combination with CFP-tagged TRAF1 in 293 cells at a ratio of 1:1 or 1:9 as indicated. A higher level of TRAF1 led to the disaggregation of TRAF2 clusters.

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