TRAF2 plays a dual role in NF-κB-dependent gene activation by mediating the TNF-induced activation of p38 MAPK and IκB kinase pathways

Isabelle Carpentier[a], Wim Declercq[a], Nikolai L. Malinin[b], David Wallach[b], Walter Fiers[a], Rudi Beyaert[a,*]

[a] *Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Ghent, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium*

[b] *Department of Membrane Research and Biophysics, The Weizmann Institute of Science, Rehovot, Israel*

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Abstract We previously demonstrated that p38 MAPK is a crucial mediator in the NF-κB-dependent gene activation induced by TNF. Here, we have studied the role of several TNF receptor-associated proteins and caspases in p38 MAPK activation by TNF. The latter appears to be dependent on TRAF2, but independent of FADD or caspases. Remarkably, p38 MAPK activation by TNF proceeds independently of the TRAF2-associated NF-κB-inducing kinase NIK, which is known to bind and activate two recently identified IκB kinases. These results demonstrate that two kinase pathways involved in NF-κB regulation, viz. NIK and p38 MAPK-mediated, diverge at the level of TRAF2.

Key words: Tumor necrosis factor receptor-associated factor 2; p38 mitogen-activated protein kinase; Nuclear factor κB

1. Introduction

Tumor necrosis factor (TNF) elicits its wide spectrum of cellular responses by binding to two distinct receptors, p55 (TNFR1) and p75 (TNFR2), the former mediating most TNF activities [1]. Recently, our understanding of the TNF-induced signaling pathways leading to gene expression or to apoptosis has considerably increased with the isolation and characterization of several TNFR-associated molecules. Activation of TNFR1 results in recruitment of a TNFR-associated death domain (TRADD) protein, which directly interacts with Fas-associated death domain (FADD) protein and TNFR-associated factor (TRAF) 2 [2–4]. FADD signals to apoptosis by binding to caspase-8, which is believed to activate a proteolytic cascade of caspases [5,6]. On the other hand, TRAF2 signals to nuclear factor (NF) κB by binding to NF-κB-inducing kinase (NIK), a mitogen-activated protein kinase (MAPK) kinase kinase-related molecule that forms a complex with IκB kinase (IKK) α and β [7–9]. The latter are directly responsible for activation of NF-κB by phosphorylating the NF-κB inhibitory protein IκB (reviewed in [10]). In addition, it was recently shown that TRAF2 also mediates the TNF-induced activation of the stress-activated protein kinase JNK [9,11]. However, in contrast to the TRAF2-mediated activation of IKK, JNK activation via TRAF2 is independent of NIK and does not seem to play any role in NF-κB-dependent gene induction by TNF [9]. p38 MAPK is another stress-activated protein kinase that is activated by TNF and stress stimuli [12]. We recently demonstrated that p38 MAPK is a crucial mediator in TNF-induced activation of NF-κB-dependent gene expression [13]. Because of the differential role of JNK and p38 MAPK in NF-κB activation, and the role of distinct MAPK kinases in their activation [12], we decided to analyze the role of several well-defined TNF receptor-associated signaling molecules in TNF-induced p38 MAPK activation.

Our results demonstrate that p38 MAPK is specifically activated by a TRAF2-dependent pathway, but which is totally independent of the FADD- and caspase-mediated pathways. Moreover, p38 MAPK activation was independent of TRAF2-associated NIK, suggesting that at least two kinase pathways involved in NF-κB regulation diverge at the level of TRAF2.

2. Materials and methods

2.1. Cells and reagents

HEK 293T and HeLa H21 cells were cultured in DMEM, and KYM cells were cultured in RPMI, each supplemented with 2 mM L-glutamine, 10% FCS (or 5% FCS and 5% newborn calf serum for HeLa H21), 10^6 U/l streptomycin, 100 mg/l penicillin and 0.4 mM sodium pyruvate. HeLa H21 cells stably expressing CrmA have been described previously [14]. SB 203580 was generously provided by Dr. J.C. Lee (SmithKline Beecham, King of Prussia, PA) and was dissolved in DMSO. Glutathione-coupled Sepharose beads were obtained from Pharmacia Biotech (Uppsala, Sweden). GST-ATF2(1–96) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A β-galactosidase assay kit originated from ICN Pharmaceuticals (Costa Mesa, CA). z-VAD-fmk was from Enzyme Systems Products (Dublin, CA). DEVD-AMC and YVAD-AMC were from Peptide Institute (Osaka, Japan).

2.2. Plasmids and antibodies

pBEG-GSTp38 was obtained from Dr. L.E. Zon (Harvard Medical School, Boston, MA [15]). pRK5-TRAF2 and pRK5-TRAF2(98–501) were a generous gift from Dr. D.V. Goeddel (Tularik, San Francisco, CA [3]). pNFconluc, which carries a luciferase gene under the control of a minimal promoter preceded by three NF-κB-binding sites, was a gift from Dr. A. Israël (Institut Pasteur, Paris, France [16]). pCDNA3-FADD, pCDNA3-FADD(80–205), pCDNA3-NIK and pCDNA3-NIK(KK429–430AA) were described previously [4,7]. pUT651, a β-galactosidase expression plasmid, was obtained from Eurogentec (Seraing, Belgium). Anti-glutathione *S*-transferase (GST)

*Corresponding author. Fax: (32) (9) 264-5348.
E-mail: RUDI@LMB.RUG.AC.BE

Abbreviations: AMC, 7-amino-4-methylcoumarin; FADD, Fas-associated death domain; GST, glutathione *S*-transferase; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; NF, nuclear factor; NIK, NF-κB-inducing kinase; TNFR, tumor necrosis factor receptor; TRAF, TNF receptor-associated factor

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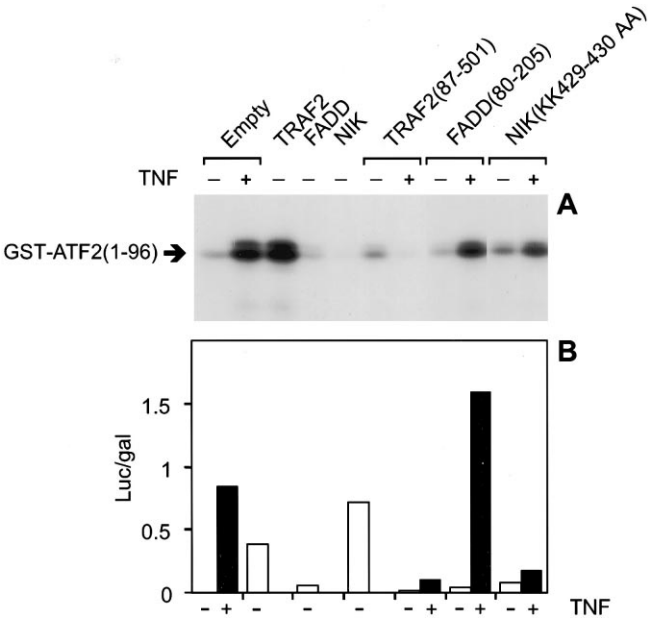

Fig. 1. Effect of overexpression of wild-type and dominant-negative mutants of TNFR-associated proteins on p38 MAPK and NF-κB activity. HEK 293T cells were transiently cotransfected with pBEG-GSTp38, pUT651, pNFconluc, and a plasmid expressing a TNFR-associated protein or an empty vector. 48 h after transfection, cells were either untreated or treated for 15 min (A) or 6 h (B) with 5000 IU/ml TNF before harvest, and analyzed for p38 MAPK activity on GST-ATF2 (A), or for NF-κB-dependent expression of a luciferase reporter gene (B). Results obtained for TNF-treated samples are shown in black bars. Equal expression of GST-p38 was verified by immunoblot analysis with anti-GST antiserum. Luciferase activities were normalized on the basis of β-galactosidase expression. Results shown are representative of three independent experiments. S.D. on luciferase expression was $<10\%$.

antiserum was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-p38 MAPK phospho-specific antiserum was from New England Biolabs (Beverly, MA).

2.3. Transient transfection and in vitro immunocomplex p38 MAPK assay

10^6 HEK 293T cells were seeded in 85-mm Petri dishes. 20 h later cells were transfected, using the calcium-phosphate precipitation method, with 0.5 μg pUT651, 0.5 μg pNFconluc, 1 μg pBEG-GSTp38 and 6 μg empty vector or a plasmid expressing a TNFR-associated protein. 48 h later, cells were either left untreated or treated with 5000 IU/ml TNF. Cell lysates were prepared and p38 MAPK activity was analyzed by an in vitro immunocomplex kinase assay with 1 μg GST-ATF-2(1–96) as a substrate [17]. After 30 min incubation at 30°C, the kinase reaction was terminated by adding 5× Laemmli sample buffer. Phosphorylated proteins were resolved by 10% SDS-PAGE, visualized by autoradiography and quantitated by phosphor imaging analysis. NF-κB-dependent reporter gene expression was parallelly analyzed as described previously [13] and normalized for β-galactosidase activity.

2.4. Caspase activity assay

10^6 KYM cells or HeLa H21 cells were seeded in 6-well plates and treated with TNF for 5 h. Some samples were pretreated for 2 h with 10 μM SB 203580. Cells were washed twice with PBS and resuspended in lysis buffer (25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-cyclohexanediaminetetraacetic acid, 10% glycerol and 1% Triton X-100), supplemented with protease inhibitors (1 mM Pefabloc and 20 μg/ml aprotinin). Lysates were centrifuged at $13\,000\times g$; cleared supernatants were collected for enzymatic assays. Aliquots (20 μg) of the lysates were incubated with 50 μM YVAD-AMC or DEVD-AMC for 60 min in a caspase reaction buffer, comprising 10 mM HEPES/NaOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM sodium pyruvate, 0.1 mM PMSF and 1 mM DTT. Protease activities were determined by spectrofluorometry (Cytofluor; PerSeptive Biosystems, Cambridge, MA), monitoring the release of 7-amino-4-methylcoumarin (AMC) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

3. Results

3.1. TNF-induced p38 MAPK activation is mediated by TRAF2 but not by FADD or NIK

To investigate the role of specific TNFR-associated proteins in TNF-induced p38 MAPK activation, HEK 293T cells were cotransfected with pBEG-GSTp38 encoding GST-tagged human p38 MAPK [15], and a mammalian expression plasmid encoding either TRAF2, NIK, FADD or dominant-negative acting mutants of these molecules, viz. TRAF2(98–501), FADD(80–205) or NIK(KK429–430AA), respectively [3,4,7]. 48 h after transfection, GST-p38 was recovered using glutathione-coupled Sepharose beads, after which p38 MAPK activity was analyzed using a solid-phase kinase assay in which GST-ATF2(1–96) was used as a substrate. In the case of transfection with dominant-negative mutants, cells were treated for 15 min with 5000 IU/ml TNF before cell lysis. FADD overexpression had no effect on p38 MAPK, while the latter was strongly activated by TRAF2 overexpression (Fig. 1A). Remarkably, the TRAF2-associated protein NIK was unable to activate p38 MAPK. In line with the above results, p38 MAPK activation by TNF was specifically inhibited by overexpression of a TRAF2 dominant-negative mutant, while a dominant-negative mutant of FADD or NIK had no effect. Similar results were obtained when p38 MAPK activation was scored by measuring p38 MAPK phosphorylation with p38 MAPK phospho-specific antibodies (data not shown). These results show that TNF activates p38 MAPK by a TRAF2-dependent, but NIK-independent pathway. We also performed parallel experiments in which an NF-κB-responsive luciferase reporter gene was cotransfected to verify the effect of the expressed TNFR-associated

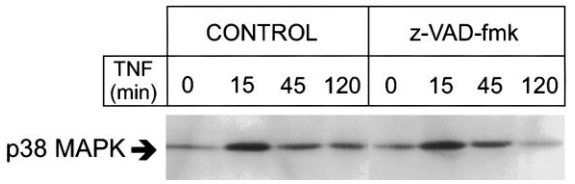

Fig. 2. Inhibition of caspases has no effect on TNF-induced activation of p38 MAPK. HeLa H21 cells were either untreated or treated for 2 h with 10 μM z-VAD-fmk before stimulation with 2000 IU/ml TNF. Cells were harvested, and 50 μg of total cellular protein was analyzed by SDS-PAGE and immunoblot analysis with p38 MAPK phospho-specific antibodies.

proteins on TNF-induced NF-κB activation. As expected, overexpression of either TRAF2 or NIK led to NF-κB activation, whereas TRAF2(98–501) or NIK(KK429–430AA) each blocked NF-κB activation by TNF (Fig. 1B). In conclusion, our results point to a bifurcation of NIK- and p38 MAPK-mediated pathways leading to NF-κB activation at the level of TRAF2.

3.2. Caspase inhibitors have no effect on TNF-induced p38 MAPK activation

Caspases play an important role in TNF- and Fas-mediated apoptosis. Recently, it was reported that Fas-activated caspases act both upstream and downstream of the p38 MAPK kinase MKK-6 [18], suggesting that p38 MAPK and caspases function along the same pathway. Our finding that p38 MAPK activation by TNF is FADD-independent suggests that at least caspases triggered by the FADD/caspase-8 pathway are not involved in p38 MAPK activation. In order to clarify the possible regulatory relationship between p38 MAPK and caspases during TNF signalization, we analyzed p38 MAPK activation by TNF in HeLa cells stably expressing the viral caspase inhibitor CrmA, or which had been pretreated for 2 h with 10 μM of the caspase inhibitor peptide z-VAD-fmk. It has previously been shown that CrmA expression or pretreatment with z-VAD-fmk protects these cells against TNF-induced apoptosis [14]. Cells were lysed at different times of TNF treatment, and p38 MAPK activation was measured by analyzing p38 MAPK phosphorylation with phospho-specific antibodies on a Western blot (Fig. 2). p38 MAPK was maximally activated 15 min after TNF stimulation. However, neither CrmA expression nor z-VAD-fmk pretreatment had any effect on p38 MAPK phosphorylation (only data for z-VAD-fmk-treated cells are shown), although the cells were completely protected against TNF-induced apoptosis (data not shown). Similar p38 MAPK data were obtained in L929 cells. These results demonstrate that TNF-induced activation of p38 MAPK is caspase-independent.

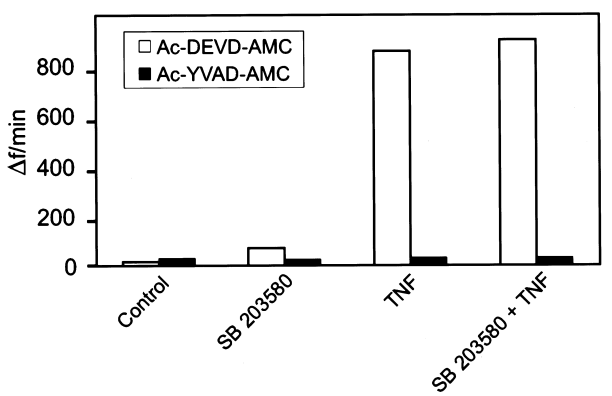

Fig. 3. SB 203580 does not affect TNF-induced caspase activity. KYM cells were either untreated or treated for 2 h with 10 μM SB 203580 before stimulation with 2000 IU/ml TNF for 5 h. Cells were harvested, and 20 μg of total cellular protein was analyzed for proteolytic activity on DEVD-AMC and YVAD-AMC. Data are expressed as an increase in fluorescence (F) as a function of time (ΔF/min).

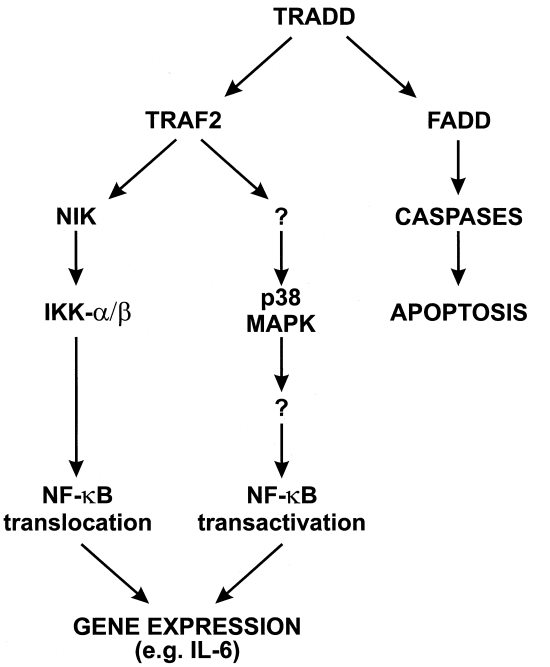

Fig. 4. Model proposed for TNFR1-initiated signaling pathways leading to p38 MAPK and NF-κB activation, showing a bifurcation at the level of TRADD and TRAF2.

3.3. TNF-induced caspase activation is p38 MAPK-independent

To investigate whether caspases might act downstream of p38 MAPK, we studied the effect of the p38 MAPK-specific inhibitor SB 203580 on TNF-induced activation of caspases. HeLa H21 and KYM cells were treated for 2 h with 10 μM SB 203580 before TNF treatment. This concentration has previously been shown to inhibit completely TNF-induced p38 MAPK activity and activation of an NF-κB-responsive reporter gene [13]. HeLa H21 cells were sensitized to TNF by treatment with cycloheximide as described previously [14]. After treatment with TNF for 5 h, the caspase activity in cell lysates was measured on the fluorogenic peptide substrates DEVD-AMC or YVAD-AMC, which are specifically cleaved by caspase-3 and caspase-1 family proteases, respectively [19]. In both cell lines, TNF treatment resulted in a significant increase in caspase-3-like activity on DEVD-AMC, while no activity could be detected on YVAD-AMC. However, pretreatment with SB 203580 had no effect on TNF-induced caspase activity (Fig. 3; only data for KYM cells are shown). These results further demonstrate that caspases and p38 MAPK act along totally independent TNF-signaling pathways.

4. Discussion

We have characterized the role of several TNF receptor-associated proteins in TNF-induced activation of p38 MAPK and conclude that there is a bifurcation of the TNF signal leading to p38 MAPK at the level of both TRADD and TRAF2 (Fig. 4). TRAF2 has been implicated in TNF-induced NF-κB activation by interacting with the MAPK kinase kinase related protein NIK, which is part of an IKK complex [10]. Our finding that TRAF2 mediates p38 MAPK activation by TNF, as well as the fact that p38 MAPK plays a crucial role in TNF-induced activation of an NF-κB-dependent reporter gene [13], could suggest that NF-κB activation was mediated by a signaling pathway consisting of TRAF2-NIK-p38 MAPK. However, whereas TRAF2 is involved in TNF-induced activation of both p38 MAPK and NF-κB, we could not obtain any evidence for a role of NIK in p38 MAPK activation by TNF. This suggests the existence of two different signals leading to NF-κB-dependent transcription, the first being NIK-mediated and the second p38 MAPK-mediated; these pathways would diverge at the level of TRAF2. Similar to p38 MAPK, also activation of JNK has recently been shown to be fully dependent on TRAF2 [9,11,20]. Divergence of pathways leading to p38 MAPK and to JNK is likely to occur after TRAF2, presumably at the level of MAPK kinases, such as MKK-3/6 and MKK-4/7, respectively. The observation that p38 MAPK and JNK have partially different substrate specificities might explain the specific role of p38 MAPK in NF-κB activation [12]. While this article was in preparation, it was reported that TNF still activates NF-κB in TRAF2-deficient cells, suggesting the existence of redundancy within the TRAF family or the role of other pathways [20].

What might be the functional role of the p38 MAPK and NIK pathways diverging at TRAF2? NIK was recently found to associate with and to activate IKK-α and -β, which directly phosphorylate the NF-κB inhibitory protein IκB. These modifications lead to the targeted degradation of IκB-α and -β via the ubiquitin-proteasome pathway and subsequent translocation of NF-κB into the nucleus. In contrast, p38 MAPK selectively modulates the transactivation potential of NF-κB without interfering with IκB phosphorylation and NF-κB translocation [13,17]. This is further suggested by the finding that the specific p38 MAPK inhibitor SB 203580 also inhibits the transactivation potential of the p65 NF-κB subunit in a mammalian one-hybrid assay [21]. TNF-induced phosphorylation of p65 is apparently not affected by SB 203580 [2], suggesting that the p65 subunit is not a direct substrate of p38 MAPK. Alternatively, p38 MAPK might phosphorylate and activate other factors, including AP-1, p300/CBP, SRF, ATF-2, steroid receptors and TFII-D, which physically and functionally interact with NF-κB, and thus might cooperate in directly activating NF-κB by a TRAF2/NIK-dependent pathway.

Our observation that a FADD dominant-negative acting mutant and several caspase inhibitors have no effect on p38 MAPK activation by TNF, as well as the p38 MAPK-independent activation of caspases, also clearly demonstrates that p38 MAPK and caspases act along totally independent TNF-activated pathways. This seems to be different from Fas-mediated signaling, in which caspases act both upstream and downstream of p38 MAPK [18]. The signaling role of p38 MAPK activation after Fas stimulation might therefore be rather different from its role in TNF signaling.

In conclusion, we have demonstrated that the TNF-signaling pathway leading to p38 MAPK activation and NF-κB-dependent gene activation is totally independent of caspases, and diverges not only at the level of TRADD, but also at the level of TRAF2. Experiments to identify the mediator linking TRAF2 to p38 MAPK are currently in progress.

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