

The cytokine-inducible zinc finger protein A20 inhibits IL-1-induced NF- κ B activation at the level of TRAF6

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Abstract The zinc finger protein A20 is encoded by an immediate early response gene whose expression is induced by different inflammatory stimuli, including interleukin-1 (IL-1). Gene induction by IL-1 is mediated by activation of the transcription factor NF- κ B, and requires the signal adapter protein TRAF6. The latter interacts with the NF- κ B-inducing kinase NIK, which is believed to be part of the I κ B kinase complex. Expression of A20 potently inhibits IL-1-induced NF- κ B activation by an unknown mechanism. Inhibition of IL-1-induced NF- κ B activation was found to be mediated by the C-terminal zinc finger-containing domain of A20. More importantly, we present evidence that A20 interferes with IL-1-induced NF- κ B activation at the level of TRAF6, upstream of NIK. Moreover, A20 was shown to directly interact with TRAF6.

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Key words: Gene expression; Interleukin-1; Nuclear factor κ B; TRAF6; Zinc finger

1. Introduction

The transcription factor NF- κ B resides in an inactive state as a cytosolic protein and is activated by different proinflammatory cytokines including interleukin-1 (IL-1). IL-1 signalling is initiated by the formation of the high-affinity complex composed of IL-1, IL-1 receptor (IL-1R) and IL-1R accessory protein (IL-1RAcP). This is followed by the recruitment of the adapter protein MyD88 and the IL-1 receptor-associated kinase (IRAK) to the receptor complex [1–4]. Then IRAK interacts with TRAF6, which finally results in NF- κ B activation [5]. TRAF6 is a member of a new family of signalling proteins that is characterised by the presence of a conserved TRAF domain of approximately 230 amino acids in the C-terminal portion, and multiple zinc finger motifs within the N-terminal half. The TRAF domain mediates homotypic and heterotypic interactions and is also involved in binding to other signalling molecules including the NF- κ B-inducing kinase NIK [6]. The latter then interacts with and activates the I κ B kinases IKK α and IKK β which are part of a multiprotein I κ B kinase complex, leading to the phosphorylation, ubiquitination and subsequent degradation of the NF- κ B inhibitory subunit I κ B [7–9]. This allows NF- κ B translocation to the nucleus and activation of the transcription of several genes involved in inflammation, immune responses, lymphoid differentiation, growth control and development.

Previously, the cytokine-inducible zinc finger protein A20 was shown to block IL-1-mediated NF- κ B activation by an unknown mechanism [10]. We could demonstrate that inter-

action of A20 with 14-3-3 proteins is not required for its NF- κ B-inhibiting activity [11]. To better understand how and at what level A20 interferes with IL-1-induced signal transduction leading to NF- κ B activation, we made use of the ability to activate NF- κ B activation by overexpression of specific signalling proteins involved in IL-1-induced NF- κ B activation. Our results clearly demonstrate that A20 blocks IL-1-induced NF- κ B activation upstream of NIK, most likely via a direct interaction with TRAF6.

2. Materials and methods

2.1. Cell lines and reagents

Human embryonic kidney cells (293T) were a kind gift of Dr M. Hall (Department of Biochemistry, University of Birmingham, UK). These cells were cultured in DMEM supplemented with 10% FCS, 0.4 mM sodium pyruvate and antibiotics.

Recombinant human tumour necrosis factor (TNF) was expressed in *Escherichia coli* and had a specific biological activity of 8.8×10^6 IU/mg purified protein, as determined with the International Standard (code 88/532) (National Institute for Biological Standards and Control, Potters Bar, UK). Recombinant murine IL-1 β was produced by *E. coli* and had a specific activity of 3.65×10^8 IU/mg purified protein, as determined with the International Standard (code 93/668) (National Institute for Biological Standards and Control, Potters Bar, UK).

Anti-Flag tag antibody was purchased from Eastman Kodak Company (New Haven, CT, USA), polyclonal anti-TRAF2 antibody from Immunotech (Marseille, France), polyclonal anti-GFP from Clontech (Palo Alto, CA, USA), monoclonal anti-E-tag horseradish peroxidase linked from Pharmacia Biotech (Uppsala, Sweden), protein A on Tris-acryl beads from Pierce Chemicals (Rockford, IL, USA), and anti-rabbit Ig antibody as well as ECL Western blot detection kit from Amersham Life Sciences (Amersham, UK).

2.2. Expression plasmids

The plasmid pCAGGS-GFP was made by inserting the GFP-encoding cDNA including a multiple cloning site from the pGFP-CI plasmid (Clontech, Palo Alto, CA) in the *EcoRI-MscI* sites of the eukaryotic expression plasmid pCAGGS [12]. In the GFP-cDNA a S65T mutation was inserted to enhance fluorescence. In the *SmaI* site of the multicloning site inserted in the GFP-cDNA, a blunted *NcoI-EcoRI* fragment of mA20 was cloned in frame with the C-terminus of GFP, resulting in the vector pCAGGS-GFP/A20. Also the *NcoI-FspI* fragment of A20 encoding the N-terminal amino acids (1–369) and the *FspI-EcoRI* fragment encoding the C-terminal amino acids (370–775) were cloned in frame with GFP, resulting in the plasmids pCAGGS-GFP/A20(ZF–) and pCAGGS-GFP/A20(ZF+), respectively.

The plasmid pNFconluc contains the luciferase reporter gene driven by a minimal NF- κ B-responsive promoter and was a kind gift of Dr A. Israel (Institut Pasteur, Paris, France) [13]. The β -galactosidase-encoding plasmid pUT651 was obtained from Cayla (Toulouse, France). Expression plasmids for TRAF1, TRAF2, Flag-tagged TRAF6 (gifts from Dr D. Goeddel, Tularik, San Francisco, CA, USA) and NIK (gift from Dr D. Wallach, Weizmann Institute of Science, Rehovot, Israel) have been described previously [5,6,14].

2.3. NF- κ B reporter gene assay

293T cells were grown in 6-well plates and transfected by DNA

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calcium phosphate precipitation [15] with 100 ng pUT651, 100 ng pNFconluc, 700 ng empty vector pCAGGS and 100 ng pCAGGS-GFP or pCAGGS-GFP/A20. After transfection cells were seeded in 24-well plates. Another 24 h later cells were either stimulated with 1000 IU/ml TNF or 7000 IU/ml IL-1 for 6 h or left untreated, followed by lysis of the cells in Tris-phosphate (25 mM pH 7.8), DTT (2 mM), CDTA (2 mM), glycerol (10%) and Triton X-100 (1%). Luciferase and β -galactosidase activities were measured as described previously [11].

When NF- κ B activation by overexpression of TRAF6 and NIK was analysed, cells were immediately seeded in 24-well plates and transfected with 20 ng pNFconluc, 20 ng pUT651, 20 ng plasmids encoding TRAF6 or NIK, 100 ng pCAGGS-GFP or pCAGGS-GFP/A20 and 40 ng empty vector. 24 h after transfection cells were lysed and processed as described above.

2.4. Coimmunoprecipitation and Western blotting

10⁶ 293T cells were grown in 10-cm Petri dishes and transiently transfected with in total 5 μ g plasmid DNA using the DNA calcium phosphate coprecipitation method as described [15]. 24 h after transfection cells were lysed in Tris-HCl (20 mM pH 7.5), Triton X-100 (1%), NaCl (137 mM), MgCl₂ (1.5 mM) and EGTA (1 mM). Immunoprecipitation was performed with polyclonal anti-TRAF2 antibody or monoclonal anti-Flag tag antibody and binding to protein A Tris-acyl beads. Beads were washed four times with lysis buffer and binding proteins were analysed by 10% SDS-PAGE and Western blotting onto nitrocellulose membranes. Detection of coimmunoprecipitating proteins was achieved with monoclonal anti-E tag antibody coupled to horseradish peroxidase and an ECL Western blotting detection kit according to the manufacturer's instructions.

3. Results

3.1. A20 inhibits IL-1-induced NF- κ B activation via its C-terminal zinc finger containing domain

The A20 protein consists of seven Cys₂/Cys₂ zinc fingers in the C-terminal half of the molecule [16]. By the use of N- and C-terminal deletion mutants, the C-terminal zinc finger-containing domain of A20 has previously already been implicated in the inhibitory effect of A20 on TNF-induced NF- κ B activation [17]. To investigate if the same domain of A20 also mediates inhibition of the IL-1 signal transduction pathway to NF- κ B, we transiently transfected 293T cells with an NF- κ B-dependent reporter gene and expression plasmids for A20, its N-terminal (A20(ZF-)) or its C-terminal (A20(ZF+)) frag-

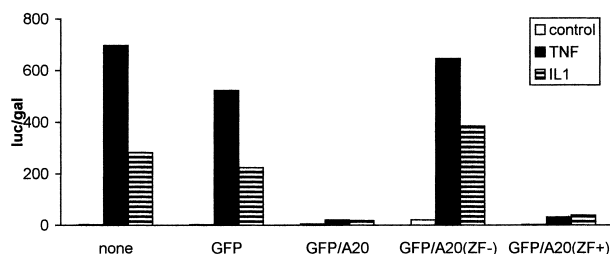


Fig. 1. Effect of overexpression of wild-type or N- and C-terminal deletion mutants of A20 on NF- κ B activation induced by TNF and IL-1. 293T cells were transiently transfected with an NF- κ B-dependent luciferase reporter gene (luc) and a β -galactosidase (gal) expression plasmid as described in Section 2. 24 h later, cells were stimulated with TNF (1000 IU/ml) or IL-1 (7000 IU/ml) during 6 h. The effect of A20 was analysed by cotransfection of GFP/A20 or fragments corresponding to the N-terminal (GFP/A20(ZF-)) or C-terminal half (GFP/A20(ZF+)) of A20. GFP was cotransfected as a negative control. Luciferase activity present in cell extracts was used as a parameter for NF- κ B activation, and is expressed as luc/gal to normalise transfection efficiencies. Data ($n=3$) are from a representative experiment with standard deviations less than 10%.

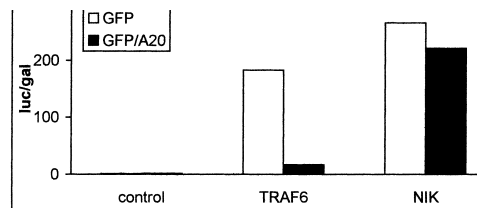


Fig. 2. Effect of A20 overexpression on NF- κ B activation induced by overexpression of TRAF6 and NIK. Cells were transfected with a TRAF6 or NIK expression plasmid, a β -galactosidase expression plasmid and an NF- κ B-dependent luciferase reporter gene. The effect of A20 was analysed by cotransfection with an expression plasmid for GFP/A20. Cotransfection with GFP served as a negative control. Cells were lysed 24 h after transfection and analysed for luciferase (luc) and β -galactosidase (gal) activity. Results from a representative experiment are expressed as luc/gal to correct for differences in transfection efficiency, and are mean values ($n=3$) with standard deviations less than 10%.

ments corresponding to amino acids 1–369 and 370–775, respectively. In all cases A20 was fused to GFP which allows detection via Western blotting. Transfected cells were treated with TNF or IL-1 for 6 h and analysed for NF- κ B activation by measuring the NF- κ B-responsive expression of the luciferase reporter gene. NF- κ B activation by TNF or IL-1 was maximal at concentrations of 1000 IU/ml and 7000 IU/ml, respectively, but could already be obtained at a TNF or IL-1 concentration as low as 50 IU/ml (data not shown). As expected, overexpression of the fusion protein GFP/A20 in 293T cells clearly prevented TNF-induced and IL-1-induced NF- κ B activation whereas overexpression of GFP as such had no effect (Fig. 1). When only the C-terminal part of A20 was expressed (GFP/A20(ZF+)), NF- κ B activation was equally well repressed. In contrast, overexpression of the N-terminal

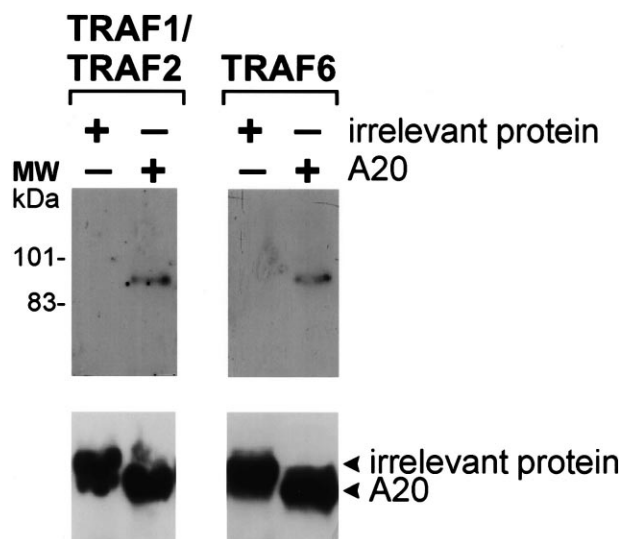


Fig. 3. Coimmunoprecipitation of A20 with TRAF6 and TRAF2/ TRAF1. 293T cells were transiently transfected with E-tagged A20 or an irrelevant E-tagged protein (p110 PITSLRE kinase) together with Flag-tagged TRAF6 or a combination of TRAF2 and TRAF1. Immunoprecipitation (upper panel) was performed with anti-Flag tag antibody or anti-TRAF2 antibody, respectively, and coimmunoprecipitating proteins were revealed by Western blot detection with anti-E tag antibody. To control expression levels of E-tagged A20 and the irrelevant protein, 10 μ l total cell lysates were subjected to 10% SDS-PAGE and immunoblotted with anti-E tag antibody (lower panel).

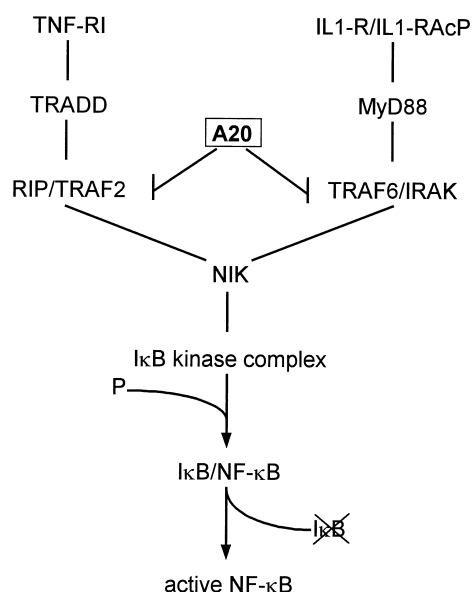


Fig. 4. Overview of TNF- and IL-1-induced signalling pathways leading to I κ B degradation and activation of NF- κ B. In the case of TNF-RI, which is the main NF- κ B-activating TNF receptor, signalling starts with the recruitment of TRADD, which serves as an adapter protein for two other components TRAF2 and RIP. These also associate with each other, possibly stabilising the complex [14,27,28]. IL-1R stimulation leads to the recruitment of MyD88 and IRAK to the IL-1 receptor complex. IRAK associates with TRAF6. TRAF2 and TRAF6 both bind with NIK which functions as a direct activator of the I κ B kinase complex. The I κ B kinases of this complex directly phosphorylate I κ B, leading to its degradation and NF- κ B activation. Interaction of A20 with TRAF2/6 might be involved in inhibition of TNF- and IL-1-induced NF- κ B activation.

part of A20 (GFP/A20(ZF-)) had no effect at all on both TNF-induced and IL-1-induced NF- κ B activation. Western blotting with anti-GFP antibodies revealed that A20 and its N- and C-terminal deletion mutants were in all cases equally well expressed (data not shown). These results demonstrate that upon overexpression the zinc finger-containing domain of A20 is sufficient for inhibition of TNF-induced as well as IL-1-induced NF- κ B activation.

3.2. A20 inhibits NF- κ B upstream of NIK

As already mentioned in Section 1, the mitogen-activated protein kinase kinase kinase NIK, which interacts with all TRAF proteins, has been placed downstream of TRAF6 in the IL-1-initiated NF- κ B signalling pathway [6]. Overexpression of TRAF6 or NIK is sufficient to induce NF- κ B activation. This allowed us to investigate at which point in the IL-1-induced signalling pathway A20 could prevent NF- κ B activation. Therefore, 293T cells were transiently transfected with TRAF6 or NIK in combination with the expression plasmids of GFP or GFP/A20 and an NF- κ B-responsive reporter plasmid. NF- κ B-dependent reporter gene expression was measured after 24 h. As expected, GFP expression had no effect on TRAF6-induced or NIK-induced NF- κ B activation (Fig. 2). In contrast, GFP/A20 strongly inhibited NF- κ B activation induced by overexpression of TRAF6. Interestingly, GFP/A20 was not able to block NIK-induced NF- κ B activation. These results clearly indicate that A20 interferes upstream of NIK in the IL-1 signal transduction pathway to NF- κ B, presumably at the level of TRAF6 itself.

3.3. A20 interacts with TRAF6

To further substantiate our hypothesis that A20 prevents IL-1-induced NF- κ B activation at the level of TRAF6, we investigated if this inhibition could be mediated by a direct interaction of A20 with TRAF6. In this respect, A20 has previously already been shown to associate with the TRAF1/TRAF2 complex in the TNF signalling pathway [17]. Therefore 293T cells were transiently transfected with expression plasmids encoding Flag-tagged TRAF6 and E-tagged A20 or an E-tagged irrelevant protein which served as a negative control. Cell lysates were immunoprecipitated with a monoclonal anti-Flag tag antibody and then immunoblotted with anti-E-tag antibodies coupled with horseradish peroxidase. This analysis demonstrated that A20 is able to bind TRAF6 (Fig. 3) further indicating that A20 prevents IL-1-induced NF- κ B activation by directly interacting with TRAF6. When we transfected TRAF2 and TRAF1 instead of TRAF6 and made the immunoprecipitate with a polyclonal anti-TRAF2 antibody, specific interaction of A20 with TRAF1/TRAF2 complex could be demonstrated as described previously [17]. Therefore, A20 is likely to prevent TNF-induced and IL-1-induced NF- κ B activation at the level of TRAF2 and TRAF6, respectively.

4. Discussion

The transcription factor NF- κ B becomes activated in several cell lines upon stimulation with the cytokines TNF and IL-1, and is responsible for the transcription of many proinflammatory genes. Its activation can be negatively regulated by the NF- κ B-associating protein I κ B and by the zinc finger protein A20. Both are TNF- and IL-1-inducible proteins whose expression is itself NF- κ B-dependent, resulting in a negative feedback regulation [18,19]. Whereas I κ B inhibits NF- κ B activation by sequestration of latent NF- κ B in an inactive form in the cytoplasm, the mechanism by which A20 abrogates NF- κ B activation is still unknown.

Recently, the signal transduction pathways leading to NF- κ B activation by TNF and IL-1 have been largely elucidated. Whereas the upstream proteins involved in these signalling pathways are different in the case of TNF and IL-1, both pathways converge at the NF- κ B-inducing kinase NIK (Fig. 4) [6]. Our results show that A20 blocks IL-1-induced NF- κ B activation upstream of NIK, most likely at the level of TRAF6. This is further suggested by our observation that A20 can directly interact with TRAF6. Similarly, A20 has been shown previously to interact with the TRAF1/TRAF2 complex, suggesting that the latter interaction might also be involved in the A20-mediated inhibition of NF- κ B activation induced by TNF [17]. The C-terminal zinc finger domain of A20 was shown to be responsible for NF- κ B inhibition. In contrast, the N-terminal domain of A20 has been shown previously to interact with TRAF2 [17]. Although we did not analyse whether this part of A20 also mediates its interaction with TRAF6, these interactions would fit with a molecular mechanism in which A20 is recruited to the NF- κ B activation cascade via its binding with TRAF2 or TRAF6. At this level A20 could prevent NF- κ B activation by disrupting essential protein-protein interactions such TRAF2-RIP, TRAF6-IRAK, TRAF2/6-TRAF2/6 or TRAF2/6-NIK. On the other hand, A20 might interfere with the activation of NF- κ B, by preventing the TRAF-mediated activation of p38 MAP ki-

nase, which has been shown previously to be involved in the transactivation of NF- κ B [20,21]. Recently, in some cell lines TRAF2 has been shown to localise in the nucleus where it can directly regulate transcription [22]. Since A20 is located in the cytosol (own unpublished results, [23]), A20 could retain TRAF2, and most likely also TRAF6, in the cytosol. Finally, we cannot exclude that A20 might still interact with other proteins which are responsible for the A20-mediated inhibition of NF- κ B activation. In this context, we recently identified a number of A20-interacting proteins which are able to inhibit NF- κ B activation by TNF and IL-1 upon overexpression (unpublished results).

TRAF2 and TRAF6 not only link the TNF-R and IL-1R to the NF- κ B activation cascade, but have also been suggested to be involved in the NF- κ B activation by CD40, IL-18, the human Toll receptor, and some other members of the TNF and IL-1 receptor superfamily [24–26]. Therefore, binding of A20 with TRAF2 and TRAF6 might provide a negative regulatory mechanism for NF- κ B activation by many members of the TNF and IL-1 receptor superfamily. However, in the case of sepsis or chronic inflammation, abundant secretion of IL-1 or TNF may override the protective effect of A20 leading to the development of the disease. Therefore, reagents which increase the cellular level of A20 might be useful tools in the treatment of many inflammatory diseases.

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