The Zinc Finger Protein A20 Inhibits TNF-induced NF-κB-dependent Gene Expression by Interfering with an RIP- or TRAF2-mediated Transactivation Signal and Directly Binds to a Novel NF-κB-inhibiting Protein ABIN

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Abstract. The zinc finger protein A20 is a tumor necrosis factor (TNF)– and interleukin 1 (IL-1)-inducible protein that negatively regulates nuclear factor-kappa B (NF-κB)–dependent gene expression. However, the molecular mechanism by which A 20 exerts this effect is still unclear. We show that A 20 does not inhibit TNF-induced nuclear translocation and DNA binding of NF-κB, although it completely prevents the TNF-induced activation of an NF-κB-dependent reporter gene, as well as TNF-induced IL-6 and granulocyte-macrophage colony-stimulating factor gene expression. Moreover, NF-κB activation induced by overexpression of TNF receptor–associated proteins TNF receptor–associated death domain protein (TRADD), receptor interacting protein (RIP), and TNF receptor–associated factor 2 (TRAF2) was also inhibited by expression of A 20, whereas NF-κB activation induced by overexpression of NF-κB–inducing kinase (NIK) or the human T cell leukemia virus type 1 (HTLV-1) Tax was unaffected. These results demonstrate that A 20 inhibits NF-κB–dependent gene expression by interfering with a novel TNF-induced and RIP- or TRAF2-mediated pathway that is different from the NIK–IκB kinase pathway and that is specifically involved in the transactivation of NF-κB. Via yeast two-hybrid screening, we found that A 20 binds to a novel protein, A BIN, which mimics the NF-κB inhibiting effects of A 20 upon overexpression, suggesting that the effect of A 20 is mediated by its interaction with this NF-κB inhibiting protein, A BIN.

Key words: A 20 • A BIN • nuclear factor-κB • tumor necrosis factor • TRAF

Tumor necrosis factor (TNF) is a pleiotropic cytokine that acts as an important mediator of inflammation and immune responses by modulating the expression of many inflammatory proteins (Barnes and Karin, 1997). Induction of specific transcriptional programs by TNF is mediated by the activation of the transcription factor nuclear factor-kappa B (NF-κB). Moreover, an antiapoptotic function for NF-κB has been described recently (for review see Van Antwerp et al., 1998). TNF-induced activation of NF-κB is initiated by the recruitment of a number of proteins to both TNF receptors, with the TNF-R55 being far more effective than the TNF-R75 (Vandenabeele et al., 1995). The TNF-R55 recruits by its death domain the death domain–containing adapter protein TRADD (TNF receptor–associated death domain protein) (Hsu et al., 1995). The TNF-R55 recruits by its death domain the death domain–containing adapter protein TRADD (TNF receptor–associated death domain protein) (Hsu et al., 1995).
with the death domain-containing proteins FADD/MORT1 and receptor interacting protein (RIP) (Hsu et al., 1996a,b). The former initiates signaling leading to apoptosis (Boldin et al., 1996; Muzzio et al., 1996), whereas the latter recently has been shown to be crucial for NF-κB activation by TNF (Kelliher et al., 1998). Moreover, TRADD can also recruit the TNF receptor-associated factor 2 (TRAF2) via a direct interaction or via the interaction of TRAF2 with RIP (Hsu et al., 1996a). TRAF2 belongs to a larger family whose members have been shown to be involved in NF-κB activation by several members of the TNF receptor family, including TNF-R55, TNF-R75, CD30, and CD40, but also by the interleukin 1 (IL-1) receptor (Cao et al., 1996; Hsu et al., 1996b; Ishida et al., 1996; Lee et al., 1996; Ng et al., 1998).

Studies with TRAF2-deficient cells recently have suggested that there is a lot of redundancy within the TRAF family (Yeh et al., 1997). TRAFs are believed to provide the signal to NF-κB activation by directly associating with the NF-κB-inducing kinase (NIK) (Malinin et al., 1997). NIK associates with and activates at least two kinases, IKK kinase (IKKα) and IKKβ, which phosphorylate the inhibitory subunit IkB leading to its degradation and the migration of NF-κB to the nucleus (Mercurio et al., 1997; Zandi et al., 1997). In addition to the NIK–IkB pathway, TRAF2 also initiates the activation of JNK and p38 mitogen-activated protein (MAP) kinases, which also have been shown to be important regulators of gene expression (Beyaert et al., 1996; Reinhard et al., 1997; Carpentier et al., 1998).

Besides IkB, NF-κB-dependent gene expression is also negatively regulated by the zinc finger protein A20 (Coo- pet al., 1996; Jäättelä et al., 1996). The latter can be negatively regulated by the zinc finger protein A20 (Coo-

Materials and Methods

Cloning of the mA20 Gene

L929r2 murine fibrosarcoma cells (Vanhaesebroeck et al., 1991) were treated for 4 h with 1,000 IU/ml mTNF and 10 μg/ml cycloheximide.
**IL-6 Assay and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) Assay**

L929A transfectants were seeded in a 6-well plate at $6 \times 10^3$ cells/500 μl medium/well. After 24 h, cells were either untreated for 6 h with 1,000 IU/ml mTNF or left untreated. Supernatants were harvested and assayed for IL-6 or GM-CSF in a 7TD1 or a FDCp1 cell proliferation assay, respectively (D El Amarter et al., 1985; Van Snick et al., 1986).

**NF-κB Gel Retardation Assay**

Cells in a 6-well plate were stimulated with 1,000 IU/ml mTNF for 5, 15, or 30 min, and nuclear fractions were prepared as described by Dignam et al. (1983). NF-κB DNA binding was analyzed by incubating 10 μg nuclear proteins for 30 min with the NF-κB specific 5′-labeled oligonucleotide probe (5′-aagctATGTGGGATTTTCCCATGAGCagct-3′) containing the NF-κB site from the promoter of the IL-6 gene. Protein–DNA complexes were separated on a 4% native polyacrylamide gel as described previously (Bayeart et al., 1996).

**Transient Transfections and Reporter Gene Assays**

L929A cells were seeded at a density of $2 \times 10^5$ cells/75-cm² flask. 24 h later, cells were transfected by the DNA – calcium phosphate cotransfection method with the plasmids pUT651 and phiL6LUC or p(xB)JuLUC. The plasmids phiL6U and p(xB)JuLUC contain the luciferase gene after the complete hIL-6 promoter or the minimal hIL-6 promoter preceded by three copies of the NF-κB recognition site, respectively. 48 h after transfection, cells were trypsinized and seeded at 1.2 x 10^5 cells/well. At 66 h after transfection, cells were either noninduced or induced for 6 h with 1,000 IU/ml hTNF. Inducible promoter activity was determined by measuring the luciferase and β-galactosidase activity present in cell extracts as described previously (De Valck et al., 1996). Transfection of 293T cells proceeded similarly, except that 293T cells were plated in 6-well dishes and transiently transfected by calcium phosphate–DNA coprecipitation. 24 h after transfection, cells were trypsinized and seeded at 1.2 x 10^5 cells/well. At 66 h after transfection, cells were either noninduced or induced for 6 h with 1,000 IU/ml hTNF. Inducible promoter activity was determined by measuring the luciferase and β-galactosidase activity present in cell extracts as described previously (De Valck et al., 1996).

**p38 MAP Kinase Phosphorylation Analysis**

10^5 L929A cells were seeded in 6-well plates. The next day, cells were either untreated or treated with 1,000 IU/ml mTNF for 5, 15, or 30 min. Cell lysates were prepared by directly adding SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue) to the cells. Proteins were separated by 10% SDS-PAGE and blotted on a nitrocellulose filter. Phosphorylated p38 MAP kinase was detected with a polyclonal p38 MAP kinase specific antibody. The total amount of p38 MAP kinase present in the same cell extracts was revealed with a polyclonal p38 MAP kinase specific antibody.

**Yeast-based Two-Hybrid Screening**

The yeast two-hybrid system was purchased from CLONTECH Laboratories. The screening of a L9292Δ DNA library with pSA2-A20 was described previously (De Valck et al., 1997). Yeast colonies expressing interacting proteins were selected by growth on minimal media lacking Trp, Leu, and His, in the presence of 5 mM 3-aminom-1,2,4-triazole and by screening for β-galactosidase activity. Plasmid DNA was extracted from the positive colonies and the pGAD424 vectors encoding candidate A20-interacting proteins were recovered by electroporation in the E. coli strain H10B1 and on media lacking Leu.

**Cell Transfection, Coimmunoprecipitation, and Western Blotting**

2 x 10^5 human embryonic kidney 293T cells were plated on 10-cm petri dishes and transiently transfected by calcium phosphate-DNA coprecipitation. 24 h after transfection, cells were lysed in 500 μl of lysis buffer (50 mM Heps, pH 7.6, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA). Lysates were incubated with 5 μl of rabbit anti-GFP antibody (CLONTECH Laboratories) and immunocomplexes were immobilized on protein A–Trisacryl (Pierce Chemical Co.). The latter were washed twice with lysis buffer and twice with lysis buffer containing 1 M NaCl. Coprecipitating proteins were separated by SDS-PAGE G E and analyzed by Western blotting with mouse anti–E-tag antibody (Pharmacia Biotech).

**Localization of GFP and GFP/A20 and ImmunoLocalization of ABIN**

Localization of GFP/A20 was analyzed by means of GFP fluorescence. Therefore, L929A cells stably expressing GFP or GFP/A20 were seeded on coverslips in 6-well plates and microscopically analyzed for GFP fluorescence (emission at 510 nm) at an excitation wavelength of 485 nm. To identify the subcellular localization of ABIN, 4 x 10^3 293T cells were seeded on coverslips in 6-well plates and transfected with 1 μg plasmid DNA. 24 h after transfection, cells were fixed on the coverslips with 3% paraformaldehyde. Upon permeabilization with 1% Triton X-100, the cells were incubated for 2 h with mouse anti–E-Tag antibody (1/1,000) followed by a second incubation with anti-mouse Ig antibody coupled to biotin (1/1,000; A Mersham). A further subsequent incubation with streptavidin coupled to Texas red (A Mersham), fluorescence can be analyzed via fluorescence microscopy (Axophot; Zeiss), using a filter set with excitation at 543 nm and emission at 600 nm. In the same cells, fluorescence of GFP can be detected at a different wavelength, namely excitation at 485 nm and emission at 510 nm.

**Results**

**A20 Inhibits TNF-induced Expression of IL-6 GM-CSF, and an NF-κB–dependent Reporter Gene**

A20 has been described as a TNF inducible inhibitor of NF-κB activation, mainly on the basis of its effect on NF-κB–dependent expression of a reporter gene (Cooper et al., 1996; J äätelä et al., 1996). Therefore, we first analyzed its effect on the TNF-induced expression of IL-6 and GM-CSF in control (L929SA-neo) and in L929SA cells overexpressing either a fusion protein of A20 with GFP (L929SA-GFP/A20) or GFP as such (L929SA-GFP). Expression of these cytokines has been shown to be NF-κB–dependent (Schreck et al., 1990; Zhang et al., 1990). Compared with the amount of IL-6 and GM-CSF found in the supernatant of TNF-treated L929SA-neo and L929SA-GFP cells, their levels were considerably decreased in L929SA-GFP/A20 20 cells (Table I). A similar inhibitory effect of A20 on TNF-induced gene activation could be seen when GFP-A20 expressing L929SA cells were transiently transfected with an expression plasmid containing the luciferase reporter gene under control of either the hIL-6 promoter (phiL6LUC) or a minimal promoter with three copies of the NF-κB recognition site, respectively. The total amount of p38 MAP kinase present in the same cell extracts was revealed with a polyclonal p38 MAP kinase specific antibody.

**A20 Does Not Prevent the Nuclear Translocation and DNA Binding of NF-κB**

NF-κB activation involves the release of the inhibitory protein IκB from NF-κB in the cytosol, leading to the nuclear translocation and binding of NF-κB to its specific recognition sequence in DNA (H enkel et al., 1993). The latter can be analyzed in a gel shift assay in which binding of active NF-κB to an NF-κB specific DNA probe leads to a slower migration of this probe in a nondenaturing polyacrylamide gel. Nuclear fractions prepared from L929SA-
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A20 completely prevented NF-κB, L929SA-GFP, and L929SA-GFP/A20 cells that were

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out affecting NF-κB expression as described above, no clear differences in TNF-

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induced DNA binding of NF-κB were observed (Fig. 1 B). Also, stimulation of these cells for shorter periods, 5 or 15 min, did not reveal an effect of A20 (data not shown). Nevertheless, TNF-induced nuclear translocation and DNA binding of NF-κB could be completely abolished by pretreatment with the proteasome inhibitor MG 132 that inhibits NF-κB activation by preventing IκB degradation (Chen et al., 1995; data not shown). These results indicate that A20 inhibits NF-κB-dependent gene induction by specifically interfering with an NF-κB transactivation signal, without affecting the nuclear translocation and DNA binding of NF-κB.

By the presence of seven zinc finger structures in A20, it has been originally suggested that A20 may bind to DNA and directly interfere with the transcriptional machinery (O pipari et al., 1990). However, more recently, it has been demonstrated that A20 transiently expressed in 293T cells exclusively localized in the cytoplasm (V incenz and D ixit, 1996). In our L929SA cells stably expressing GFP/A20 fusion proteins, a similar speckled cytoplasmic staining was observed via GFP fluorescence and confocal microscopy (Fig. 2). In contrast, GFP as such was detected both in the
cytoplasm and the nucleus. Furthermore, treatment of the cells with TNF did not induce a detectable relocalization of A20 (data not shown). These results suggest that A20 cannot directly interfere with NF-κB transcriptional activity, but indicate that A20-mediated inhibition of NF-κB transactivation is a cytosolic event.

A20 Inhibits a TNF-induced NF-κB Transactivation Pathway That Is Initiated at the Level of RIP/TRAF2 and That Is Independent of NIK

TNF-induced activation of NF-κB involves the recruitment of several cytoplasmic signaling proteins to the TNF-R55 (for review see D arrny and Aggarwal, 1997), which is the main signaling receptor for TNF. Moreover, overexpression of some of these proteins is sufficient to induce NF-κB activation. To investigate at which step A20 interferes with NF-κB activation, we tested the effect of A20 on NF-κB-dependent gene expression induced by TNF treatment or by overexpression of the TNF receptor associating proteins TRADD, RIP, TRAF2, and NIK in 293T cells. As expected, A20 completely prevented NF-κB-dependent luciferase expression induced by TNF (Fig. 3). A similar protective effect of A20 could be observed when NF-κB was activated by overexpression of TRADD, RIP, or TRAF2. However, NF-κB activation by overexpression of downstream acting NIK was not sensitive to A20. These results clearly demonstrate that A20 interferes with an RIP- or TRAF2-initiated NF-κB transactivation signal, which is different from the NIK-mediated pathway leading to nuclear translocation of NF-κB.

Inhibition of NF-κB Activation by A20 Is Stimulus-dependent

Recently, it was shown that activation of NF-κB induced by the HTLV transactivator Tax protein is mediated by NIK and both I K K α and I K K β (Chu et al., 1998; U hlik et al., 1998; Y in et al., 1998). In contrast, overexpression of a TRAF2 dominant negative mutant was not able to block Tax-induced NF-κB activation, excluding a role for TRAF2 in this pathway (Geleziunas et al., 1998; Y in et al., 1998). Therefore, we tested the effect of GFP/A20 overexpression on Tax-induced expression of an NF-κB-dependent reporter gene in 293T cells. Whereas TNF-induced NF-κB activation was completely prevented by A20, the latter could not block the gene-inducing effects mediated

Table I. Effect of A20 Expression on TNF-induced Expression of IL-6 and GM-CSF in L929SA Transfectants

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Cells were stimulated for 6 h with 1,000 IU/ml mTNF and supernatants were tested for the presence of IL-6 and GM-CSF as described in Materials and Methods. Results are shown for two representative subclones (total number of experiments, 2) are expressed as the mean value (n = 3) with SD < 10%.
by Tax (Fig. 4 A). These results further indicate that A20 interferes with TNF-induced NF-κB-dependent gene expression at the level of an RIP- or TRAF2-initiated NF-κB transactivation pathway that is independent of NIK.

To further substantiate this hypothesis, we also analyzed the effect of A20 on the activation of NF-κB by a number of other stimuli for which NF-κB activation has been shown to be mediated by TRAF2 or TRAF family members. IL-1 engages TRAF6 in its signaling pathway (Cao et al., 1996), whereas CD40 initiates NF-κB activation via TRAF2, TRAF5, and TRAF6 (Hu et al., 1994; Ishida et al., 1996a, b). Overexpression of A20 completely prevented IL-1-induced NF-κB activation in 293T cells (Fig. 4 B), which confirms previously published data (Jäättelä et al., 1996). Similarly, NF-κB activation induced by overexpression of CD40 was also abolished when A20 was coexpressed (Fig. 4 C). These results are in agreement with the above suggested role for a TRAF-initiated signaling pathway as target for A20.

Surprisingly, A20 also inhibited NF-κB activation by the protein kinase C activator TPA (Fig. 5). The latter also activates A P1- and SRE-dependent gene expression in 293T cells. Therefore, we analyzed whether A20 could also prevent the TPA-induced expression of a luciferase reporter gene whose expression is controlled by A P1- or SRE-binding proteins. However, in contrast to NF-κB-dependent gene expression, the TPA-induced activation of A P1- or SRE-dependent transcription was not sensitive to A20. These results demonstrate that A20 can also act as a specific inhibitor of NF-κB-dependent gene expression induced by TPA. It remains to be seen whether TRAFs or downstream signaling proteins are also involved in NF-κB activation by this stimulus.

**A20 Does Not Prevent the TNF-induced Activation of p38 MAP Kinase**

Recently, we and others demonstrated an important role for TNF-induced activation of p38 MAP kinase in the transactivation of NF-κB (Beyaert et al., 1996; Bergmann et al., 1998; Vanden Berghe et al., 1998). Moreover, TNF-induced p38 MAP kinase activation was shown to be mediated by TRAF2 (Carpentier et al., 1998). To investigate whether A20 inhibited the TNF-induced transactivation of NF-κB by preventing p38 MAP kinase activation, we analyzed the TNF-induced activation of p38 MAP kinase in GFP/A20-transfected versus GFP-transfected L929SA cells. p38 MAP kinase activation was revealed by immunodetection of phosphorylated p38 MAP kinase with p38 MAP kinase phosphospecific antibodies. TNF induced a
transient phosphorylation of p38 MAP kinase in L929SA-GFP as well as in L929SA-GFP/A20 cells (Fig. 6). Time kinetics of TNF-induced p38 MAP kinase phosphorylation/activation were similar in both cell lines, although in some experiments dephosphorylation occurred slightly faster in A20-expressing cells. These results make it unlikely that A20 prevents NF-κB transactivation by inhibiting the TNF-induced activation of p38 MAP kinase.

NF-κB Activation Cannot Be Rescued from A20 Inhibition by CBP/p300 Overexpression

CREB-binding protein (CBP) and p300 are coactivators that link transcriptional activators to the basal transcriptional apparatus. Both CBP and p300 were shown to act as coactivators of NF-κB-dependent gene expression by a direct interaction with the p65 subunit (Gerritsen et al., 1997; Perkins et al., 1997). The latter interaction was recently shown to be enhanced by phosphorylation of p65 by protein kinase A (Zhong et al., 1998). To analyze whether A20 would prevent NF-κB transactivation by inhibiting the TNF-induced activation of p38 MAP kinase.

A20 Binds to a Novel NF-κB Inhibitory Protein ABIN

Although it is known that the NF-κB inhibiting potential of A20 resides in its COOH-terminal zinc finger-containing domain (Song et al., 1996; Natoli et al., 1998; Heyninck et al., 1999), the underlying mechanism is still unclear. Therefore, we used the yeast two-hybrid system to screen an L929r2 cDNA library for A20 interacting proteins that might be involved in the negative regulation of NF-κB. From 1.3 × 10^6 transformants, 10 clones expressed A20-interacting proteins, including A20 itself (D e V alck et al., 1996) and 14-3-3 proteins (D e V alck et al., 1997). Three clones contained COOH-terminal fragments of the same cDNA encoding a protein that we named A20. Full-length A20 cDNA was subsequently isolated from the L929r2 cDNA library by colony hybridization with a fragment cloned by two-hybrid analysis as a probe. Several cDNAs were isolated and in the longest cDNA stop co-
were identified in all three frames 5' of a potential initiator methionine. Two different splice variants were found of z2,800 and 2,600-nt long. Northern blot analysis revealed that ABIN is expressed in all murine tissues tested as an mRNA of z2,800 bp that is in accordance with the length of the cloned full-length cDNA (data not shown). In contrast to A20, ABIN mRNA is constitutively expressed in both TNF-sensitive and TNF-resistant subclones derived from the parental cell line L929s (Vanhaesebroeck et al., 1991), irrespective of TNF stimulation.

The two splice variants of the ABIN cDNA contained an open reading frame of 1,941 and 1,782 nucleotides respectively, initiating at two different methionines [ABIN (1-647) and ABIN (54-647)] (GenBank/EMBL/DDBJ accession numbers AJ242777 and AJ242778). These cDNAs encode proteins of 72 and 68 kD, containing an amphiphatic helix with four consecutive repeats of a leucine followed by six random amino acid residues, which is characteristic of a leucine zipper structure. Also, full-length ABIN (1-647) and ABIN (54-647) bound A20 in a yeast two-hybrid assay, confirming the original interaction found with the 3' COOH-terminal fragments ABIN (390-599), ABIN (249-647), and ABIN (312-647). The latter all contain the putative leucine zipper protein interaction motif (397-420). In addition, ABIN also interacted with A20 in mammalian cells since ABIN was able to coimmunoprecipitate with A20 in 293T cells that were transiently transfected with an expression plasmid for chimeric GFP-A20 protein and ABIN with an NH2-terminal E-tag (Fig. 8).

Interestingly, interaction of ABIN with A20 required the COOH-terminal, zinc finger–containing part of A20 [A20(369-775)]. This domain was shown previously to be required for dimerization of A20 and for the interaction of A20 with 14-3-3 proteins (De Valck et al., 1996, 1997). Furthermore, overexpression of this domain is sufficient for the NF-κB inhibiting effects (Song et al., 1996; Natoli et al., 1997).
et al., 1998; Heyninck et al., 1999). In contrast, the NH$_2$-terminal part of A20 has been shown to interact with TRAF2 (Song et al., 1996), suggesting that A20 acts as an adapter protein between TRAF2 and ABIN. The interaction between A20 and ABIN was not influenced by stimulation with TNF (data not shown). To characterize the subcellular distribution of ABIN, we transiently transfected GFP-A20 and E-tagged ABIN cDNA in 293T cells and analyzed their expression by means of GFP fluorescence and immunofluorescence via the anti–E-tag antibody, respectively. ABIN colocalized with A20 throughout the cytoplasm, both in unstimulated and in TNF-stimulated cells (data not shown).

Database similarity searches (BLAST) showed that ABIN is the murine homologue of the human cDNA encoding a human immunodeficiency virus (HIV) Nef-associating factor, NAF1 (Fukushi et al., 1999). HIV–Nef contributes substantially to disease pathogenesis by augmenting virus replication and markedly perturbing T cell function. Interestingly, the effect of Nef on host cell activation has been explained in part by its interaction with specific cellular proteins involved in signal transduction (for review see Harris, 1996), of which ABIN might be an example.

**ABIN Mimics the NF-κB Inhibiting Effects of A20**

Since both A20 and Nef were shown previously to block the signal transduction pathway leading to NF-κB activation upon stimulation with TNF or IL-1 and T cell receptor stimulation, respectively (Niederman et al., 1992; Bandres et al., 1994; Jäättelä et al., 1996; Song et al., 1996), we investigated the effect of ABIN on NF-κB–dependent reporter gene expression in transiently transfected 293T cells. GFP and GFP-A20 served as negative and positive controls, respectively. Similar to A20, both splice variants of ABIN were able to block TNF-induced NF-κB activation in these cells, with the shorter NH$_2$-terminal truncated isoform being slightly more effective (Fig. 9 A). Overexpression of a combination of suboptimal doses of A20 and ABIN, on their own not sufficient to inhibit NF-κB activation, diminished NF-κB activation upon stimulation with TNF considerably (Fig. 9 B). This suggests that ABIN might mediate the NF-κB inhibiting effect of A20. Furthermore, cotransfection of expression plasmids encoding the TNF receptor–associated signaling proteins TRADD, RIP, TRAF2, and NIK together with the expression plasmid encoding A20, showed that the latter completely inhibited NF-κB activation induced by TRADD or RIP, and partially TRAF2-induced NF-κB activation. In contrast, no effect of ABIN was observed when NF-κB–dependent reporter gene expression was induced by NIK or more directly by overexpression of the p65 subunit of NF-κB (Fig. 9 C). Similarly, Tax-induced NF-κB activation that is TRAF-independent and is insensitive to A20, was also not affected by coexpression of ABIN (Fig. 9 D). These results suggest that ABIN, like A20, inhibits TNF-induced NF-κB activation.

![Figure 9](link)
activation at the level of RIP or TRAF2 proteins, preceding the activation of the NIK-IkB kinase steps.

Discussion

The zinc finger protein A20 is encoded by an immediate early response gene and acts as an inhibitor of NF-kB-dependent gene expression induced by different stimuli including TNF and IL-1 (Cooper et al., 1996; Jäätelä et al., 1996; Song et al., 1996). Here we show that the TNF-induced expression of GM-CSF and IL-6, as well as the TNF-induced expression of a luciferase reporter gene that is expressed under control of the complete hIL-6 promoter or the minimal hIL-6 promoter preceded by 3 NF-kB recognition sequences, are clearly inhibited in L929sA cells stably transfected with A20. These results are consistent with the fact that NF-kB is required for IL-6 and GM-CSF gene transcription (Schreck et al., 1990; Zhang et al., 1990). Surprisingly, gel retardation assays revealed that overexpression of A20 had no effect on the TNF-induced nuclear translocation and DNA binding of NF-kB. A iso the constitution of the NF-kB complex was not altered in cells overexpressing A20, and consisted in both cases of a p65 and a p50 subunit as revealed by gel supershift assays (data not shown). Therefore, the inhibition of NF-kB-dependent gene expression by A20 cannot be explained by an A20-induced alteration in the subunits of NF-kB. Ferran et al. (1998) showed recently that A20 acts upstream of IkB degradation and prevented the nuclear translocation of NF-kB. The reason for the discrepancy with our results is still unclear. Because activation of NF-kB is an early response after stimulation with TNF, we analyzed NF-kB translocation at 5, 15, and 30 min after TNF stimulation, whereas results of Ferran et al. were obtained 2 h after TNF stimulation. The latter is quite late and might already be regulated by secondary factors that are A20-sensitive. Moreover, NF-kB activation at later times is also regulated by TNF-induced negative regulatory proteins such as IkBα and A20 whose expression is itself under the control of NF-kB, further raising the complexity of NF-kB activation at later time points (Krikos et al., 1992; Le Bail et al., 1993). Alternatively, we cannot exclude cell type-dependent differences.

Until now, the mechanism by which A20 blocks the activation of NF-kB-dependent gene regulation was not known. Up to now, the NIK-IkB kinase pathway has been assumed to be responsible for NF-kB activation upon TNF treatment. However, our finding that A20 has no effect on the translocation of NF-kB to the nucleus argues against this pathway as the target for A20. Moreover we demonstrated that A20 could inhibit RIP- and TRAF2- but not NIK-induced NF-kB-dependent reporter gene activation, suggesting that A20 interferes with NF-kB activation upstream of NIK. These conclusions were confirmed by the inability of A20 to block Tax-induced NF-kB activation since the latter was shown to activate NF-kB by directly interfering with the downstream kinases NIK, IKKα, and IKKβ, independent of TRAF2 (Chu et al., 1998; Uhlík et al., 1998; Y in et al., 1998).

The above results suggest that TNF-induced NF-kB-dependent gene activation requires at least two different pathways: an NIK-mediated pathway leading to translocation of NF-kB to the nucleus, and an NIK-independent pathway leading to transactivation of NF-kB. Our results demonstrate that A20 specifically interferes with the NF-kB transactivation pathway. A20 has also been shown to interact with the TNF receptor-associated protein TRAF2 (Song et al., 1996). Interestingly, TRAF2 as well as some other members of the TRAF protein family, including TRAF5 and TRAF6, were shown to play a positive role in NF-kB activation induced by different cytokines, such as TNF, IL-1, and CD40 ligand, via their interaction with the NF-kB inducing kinase NIK (Cao et al., 1996; Malinin et al., 1997). The fact that A20 directly associates with TRAF2, as well as our observation that A20 not only prevents NF-kB activation by TNF but also by IL-1 and CD40 overexpression, points to a role of a TRAF2-mediated signaling pathway as a target for A20. However, gene knockout studies have recently shown that TRAF2 is not absolutely required for NF-kB activation by TNF, although this probably is a consequence of redundancy within the TRAF protein family (Ye et al., 1997). Alternatively, RIP may be more important than TRAF2 in mediating activation of NF-kB upon TNF stimulation (Keller et al., 1998).

The nature of the RIP/TRAF-initiated NF-kB transactivation signal is still unclear. Recently, an important role in the transactivating potential of NF-kB upon TNF stimulation was demonstrated for p38 MAP kinase (Beyaert et al., 1996; Bergmann et al., 1998; Vanden Berghe et al., 1998). This kinase becomes activated by stimulation of cells with TNF as well as by overexpression of TRAF2, which might interfere with the TRAF2-p38 MAP kinase pathway. It is still possible that A20 interferes with the TRAF2-p38 MAP kinase pathway, as these results suggested that A20 might interfere with the activation of p38 MAP kinase (Carpentier et al., 1998). Similar to the effect of A20 overexpression, inhibition of p38 MAP kinase with the specific inhibitor SB203580 also prevented NF-kB-dependent gene expression without altering the translocation of NF-kB to the nucleus (Beyaert et al., 1996; Bergmann et al., 1998). A20 might interfere with the TRAF2-p38 MAP kinase pathway downstream of p38 MAP kinase. However, no significant effect of A20 on p38 MAP kinase phosphorylation, which is a marker for its activation, could be observed. Although these results indicate that A20 does not act upstream of p38 MAP kinase activation, it is still possible that A20 interferes with the TRAF2-p38 MAP kinase/NF-kB transactivation pathway downstream of p38 MAP kinase. The validation of this possibility awaits the identification of the p38 MAP kinase substrate that is involved in NF-kB transactivation. Alternatively, our results might also fit with the existence of another RIP- or TRAF2-initiated pathway that contributes to NF-kB-dependent transcription, and which is blocked by A20.

Our observation that A20 also prevents NF-kB activation by TPA indicates that the A20-sensitive pathway might also be activated by protein kinase C, at least in some cell lines. Similar results were obtained by Cooper et al. (1996). In contrast, stable expression of A20 has been reported to be unable to prevent TPA-induced NF-kB activation in breast carcinoma MCF cells (Jäätelä et al., 1998).
activation of NF-κB has been primarily attributed to the p65 subunit, whose transactivating potential resides in its COOH-terminal portion (Ballard et al., 1992; Schmitz et al., 1994). Furthermore, the p65 subunit becomes phosphorylated during the activation of NF-κB upon TNF stimulation (Naumann and Scheidereit, 1994; Schmitz et al., 1995). Indeed, a p65 phosphorylating activity was found in the IκB kinase complex (Merciruo et al., 1997). Moreover, it was also shown that IκB is associated with the protein kinase A catalytic subunit that can phosphorylate the p65 in its ret homology domain resulting in enhanced activity of NF-κB (Zhong et al., 1997). Recently, p65 phosphorylation was shown to promote an interaction between p65 and the coactivators CBP/p300 (Zhong et al., 1998). The latter were previously shown to synergistically enhance the transcription activating potential of NF-κB (Gerritsen et al., 1997; Perkins et al., 1997). However, it is unlikely that A20 interferes with protein kinase A or another signaling pathway leading to the engagement of the coactivators CBP/p300 in the transcription activation of NF-κB because we were unable to rescue NF-κB activation from A20 inhibition by overexpression of CBP/p300. Moreover, activation of the protein kinase A catalytic subunit requires degradation of IκB and the activation of the NIK-IκB kinase pathway which is, however, not modulated by A20. A iso, A20 did not interfere with NF-κB-dependent gene expression obtained by overexpression of the p65 subunit as such (Cooper et al., 1996).

In endothelial cells, TRAF2 has been recently shown to translocate to the nucleus, where it might directly regulate transcription (Min et al., 1998). Because A20 can bind to TRAF2 (Song et al., 1996), and exclusively resides in the cytosol, A20 might prevent nuclear localization of TRAF2.

Screening of a cDNA library for A20 interacting proteins by the yeast two-hybrid system has revealed some isoforms of the 14-3-3 proteins that interact with the COOH-terminal zinc finger domain of A20 (Vincenz and Dixit, 1996; De V alck et al., 1997). 14-3-3 proteins were shown to function as adapter proteins between A20 and c-Raf. Moreover, 14-3-3 also functioned as a chaperone in these studies (Vincenz and Dixit, 1996). However, by mutation analysis we previously demonstrated that the interaction of 14-3-3 proteins with A20 is not involved in the effect of A20 on NF-κB activation (De V alck et al., 1997). By the yeast two-hybrid screening system, we also identiﬁed A2IN as a novel A20-interacting leucine zipper protein. The interaction of A2IN with A20 was conﬁrmed in human cells and shown to map to the functional COOH-terminal zinc ﬁnger-containing domain of A20. Upon overexpression, A2IN potently inhibits NF-κB activation induced by TNF. Furthermore, A2IN interferes with TNF-induced NF-κB activation at the level of RIP/TRAF2. Therefore, the ability of A20 to block TNF-mediated NF-κB activation is likely to involve the binding of the NF-κB inhibitory protein A2IN to the COOH-terminal zinc ﬁnger domain of A20. Moreover, the fact that A20 can also interact via its NH2-terminal domain with TRAF1 and TRAF2 (Song et al., 1996) suggests that A20 can recruit A2IN to the TRAF2 complex in the TNF signaling pathway.

In conclusion, A20 appears to prevent NF-κB-dependent gene expression by specifically interfering in the cytosol with a novel RIP/TRAF2-initiated transactivation pathway, thus inhibiting the TNF-induced expression of several cytokines and proinflammatory proteins. Since A20 also inhibits NF-κB activation by IL-1 and CD40, which all signal to NF-κB activation via members of the TRAF family, further identiﬁcation of TRAF-mediated NF-κB transactivation signals may provide means of achieving more speciﬁc antiinflammatory treatments.

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