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A20 and A20-Binding Proteins as Cellular Inhibitors of Nuclear Factor-κB-Dependent Gene Expression and Apoptosis

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ABSTRACT. Proper gene expression and cell growth are critical for the survival of all organisms. Nuclear factor-kappaB (NF-κB)-dependent gene expression and apoptosis play crucial roles in numerous cellular processes, and defects in their regulation may contribute to a variety of diseases including inflammation and cancer. Although there has recently been tremendous progress in our understanding of the signaling pathways that lead to NF-κB activation and apoptosis, signaling mechanisms that negatively regulate these processes are only partially understood. This review deals with the zinc finger protein A20, which has been characterized as a dual inhibitor of NF-κB activation and apoptosis. Its inducible expression by a wide variety of stimuli, including cytokines such as tumor necrosis factor, interleukin-1, and CD40, as well as bacterial and viral products such as lipopolysaccharide, Epstein–Barr virus latent membrane protein 1, and human T-cell leukemia virus type I Tax, suggests that it is involved in the negative feedback regulation of signaling. We will discuss the possible underlying mechanisms, placing emphasis on the role of several A20-binding proteins that have recently been described. Moreover, evidence is presented that A20 and A20-binding proteins are potential novel therapeutic tools in the treatment of a variety of diseases. BIOCHEM PHARMACOL **60**;8:1143–1151, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. NF-κB; apoptosis; A20; zinc finger; tumor necrosis factor; protein–protein interaction

Uncontrolled gene expression and apoptosis result in the development of various diseases. Inflammatory diseases such as rheumatoid arthritis and asthma are due to the induction of proinflammatory cytokines, chemokines, inflammation-promoting enzymes, immunoreceptors, and adhesion molecules [1]. Failure of cells to undergo apoptosis can lead to cancer and autoimmune diseases, whereas an excess of apoptosis plays a role in the pathogenesis of AIDS and neurological diseases such as Alzheimer's disease, muscular atrophy, and Huntington's disease [2]. In these processes, the transcription factor NF-κB† plays a key role, more particularly because the expression of many mediators of inflammation is NF-κB-dependent [3]. In addition, NF-κB also fulfils an antiapoptotic function, most likely by inducing the expression of antiapoptotic genes [4]. Because of the potential role of NF-κB in the above-mentioned diseases, signaling molecules that are involved in the activation and regulation of NF-κB-dependent gene expression have drawn much interest as potential targets for the treatment of several diseases. The best-characterized signaling pathways that lead to the activation of NF-κB are those stimulated by TNF and members of the IL-1/toll receptor family (Fig. 1) [5]. TNF binding to the 55-kDa TNF receptor (TNF-R1) activates the recruitment of TRAF2 and RIP to the TNF-R via their binding to the adaptor protein TRADD. The latter also recruits FADD, which initiates the activation of a caspase cascade that leads to apoptosis [6]. TRAF2 and RIP can also interact with each other, and have both been shown to be involved in the activation of NF-κB by TNF. Similarly, IL-1 binds to the IL-1 receptor/IL1-RAcP complex, leading to the recruitment of the adaptor protein MyD88 and the activation of IRAK and TRAF6, which are crucial for NF-κB activation by IL-1. More recently, LPS-induced activation of NF-κB has been shown to involve a TLR4-initiated pathway which is common to the IL-1 pathway [7]. The TNF-R and IL1-R/TLR4-induced pathways converge at the level of the IKK complex, which consists of a modulatory subunit (IKK-γ/NEMO) and two protein kinases (IKK-α and IKK-β) that are responsible for phosphorylation of the NF-κB-inhibitory protein IκB. This leads to IκB ubiquitination and its proteolytic degradation by the proteasome.

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† *Abbreviations:* ABIN, A20-binding inhibitor of NF-κB activation; cIAP, cellular inhibitor of apoptosis protein; EBV-LMP1, Epstein–Barr virus latent membrane protein 1; FADD, Fas-associated death domain; HTLV-I, human T-cell leukemia virus type-I; IKK, IκB kinase; IL-1, interleukin-1; IL1-RAcP, IL-1 receptor accessory protein; IRAK, IL-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappaB; NIK, NF-κB-inducing kinase; RIP, receptor interacting protein; TLR4, toll-like receptor 4; TNF, tumor necrosis factor; TNF-R, TNF receptor; TRADD, TNF-R-associated death domain; and TRAF, TNF-R-associated factor.

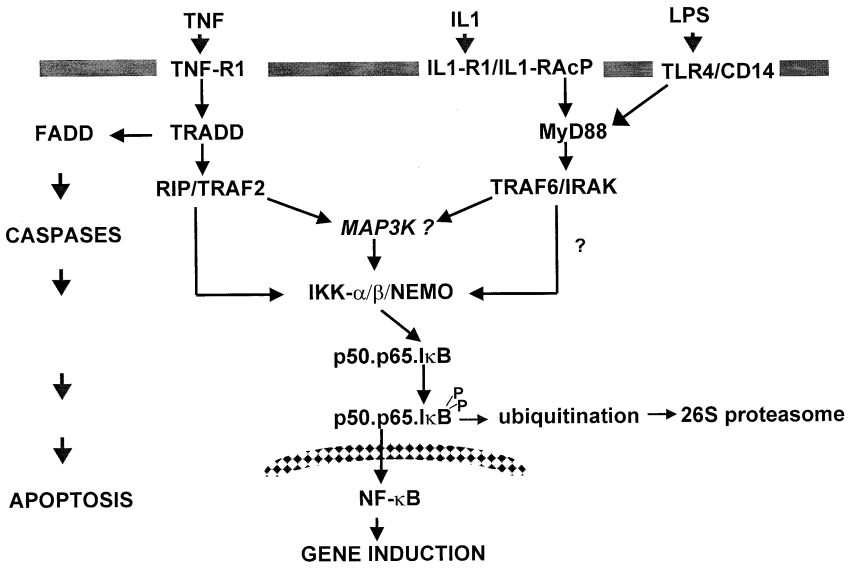

FIG. 1. TNF-, IL-1-, and LPS-induced signaling towards NF-κB-dependent gene expression. TNF binding to TNF-R1 activates the recruitment of TRAF2 and RIP to the TNF-R via their binding to the adaptor protein TRADD. The latter also binds FADD, which initiates a caspase cascade that finally leads to apoptosis. IL-1 binds to the IL-1 receptor/IL-1 receptor accessory protein complex (IL1-R1/IL1-RAcP), leading to the recruitment of the adaptor protein MyD88 and the activation of IRAK and TRAF6. The MyD88/TRAF6/IRAK pathway is also triggered in response to LPS binding to TLR4 and CD14. The TNF-, IL-1-, and LPS-induced pathways converge at the level of the IKK complex, which is believed to be further regulated by stimulus-specific MAPK kinase kinases (MAP3K) (e.g. NIK, MEKK1). The IKK complex consists of two protein kinases (IKK-α and IKK-β) and a modulatory subunit (IKK-γ/NEMO). IKK-α and -β are directly responsible for phosphorylation of the inhibitory protein IκB, with subsequent ubiquitination and proteolytic degradation by the proteasome. Free NF-κB then translocates to the nucleus, where it binds to κB sites in the promoter region of NF-κB-responsive genes. In the case of TNF-induced NF-κB activation, association of RIP with IKK-γ mediates the recruitment of the IKK complex to the TNF-R complex in response to TNF. A similar mechanism is likely to exist for IL-1- and LPS-induced NF-κB activation.

The free NF-κB then translocates to the nucleus, where it binds to κB sites in the promoter region of NF-κB-responsive genes. TRAF2 and TRAF6 also activate p38 MAPK and JNK, which are involved in the transactivation of NF-κB [8–10]. The identity of the direct activators of the IKK complex is still unclear, although specific MAPK kinase kinase (MAP3K)-related enzymes have been claimed to be involved in the activation of IKK by TNF and IL-1 [11].

Whereas members of the IκB family have been carefully studied as direct inhibitors of NF-κB [12], a number of other proteins (e.g. TANK/I-TRAF, TRAF-interacting protein TRIP, A20) have been reported to negatively regulate NF-κB-dependent gene expression by interfering with upstream signaling pathways that are involved in the activation and regulation of NF-κB [13–15]. The zinc finger protein A20 is peculiar because it seems to have a dual activity, being not only an inhibitor of NF-κB activation, but also behaving as an antiapoptotic molecule in some cell systems (see below). In the present paper, we will review and discuss our current knowledge of this protein.

A20: STRUCTURE AND EXPRESSION

A20 is encoded by a primary response gene originally identified as a TNF-inducible gene in human umbilical vein endothelial cells [16]. The induction of A20 by TNF is transient, being detectable after 15 min, and is maximal after 1-hr stimulation. Costimulation with cycloheximide results in stabilization of the A20 mRNA, consistent with the fact that the 3′-untranslated region of A20 mRNA contains four copies of the canonical sequence ATTTA, which is known to confer instability on a number of short-lived transcripts. In the original studies by Dixit and colleagues, IL-1 and LPS were also found to up-regulate A20 in endothelial cells [16]. Subsequent research demonstrated that A20 is also induced in many other cell types and by a wide range of other stimuli including phorbol 12-myristate 13-acetate [17], activation of the B-cell surface receptor CD40 [18], as well as overexpression of HTLV-I Tax [19] and EBV-LMP1 [20, 21]. Remarkably, the gene encoding A20 has also been isolated as a cDNA clone that is induced in monocytes upon adherence to collagen or endothelial cells, and has also been called MAD6 [22]. This adhesion-mediated induction of A20 was shown to involve very late antigen-4 [23].

In contrast to the inducible expression of A20 in most cells, constitutive expression of A20 could be observed in thymocytes and resting peripheral T cells. Moreover, activation of these cells led to the down-regulation of A20 expression [24]. Constitutive expression of A20 was also found in the differentiated monocyte cell line THP-1 [19]. Because A20 was not expressed in the U937 promonocytic cell line, a direct correlation between the activation state of monocytes and A20 expression has been proposed.

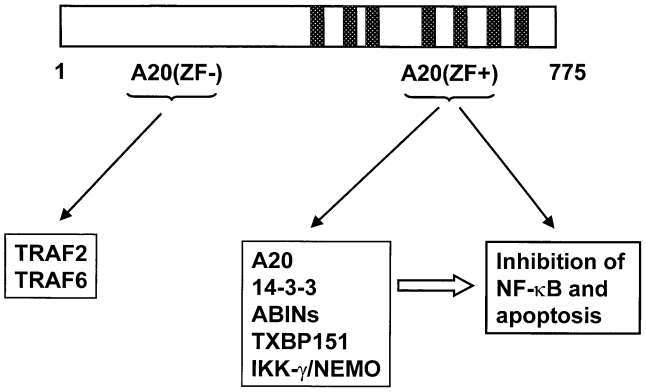

FIG. 2. Structure of A20 showing the location of the 7 zinc finger structures. The N-terminal A20(ZF−) region is sufficient for binding to TRAF2 and TRAF6. The C-terminal A20(ZF+) region, containing the 7 zinc finger structures, mediates A20 dimerization and the binding to several other proteins that might be involved in the inhibition of NF-κB activation and apoptosis by A20.

Expression of A20 RNA has been examined during murine development [24]. In developing embryos at day 15 post-coitum (dpc), the highest levels of A20 expression were detected in the olfactory epithelium, in the tooth germ epithelium at the bell stage, along the nasopharynx, and in the thymus. Low-level expression was also apparent in many other tissues including the central nervous system, spinal cord, liver, lung, and heart. Additional expression in the thyroid gland and in chondrocytes within ossifying vertebrae could be observed 15 dpc. By 19 dpc, high-level expression was restricted to the thymus and thyroid gland. Patchy expression of A20 was also apparent in the basal layer of the forming skin at 15 dpc and in epithelial cells at the deepest aspect of epidermal invaginations. A20 may therefore play important roles in multiple cell compartments, and in addition to critical roles in the function of the lymphoid system, may be important for the development of skin epidermis and hair follicles. A20 RNA is not detectable in normal adult skin epidermis or oral mucosa, suggesting that A20 does not play a role in the maintenance of normal skin architecture [25]. In contrast, high expression of A20 was observed in undifferentiated nasopharyngeal carcinoma and poorly differentiated head and neck squamous cell carcinoma [25], suggesting a role for A20 in the pathogenesis of these tumors.

The human A20 gene encodes a 790-amino acid-containing protein of 90 kDa, whereas murine A20 is 15 amino acids shorter [24, 26]. The protein sequences of human and murine A20 are 90% identical and 96% conserved. The C-terminal half of A20 contains seven Cys_2/Cys_2 zinc fingers that are completely identical in murine and human A20, except for a single substitution of methionine for valine in the fourth finger. They consist of 6 times a Cys-X4-Cys-X11-Cys-X2-Cys zinc finger motif and once a Cys-X2-Cys-X11-Cys-X2-Cys motif. Because of this unusual spacing of the cysteines, A20 has been considered as a new type of zinc finger protein (Fig. 2). The murine A20 gene has been mapped to mouse chromosome 10, 3.5 centimorgans proximal to the c-*myb* locus [24].

FUNCTION OF A20

A20 was initially characterized as an inhibitor of TNF-induced apoptosis [27]. Stable overexpression of A20 in a number of cell lines was shown to result in partial resistance to TNF-induced apoptosis. It should be noted that A20-mediated inhibition of apoptosis has not been observed in all cell lines studied. Hence, protection against TNF cytotoxicity exists for human breast carcinoma MCF7 cells, murine fibrosarcoma WEHI164 cells, murine embryonic fibroblast NIH3T3 cells, and human umbilical vein endothelial cells, but not for human cervix carcinoma HeLa cells, lung epithelial A549 cells, or human hepatoma HepG2 cells [27–33]. The reason why some cell lines are protected by A20 and others not is still unclear. Inhibition of TNF-induced apoptosis by A20 was shown to be correlated with inhibition of phospholipase A2 activation, reduced production of reactive oxygen species, reduced collapse of mitochondrial membrane potential, and decreased activation of caspase-3-like proteases [29, 34]. Similarly, A20 has recently also been shown to prevent TNF-induced cell death by necrosis, which was also associated with a delayed production of oxygen radicals and diminished activation of phospholipase A2, C, and D [35]. Apart from TNF-induced cell death, A20 has also been shown to inhibit cell death in a number of other systems: serum depletion-induced apoptosis of Louckes and BJAB B cells [18] as well as human umbilical vein endothelial cells [36], apoptosis of H1299 epithelial cells induced by p53 overexpression [37], and LPS-induced death of the human microvascular endothelial cell line HMEC-1 [38]. In contrast, overexpression of A20 in MCF7 and WEHI-S cells has no effect on apoptosis induced by the Fas receptor, lymphokine-activated killer cells, serum depletion, or oxidative stress [29].

Besides being an antiapoptotic molecule, A20 can also function as a potent inhibitor of NF-κB-dependent gene expression. The first evidence for this was obtained from the observation that A20 can negatively regulate its own NF-κB-dependent expression [15]. Overexpression of A20 was subsequently shown to block the activation of NF-κB by TNF, IL-1, LPS, phorbol esters, and hydrogen peroxide in different cell types [29, 39–43]. Consistent with these results, overexpression of A20 is able to prevent TNF-induced production of NF-κB-dependent proteins such as E-selectin, vascular cell adhesion molecule-1, IκB-α, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor [42, 43]. Furthermore, A20 can also prevent NF-κB activation induced by overexpression of EBV-LMP1 in epithelial cells [44], as well as CD40 in human embryonic kidney HEK293 cells [40].

The fact that expression of A20 is itself under the control of NF-κB [15] suggests that A20 is involved in the negative feed-back regulation of NF-κB activation. A similar effect has been shown for IκB-α, which directly binds NF-κB and

retains it in the cytoplasm in a dormant form. This protein has itself several κB sequences in its promoter region, so that NF-κB induces its synthesis. IκB-α can then enter the nucleus to bind to activated NF-κB and cause the return of NF-κB to the cytoplasm, thus terminating activation [45]. Targeted gene disruption of IκB-α results in prolonged NF-κB activation, and the animals die of severe inflammation, including widespread dermatitis [46]. In cell types where uninducible IκB subtypes predominate, e.g. IκB-β, NF-κB is likely to be activated for a more prolonged period. In contrast to IκB-α and IκB-β, the function of the other members of the IκB family is not yet clear, but it is likely that they are important in balancing the NF-κB activation and specific gene regulation.

More recently, an antiproliferative effect of A20 in smooth muscle cells has been described [47]. Because A20 also affects NF-κB activation in these cells, it is still unclear whether the two effects of A20 are related.

A20-BINDING PROTEINS

A20

A20 has been shown to self-associate via its zinc finger domain [48]. Whether A20 forms homodimers or multimers is still not known. As well, the functional significance of A20 oligomerization is still unclear.

TRAFs

A20 has been shown to interact via its N-terminal domain with TRAF1 and TRAF2, which are part of the NF-κB activation cascade initiated by TNF [40], as well as with TRAF6 [49], which is part of the IL-1- and LPS-induced signaling pathway to NF-κB. At the moment, six members of the TRAF protein family are known (TRAF1–6) [50]. Whereas the N-terminal domain of A20 seems to be involved in its interaction with specific TRAF proteins, its NF-κB-inhibiting potential resides in the C-terminal zinc finger-containing domain (Fig. 2). Therefore, it is assumed that the interaction of A20 with TRAFs might serve to recruit the inhibitory zinc finger domain to the NF-κB activation cascade. It should be noted that this hypothesis is contradicted by the observation that overexpression of an A20 mutant which lacks the N-terminal TRAF-interacting domain is still able to prevent TNF- and IL-1-induced NF-κB activation [49]. However, the requirement for TRAF binding to localize A20 to its target might be lost upon overexpression of A20.

14-3-3

Some isoforms of the 14-3-3 protein family have been shown to interact with the C-terminal zinc finger domain of A20 [41, 51]. 14-3-3 proteins have been reported to function as adaptor proteins between A20 and c-raf. Moreover, 14-3-3 also functions as a chaperone, promoting the transition of A20 from insoluble punctuate cytoplasmic structures to the soluble cytoplasmic compartment [51]. Mutagenesis of the 14-3-3-binding site in A20 revealed that the interaction of A20 with 14-3-3 proteins is not involved in its effect on NF-κB activation [41]. It remains to be seen whether binding to 14-3-3 proteins is involved in the antiapoptotic effect of A20.

ABINs

By yeast two-hybrid screening for A20-interacting proteins, we recently isolated a novel A20-binding protein that was given the name ABIN (A20-binding inhibitor of NF-κB activation) [43]. Two different splice variants approximately 2800 and 2600 nucleotides long were found, with an open reading frame of 1941 and 1781 nucleotides, respectively, initiating at two different methionines. These cDNAs encode proteins of 72 and 68 kDa containing an amphipathic helix with 4 consecutive repeats of a leucine followed by 6 random amino acid residues, which is characteristic of a leucine zipper structure. ABIN is the murine homologue of human immunodeficiency virus Nef-associated factor 1 [52]. Upon overexpression, ABIN blocks NF-κB activation induced both by TNF or IL-1 and by overexpression of the TNF-R-associating factors TRADD, RIP, and TRAF2, but not that induced by overexpression of NIK or IKK-β. These results suggest that ABIN blocks the NF-κB activation induced by TNF and IL-1 upstream of the IKK complex, possibly at the level of TRAF2 and TRAF6, respectively.

Yeast two-hybrid screening also revealed another A20-binding protein that shows no homology with any other known protein, except for the presence of two stretches of approximately 20 amino acids that can also be found in ABIN. Because overexpression of this novel protein is also able to inhibit NF-κB activation by TNF and IL-1,* it was named ABIN-2. Mutagenesis of the conserved regions in ABIN and ABIN-2 showed that the second region is essential for NF-κB inhibition.†

TXBP151

Yeast two-hybrid screening with A20 also revealed its interaction with TXBP151/TAX1BP1 [33], which was independently isolated as an HTLV-I Tax-binding protein [53]. The full-length 2386-bp TXBP151 mRNA encodes a protein of 86 kDa. Besides a putative 14-3-3-binding motif (RGASTP), no distinctive protein domains were found. No significant homology with other known proteins could be detected, except for the N-terminal region of TXBP151 which was found to be homologous to nuclear domain protein 52 [54]. While A20 is only expressed in stimulated cells, TXBP151 is constitutively expressed in several human cell lines. A20 associates with TXBP151 through the C-terminal zinc finger-containing domain of A20 (Fig. 2).

* Van Huffel S, Heyninck K and Beyaert R, manuscript in preparation.
† Heyninck K, Van Huffel S and Beyaert R, manuscript in preparation.

LMP1

A20 has been shown to form a complex with EBV-LMP1 [37]. LMP1 induces profound changes in cellular gene expression, in part due to activation of NF-κB. LMP1 can initiate NF-κB activation via two separate domains, a carboxy-terminal-activating region 1, which interacts with TRAFs, and a carboxy-terminal-activating region 2, which interacts with the adaptor protein TRADD. The interaction of A20 with LMP1 significantly decreases the interaction between LMP1 and the TRAF molecules and TRADD, affecting the activation of NF-κB and JNK.

IKK-γ/NEMO/IKKAP1

The binding of A20 to IKK-γ (also named NEMO or IKKAP1) via its C-terminal zinc finger-containing domain has very recently been reported [39]. IKK-γ is part of the IKK complex, which also contains two kinases (IKK-α and IKK-β) that can directly phosphorylate IκB. In contrast to IKK-α and IKK-β, IKK-γ lacks enzymatic activity, but is believed to transfer the upstream activator signal to IKK-α and IKK-β [55, 56]. Interestingly, A20 binds to IKK-γ only after TNF application or in response to overexpression of the TNF-R1, and is recruited together with the IKK complex to the TNF-R1 [39].

A20: MECHANISM OF ACTION

As A20 is capable of inhibiting both TNF-induced cell death and NF-κB activation, it is tempting to speculate that it may interfere with TRADD binding to the TNF-R1. However, it should be mentioned that in contrast to the NF-κB modulatory effect of A20, its antiapoptotic activity can only be observed in a limited number of cell lines [31, 33], suggesting at least two different targets for A20. Alternatively, one cannot exclude the cell line-specific existence of different proapoptotic signaling pathways that can be initiated by a single stimulus or the cell line-specific expression of proteins that mediate the antiapoptotic effect of A20. The fact that A20 can prevent TNF-induced cell death, TNF-induced gene activation, and TNF-induced activation of several TNF signaling pathways suggests that it interferes with an early step in TNF signaling. In this context, it will be interesting to analyze the functional relationship between the inhibitory effect of A20 on TNF responses and its demonstrated binding to proteins that are part of the TNF-R complex. These include TRAF1 and TRAF2, which have been implicated in inhibition and activation of NF-κB, respectively [57, 58], as well as in the negative regulation of TNF cytotoxicity [31, 59, 60]. In addition, the recently described interaction of A20 with IKK-γ, which is itself recruited to the TNF-R complex via binding to RIP, is likely to contribute to the inhibition of NF-κB by A20 [39]. In fact, there is evidence that A20 affects the function of the TNF-R by more than one mechanism. While a number of studies have shown that overexpression of A20 can inhibit IκB phosphorylation in response to TNF [39, 42], we have observed that A20 can also inhibit the function of NF-κB at a step subsequent to IκB degradation [43]. The mechanisms for these effects remain to be clarified. The observation that the inhibitory effect of A20 on NF-κB activation can be overcome by overexpressing the IKK-activating kinase NIK [43] raises the possibility that A20 restricts the accessibility of the IKKs to IKK-activating kinases. In the case of LMP1 signaling, the alteration of LMP1/TRAF and LMP1/TRADD complex formation by A20 has been proposed to be responsible for its inhibition of LMP1 activation of NF-κB and JNK [37]. Alternatively, the physical association of A20 with the TNF-R complex may allow proteins associated with A20 (e.g. ABINs, TXBP151) to exert inhibitory effects on IKK function [33, 43]. Interestingly, part of the homologous amino acid region of ABIN and ABIN-2 is also present in IKK-γ.* Therefore, it is possible that ABINs might compete with IKK-γ for binding to an upstream activator protein (Fig. 3).

Apart from a pathway leading to IKK activation, TNF also initiates a TRAF2-mediated pathway that leads to activation of p38 MAPK and is involved in the transactivation of NF-κB [8, 9]. However, although A20 prevented the TNF-induced and TRAF2-mediated activation of NF-κB in L929 cells, it had no effect on the TNF-induced activation of p38 MAPK and JNK in these cells [43]. These results suggest that A20 specifically interferes with the TRAF–IKK pathway. Nevertheless, inhibition of LMP1-induced JNK activation could be observed in H1299 epithelial cells [61], suggesting stimulus-specific effects of A20 on MAPK activation.

Inhibition of TNF-induced apoptosis by A20 is at least partially mediated by the A20-binding protein TXBP151. Like A20, overexpression of TXBP151 inhibited apoptosis induced by TNF in NIH3T3 cells [33]. Moreover, transfection of antisense TXBP151 partially abolished the antiapoptotic effect of A20. Although the above results indicate a role for TXBP151 in the antiapoptotic effect of A20, the underlying mechanism is still unknown. Apoptosis induced by TNF-R or Fas triggering has been associated with proteolysis of TXBP151 by members of the caspase-3-like subfamily, i.e., caspase-3, caspase-6, and caspase-7 [33]. As with most identified caspase substrates, the functional significance of the cleavage of TXBP151 is still unclear. Because TXBP151 cleavage leads to multiple processed products, it is tempting to speculate that caspase-mediated cleavage of TXBP151 serves to turn off its antiapoptotic function, as has already been described for a number of other antiapoptotic proteins [62]. Future studies with uncleavable mutants of TXBP151 will be required to evaluate this hypothesis. The protective effect of A20 on TNF-induced apoptosis may also involve other proteins such as cIAPs. In this context, it is interesting to note that A20 and cIAP interact with a common region in TRAF2 [63].

* Heyninck K, Van Huffel S and Beyaert R, manuscript in preparation.

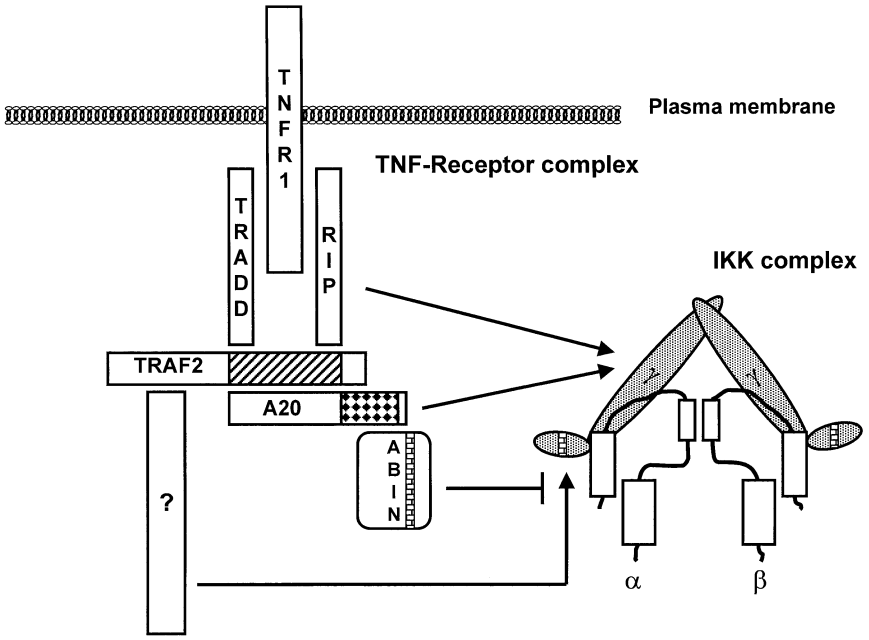

FIG. 3. Model for the inhibition of TNF-induced NF-κB activation by A20 and ABIN. A20 is recruited to the TNF-R complex by binding of its N-terminal domain to the so-called "TRAF domain" (hatched bars) of TRAF2. The zinc finger-containing region of A20 (checked) binds to IKK-γ, which also binds to RIP in response to TNF, and enhances the recruitment of the IKK complex to the TNF-R. IKK-γ is believed to act as a linker between IKK-α/β and a hitherto unknown upstream activator of the IKK complex (indicated by "?"). The partial homology between the C-terminal part of IKK-γ and the A20-binding protein ABIN might allow the latter to compete with IKK-γ for binding to the same upstream activator protein of the IKK complex, thus leading to inhibition of NF-κB activation.

Therefore, it is an intriguing possibility that A20 releases cIAP from the TRAF-signaling complex, thereby enabling these proteins to exert their antiapoptotic effects.

THERAPEUTIC IMPLICATIONS

The increased understanding of the activation and regulation of NF-κB has opened the way for the development of new treatments for inflammatory diseases in the future. Several synthetic and naturally occurring inhibitors of NF-κB-dependent gene expression have been described [64]. Some of these have recently been shown to directly target the IKK complex [65, 66], while many others are certainly in the pipeline. Antisense oligonucleotides against the p65 subunit of NF-κB have been evaluated successfully in a number of disease model systems [67]. Alternatively, adenovirus-mediated expression of IκB-α or a dominant negative mutant of p65 has been used to inhibit proinflammatory gene expression in rheumatoid synovial tissue and endothelial cells, respectively [68, 69]. Since NF-κB plays such a critical role in the immune and other defense responses, it may be unwise to block NF-κB for prolonged periods. Indeed, targeted disruption of the p65 subunit of NF-κB is lethal in animal models because of associated developmental abnormalities [70], whereas lack of the p50 component results in immune deficiency and increased susceptibility to infection [71]. Topical application of NF-κB inhibitors may prove to be safe. Alternatively, inhibitors that target specific cytokine receptor-associated molecules involved in NF-κB activation might provide NF-κB inhibition initiated by specific cytokines. In this context, A20 and ABINs might be interesting candidates for gene therapy. Because these molecules seem to interfere with an early step in the activation of NF-κB which is upstream of IKK, they are likely to only affect NF-κB activation by specific stimuli. Indeed, no effect of A20 and ABIN could be observed on HTLV-I Tax-induced NF-κB activation [43], which is believed to result from a direct interaction of Tax with the IKK complex [72]. Alternatively, strategies that aim to mimic the activity of endogenous cellular inhibitors of NF-κB (e.g. A20) might provide us with additional tools.

Although inhibition of NF-κB by IκB-α overexpression renders many cells susceptible to TNF-induced apoptosis, A20 inhibits NF-κB activation in many cell lines without sensitizing to TNF-mediated apoptosis [42, 43]. From a therapeutic perspective, genetic engineering of endothelial cells to express an NF-κB inhibitor such as A20 offers the means of achieving an anti-inflammatory effect without sensitizing the cells to TNF-mediated apoptosis. A dual antiapoptotic and anti-inflammatory function has also been described for A20 in β-cells within the islet of Langerhans [73]. Overexpression of A20 by means of adenovirus-mediated gene transfer was shown to protect islets from IL-1β- and interferon-γ-induced apoptosis. This was due to inhibition of cytokine-induced nitric oxide production, resulting from a blockade of NF-κB-mediated induction of inducible nitric oxide synthase. Because destruction of

transplanted islets by apoptosis is a major problem in the treatment of insulin-dependent diabetes mellitus, A20 may have therapeutic potential as a gene therapy candidate for successful islet transplantation.

A correlation has been found between A20 expression in endothelial cells of a heart xenograft and the reduced rejection of this transplant [74, 75]. Moreover, A20 expression was associated with the absence of arteriosclerosis that usually develops in rejected hearts [74, 76]. This is most likely due to the inhibition of NF-κB activation in endothelial cells in response to proinflammatory stimuli, as well as to an antiproliferative effect on smooth muscle cells, as has been observed *in vitro* upon overexpression of A20 [47]. Therefore, A20 could represent a potential therapeutic tool to genetically engineer donor smooth muscle cells to impact on development of transplant arteriosclerosis.

CONCLUDING REMARKS

Over the last 2–3 years, our understanding of the signaling pathways involved in the regulation of NF-κB-dependent gene expression and apoptosis in response to diverse stimuli has increased enormously. A20 promises to be an interesting molecule because of its dual activity on NF-κB activation and cell death. Nevertheless, intriguing questions regarding the mechanism of action, regulation, and function of A20 and A20-binding proteins remain to be answered:

1. What is the molecular basis for the fact that only a limited number of cell lines are sensitive to the anti-apoptotic effect of A20?
2. Why is inhibition of NF-κB activation by A20 not associated with sensitization to TNF-induced cell death, as suggested by the antiapoptotic function that has been attributed to NF-κB [4]?
3. Is the interaction of A20 with TRAFs and other A20-binding proteins essential for its function? What are the direct targets of A20 and A20-binding proteins?
4. Is inducible expression of A20 important in the negative feedback regulation of NF-κB activation and apoptosis?
5. Is the observation that two of the A20-binding proteins also bind to specific retroviral proteins a coincidence, or is it significant? First, TXBP151 has been shown to bind to the HTLV-I transactivator protein Tax, which appears to play a central role in cell transformation and is known to alter the expression of a large number of cellular genes. Secondly, ABIN has been described as an HIV-Nef interacting protein. 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 [77], of which ABIN might be an example. Future experiments which analyze the effect of these proteins, as well as A20, on HTLV-I and HIV pathogenesis will certainly be of interest.
6. Is there a role for A20 and A20-binding proteins in normal development?
7. Is deregulated expression of A20 linked to disease?

This knowledge, together with advances in gene therapy and drug development by the pharmaceutical industry, should pave the way for the introduction of a rational therapy based on A20 and A20-binding proteins for several diseases in the 21st Century.

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