Functional redundancy of the zinc fingers of A20 for inhibition of NF-κB activation and protein–protein interactions

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Abstract The tumor necrosis factor (TNF) inducible protein A20 is a potent inhibitor of nuclear factor-κB (IκB)-mediated gene expression in response to TNF and several other stimuli. The C-terminal domain of A20 is characterized by seven zinc finger structures. Here, we show that a minimum of four zinc fingers is required to inhibit TNF-induced nuclear factor-κB (NF-κB) activation to a level that is comparable to that obtained with the wild-type A20 protein. However, there was no strict requirement for a particular zinc finger structure, since a mutant A20 protein containing only the first four zinc fingers was as potent as a mutant protein containing only the last four zinc fingers. A similar functional redundancy of the A20 zinc fingers was also observed for binding of A20 to a number of other proteins, including two novel NF-κB inhibitory proteins (ABIN-1, ABIN-2), A20 itself, the anti-apoptotic protein TXBP151, and a regulatory component of the IκB kinase complex, IKKγ. Moreover, we demonstrate that complete loss of binding of any of these proteins correlates with complete loss of A20's ability to inhibit TNF-induced NF-κB activation. However, binding of IKKγ as such is not sufficient for inhibition of NF-κB dependent gene expression in response to TNF.

Key words: A20; Tumor necrosis factor; Inhibitor of nuclear factor-κB kinase γ; Nuclear factor-κB; Zinc finger; A20-binding inhibitor of NF-κB activation

1. Introduction

A20 was originally identified as a tumor necrosis factor (TNF) responsive gene in human endothelial cells [1]. A large number of other stimuli also induce the expression of the A20 gene. These include the binding of human cytomegalovirus to unactivated monocytes [2], CD40 ligand [3], integrins [4], interleukin-1 (IL-1) [5], lipopolysaccharide [6], the latent membrane protein 1 of Epstein–Barr virus [7], the human T-cell leukemia virus type I Tax protein [8], and polyribocytidilic-polyriboinosinic acid [9]. A20 protects cells from TNF-induced apoptosis as well as necrosis [3,10,11]. It should be mentioned that an anti-apoptotic activity of A20 cannot be observed in all cells, suggesting cell-line specific mechanisms. Interestingly, A20 also strongly inhibits nuclear factor-κB (NF-κB) dependent gene expression in response to TNF and several other stimuli [5,12,13]. A20 expression itself is regulated by NF-κB, and is mediated by two adjacent NF-κB-binding sites in the A20 promoter [7,14]. The latter suggests that A20 is involved in the negative feedback regulation of NF-κB activation. A20 deficient cells, indeed, fail to terminate TNF-induced NF-κB activation [15].

We previously identified a number of A20 associating proteins, including ABIN-1 (A20-binding inhibitor of NF-κB activation; previously referred [12] to as ABIN), ABIN-2, A20 itself, and TXBP151 [16]. ABIN-1 and ABIN-2, upon overexpression, inhibit NF-κB activation induced by both TNF and IL-1, and have therefore been proposed to be involved in the NF-κB inhibitory effect of A20 ([12], Van Huffel et al., manuscript in preparation). TXBP151 has anti-apoptotic properties, and has been shown to mediate at least partially the anti-apoptotic effect of A20 [17]. The functional relevance of A20 oligomerization is not known [18]. Zhang et al. have shown that A20 also binds inhibitor of NF-κB kinase γ (IKKγ) [19], which was identified as a component of the purified IKK complex and is essential for NF-κB activation [20]. Finally, A20 also binds to TNF receptor-associated factors (TRAFs), including TRAF2 and TRAF6 [13,21]. The C-terminal domain of A20 contains seven novel zinc finger motifs of the type $CX_{2-4}CX_{11}CX_2C$ [22] (Fig. 1). All of the A20 interacting proteins, except TRAF2 and TRAF6, interact with this C-terminal domain of A20 [16]. An A20 zinc finger was also identified in the GDP/GTP exchange protein rab5 [23] and the AWP1 (associated with PRK1) protein [24]. In this study, we examined in detail the role of the seven zinc fingers of A20 in both NF-κB inhibition and in the binding of A20 associating proteins. We show a functional redundancy of the seven zinc finger elements and a minimal requirement of four zinc fingers for full activity. Moreover, we demonstrate that binding of IKKγ as such is not sufficient for inhibition of NF-κB dependent gene expression in response to TNF.

2. Materials and methods

2.1. Cells and reagents

HEK293T cells (adenovirus-transformed human embryonic kidney cells, expressing SV40 large T antigen) (a kind gift of Dr. M. Hall, Department of Biochemistry, University of Birmingham, UK) were

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Abbreviations: ABIN, A20-binding inhibitor of NF-κB activation; IKK, inhibitor of nuclear factor-κB kinase; IL-1, interleukin-1; NF-κB, nuclear factor-κB; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor

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cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10^6 U/l streptomycin, 100 mg/ml penicillin and 0.4 mM sodium pyruvate. Recombinant human TNF was expressed in *Escherichia coli* and had a specific biological activity of 2.3×10^7 IU/mg purified protein, as determined with the International Standard (code 88/532) (National Institute for Biological Standards and Control, Potters Bar, UK). Protein A on Trisacryl beads waas obtained from Pierce Chemicals (Rockford, IL, USA). Anti-FLAG M2 monoclonal antibody was obtained from Sigma (St. Louis, MO, USA) and a monoclonal anti-E tag, horseradish peroxidase (HRP)-conjugated, antibody was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The polyclonal antibody directed against a C-terminal peptide of TXBP151 has been described previously [17]. A monoclonal antibody to anti-HA, HRP-conjugated, was obtained from Roche Molecular Biochemicals – Research (Basel, Switzerland).

2.2. Expression plasmids

The plasmid pNFconluc, containing the luciferase gene under the control of a minimal promoter, preceded by three NF-κB sites, was a gift from Dr. A. Israël (Institut Pasteur, Paris, France). pUT651, a β-galactosidase expression plasmid, was obtained from Cayla (Toulouse, France). Full-length FLAG-tagged murine A20 and its mutants were generated by standard PCR methods, and cloned into the eukaryotic expression vector pCAGGS [25] by means of *Xho*I/*Bgl*II digestion. A *Xho*I restriction site was included in the forward primer that also incorporated the FLAG epitope sequence in frame with the N-terminus of A20. The reverse primer contained a *Bgl*II restriction site and the stop codon of the murine A20 cDNA [12]. All constructs were confirmed by entirely sequencing the open reading frame of each individual mutant construct. The plasmids pCAGGS/ABIN-1 (containing the largest identified splice variant of ABIN-1), pCAGGS/TXBP151, and pCAGGS/A20 (E-tagged) have been described previously [12,17,26]. The plasmid pCAGGS/ABIN-2 will be described elsewhere (Van Huffel et al., manuscript in preparation). The plasmid pM-IKKγ was kindly provided by Dr. Kuan-Teh Jeang (NIH, Bethesda, MD, USA) and has been described [27].

2.3. NF-κB dependent reporter assays

HEK293T cells were seeded at a concentration of 2×10^5 cells/well on 6-well (35 mm) plates. Cells were transfected the next day by DNA calcium phosphate coprecipitation with 0.1 μg pUT651, 0.1 μg pNFconluc, and 0.1 μg of each of the FLAG-tagged A20 expression plasmids. The total amount of DNA (1 μg) was kept constant by supplementing with the empty vector, pCAGGS. 16–18 h after transfection, cells were seeded in 24-well plates. 24 h later, cells were either treated with TNF (1000 IU/ml) or left untreated for 6 h, prior to lysis in luciferase assay lysis buffer [26]. The β-galactosidase expression vector pUT651 was used to normalize transfection efficiencies.

2.4. Coimmunoprecipitation and immunoblotting

HEK293T cells were seeded at a concentration of 1.5×10^6 on 900 mm plates and transfected with a total of 4 μg of various constructs. 16–18 h after transfection, the medium was replaced. 36 h after transfection, the cells were harvested, washed twice with cold phosphate-buffered saline and lysed for 15 min at 4°C in 1 ml of E1A buffer (50 mM HEPES (pH 7.6), 250 mM NaCl, 5 mM EDTA and 0.1% NP-40) [28]. The samples were centrifuged at 14 000 rpm for 10 min, and 9/10 of the lysate was incubated with 5 μg of monoclonal anti-FLAG antibody for 2 h at 4°C, then mixed with 50 μl of a 1:1 slurry of protein A on Trisacryl beads, and incubation was continued for an additional 2 h. The beads were washed twice with 0.5 ml E1A buffer, twice with 0.5 ml E1B buffer, and twice again with 0.5 ml E1A buffer. E1B buffer is identical to E1A buffer, except for the presence of 1 M NaCl [28]. Protease inhibitors (100 μg/ml PMSF, 0.27 trypsin inhibitory U/ml aprotinin and 10 μg/ml leupeptin) and phosphatase inhibitors (2 mM Na_3VO_4, 10 mM NaF, and 26 mM β-glycerophosphate) were included in both E1A and E1B buffer throughout the entire procedure. In the case of the A20–IKKγ interaction, the beads were washed at least four times with 0.5 ml of E1A buffer, but not with E1B buffer [19]. The remainder of each cell lysate was used for the detection of the expressed proteins. The immunoprecipitates were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany). Western blot analysis was performed as described previously [21]. The anti-TXBP151 antibody was used at a 1:2000 dilution, the anti-FLAG antibody at 1 μg/ml, the anti-E tag–HRP conjugate at a 1:5000 dilution, and the anti-HA–HRP conjugate at a 1:3000 dilution. Secondary antibodies were used at a 1:2000 dilution.

3. Results

It is well established that the C-terminal zinc finger containing domain of both human and murine A20 is responsible for the inhibitory effect of A20 on TNF-induced NF-κB activation [13,21]. To investigate how many, and which zinc fingers in particular, are required for this activity, we generated a series of C-terminal deletion mutants of murine A20. Fig. 2A shows the relative expression level of the various A20 mutants, after transient expression in HEK293T. To analyze the inhibitory potential of each mutant on TNF-induced NF-κB activation, HEK293T cells were cotransfected with the corresponding expression plasmid, a NF-κB dependent luciferase reporter plasmid and a β-galactosidase expression plasmid. 36 h after transfection, cells were either untreated or stimulated with TNF for 6 h. Subsequently, cell lysates were prepared and analyzed for NF-κB dependent reporter gene expression. As shown in Fig. 2B, deletion of the two C-terminal zinc fingers (A20[1–699]) did not affect the potential of A20 as an inhibitor of TNF-induced NF-κB activation. Deletion of the three C-terminal zinc fingers (A20[1–640]) reduced the activity of A20 slightly, while a further deletion of one more zinc fingers (A20[1–590]) drastically affected A20's function. As shown previously, the N-terminal domain of A20 (A20[1–367]) was ineffective as an inhibitor of TNF-induced NF-κB activation [13,21]. These results show that a minimum of three zinc fingers is sufficient, but that a total of four zinc fingers is most effective for A20's function as an inhibitor of TNF-induced NF-κB activation.

Since the zinc fingers and the surrounding sequences are conserved in both human and murine A20 [22] (Fig. 1), we wondered if different zinc fingers could substitute for one another. To test this hypothesis, we created a series of internal

	aa				aa
Zf1	386	KCETPNCPFF MSVNTQPLCH ECSERRQKNQ SKLPKLNSKL	GPE------- ----------	----------	428
Zf2	469	KCRSPGCPFT LNVQHNGFCE RCHARQINAS HTADPG----	---------- ----------	----------	504
Zf3	505	KCQ--ACLQD VTRTFNGICS TCFKRTTAEP SSSLTSSIPA	SCHQ------ ----------	----------	546
Zf4	591	KCRKAGCMYF GTPENKGFCT LCFIEYRENK QSVTASAKAG	SPAPRFQNNV ----------	----------	640
Zf5	641	PCLGRECGTL GSTMFEGYCQ KCFIEAQNQR FHEARRTEEQ	LRSSQHRDMP RTTQVASRP-	----------	699
Zf6	700	KCARASCKNI LACRSEELCM ECQHLSQRVG SVAHRGEPTP	EEPPKQ---- ----------	----------	745
Zf7	746	RCRAPACDHF GNAKCNGYCN ECYQFKQMYG ----------	---------- ----------	----------	775

Fig. 1. Schematic representation of murine A20 and the localization of the seven zinc fingers (Zf) in its C-terminal domain (top). Amino acid sequence alignment of the seven zinc finger elements by ClustalW [29] analysis (bottom). The cysteine residues of the zinc finger structures are indicated in bold.

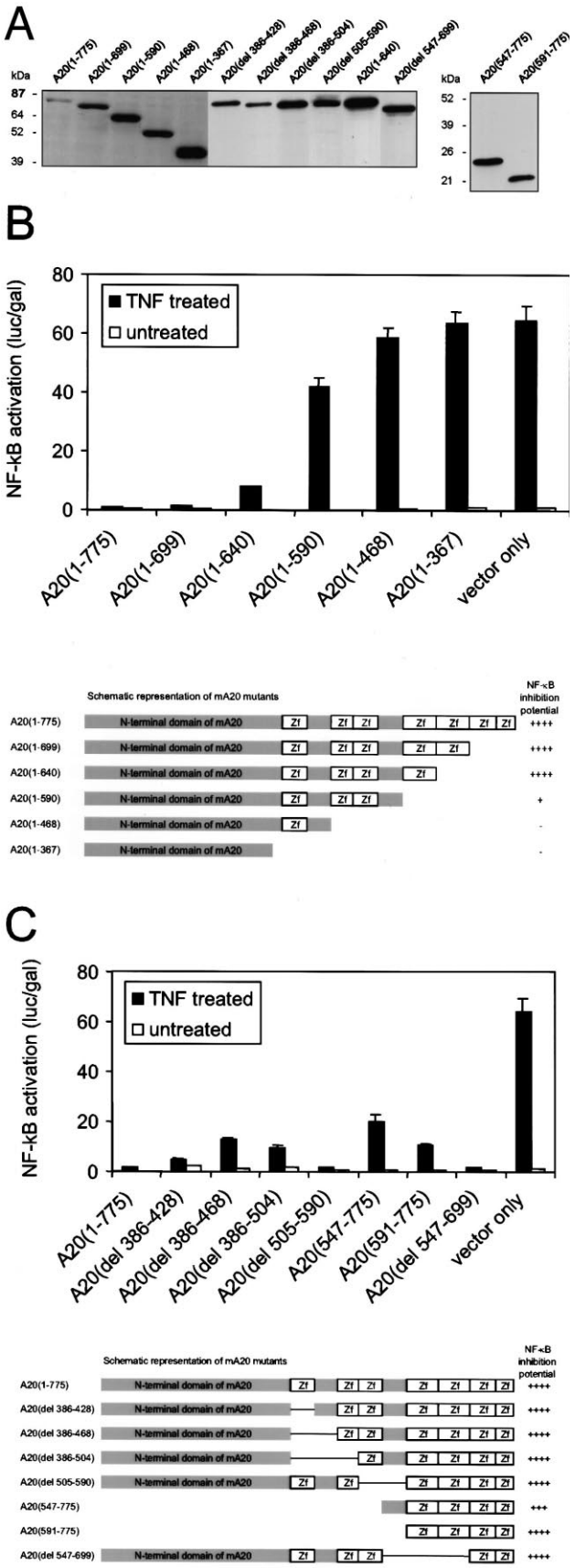

Fig. 2. Effect of A20 mutants on TNF-induced NF-κB activation. HEK293T cells were transiently cotransfected with the indicated FLAG-tagged mutant A20 expression plasmids, a NF-κB dependent luciferase reporter plasmid, and a β-galactosidase expression plasmid. 36 h after transfection, cells were analyzed for A20 expression by SDS–PAGE and immunoblotting (A). The effect of C-terminal (B) or internal (C) deletion mutants of A20 on NF-κB dependent gene expression in response to 6 h TNF treatment was measured as described in Section 2. Values correspond to means ± S.E.M. of three cell lysates. The results are representative for at least three independent experiments.
←

deletions in the C-terminal domain of murine A20. As shown in Fig. 2C, deletion of none of the three N-terminal zinc fingers (A20[del 386–428], A20[del 386–468], A20[del 386–504], and A20[del 505–590]) significantly affected the function of A20. Moreover, a combination of the last four zinc fingers (A20[591–775]) was almost as effective as the wild-type A20 protein. These results further demonstrate that a total of four A20 zinc fingers is sufficient to inhibit TNF-induced NF-κB activation, and that the intervening non-zinc finger containing elements are not essential. Moreover, two regions in the C-terminal domain of A20, corresponding to either the first four zinc fingers or the last four zinc fingers, can mediate its NF-κB inhibitory potential. Because all of the above mentioned A20 mutants that inhibited TNF-induced NF-κB activation still contained the fourth zinc finger of A20, it was still possible that this specific zinc finger was responsible for NF-κB inhibition. However, a mutant in which zinc fingers 4 and 5 were deleted, thereby fusing zinc finger 3 with zinc finger 6 (A20[del 547–699]), was as potent as the wild-type murine A20 to inhibit TNF-induced NF-κB activation. This ruled out the

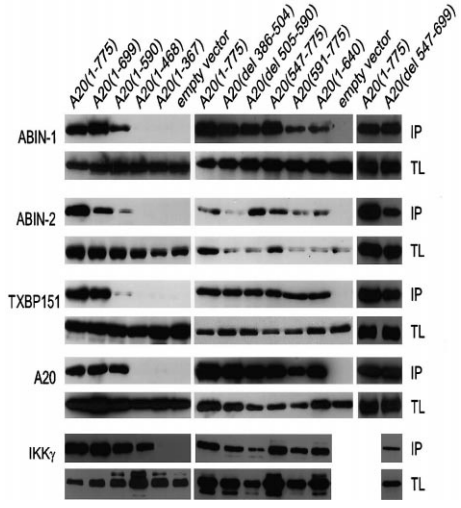

Fig. 3. Binding of A20 mutants with ABIN-1, ABIN-2, A20, TXBP151 and IKKγ. FLAG-tagged A20 and its deletion mutants, as indicated, were cotransfected with either E-tagged ABIN-1, E-tagged ABIN-2, TXBP151, E-tagged A20, or HA-tagged IKKγ into HEK293T cells. 36 h after transfection, cell extracts were prepared and A20 and A20 mutants were immunoprecipitated with anti-FLAG antibody M2. Coimmunoprecipitating proteins were revealed by SDS–PAGE and immunoblotting. For each coimmunoprecipitating protein, the upper panel shows the amounts of coprecipitating protein (IP), while the lower panel shows the expression of each of the coprecipitating proteins in total lysates (TL). Similarly, equal expression of the various A20 mutant proteins was confirmed by immunoblotting total cell extracts with anti-FLAG antibody (data not shown).

possibility that zinc finger four was essential for A20's function. Overall, we conclude that there is redundancy of the zinc fingers of A20 for inhibition of NF-κB activation.

We next examined if a similar redundancy exists for binding of A20 to several of the A20 associating proteins, and whether loss of inhibition of TNF-induced NF-κB activation by deletion of specific zinc fingers is associated with loss of A20's capacity to bind specific proteins. For this purpose, we transiently transfected the A20 mutant expression plasmids (which all contained an N-terminal FLAG tag) individually with either an expression plasmid encoding ABIN-1, ABIN-2, or TXBP151. All of these proteins have been shown previously to bind to the C-terminal zinc finger containing domain of A20 ([12,17], Van Huffel et al., manuscript in preparation). 36 h after transfection, cell lysates were prepared and an antibody directed against the FLAG epitope was used to immunoprecipitate the various A20 proteins. Coimmunoprecipitating proteins were revealed by SDS–PAGE and immunoblotting. Deletion of the last four zinc fingers in A20 (A20[1–590]) significantly reduced its association with ABIN-1, ABIN-2, and TXBP151, but a mutant having only the last four zinc fingers of A20 (A20[591–775]) still retained its ability to bind these three proteins (Fig. 3). These results demonstrate that the A20 zinc fingers also have a redundant role in the binding to other proteins. Although comparison of the effect of the different A20 mutants on NF-κB activation and their ability to bind to other proteins does not allow us to make any conclusions about the role of these proteins in NF-κB inhibition, our results clearly demonstrate that a correlation exists between the loss of their binding and A20's ability to inhibit NF-κB activation.

To analyze the role of specific zinc fingers in A20 oligomerization, we cotransfected FLAG-tagged mutant A20 expression plasmids with an E-tagged A20 expression plasmid. Fig. 3 shows that deleting the first three (A20[547–775]) or the last four zinc fingers (A20[1–590]) considerably compromised A20's ability to inhibit TNF-induced NF-κB activation, we conclude that A20 oligomerization as such is not sufficient for NF-κB inhibition.

Finally, we examined the interaction of A20 with IKKγ. Zhang et al. have demonstrated that both the N-terminal and C-terminal domains of A20 are involved in binding to IKKγ [19]. However, we were unable to show any interaction of IKKγ with the N-terminal domain of A20 (which lacks any zinc finger) (Fig. 3). Extending the N-terminal domain with as little as one zinc finger (A20[1–468]) was sufficient to restore IKKγ-binding almost completely. Interestingly, the latter mutant had no NF-κB inhibitory activity, suggesting that binding of A20 to IKKγ as such is not sufficient for its NF-κB inhibitory potential.

4. Discussion

In this study, we have investigated by deletional analysis the role of the seven zinc fingers of A20, for its NF-κB inhibitory potential and capacity to bind a number of proteins. Remarkably, either the first three or last four zinc fingers are sufficient for NF-κB inhibition, indicating that two regions in the C-terminal domain of A20 can mediate its NF-κB inhibitory effect. In contrast to our study, Natoli et al. have demonstrated that the last zinc finger of human A20 is absolutely required for inhibition of TNF-induced NF-κB activation [30]. However, we show that deletion of zinc fingers 6 and 7 does not affect the NF-κB inhibitory potential of murine A20. The reason for these controversial results is still unclear, but might be due to differences in expression levels and the cells used in both studies (HEK293T versus HeLa cells). Natoli et al. also demonstrated that a four times fusion of zinc finger 7 of human A20 is sufficient to inhibit TNF-induced NF-κB activation [30]. Together with our finding that the last four zinc fingers of murine A20 are sufficient for NF-κB inhibition, these results further demonstrate the minimal requirement for a particular number of zinc fingers and their functional redundancy in inhibiting TNF-induced NF-κB activation.

A similar redundancy of the zinc fingers could be demonstrated for A20's ability to bind to ABIN-1, ABIN-2, TXBP151, IKKγ, and A20 itself. All these proteins are able to bind a mutant A20, lacking the last four zinc fingers, as well as to a mutant consisting of only the last four zinc fingers, albeit with different affinity. This suggests that at least two different proteins might bind simultaneously to the C-terminal domain of a single A20 protein. Whether A20 oligomerization is involved in its inhibition of NF-κB activation is still unclear. However deletion of the last four zinc fingers strongly reduced the activity of A20, whereas its oligomerization was unaffected. Therefore, it is unlikely that A20 oligomerization as such is sufficient for NF-κB inhibition. Similarly, A20's potential to bind IKKγ does not correlate with inhibition of TNF-induced NF-κB activation. In this case, a mutant A20 protein containing only the N-terminal domain and the first zinc finger was still able to bind IKKγ, but was unable to prevent NF-κB activation. These results clearly indicate that binding of A20 to the IKK complex might be involved, but is certainly not sufficient for NF-κB inhibition.

In conclusion, we have provided evidence that the zinc fingers of A20 have a redundant role in the inhibition of TNF-induced NF-κB activation, as well as in the binding of several proteins. The latter also suggests that the C-terminal domain of A20 might have the potential to simultaneously bind at least two different proteins, and act as a scaffold protein.

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