

# Structure–function analysis of the A20-binding inhibitor of NF- $\kappa$ B activation, ABIN-1

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**Abstract** Nuclear factor  $\kappa$ B (NF- $\kappa$ B)-dependent gene expression plays an important role in numerous cellular processes including stress responses, inflammation and cell proliferation. Therefore, the activity of this transcription factor needs to be tightly regulated. Among others, the NF- $\kappa$ B-dependent zinc finger protein A20 is involved in the negative feedback regulation of NF- $\kappa$ B activation in response to tumor necrosis factor (TNF). We previously demonstrated that A20 can interact with A20-binding inhibitors of NF- $\kappa$ B activation (ABINs), which have the potential to inhibit TNF-induced activation of NF- $\kappa$ B upon overexpression. The ABIN proteins were therefore proposed to mediate the NF- $\kappa$ B inhibiting function of A20. Here we demonstrate the presence of a short homologous region in ABINs and I $\kappa$ B kinase  $\gamma$ , the regulatory subunit of the I $\kappa$ B kinase complex. Site-specific mutagenesis of this region abolished the NF- $\kappa$ B inhibiting function of ABIN-1, without affecting the interaction with A20. Furthermore, coexpression of these ABIN-1 mutants interfered in a dominant negative manner with the NF- $\kappa$ B inhibiting function of ABIN-1, whereas the A20-mediated inhibition was unaffected. These results suggest that A20 and ABIN-1 probably act independently of their mutual interaction.

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**Key words:** Inflammation; Nuclear factor  $\kappa$ B; Gene expression; Signal transduction

## 1. Introduction

The transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) is one of the main regulators involved in inflammatory responses, as well as several other cellular functions including cell growth and differentiation. Uncontrolled NF- $\kappa$ B activity is a major hallmark of several inflammatory diseases. Therefore, activation of this transcription factor is tightly regulated and is only transient. Negative feedback regulation of NF- $\kappa$ B activity requires, besides the well studied NF- $\kappa$ B interacting protein I $\kappa$ B, also the zinc finger protein A20: mice deficient for A20 develop cachexia, are hyperresponsive to tumor necrosis fac-

tor (TNF) and suffer from severe inflammation correlated with enhanced NF- $\kappa$ B activity [1]. A20 is a primary response gene that was originally identified as a cytokine-inducible gene in human umbilical vein endothelial cells, and which encodes a protein of approximately 80 kDa of which the C-terminal domain consists of seven Cys2/Cys2 zinc finger structures [2]. In the meantime, A20 has been shown to be an NF- $\kappa$ B-responsive gene that can be induced in several cell lines by a variety of stimuli including TNF, interleukin-1 (IL-1), bacterial lipopolysaccharide (LPS), the B-cell surface receptor CD40, phorbol 12-myristate 13-acetate, as well as by overexpression of the Epstein–Barr virus LMP1 protein and the human T-cell leukemia virus Tax protein [2–6].

Although A20 was originally described as an inhibitor of TNF-induced apoptosis [7], it is best characterized as a potent cellular inhibitor of NF- $\kappa$ B activation [8–12]. However, the underlying molecular mechanisms still remain largely unknown. A20 has been described to interact with the TNF receptor-associated factors TRAF1 and TRAF2 [13]. The latter proteins form an oligomeric complex that associates with the cytoplasmic domains of various members of the TNF receptor superfamily. Gene targeting studies indicated that TRAF2 is required for optimal TNF-induced activation of the I $\kappa$ B kinase (IKK) complex [14]. The latter complex contains two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , which upon activation phosphorylate the NF- $\kappa$ B inhibiting protein I $\kappa$ B, making I $\kappa$ B a substrate for ubiquitination and proteasomal degradation, thus allowing translocation of NF- $\kappa$ B to the nucleus [15]. Another core component of the IKK complex is the regulatory subunit IKK $\gamma$ , which upon TNF triggering interacts with the death domain kinase RIP (receptor interacting protein), resulting in dimerization and activation of the IKK complex [16,17]. More recently, A20 was also shown to interact with IKK $\gamma$  in a TNF-dependent manner [18]. However, direct interaction of A20 with TRAF proteins or with IKK $\gamma$  is probably not sufficient for its NF- $\kappa$ B inhibiting function: structure–function studies using deletion mutants of A20 indicated that interaction of A20 with these NF- $\kappa$ B intermediates is mediated by the N-terminal, non-zinc finger-containing part of A20 or with the N-terminal domain extended by one zinc finger, respectively, whereas the C-terminal zinc finger-containing part of A20 containing at least four zinc finger structures is essential for inhibition of NF- $\kappa$ B activation [13,19]. Yeast two-hybrid studies revealed the binding of the zinc finger domain of A20 with two novel proteins, named ABIN-1 (previously called ABIN, for A20-binding inhibitor of NF- $\kappa$ B activation) and ABIN-2 [9,20]. Overexpression of each ABIN protein mimics the NF- $\kappa$ B inhibiting effect of

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**Abbreviations:** ABIN, A20-binding inhibitor of NF- $\kappa$ B activation; AHD, ABIN homology domain; I $\kappa$ B, inhibitor of  $\kappa$ B; IKK, I $\kappa$ B kinase; IL-1, interleukin-1; LPS, lipopolysaccharide; NF- $\kappa$ B, nuclear factor kappa B; RIP, receptor interacting protein; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor

A20, suggesting that this function of A20 may at least partially be mediated by ABINs. In the present study, we provide evidence that interaction of A20 and ABIN-1 is not essential for TNF-induced NF- $\kappa$ B inhibition.

## 2. Materials and methods

### 2.1. Cell lines and reagents

Human embryonic kidney HEK293T cells were a kind gift of Dr. M. Hall (Department of Biochemistry, University of Birmingham, UK). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.4 mM sodium pyruvate and antibiotics. Recombinant human TNF was expressed in *Escherichia coli*, purified to at least 99% homogeneity and had a specific biological activity of  $2.3 \times 10^7$  IU/mg purified protein, as determined with the international standard (code 87/650; National Institute for Biological Standards and Control, Potters Bar, UK). Monoclonal anti-E tag antibody and monoclonal anti-E tag horseradish peroxidase-linked antibody were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden), monoclonal anti-FLAG horseradish peroxidase-linked antibody from Sigma-Aldrich (St. Louis, MO, USA), monoclonal anti-HA antibody from CRP (Richmond, CA, USA). Anti-mouse horseradish peroxidase-linked antibodies were obtained from Amersham Pharmacia Biotech.

### 2.2. Expression vectors

The eukaryotic expression vectors pUT651, pNF $\kappa$ conluc, pCAGGS-E-A20, pCAGGS-FLAG-A20, pCAGGS-E-ABIN-1 have been described earlier [9,19,21,22]. The vector pCAGGS-HA-ABIN-1 was obtained by cloning the full-length coding sequence of ABIN-1 in frame with an N-terminally located HA tag in the eukaryotic expression vector pCAGGS. Fragments of ABIN-1 were amplified by polymerase chain reaction (PCR) using specific primers and were cloned in frame with an N-terminal E tag in pCAGGS. Site-specific mutations in ABIN-1 were introduced by overlap PCR using specific primers (5'-GTTGCTGAAAGAGGACGTCAAATCTTTGA-3' for ABIN-1-MUT1; 5'-GCAGGTAAAAATCTTTGAAGAGAATGCCAGAGGGAACG-3' for ABIN-1-MUT2; 5'-GCAGGTAAAAATCTTTGAAGAGGACTTCCAGAGGGAACG-3' for ABIN-1-MUT3). Mutated ABIN-1 cDNAs were cloned in the eukaryotic expression vector pCAGGS C-terminal of the E tag.

### 2.3. Coimmunoprecipitation and Western blotting

HEK293T cells ( $1.5 \times 10^6$ ) were plated on 10-cm Petri dishes and transfected by calcium phosphate precipitation with 5  $\mu$ g of expression plasmids. Twenty-four hours after transfection cells were lysed in

500  $\mu$ l of lysis buffer (50 mM HEPES pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40 and 5 mM EDTA, supplemented with protease and phosphatase inhibitors). Lysates were incubated with the indicated antibodies and immobilized protein A on Trisacryl (Pierce Chemicals, Rockford, IL, USA). Beads were washed twice with lysis buffer, twice with lysis buffer containing 1 M NaCl and twice with lysis buffer. Coprecipitating proteins were separated by SDS-PAGE and analyzed by Western blotting using ECL detection (Perkin Elmer Life Sciences).

### 2.4. NF- $\kappa$ B reporter gene assay

HEK293T cells were seeded in 24-well plates and transiently transfected by a standard calcium phosphate coprecipitation method using 200 ng DNA per well. Each transfection contained 20 ng of pNF $\kappa$ conluc plasmid and 20 ng pUT651 plasmid. Twenty-four hours after transfection cells were either stimulated with 1000 IU/ml hTNF or left untreated. After 6 h stimulation, cells were lysed in 200  $\mu$ l lysis buffer and NF- $\kappa$ B-dependent luciferase activity was measured as previously described [22]. Constitutively expressed  $\beta$ -galactosidase activity was measured to normalize for differences in transfection efficiency.

## 3. Results and discussion

### 3.1. A region that is conserved between ABINs and IKK $\gamma$ is necessary but not sufficient for the NF- $\kappa$ B inhibiting function of ABIN-1

The underlying mechanism of negative feedback regulation of NF- $\kappa$ B activation by A20 is still unclear. Structure–function analysis of A20 indicated that similar domains of A20 are required for the NF- $\kappa$ B inhibiting potential of A20 as well as for its interaction with the NF- $\kappa$ B inhibiting proteins ABIN-1 and ABIN-2. Therefore ABIN proteins might at least partially mediate the effect of A20 [19]. Amino acid sequence comparison of the A20 interacting proteins ABIN-1 and ABIN-2 indicated the presence of two homologous regions of approximately 20 amino acids, further designated AHD1 and AHD2 (ABIN homology domain [20]). PSI-BLAST searches at NCBI led to the identification of a homologous AHD2 sequence in the C-terminal part of IKK $\gamma$ , as well as in LIND (which we refer to as ABIN-3) and the IKK $\gamma$ -related protein, NRP [23,24]. Furthermore, for these proteins the AHD2

<b>ABIN-1</b>	<b>Hs</b>	<b>AAG42154</b>	461	<b>LLKQQVKI<b>FEEDFQ</b>RE<b>RS</b>DRE<b>RM</b></b>	483
	<b>Mm</b>	<b>CAB44240</b>	474	<b>LLKQQVKI<b>FEEDFQ</b>RE<b>RS</b>DRE<b>RM</b></b>	496
	<b>Gg</b>	<b>AAG42156</b>	183	<b>LLKQQVKI<b>FEEDFQ</b>RE<b>RS</b>DRE<b>RM</b></b>	205
<b>ABIN-2</b>	<b>Hs</b>	<b>CAC34835</b>	298	<b>MLEQQILAYKDD<b>FMSE</b>RA<b>DR</b>ER<b>A</b></b>	320
	<b>Mm</b>	<b>CAC34841</b>	299	<b>MLEQQILAYKDD<b>FKSE</b>RA<b>DR</b>ER<b>A</b></b>	320
<b>LIND/ABIN-3</b>	<b>Hs</b>	<b>AAL02151</b>	199	<b>VLKQQVQI<b>YEEDF</b>KK<b>ER</b>S<b>DRE</b>RL</b>	221
<b>IKK<math>\gamma</math></b>	<b>Hs</b>	<b>CAB93146</b>	300	<b>VLKAQADI<b>YKAD</b>FQ<b>AE</b>RQ<b>ARE</b>KL</b>	322
	<b>Mm</b>	<b>AAC40153</b>	293	<b>VLKAQADI<b>YKAD</b>FQ<b>AE</b>RH<b>ARE</b>KL</b>	315
	<b>Bt</b>	<b>CAC93688</b>	300	<b>VLKAQADI<b>YKAD</b>FQ<b>AE</b>RQ<b>ARE</b>KL</b>	322
	<b>Dm</b>	<b>AF294396</b>	269	<b>GLQIQNDI<b>YRRD</b>F<b>EMER</b>AD<b>RE</b>KN</b>	291
<b>NRP</b>	<b>Hs</b>	<b>AAG00497</b>	463	<b>ILRAQMEV<b>YCSDF</b>HA<b>ERA</b>ARE<b>KI</b></b>	485
	<b>Mm</b>	<b>AAL61853</b>	466	<b>VLRAQMEV<b>YCSDF</b>HA<b>ERA</b>ARE<b>KI</b></b>	488
	<b>Rn</b>	<b>BAB84696</b>	468	<b>VLRAQMEV<b>YCSDF</b>HA<b>ERA</b>ARE<b>KI</b></b>	490
	<b>Mf</b>	<b>BAB33067</b>	457	<b>VLRAQMEV<b>YCSDF</b>HA<b>ERA</b>ARE<b>KI</b></b>	479
<b>IKK<math>\gamma</math>(D311N)</b>	<b>Hs</b>		300	<b>VLKAQADI<b>YKAN</b>FQ<b>AE</b>RQ<b>ARE</b>KL</b>	322

Fig. 1. Sequence alignment of the conserved AHD2 region found in ABIN-1, ABIN-2, LIND/ABIN-3, IKK $\gamma$  and NRP of different species. Identical and homologous amino acid residues present in all proteins of the different species are indicated in bold or underlined, respectively (Bt: *Bos taurus*; Dm: *Drosophila melanogaster*; Gg: *Gallus gallus*; Hs: *Homo sapiens*; Mf: *Macaca fascicularis*; Mm: *Mus musculus*; Rn: *Rattus norvegicus*). Also the sequence of the AHD2 domain of the IKK $\gamma$ (D311N) mutant, found in a family suffering from X-linked anhydrotic ectodermal dysplasia with immunodeficiency, is indicated [25].

sequence is highly conserved throughout different species (Fig. 1).

To unravel the importance of this AHD2 sequence in ABIN-1, deletion mutagenesis and site-specific mutagenesis was applied. Previously we demonstrated that the NF- $\kappa$ B inhibiting function is located in the C-terminal part of ABIN-1 (aa 390–647) that contains this homology sequence. Therefore we generated further N- and C-terminal deletion mutants starting from ABIN-1(390–647) (Fig. 2A). On the other hand, three site-specific mutants of ABIN-1, indicated by the extensions MUT1, MUT2 and MUT3, were made in which each time two conserved amino acids of AHD2 were replaced (Fig. 2B).

To analyze the contribution of AHD2 to the NF- $\kappa$ B inhibiting effects of ABIN-1, we transiently transfected HEK293T cells with expression plasmids encoding ABIN-1, ABIN-1 deletion mutants or ABIN-1-MUTs and studied their effects on TNF-induced activation of NF- $\kappa$ B by luciferase reporter tests. Overexpression of deletion mutants of ABIN-1 indicated that the shortest ABIN-1 fragment that still inhibits TNF-induced NF- $\kappa$ B activation was composed of amino acids 444–601 containing the AHD2 domain with an extra 30 amino acids N-terminally and 105 amino acids C-terminally (Fig. 2C).

Further N- or C-terminal deletions abolished this function indicating that besides the AHD2 sequence N- and C-terminal sequences are required for the function of ABIN-1. In addition, AHD2 site-specific mutants of ABIN-1 were not able to inhibit TNF-induced activation of NF- $\kappa$ B, even at high expression levels (Fig. 2D), demonstrating an essential role for a short region (AHD2) in ABIN-1 that is closely related to a region in the C-terminal part of IKK $\gamma$ , as well as to a region in ABIN-2, ABIN-3 and NRP [20,23,24]. Whereas overexpression of ABIN-1, ABIN-2 or ABIN-3 abrogates TNF-induced NF- $\kappa$ B activation, no effect was observed upon overexpression of NRP [9,20,24]; our unpublished data). Interestingly, in families suffering from X-linked anhydrotic ectodermal dysplasia with immunodeficiency, a disease correlated with impaired NF- $\kappa$ B signalling, several point mutations in the C-terminus of IKK $\gamma$  have been found. One of these mutations corresponds to a conserved aspartic acid residue in AHD2, suggesting also an important role of this homologous region in IKK $\gamma$  [25]. Taking into account the above findings we hypothesize that ABIN-1 competes with IKK $\gamma$  for an upstream IKK $\gamma$  activator that binds the AHD2 region. However, activation mechanisms of IKK $\gamma$  are still largely unclear. A role for RIP, CIKS/Act1 and TANK/I-TRAF has been proposed,

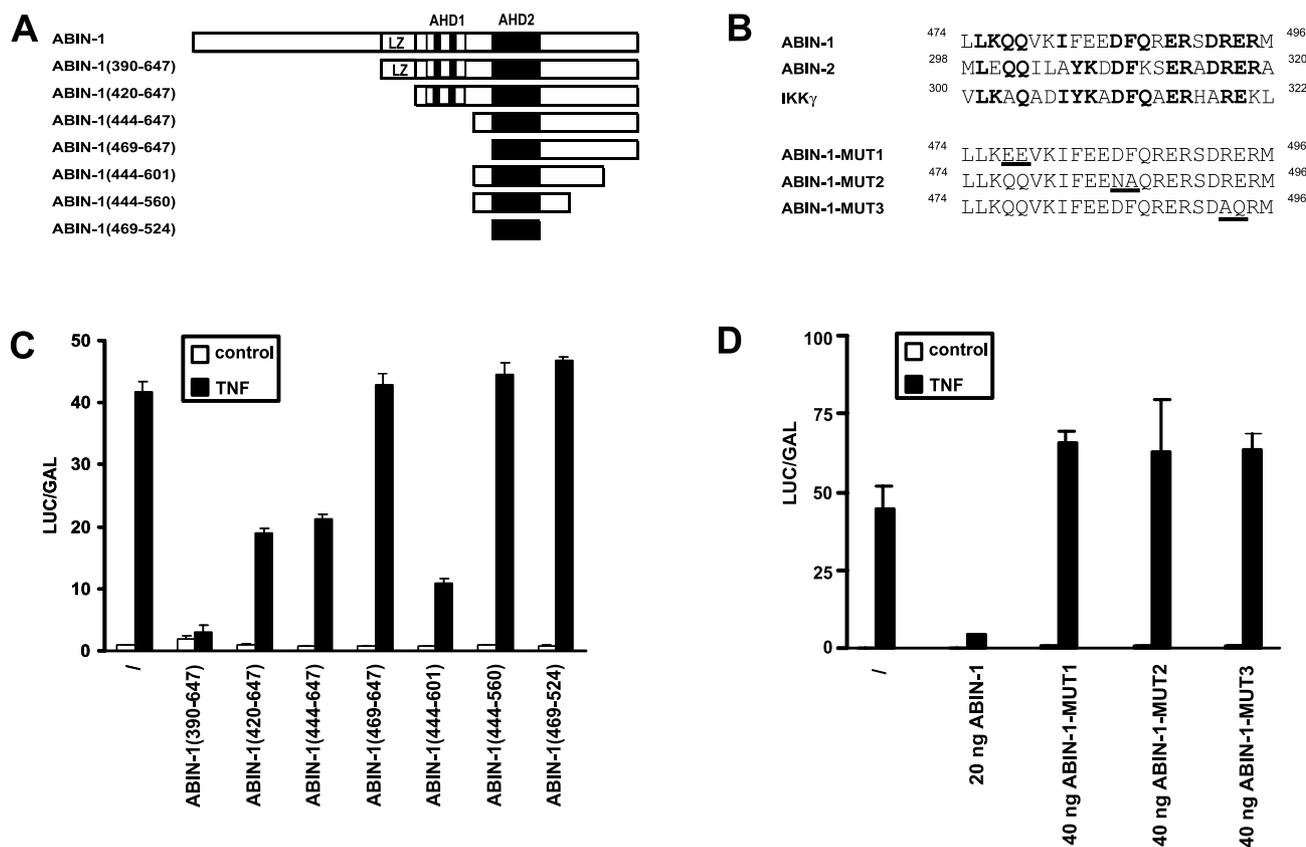


Fig. 2. Structure–function analysis of the NF- $\kappa$ B inhibiting capacity of ABIN-1. A: Schematic overview of deletion mutants of ABIN-1. Homologous regions AHD1 and AHD2 are indicated. B: Comparison of AHD2 sequences of ABIN-1, ABIN-2 and IKK $\gamma$ . Identical amino acids are indicated in bold. Conserved amino acids were mutated in the AHD2 sequence of ABIN-1 by overlap PCR, generating ABIN-1-MUT1, ABIN-1-MUT2 and ABIN-1-MUT3 (mutated amino acids are underlined), and confirmed by sequence analysis. C,D: Effect of ABIN-1 mutants on TNF-induced NF- $\kappa$ B activation. HEK293T cells were transiently transfected with 20 ng expression vectors encoding the different ABIN-1 mutants together with 20 ng pNFconluc and 20 ng pUT651. After 24 h, cells were left untreated (open bars) or stimulated for 6 h with 1000 IU/ml TNF (filled bars). NF- $\kappa$ B activity was determined via luciferase and  $\beta$ -galactosidase reporter gene tests and is shown as luc/gal values. Results are representative of at least three independent experiments. Each bar represents the mean of three samples.

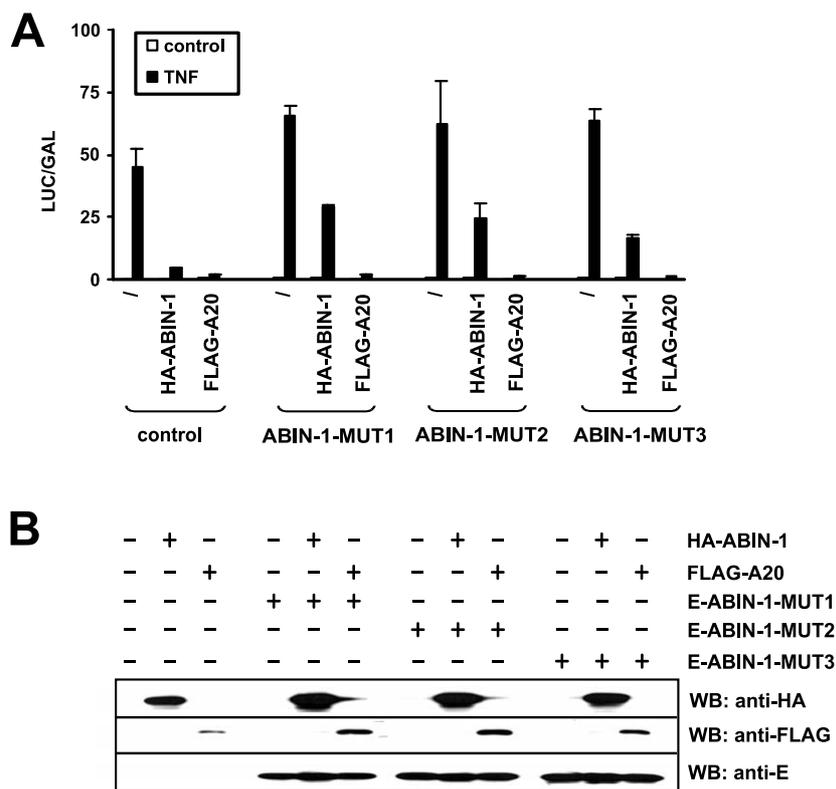


Fig. 3. Coexpression of ABIN-1 AHD2 site-specific mutants interferes in a dominant negative manner with the NF-κB inhibiting function of wild type ABIN-1. To analyze if overexpression of the site-specific mutants of ABIN-1 interfered with the NF-κB inhibiting function of wild type ABIN-1 or A20, an excessive amount of E-tagged ABIN-1-MUTs (40 ng) was cotransfected with either HA-tagged ABIN-1 (20 ng) or FLAG-tagged A20 (20 ng) in HEK293T cells. Cells were left untreated (open bars) or stimulated with 1000 IU/ml TNF (filled bars) for 6 h. Cells were lysed and NF-κB-dependent luciferase reporter gene expression and constitutive β-galactosidase expression were analyzed. Each luciferase value represents the mean of three samples (A). The corresponding expression levels were analyzed by Western blotting (B). Results are representative of at least three independent experiments.

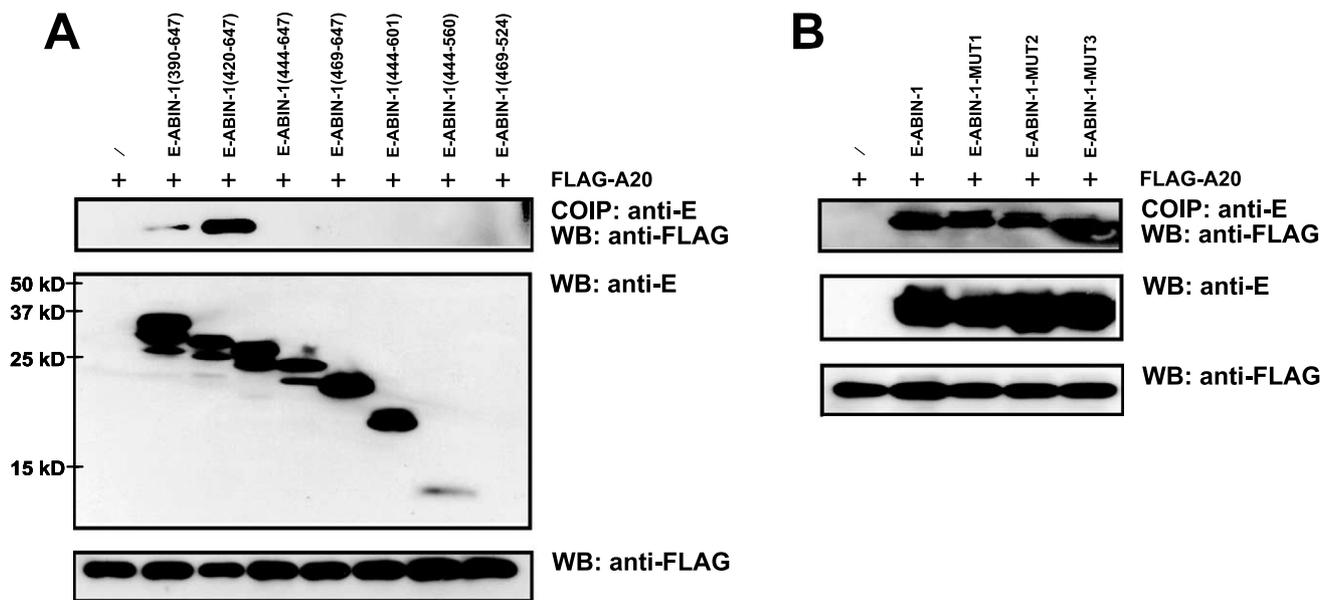


Fig. 4. Interaction of ABIN-1 mutants with A20.  $1.5 \times 10^6$  HEK293T cells were transiently transfected with 1 μg pCAGGS-FLAG-A20 together with 1 μg empty vector or pCAGGS vectors encoding ABIN-1 deletion (A) or site-specific mutants (B). Twenty-four hours after transfection, ABIN-1 variants were immunoprecipitated using anti-E tag antibodies, and the presence of coimmunoprecipitated A20 was revealed by immunoblotting using anti-FLAG antibodies, as shown in the upper panels. Aliquots of total lysates were analyzed to confirm expression of transfected ABIN-1 proteins or A20 using anti-E tag and anti-FLAG tag, respectively, as shown in the lower panels. The cause of the appearance of multiple bands after overexpression of ABIN-1(390–647), ABIN-1(420–647), ABIN-1(444–647) and ABIN-1(469–647) is still unclear.

but these proteins interact with an intermediate domain of IKK $\gamma$  [18,26,27]. So far no IKK $\gamma$ -activating signalling intermediate that specifically binds to the AHD2 region has been identified.

### 3.2. Coexpression of ABIN-1 AHD2 mutants prevents the NF- $\kappa$ B inhibiting function of ABIN-1

Since AHD2 site-specific mutants of ABIN-1 were unable to inhibit TNF-induced activation of NF- $\kappa$ B, we analyzed if these mutants can interfere in a dominant negative manner with the NF- $\kappa$ B inhibiting effects of ABIN-1 or A20. Therefore an excessive amount of plasmids containing these E-tagged ABIN-1-MUTs was cotransfected with expression vectors containing HA-tagged ABIN-1 or FLAG-tagged A20 in HEK293T cells. NF- $\kappa$ B activation was analyzed in a luciferase reporter test after stimulation with TNF for 6 h. Coexpression of the ABIN-1-MUTs with wild type ABIN-1 clearly interferes with the NF- $\kappa$ B inhibiting effect of ABIN-1. In contrast, A20 retains its NF- $\kappa$ B inhibiting function even in the presence of high expression levels of ABIN-1-MUTs (Fig. 3A). As shown by Western blotting, these effects are not due to altered expression levels of wild type ABIN-1 or A20 (Fig. 3B). These results further demonstrate the importance of the AHD2 sequence in the NF- $\kappa$ B inhibitory function of ABIN-1. Furthermore, the observation that coexpression of inactive ABIN-1 mutants carrying a specific mutation in their AHD2 region with wild type ABIN-1 or A20 specifically prevents the effect of ABIN-1 but not of A20 indicates that A20 can function independently of ABIN-1.

### 3.3. A20 binding is not required for NF- $\kappa$ B inhibition by ABIN-1

To further analyze if the NF- $\kappa$ B inhibiting function of the ABIN-1 fragments is correlated with their interaction with A20, coimmunoprecipitation analysis was performed. Therefore HEK293T cells were transiently transfected with FLAG-tagged A20 with an empty vector as a negative control, or with vectors encoding the different E-tagged ABIN-1 mutants. Coimmunoprecipitation was performed using anti-E tag antibodies and coimmunoprecipitating A20 was revealed with anti-FLAG-tagged antibodies. This demonstrated that ABIN-1(420–647), which corresponds to the C-terminal domain lacking the leucine zipper structure, interacts with higher efficiency with A20 compared to ABIN-1(390–647), which was previously designated the A20 interacting domain [9]. In contrast, the minimal ABIN-1 fragment that still inhibits NF- $\kappa$ B activation (ABIN-1(444–601)) does not interact with A20 (Fig. 4A), indicating that interaction with A20 is not required for the NF- $\kappa$ B inhibiting function of ABIN-1. On the other hand, site-specific mutants of ABIN-1 without NF- $\kappa$ B inhibiting activity still interact with A20 (Fig. 4B). Consequently, although the AHD2 domain is necessary for the NF- $\kappa$ B inhibiting function of ABIN-1, this region is not the A20 interacting domain. Similarly, also the AHD2-like sequence in IKK $\gamma$  is not involved in A20 IKK $\gamma$  binding. In this case, A20 binding was localized in a region spanning amino acids 95–218, which does not include the homologous AHD2 region (aa 300–322) [18]. Conversely, the interaction of A20 with IKK $\gamma$  and ABIN-1 seems to be mediated by different domains in A20 [19], further demonstrating that the homologous AHD2 region is not an A20 interaction domain. Alignment of the amino acid sequences of the ABIN proteins revealed

the presence of another conserved amino acid stretch, referred to as AHD1, which is however absent in IKK $\gamma$  and NRP. Via deletion analysis of ABIN-1 we obtained evidence that AHD1 is involved in the interaction of ABIN-1 with A20 (Fig. 4A). The absence of a correlation between A20 binding and NF- $\kappa$ B inhibition further indicates that interaction with A20 is not required for NF- $\kappa$ B inhibition by ABIN-1. At this moment, the biological significance of the interaction of A20 with ABIN-1 remains unknown.

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