ABIN-1 Binds to NEMO/IκKB and Co-operates with A20 in Inhibiting NF-κB*

Received for publication, February 16, 2006, and in revised form, April 26, 2006. Published, JBC Papers in Press, May 9, 2006, DOI 10.1074/jbc.M601502200

Claudio Mauro†§, Francesco Pacifico†§, Alfonso Lavoroga●, Stefano Mellone●, Alessio Iannetti●, Renato Acquaviva●, Silvestro Formisano●, Pasquale Vito● and Antonio Leonardi‡

From the †Dipartimento di Biologia e Patologia Cellulare e Molecolare, “Federico II,” University of Naples, via S. Pansini, 5, 80131 Naples, the ●Istituto di Endocrinologia ed Oncologia Sperimentale, CNR, via S. Pansini, 5, 80131 Naples, and the §Dipartimento di Scienze Biologiche ed Ambientali, Università degli Studi del Sannio, via Port’Arsa, 11, 82100 Benevento, Italy

Nuclear factor κB (NF-κB) plays a pivotal role in inflammation, immunity, stress responses, and protection from apoptosis. Canonical activation of NF-κB is dependent on the phosphorylation of the inhibitory subunit IκBα that is mediated by a multimeric, high molecular weight complex, called IκB kinase (IKK) complex. This is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO/IκKB. The latter particle is essential for the activation of IKKs and NF-κB, but its mechanism of action is not well understood. Here we identified ABIN-1 (A20 binding inhibitor of NF-κB) as a NEMO/IκKγ-interacting protein. ABIN-1 has been previously identified as an A20-binding protein and it has been proposed to mediate the NF-κB inhibiting effects of A20. We find that both ABIN-1 and A20 inhibit NF-κB at the level of the IKK complex and that A20 inhibits activation of NF-κB by de-ubiquitination of NEMO/IκKγ. Importantly, small interfering RNA targeting ABIN-1 abrogates A20-dependent de-ubiquitination of NEMO/IκKγ and RNA interference of A20 impairs the ability of ABIN-1 to inhibit NF-κB activation. Altogether our data indicate that ABIN-1 physically links A20 to NEMO/IκKγ and facilitates A20-mediated de-ubiquitination of NEMO/IκKγ, thus resulting in inhibition of NF-κB.

NF-κB is a ubiquitously expressed family of transcription factors that controls the expression of numerous genes involved in immune and inflammatory responses (1). NF-κB also plays an important role during cellular stress responses, due to its anti-apoptotic and proliferation-promoting functions (2). Abrupt activation of NF-κB is a major hallmark of several inflammatory diseases such as arthritis (3, 4), and a variety of human cancers (5, 6). In resting cells, NF-κB is sequestered in the cytoplasm in an inactive form by members of the inhibitory family of IκB proteins (1). Various stimuli including pathogens, pathogen-related factors, and cytokines lead to phosphorylation of the inhibitory subunit IκBα on specific serine residues (Ser32 and Ser36) (7) catalyzed by two IκB kinases (IKKs),3 namely IKKα and IKKβ (8–12). This step marks the IκB protein for ubiquitination and subsequent degradation through a proteasome-dependent pathway (1). The active NF-κB is then free for translocation to the nucleus, where it binds the κB sequences present in the promoters of responsive genes.

IKKα and IKKβ reside in a larger kinase complex (700–900 kDa), called the IκB kinase complex (IKK complex), that also contains the essential regulatory subunit NEMO (also known as IKKγ) (13, 14). Genetic studies suggest that NEMO/IκKγ is absolutely required for the activation of IKKs and NF-κB in response to different stimuli (13, 15). NEMO/IκKγ contains several coiled-coil domains, a leucine zipper, and a C-terminal zinc finger domain. These motifs are required for the correct assembly of the IKK complex (13) and recruitment of upstream signaling mediators (16). Numerous proteins have been demonstrated to interact with NEMO/IκKγ, as the kinase RIP and the inhibitor of NF-κB A20 (17), the viral trans-activator TAX (18–20), and the adaptor proteins CIKS/Act-1, TANK, and CARMA (21–23). Therefore, NEMO/IκKγ represents the point where most NF-κB signaling pathways converge. Despite this information, the molecular mechanism regulating IKK complex function is not fully understood.

Ubiquitin conjugation has been most prominently associated with protein degradation through a proteasome-dependent pathway, but it is becoming increasingly evident that ubiquitination plays a key role in the signal transduction pathway leading to activation of NF-κB (24, 25). Recent reports show that lysine 63-linked ubiquitination of NEMO/IκKγ is an important step for the activation of IKKs and NF-κB following various stimuli, such as TNF, lipopolysaccharide, and antigen receptor (26–28). In contrast, the tumor suppressor CYLD is reported as a negative regulator of NF-κB by specific de-ubiquitination of NF-κB signaling molecules, such as TRAF2, TRAF6, and NEMO/IκKγ (29). Also A20 functions as an inhibitor of the NF-κB pathway by removing Lys63-linked ubiquitin chains from RIP, an essential mediator of the proximal TNF-Receptor-1 signaling complex. Then A20 targets RIP for Lys48-linked polyubiquitination and proteasomal degradation (30). Furthermore, A20 terminates Toll-like receptor-induced NF-κB signaling, by cleaving ubiquitin chains from TRAF6 (27). The central role played by A20 in terminating NF-κB activation is further demonstrated by the fact that A20−/− mice develop severe inflammation and cachexia, are hypersensitive to both lipopolysaccharide and TNF, and die prematurely (27, 31). Here we used NEMO/IκKγ as bait in yeast two-hybrid screening, and identified ABIN-1 (A20 binding inhibitor of NF-κB) as a NEMO/IκKγ-interacting protein.

EXPERIMENTAL PROCEDURES

Cell Culture and Biological Reagents—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 1% glutamine.

*This work was supported by grants from the Associazione Italiana Ricerca sul Cancro (AIRC), MIUR-PRIN 2005051307, European Molecular Imaging Laboratory Network Grant LSCH-2004-503569, and Fondazione Italiana Sclerosi Multipla (2003/R6). The members of this group hold a number of patents. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Both authors equally contributed to this work.

2 To whom correspondence should be addressed. Tel.: 39-081-7463606; Fax: 39-081-7701016; E-mail: leonardi@unina.it

3 The abbreviations used are: IκB, IκB kinases; TNF, tumor necrosis factor; ABIN-1, A20 binding inhibitor of NF-κB; HA, hemagglutinin; GST, glutathione S-transferase; siRNA, small interfering RNA; CIKS, connection to IκB kinase and SAPK.
Anti-ABIN-1 polyclonal antibodies were generated in rabbits, by using a recombinant peptide encompassing amino acids 380–636 of human ABIN-1. Other antibodies used for this study were: FLAG epitope (Sigma), A20 (BD Pharmpingen), HA epitope, NEMO/IKK-γ, IKKβ, IκBα, and tubulin (Santa Cruz Biotechnologies). Human TNF-α (Peprotech Inc.) was used at 2,000 units/ml.

Human ABIN-1 was amplified by PCR from a human liver cDNA library (Clontech) and cloned into pcDNA3.1-HA, -FLAG, and -His expression vectors (Invitrogen) for expression in mammalian cells. A20, TAX, and ubiquitin expression vectors were gifts from G. Natoli, T. K. Jeang, and G. Courtois, respectively. NEMO/IKK-γ, IKKβ, CIKS, and TRAF2 expression vectors were previously described (21, 32). All deletion mutants were prepared by conventional PCR and cloned into pcDNA3.1-HA or -FLAG vectors. Point mutants of A20 (CI103S and D100A/C103S) were generated by the QuikChange Site-directed Mutagenesis kit (Stratagene), according to the manufacturer’s protocol.

**Yeast Two-hybrid Screening**—The cDNA encoding the N-terminal part of mouse NEMO/IKK-γ (amino acids 1–311) was cloned in-frame into the GAL-4 DNA-binding domain vector pGBK77 (Clontech). The resulting plasmid pGBK77-NEMO/IKK-γ was used as bait in a yeast two-hybrid screen of a human liver cDNA library (Clontech) in Saccharomyces cerevisiae strain AH109. The NEMO/IKK-γ deletion mutants for two-hybrid mapping were made by conventional PCR and cloned into the pGBK77 vector.

**Gel Filtration of Cellular Extracts**—Gel filtration procedures were performed as previously described (42). Fractions were analyzed by Western blotting for ABIN-1, NEMO/IKK-γ, and IKKβ.

**In Vitro Translation and GST Pull-down Assays**—In vitro transcription and translation were carried out with 1 μg of ABIN-1 constructs according to the TNT Quick Coupled Transcription/Translation System protocol (Promega) in the presence of [35S]methionine.

**GST-NEMO/IKK-γ fusion protein was produced and purified as described** (33). GST pull-down assays were performed by incubating an aliquot of GST-NEMO/IKK-γ bound to glutathione-Sepharose beads (Amersham Biosciences) together with 10 μl of in vitro translated ABIN-1 protein in phosphate-buffered saline, 1% Triton X-100 buffer (including Complete Protease Inhibitor mixture (Roche)) for 2 h at 4 °C. Beads were then washed five times with the same buffer, resuspended in Laemmli buffer, and run on a SDS-polyacrylamide gel before autoradiography.

**Transfection, Immunoprecipitation, and Luciferase Assay**—Lipofectamine-mediated transfections were performed according to the manufacturer’s instructions (Invitrogen). All transfections included supplemental empty vector to ensure that the total amount of transfected DNA was kept constant in each dish culture.

For immunoprecipitation of transfected proteins, HEK293 cells (3 × 10⁶) were transiently transfected and 24 h after transfection cells were lysed in Triton X-100 lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and Complete Protease Inhibitor mixture (Roche)). After an additional 15 min on ice, cell extracts were centrifuged for 10 min at 14,000 × g at 4 °C and supernatants were incubated for 4 h at 4 °C with anti-FLAG antibodies bound to agarose beads (M2, Sigma). The immunoprecipitates were washed five times with Triton X-100 lysis buffer and subjected to SDS-PAGE.

For luciferase assay, HEK293 cells (4 × 10⁶) were seeded in 6-well plates. After 12 h cells were transfected with 0.5 μg of Igk-κB-luciferase reporter plasmid and various combinations of expression plasmids. 24 h after transfection, cells were stimulated with TNF-α for 3 h or left untreated. Cell extracts were prepared and reporter gene activity was determined via the luciferase assay system (Promega). Expression of the pRSV-β-galactosidase vector (0.2 μg) was used to normalize transfection efficiencies.
**ABIN-1 Binds to NEMO/IKKγ**

In Vivo Ubiquitination and De-ubiquitination Assays—HEK293 cells (3 \( \times \) 10^6) were co-transfected with expression vectors containing epitope-tagged ubiquitin (1 mg) and NEMO/IKKγ (200 ng), plus various constructs encoding A20 or ABIN-1 proteins. 24 h after transfection, cell lysates were prepared as above and analyzed for polyubiquitination of NEMO/IKKγ either by Western blot anti-NEMO/IKKγ (FLAG) on total extracts or by immunoprecipitating FLAG-NEMO/IKKγ with anti-FLAG beads followed by Western blot anti-HA-ubiquitin.

**RESULTS**

**ABIN-1 Binds to NEMO/IKKγ**—The regulatory subunit of the IKK complex, NEMO/IKKγ, has an essential role in NF-κB activation. To gain insights into how NEMO/IKKγ modulates the activation of NF-κB, we screened a human liver cDNA library for NEMO/IKKγ interacting proteins, via the yeast two-hybrid system. 25 clones were identified that expressed NEMO/IKKγ-interacting proteins, including IKKε and CARMA (23). Three clones encoded for overlapping oligonucleotides were cloned into the pcRNAi vector that we derived from the pDNA3.1 vector (Invitrogen) by replacing the viral promoter cassette with the H1 gene promoter that is specifically recognized by RNA polymerase III. The plasmids used to knockdown A20 expression (pU6-A20) and the pU6) were a kind gift of Dr. S. Yamaoka and have been previously described (43).

**ABIN-1 Small Interfering RNA (siRNA) Expression Vectors**—To knockdown ABIN-1 expression, we designed double-stranded oligonucleotides containing sequences derived from the human ABIN-1 open reading frame (nucleotides 1136–1156 and 1685–1705) in forward and reverse orientations separated by a 7-base pair spacer region (caagaga) to allow the formation of the hairpin structure in the expressed siRNAs; ABINi-370: sense strand, 5'-taattcGAGGAGACCGACAAGGAGCAGtctcttgCTGCTCCTTGTCGTCTCTCGg; ABINi-560: sense strand, 5'-taattcCCACACCATGCGCTCAGAGGACcaagagaCTGCTCCTTGTCGTCTCTCtttttc; antisense strand, 5’-tcagaaaaGAGGAGACCGACAAGGAGCAGtctcttgCTGCTCCTTGTCGTCTCTSEg; ABINi-560: sense strand, 5’-taattcCCACACCATGCGCTCAGAGGACcaagagaCTGCTCCTTGTCGTCTCTCtttttc; antisense strand, 5’-tcagaaaaGAGGAGACCGACAAGGAGCAGtctcttgCTGCTCCTTGTCGTCTCTSg.

ABIN-1 and A20 are inhibitors of NF-κB—A and B, ABIN-1 and A20 inhibit NF-κB at level of the IKK complex. Relative reporter activity was evaluated in HEK293 cells co-transfected with the Ig-κB-luciferase plasmid and the indicated expression vectors. 24 h after transfection cells were stimulated with TNF-α for 3 h or left untreated, as indicated. Values shown in arbitrary units represent the mean ± S.D. of three experiments done in triplicate, normalized for β-galactosidase expression of a co-transfected pRSV-β-galactosidase plasmid.

**FIGURE 2.** Mapping of the NEMO/IKKγ and the A20 binding domains on ABIN-1. A and B, GST pull-down assays: GST-NEMO/IKKγ was incubated with in vitro translated full-length (FL) or deletion mutants of ABIN. Aliquots of in vitro translated constructs and GST-NEMO/IKKγ stained by Coomassie Blue are shown. C, co-immunoprecipitation of FLAG-A20 with HA-ABIN and either HA-ABIN or ABINΔ407–431. Cell extracts were immunoprecipitated with anti-FLAG antibodies (A20) followed by Western blot (WB) anti-HA (ABIN). The presence of HA and −FLAG proteins in total extracts is shown.

**FIGURE 3.** ABIN-1 and A20 are inhibitors of NF-κB. A and B, ABIN-1 and A20 inhibit NF-κB at level of the IKK complex. Relative reporter activity was evaluated in HEK293 cells co-transfected with the Ig-κB-luciferase plasmid and the indicated expression vectors. 24 h after transfection cells were stimulated with TNF-α for 3 h or left untreated, as indicated. Values shown in arbitrary units represent the mean ± S.D. of three experiments done in triplicate, normalized for β-galactosidase expression of a co-transfected pRSV-β-galactosidase plasmid.
NEMOΔN91). Immunoprecipitates of FLAG-NEMO/IKKγ contained HA-ABIN-1 only if both proteins were co-expressed (Fig. 1B, compare lanes 3 and 4). In agreement with the data obtained in yeast, ABIN-1 did not co-immunoprecipitate with NEMOΔN91 (lane 6, Fig. 1B). We were unable to detect the association between endogenous NEMO/IKKγ and ABIN-1, probably because of the transient nature of the association and/or the high stringent conditions we used to perform co-immunoprecipitation experiments. However, gel filtration experiments showed that endogenous ABIN-1 was eluted from the column in the same fractions containing endogenous NEMO/IKKγ and other components of IKK complex (Fig. 1C).

Mapping of the NEMO/IKKγ and the A20 Binding Domains on ABIN-1—To define the domain of ABIN-1 required for its interaction with NEMO/IKKγ, we performed pull-down assays by using recombinant GST-NEMO/IKKγ and in vitro translated [35S]ABIN-1 (Fig. 2A). ABIN-1 binds to GST-NEMO/IKKγ, indicating a direct interaction between the two proteins. Furthermore, amino acids 500–588 of ABIN-1 represent the minimal region that binds to NEMO/IKKγ (Fig. 2A, upper panel). To confirm that the region between amino acids 500 and 588 of ABIN-1 was responsible for interaction with NEMO/IKKγ, we generated an internal deletion mutant of ABIN-1 (∆500–588) and evaluated its ability to interact with NEMO/IKKγ. As expected, the internal deletion of 89 amino acids from ABIN-1 abolished the interaction with NEMO/IKKγ (Fig. 2B). Because ABIN-1 was identified as an A20-interacting protein (35), we confirmed that the region between amino acids 407 and 431 of ABIN-1 is responsible for interaction with A20 (Fig. 2C).

Both ABIN-1 and A20 Inhibit NF-κB at the Level of the IKK Complex by Associating with NEMO/IKKγ—Both ABIN-1 and A20 are inhibitors of NF-κB. It has been proposed that they interfere with a RIP and TRAF2-mediated transactivation signal (34). The identification of the interaction between ABIN-1 and NEMO/IKKγ prompted us to investigate if ABIN-1 was involved in controlling NF-κB activation not only upstream but also at the level of the IKK complex. To this aim, we performed reporter assays by transfecting HEK293 cells with the Ig-κ-luciferase reporter plasmid and the indicated combinations of expression plasmids. 24 h after transfection cells were stimulated with TNF-α for 3 h or left untreated, as indicated. Analysis was done as in Fig. 3. Lower panels in A–C show relative expression levels of each of the transfected proteins. D, ABIN-1 forms a complex with NEMO/IKKγ and A20. HEK293 cells were transfected with constructs encoding NEMO/IKKγ, A20, and a deletion mutant of ABIN lacking the NEMO/IKKγ-binding domain (ABINΔ500–588). Cell extracts were immunoprecipitated with anti-FLAG antibodies (NEMO/IKKγ and A20) and Western blotted (WB) anti-HA to reveal the co-precipitation of A20 and ABINΔ500–588. The presence of anti-HA and anti-FLAG proteins (NEMO/IKKγ and A20) and A20 in the whole cell lysate is shown.

NEMO/IKKγ and A20 act at the level of the IKK complex to inhibit NF-κB activity. A–C, the deletion mutant of ABIN-1 lacking both NEMO/IKKγ- and A20-binding domains (ABINΔ407–431/Δ500–588) does not block NF-κB activation (C), in contrast to ABINΔ500–588 (A) and ABINΔ407–431 (B). HEK293 cells were co-transfected with the Ig-κ-luciferase reporter plasmid and the indicated combinations of expression plasmids. 24 h after transfection cells were stimulated with TNF-α for 3 h or left untreated, as indicated. Analysis was done as in Fig. 3. Lower panels in A–C show relative expression levels of each of the transfected proteins. D, ABIN-1 forms a complex with NEMO/IKKγ and A20. HEK293 cells were transfected with constructs encoding NEMO/IKKγ, A20, and a deletion mutant of ABIN lacking the NEMO/IKKγ-binding domain (ABINΔ500–588). Cell extracts were immunoprecipitated with anti-FLAG antibodies (NEMO/IKKγ and A20) and Western blotted (WB) anti-HA to reveal the co-precipitation of A20 and ABINΔ500–588. The presence of anti-HA and anti-FLAG proteins (NEMO/IKKγ and A20) and A20 in the whole cell lysate is shown.
ABIN-1 binds to NEMO/IKKγ

FIGURE 5. A20 inhibits NF-κB by de-ubiquitinating NEMO/IKKγ. A, A20 but not ABIN-1 de-ubiquitinates NEMO/IKKγ. HEK293 cells were transfected with FLAG-NEMO/IKKγ and HA-ubiquitin, plus increasing amounts of either HA-A20 or HA-ABIN. Cell extracts were immunoprecipitated with anti-FLAG antibodies (NEMO/IKKγ) followed by Western blot analysis with anti-HA antibodies to reveal the polyubiquitinated forms of NEMO/IKKγ. Western blot analyses with anti-FLAG, -HA, -A20, and -tubulin antibodies were performed on total extracts. B, A20 blocks the ubiquitination of NEMO/IKKγ and the degradation of IκBα induced by TNF-α. HEK293 cells were transfected with FLAG-NEMO/IKKγ, HA-ubiquitin, and HA-A20; 24 h after transfection cells were stimulated with TNF-α for 5 min or left untreated, as indicated. Cell extracts were immunoprecipitated with anti-FLAG antibodies (NEMO/IKKγ) and Western blotted with anti-HA antibodies. Western blots of anti-NEMO/IKKγ, -A20, -HA, and -tubulin are shown. C, a catalytically inactive form of A20 (D100A/C103S) does not de-ubiquitinate NEMO/IKKγ. Conditions were similar to those in A, except for the plasmids encoding HA-A20 C103S, or D100A/C103S. D, A20 D100A/C103S does not inhibit NF-κB activation dependent on TRAF2 in contrast to wild type A20. Reporter assay was performed by co-transfection of the Ig-κBα-luciferase plasmid with combinations of TRAF2, plus A20-WT, or −D100A/C103S. Values shown in arbitrary units represent the mean ± S.D. of three experiments done in triplicate, normalized for β-galactosidase expression of a co-transfected pSV-β-galactosidase plasmid.

either the NEMO/IKKγ binding domain (ABINΔ500–588) or the A20 binding domain (ABINΔ407–431) were still able to inhibit the activity of a NF-κB-driven luciferase reporter following different stimuli (Fig. 4, A and B). In contrast, a mutant of ABIN-1 in which both the NEMO/IKKγ- and the A20-binding domains were deleted (ABINΔ407–431/Δ500–588) lost the ability to block activation of NF-κB (Fig. 4C). These data were consistent with the hypothesis that ABIN-1 forms a complex with both NEMO/IKKγ and A20. To address this hypothesis, we immunoprecipitated FLAG-NEMO/IKKγ and monitored the co-precipitation of the ABIN-1 mutant lacking the NEMO/IKKγ-binding domain (HA-ABINΔ500–588) either in the presence or absence of A20 (Fig. 4D). ABINΔ500–588 co-immunoprecipitated with NEMO/IKKγ only in the presence of A20 (Fig. 4D). To further support the idea that ABIN-1 promotes association of A20 with NEMO/IKKγ, we transfected A20 and NEMO/IKKγ in the presence of an increasing amount of overexpressed ABIN-1. As expected, the amount of A20 co-immunoprecipitating with NEMO/IKKγ increased in the presence of ABIN-1 (Fig. 4E).

Taken together, these data indicated that ABIN-1 interferes with activation of NF-κB at the level of the IKK complex, and support the idea that ABIN-1 promotes association of A20 with NEMO/IKKγ.

A20 Inhibits NF-κB by De-ubiquitinating NEMO/IKKγ—To explore the mechanism by which the interactions of both A20 and ABIN-1 with NEMO/IKKγ down-regulate NF-κB signaling, we assessed the effect of either A20 or ABIN-1 on NEMO/IKKγ ubiquitination. Transfection of FLAG-NEMO/IKKγ in the presence of HA-ubiquitin results in the polyubiquitination of NEMO/IKKγ (Fig. 5A, lane 3). Co-transfection of A20 and NEMO/IKKγ resulted in a dose-dependent disappearance of the ubiquitinated forms of NEMO/IKKγ (Fig. 5A, lanes 4 and 5). In contrast, co-transfection of ABIN-1 did not affect NEMO/IKKγ ubiquitination (Fig. 5A, lanes 6 and 7). We did not observe reduction in the overall level of ubiquitinated cellular proteins in the presence of A20, indicating that A20 does not have a global de-ubiquitinating activity in
cultured cells (Fig. 5A). Importantly, A20 also blocks IκBα degradation and NEMO/IKKγ ubiquitination induced by TNF-α (Fig. 5B). To demonstrate that the de-ubiquitinating activity of A20 was required for the observed reduction in NEMO/IKKγ ubiquitination, we generated two mutants in the OTU domain of A20, which is the domain responsible for the de-ubiquitinating activity of A20 (36). We replaced the cysteine residue of the DXXC motif with serine (C103S), and both the aspartic acid and the cysteine residues (D100A/C103S) with alanine and serine, respectively. The mutation C103S affected the ability of A20 to de-ubiquitinate NEMO/IKKγ compared with wild type A20, whereas the double mutant D100A/C103S resulted in the complete loss of the de-ubiquitinating activity of A20 on NEMO/IKKγ (Fig. 5C). As expected, the D100A/C103S mutant was not able to block the NF-κB activity induced by different stimuli, such as TRAF2 (Fig. 5D and data not shown).

These findings strongly suggest that NEMO/IKKγ is a target of the de-ubiquitinating activity of A20 and confirmed that the ubiquitination of NEMO/IKKγ is a crucial step in the mechanisms of NF-κB activation.

**ABIN-1 Mediates the De-ubiquitinating Activity of A20 on NEMO/IKKγ**—Next, we explored whether ABIN-1 was involved in the A20-dependent de-ubiquitination of NEMO/IKKγ. To this purpose, we transfected HEK293 cells with suboptimal amount of A20 and an increasing amount of ABIN-1 and checked for NEMO/IKKγ ubiquitination. We found that ABIN-1 increases the ability of A20 to de-ubiquitinate NEMO/IKKγ (Fig. 6A). To demonstrate a role for ABIN-1 in the A20-mediated de-ubiquitination of NEMO/IKKγ, we generated siRNA constructs targeting ABIN-1 (ABINi-370 and i-560). Fig. 6B shows that the construct i-370 knocked-down ABIN-1 expression, whereas the i-560 construct did not. Then, we evaluated whether interference of ABIN-1 impairs the de-ubiquitinating activity of A20 on NEMO/IKKγ. We co-transfected HEK293 cells with FLAG-NEMO/IKKγ and HA-ubiquitin and assessed the de-ubiquitinating activity of A20 alone or in the presence of either i-370 or i-560 constructs. The A20-dependent de-ubiquitination of NEMO/IKKγ decreased only in the presence of the i-370 construct (Fig. 6C). The i-370 construct led to a 2-fold increase of both basal and induced (TRAF2 and CIKS) NF-κB activity compared with the empty vector or the i-560 construct, which we used as controls (Fig. 6D). Accordingly with the data shown in Fig. 3A, interference of ABIN-1 did not influence the activation of the NF-κB-dependent transcribed IKKβ. Also in this case, NF-κB activity correlated with the levels of NEMO/IKKγ ubiquitination. In fact, transfected i-370 increased the ubiquitination of NEMO/IKKγ with respect to both empty vector and i-560 (data not shown). From these experiments, we concluded that reduced levels of the ABIN-1 protein affect the ability of
ABIN-1 Binds to NEMO/IKKβ

A20 to de-ubiquitinate NEMO/IKKβ, and consequently the A20-mediated inhibition of NF-kB. To further support the functional interplay between ABIN-1 and A20, we knocked-down A20 (43) and evaluated the ability of ABIN-1 to interfere with NF-kB activation. As shown in Fig. 6E, ABIN-1 requires A20 to efficiently block NF-kB activation induced by TNF and TRAF2.

DISCUSSION

In the present study, we have performed experiments in yeast, in vitro and in transfected cells demonstrating that ABIN-1 physically associates with NEMO/IKKβ. The functional consequence of this interaction is that overexpression of ABIN-1 blocks not only activation of NF-kB upstream of the IKK complex, but also NF-kB activation mediated by proteins directly contacting the IKK complex, such as CIKS and TAX. Indeed, activation of NF-kB mediated by overexpression of IKKβ, which may be considered functionally downstream of NEMO/IKKβ, is not affected by ABIN-1. ABIN-1 has been identified as an A20-binding protein (3). The functional consequence of this interaction is that overexpression of ABIN-1 blocks not only activation of NF-kB upstream of the IKK complex, but also NF-kB activation mediated by proteins directly contacting the IKK complex, such as CIKS and TAX. Indeed, activation of NF-kB mediated by overexpression of IKKβ, which may be considered functionally downstream of NEMO/IKKβ, is not affected by ABIN-1. ABIN-1 has been identified as an A20-binding protein (3).

In summary, we have identified a previously unreported association between ABIN-1 and NEMO/IKKβ and we provide evidence that ABIN-1 co-operates with A20 in inhibiting NF-kB at the level of the IKK complex. In addition, we propose that this association could target A20 on NEMO/IKKβ and interfere with NEMO/IKKβ ubiquitination, to negatively regulate NF-kB activation.

REFERENCES

1. Hayden, M. S., and Ghosh, S. (2004) Genes Dev. 18, 2195–2224
2. Karin, M., and Lin, A. (2002) Nat. Immunol. 3, 221–227
3. Walsh, N. C., Crotti, T. N., Goldring, S. R., and Gravallese, E. M. (2005) Immunol. Rev. 208, 228–251
4. Orange, J. S., Levy, O., and Geha, R. S. (2005) Immunol. Rev. 203, 21–37
5. Heyninck, K., De Valck, D., Vanden Berghe, W., Van Criekinge, W., Contreras, R., Fierz, W., Haegeman, G., and Beyaert, R. (1999) FEBS Lett. 536, 155–140
6. Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., and Rothe, M. (2000) Immunity 12, 301–311
7. Chu, Z. L., Shin, Y. A., Yang, J. M., Di Donato, J. A., and Ballard, D. W. (1999) J. Biol. Chem. 274, 15297–15300
8. Bourou, E. W., and Sun, S. C. (1999) J. Biol. Chem. 274, 22911–22914
9. Jin, D. Y., Giordano, V., Kibler, K. V., Nakano, H., and Jeang, K. T. (1999) J. Biol. Chem. 274, 17402–17405
10. Dong, D., Turer, E. E., Lee, E. G., Ahmad, R. C., Wheeler, M. T., Tsui, C., Hurley, P., Chien, M., Chai, S., Hitotsumatsui, O., McNally, E., Pickart, C., and Ma, A. (2004) Nat. Immunol. 5, 1052–1060
11. Zhou, H., Wertz, I. E., O’Rourke, K. M., Ulcsch, M., Seshgiri, S., Eby, M., Xiao, W., and Dixit, V. M. (2004) Nature 427, 167–171
12. Kovalenko, A., Chable-Bessia, C., Cantarella, G., Israel, A., Wallach, D., and Courtine, G. (2003) Nature 424, 801–805
13. Wertz, I. E., O’Rourke, K. M., Zhou, H., Eby, M., Ardavin, L., Seshgiri, S., Wu, W., Wiesmann, C., Baker, R., Boone, D. L., Ma, A., Koonin, E. V., and Dixit, V. M. (2004) Nature 430, 694–699
14. Lee, E. G., Boone, D. L., Chai, S., Libby, S. L., Chien, M., Lodolce, G. P., and Ma, A. (2000) Science 289, 2350–2354
15. Leonard, A., Ellinger-Zieglerbauer, H.,Franzoso, G., Brown, K., and Siebenlist, U. (2000) J. Biol. Chem. 275, 271–278
16. Chariot, A., Princen, F., Gielen, J., Mercurio, F., and Siebenlist, U. (2000) J. Cell Biol. 149, 1471–1482
17. Heyninck, K., Kreike, M. M., and Dixit, V. (2003) FEBS Lett. 536, 155–140
18. Evans, P. C., Smith, T. S., Lai, M. J., Williams, M. G., Burke, D. F., Heyninck, K., Kreike, M. M., Baer, R., Blundell, T. L., and Kishishima, P. J. (2003) J. Biol. Chem. 278, 23180–23186
19. Dixit, V. M., Green, S., Sarma, V., Holzman, L. B., Wolf, F. W., O’Rourke, K., Ward, P. A., Prochownik, E. V., and Marks, R. M. (1990) J. Biol. Chem. 265, 2973–2978
20. Jaittela, M., Mouton, R., Elling, F., and Bastholm, L. (1996) J. Immunol. 156, 1166–1173
21. Hu, X., Lee, E., Harlan, J. M., Wong, F., and Karsan, A. (1998) Blood 92, 2759–2765
22. Laherty, C. D., Hu, H. M., Opperhi, A. W., Wuang, F., and Dixit, V. M. (1992) J. Biol. Chem. 267, 24157–24160
23. Krikos, A., Lahteri, C. D., and Dixit, V. M. (1992) J. Biol. Chem. 267, 17971–17976
24. Mauro, C., Vito, P., Melleoni, S., Pacifico, P., Chariot, A., Formisano, S., and Leonard, A. (2003) Biochem. Biophys. Res. Commun. 309, 84–90
25. Saitoh, T., Yamamoto, M., Miyagishi, M., Taira, K., Nakanishi, M., Fujita, T., Akira, S., Yamamoto, N., and Namba, S. (2005) J. Immunol. 174, 1507–1512