The inhibitor ABIN-2 disrupts the interaction of receptor-interacting protein with the kinase subunit IKKγ to block activation of the transcription factor NF-κB and potentiate apoptosis

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NF-κB (nuclear factor κB) proteins are key transcription factors that regulate gene expression in response to various extracellular stimuli. The pathway leading to the activation of NF-κB involves a complicated network that includes a number of signalling molecules. The recent identification of a wide range of negative regulators of NF-κB has given another layer of complexity in NF-κB activation. We and others have previously identified the protein ABIN-2 (A20 binding inhibitor of NF-κB 2) as an inhibitor of NF-κB activation. In the present paper, we demonstrate that ABIN-2 exerts its inhibitory function by blocking the interaction of RIP (receptor-interacting protein) with the downstream effector IKKγ, a non-kinase component of the IκB (inhibitory κB) kinase complex. When overexpressed in cells, ABIN-2 bound to IKKγ and prevented the association of IKKγ with RIP. By a deletion mapping, a stretch of 50 amino acids on ABIN-2 is found to be essential for its interaction with IKKγ. The ABIN-2 mutant that lacked these 50 amino acids did not interact with IKKγ and, consequently, failed to inhibit NF-κB activation. Strikingly, a portion of RIP, which is similar to this 50-residue domain of ABIN-2, is also essential for RIP interaction with IKKγ. The RIP mutant with deletion of this similar region did not associate with IKKγ and had substantial reduction of its ability to mediate NF-κB activation. Taken together, these conserved 50 residues of ABIN-2 and RIP define a novel structural domain in mediating a key step in the NF-κB signalling pathway through the interaction with IKKγ. Finally, the signalling pathway of NF-κB activation is known to promote survival in many cellular events. The mechanism for decision between cell death and survival is under fine regulation. In the present paper, we demonstrated further that the expression of ABIN-2 could promote the RIP-mediated apoptosis by presumably suppressing the anti-apoptotic effect of NF-κB.

Key words: A20 binding inhibitor of nuclear factor κB 2 (ABIN-2), apoptosis, inhibitory κB kinase (IKKγ), nuclear factor κB (NF-κB), receptor-interacting protein (RIP).

INTRODUCTION

NF-κB (nuclear factor κB) proteins are transcription factors that are expressed ubiquitously and play an essential role in a number of cellular functions, including inflammatory and immune responses, apoptosis, cell proliferation and differentiation [1]. One critical step in the pathway leading to the activation of NF-κB is the phosphorylation of IκB (inhibitory κB). The phosphorylation of IκB, which is mediated by a high-molecular-mass IκB kinase (IKK) complex, results in the degradation of IκB and release of active NF-κB [2,3]. The IKK complex contains two catalytic subunits, IKKα and IKKβ [1,2,4,5], and a non-kinase protein IKKγ/NEMO (NF-κB essential modulator) [1,6,7]. Evidence from IKKγ-knockout mice has demonstrated that IKKγ is essential for activation of the IKK complex triggered by TNFα (tumour necrosis factor α) [8–10].

It has been known that, upon stimulation by TNFα, IKKγ recruits the IKK complex to TNFα receptors, TNFR1 and/or TNFR2, where IKKs may be activated further by rather differential mechanisms [11–15]. For recruiting the IKK complex, IKKγ first interacts with an upstream activator, RIP (receptor-interacting protein), which is a protein serine/threonine kinase and is essential for TNFα-induced NF-κB activation [6,16,17]. Alternatively, IKKγ may facilitate the association of IKKβ and IκB in the high-molecular-mass IKK complex and expedite IκB phosphorylation [16,18].

To elucidate the mechanisms of NF-κB activation, several inhibitors of NF-κB have been uncovered. Using different approaches, we and others have characterized ABIN-2 (A20 binding inhibitor of NF-κB 2), also known as FLIP1, as an inhibitor to NF-κB activation [19–21]. It has been indicated previously that ABIN-2 could interfere with the NF-κB function at a step upstream of IKK complex activation [19]. Although IKKγ is required absolutely for the activation of IKK complex by various stimuli, whether or not it is subject to negative regulation remains unclear. In the present paper, we demonstrate that ABIN-2 exerts

Abbreviations used: ABIN-2, A20 binding inhibitor of nuclear factor κB 2; CARD, caspase recruitment domain; CMB, core motif for binding; CYLD, cylindromatosis; DMEM, Dulbecco's modified Eagle's medium; FLIP1, foetal liver LKB1-interacting protein; GST, glutathione S-transferase; IκB, inhibitory κB; IKK, IκB kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NF-κB, nuclear factor κB; RIP, receptor-interacting protein; TNF, tumour necrosis factor; TNFR, TNF receptor; TRADD, TNFR1-associated death-domain protein.

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its inhibitory function by binding directly to IKKγ and hence disrupting the RIP–IKKγ complex formation, and that in turn results in the failure of NF-κB activation.

EXPERIMENTAL

Cell culture

HEK-293 and HEK-293T cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10 % (v/v) foetal calf serum (Hyclone), 10 mM Hepes and 2 mM L-glutamine at 37 °C.

Plasmid construction and recombinant DNA technique

For GST (glutathione S-transferase)-tagged recombinant protein expression in bacteria, the coding regions of ABIN-2 and IKKγ were amplified and incorporated with appropriate restriction enzyme sites by PCR and ligated into pGEX4T-2 (Amersham Biosciences). The plasmids were then transformed into *Escherichia coli* strain BL21(DE3) (Novagen) for protein expression.

Schematic diagrams of the full-length and truncated ABIN-2 and IKKγ DNA fragments used in the present study are shown in Figures 3(A) and 4(D), below. The mammalian expression constructs of ABIN-2 and IKKγ, including pFLAG-CMV-ABIN-2, pcDNA3-Myc-ABIN-2, pFLAG-CMV-IKKγ, pcDNA3-Myc-IKKγ and pFLAG-CMV-RIP, were generated by subcloning DNA fragments into each vector accordingly and by general molecular cloning procedures [22]. For the truncated ABIN-2s, including ABIN-$2_{aa90-429}$, ABIN-$2_{aa196-429}$, ABIN-$2_{aa253-429}$, ABIN-$2_{aa262-429}$, ABIN-$2_{aa1-261}$ and ABIN-$2_{aa196-346}$, DNA fragments were produced first by PCR-amplification with specific primers designed to match the ends of each DNA fragment and ligated into pFLAG-CMV2 (Kodak) to form pFLAG-ABIN-$2_{aa90-429}$, pFLAG-ABIN-$2_{aa196-429}$, pFLAG-ABIN-$2_{aa253-429}$, pFLAG-ABIN-$2_{aa262-429}$, pFLAG-ABIN-$2_{aa1-261}$ and pFLAG-ABIN-$2_{aa196-346}$ respectively. The FLAG–ABIN-2ΔI was made by first subcloning the PCR-amplified ABIN-2ΔI (with amino acids 1–89 and 135–287 deleted) DNA fragment into pBluescript II SK (Stratagene) to form pBluescriptII SK-ABIN-2ΔI and to take advantage of multiple cloning sites on the vector. The insert DNA was then excised with restriction enzymes according to the subsequent cloning plasmid and ligated to the plasmids. For the truncated IKKγ, including IKK$\gamma_{aa1-396}$, IKK$\gamma_{aa1-306}$, IKK$\gamma_{aa1-174}$ and IKK$\gamma_{aa175-419}$, DNA fragments were generated by PCR amplification and ligated into pFLAG-CMV2 to form pFLAG-IKK$\gamma_{aa1-396}$, pFLAG-IKK$\gamma_{aa1-306}$, pFLAG–IKK$\gamma_{aa1-174}$ and pFLAG–IKK$\gamma_{aa175-419}$ respectively. FLAG–IKK$\gamma\Delta$I was made, again, by creating specific DNA fragments and subcloning into pBluescript II SK first. The insert DNA was then excised and religated into pFLAG-CMV-2. TRADD (TNFR1-associated death-domain protein), RIP, IκB and RelA were amplified by PCR from a human cDNA library and cloned directly into pFLAG-CMV-2. RIP-ΔCMB (lacking amino acids 291–305) was generated first by sequential PCR procedures to create two DNA fragments covering amino acids 1–291 and 305–671, and religated to form the RIP-ΔCMB fragment and subsequently cloned into appropriate vectors. The construction of pFLAG-IKKα and pFLAG-IKKβ has been described elsewhere [20,23].

Pull-down analysis

The mammalian expression constructs of FLAG–IKKγ, FLAG–TRADD, FLAG–IκBα or FLAG–RIP were transiently transfected into HEK-293T cells. Cells were lysed in lysis buffer [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 % (w/v) Nonidet P-40, 0.5 % (w/v) sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol and 1:100 (v/v) of a protease inhibitor mixture]. Cell lysate (200 μg) was then mixed either with GST or with GST–ABIN-2 bound to glutathione–agarose beads (Sigma) in lysis buffer in a total volume of 500 μl at 4 °C for 3 h. Beads were then washed three times with lysis buffer, resuspended in SDS sample buffer [60 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 0.025 % (w/v) Bromphenol Blue, 0.355 M 2-mercaptoethanol and 10 % (v/v) glycerol], boiled for 3 min, and analysed by SDS/PAGE. Gels were transferred on to nitrocellulose membranes (Schleicher & Schuell). Blotted membranes were processed for immunoreactions, and visualized by a enhanced chemiluminescence (ECL®) system (Amersham Biosciences).

Co-immunoprecipitation and antibodies

HEK-293T cells were seeded in 10-cm-diameter dishes (2×10^7 cells/dish) and transfected using the calcium phosphate precipitation method with the indicated cDNA expression vectors. After 24 h, the cells were lysed in 300 μl of lysis buffer. After 30 min of incubation on ice, the lysates were clarified by centrifugation at 15 000 ***g*** for 30 min and incubated with antibodies preabsorbed on Protein A/G–Sepharose (Amersham Biosciences) for 4 h at 4 °C. After washing four times with lysis buffer, the immunocomplexes were denatured in SDS sample buffer and separated by SDS/PAGE. Immunoblotting was performed as follows: 20 μl of 2 × SDS sample buffer was added to the immunopurified or affinity-purified protein samples. After boiling for 5 min, the samples were separated by SDS/PAGE and transferred on to nitrocellulose membranes. The membranes were first blocked with 10 % (w/v) BSA in TBST [25 mM Tris/HCl, pH 7.4, 0.14 M NaCl, 3 mM KCl and 0.05 % (v/v) Tween 20] for 30 min and then incubated with the primary antibody in 5 % (w/v) BSA for 1 h at room temperature (25 °C). In addition to the anti-FLAG (Sigma) and anti-Myc antibodies (Upstate Biotechnology), in some experiments, rabbit polyclonal antiserum against IKKγ (Santa Cruz Biotechnology) was also used. The membranes were then washed four times in TBST and subsequently incubated with anti-mouse or anti-rabbit horseradish-peroxidase-conjugated secondary antibodies (Promega) in TBST for 1 h at room temperature. After four 10 min washes, the membranes were incubated with chemiluminescent substrates (Amersham Biosciences) for 3 min and then subjected to autoradiography.

Indirect fluorescence microscopy

HEK-293T cells were seeded into 12-well plates (2×10^5 cells/well) and transfected with 1 μg of each of the indicated plasmids by calcium phosphate precipitation. For immunofluorescence staining, the cells were re-plated 24 h later on to serum-coated glass coverslips for an additional 24 h, then were washed with PBS, fixed in 2 % (v/v) freshly prepared paraformaldehyde in PBS at room temperature for 20 min and in acetone at − 20 °C for 3 min, and finally washed twice in PBS. Blocking was performed with 5 % (v/v) donkey serum and 10 % (v/v) foetal bovine serum in PBS. In all cases, the coverslips were then incubated 1 h at room temperature with the specific primary antibody in PBS. After washing further with PBS three times, the cells were incubated with Rhodamine, FITC-conjugated goat anti-IgG antibody (Jackson ImmunoResearch) or 0.05 % (w/v) DAPI (4,6-diamidino-2-phenylindole; Sigma) for 1 h. The cells were washed with PBS again, and the fluorescence was analysed by confocal microscopy using a 488 nm laser and a 535 nm narrow-band filter for the FITC signal.

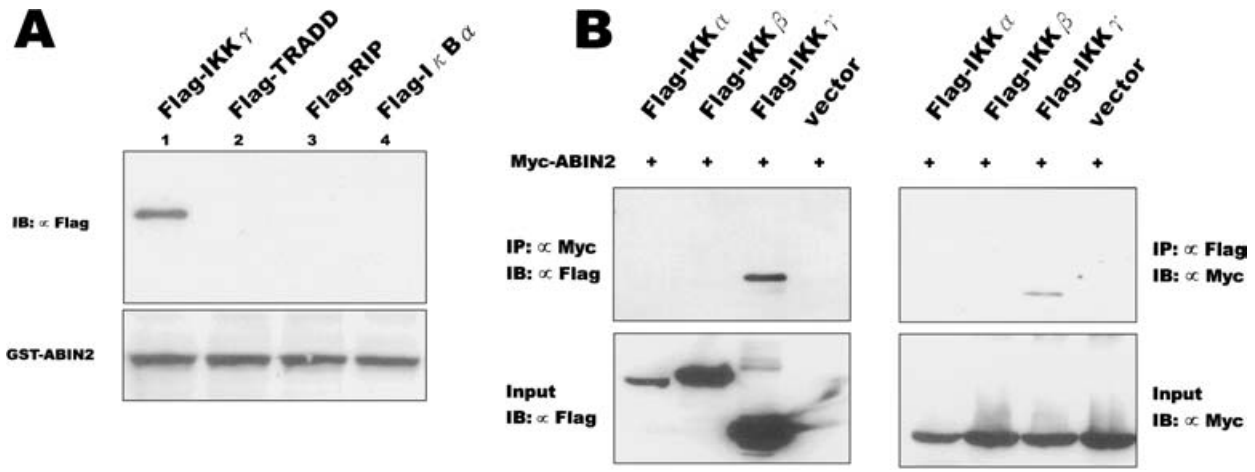


Figure 1 Association of ABIN-2 and IKKγ

(**A**) Pull-down assay by GST–ABIN-2 detects components of the IKK complex. The GST-tagged ABIN-2 was immobilized on to glutathione beads and incubated with various FLAG-tagged proteins expressed in HEK-293T cells as indicated. Pulled-down proteins were then visualized by immunoblotting (IB) with anti-FLAG (α Flag) antibodies. (**B**) Co-immunoprecipitation of ABIN-2 and IKKγ. The FLAG-tagged IKK components and Myc–ABIN-2 were co-expressed in HEK-293T cells as indicated. Cell lysates were prepared and immunoprecipitated (IP) with anti-Myc (α Myc) antibodies. The associated proteins from immunoprecipitates were detected by immunoblotting with anti-FLAG (α Flag) antibodies (left-hand panel). For a control, experiments were also performed in a reverse way with anti-FLAG antibodies in the precipitation step from cell lysates first, and then detected by immunoblotting with anti-Myc antibodies (right-hand panel).

Analysis of NF-κB activation

HEK-293T cells were maintained in DMEM supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine and antibiotics. Cells were plated in 12-well dishes at 2×10^5 cells/well the evening before transfection. The cells were transfected with 300 ng of NF-κB-dependent luciferase reporter plasmid along with various effector constructs, as indicated. The transfection efficiency was consistently greater than 80%. Cells were lysed 24 h after transfection, and luciferase measurement of cell lysates was carried out using the luciferase reporter kit (Promega) according to the manufacturer's protocol. Luciferase activity was measured by mixing 5 μl of extract with 100 μl of luciferase substrate and analysed with a luminometer.

Apoptotic analysis

To assess the viability of HEK-293 cells, we performed the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; Sigma] reduction assay. The measurement was carried out essentially as described in [24]. Briefly, HEK-293 cells were seeded in 24-well plates overnight in DMEM containing 10% (v/v) foetal BSA. Following transfection as indicated for each plasmid, MTT was added to a final concentration of 0.5 mg/ml, and incubation was continued for another 4 h. Cell lysis buffer [20% (w/v) SDS and 50% (v/v) *N*,*N*-dimethylformamide, pH 7.4] was then added (1 ml per well) and mixed. The colorimetric determination of MTT reduction was performed at the wavelength of 570 nm.

RESULTS

ABIN-2 physically associates with IKKγ

From independent work, we and others have previously identified ABIN-2/FLIP1 as an inhibitor of NF-κB activation, although the underlying mechanisms of its inhibitory function remained to be determined [19,20]. ABIN-2 represents a novel protein and shares no significant similarity with any other known protein in the Protein Data Base, except it is predicted to have four putative coiled-coil structures [20]. By computer-aided sequence and structure analysis, ABIN-2 was later shown to share slight similarity with IKKγ [20]. Given the fact that IKKγ is one of the components of the IKK complex, this information prompted us to test whether ABIN-2 could physically associate with the IKK complex *in vivo*.

First, we expressed a GST–ABIN-2 fusion protein in bacteria and performed pull-down experiments to examine whether ABIN-2 was able to associate with IKKs or other relevant proteins in the NF-κB signalling pathway. Cell lysates from HEK-293T cells transfected with expression constructs of the FLAG-tagged IKKγ and IκBα, TRADD or RIP were prepared individually and were used as a source for the protein-binding assay. Only IKKγ could be detected in the complex pulled-down by GST–ABIN-2 (Figure 1A, lane 1). In contrast, ABIN-2 did not bind to the expressed TRADD, IκBα or RIP proteins (Figures 1A, lanes 2–4). This result indicated ABIN-2 interacted physically with IKKγ *in vitro*.

To examine further the significance of ABIN-2 and IKKγ interaction, we co-transfected the Myc-tagged ABIN-2 with one of the FLAG-tagged IKK complex subunits, including IKKα, IKKβ and IKKγ, into HEK-293T cells and performed co-immunoprecipitation. As shown in Figure 1(B), only IKKγ, but not IKKα or IKKβ, was co-immunoprecipitated with ABIN-2. Since all three components of IKK were known to form a complex *in vivo*, it is likely that ABIN-2 was part of the IKK complex. Our results also suggested that the component in the IKK complex responsible for physical interaction with ABIN-2 was IKKγ. The association was confirmed further by indirect immunofluorescence microscopic examination to demonstrate that ABIN-2 and IKKγ were indeed co-localized in cells (Figure 2). When expressed individually, ABIN-2 was localized uniformly in cytoplasm and IKKγ formed unique punctate spherical structures of varying size in cytoplasm (Figure 2A). However, when ABIN-2 was co-expressed with IKKγ, ABIN-2 was redistributed completely to the punctate structures of IKKγ (Figure 2B). The punctate spherical structures of IKKγ have been widely observed and reported, although the nature of these punctate spherical structures is not yet clear [13,25]. The co-localization of ABIN-2 and IKKγ was not seen when a non-ABIN-2-interacting construct of IKKγ, $IKK\gamma_{aa1-174}$, was used in the assay (Figure 2C; and see below).

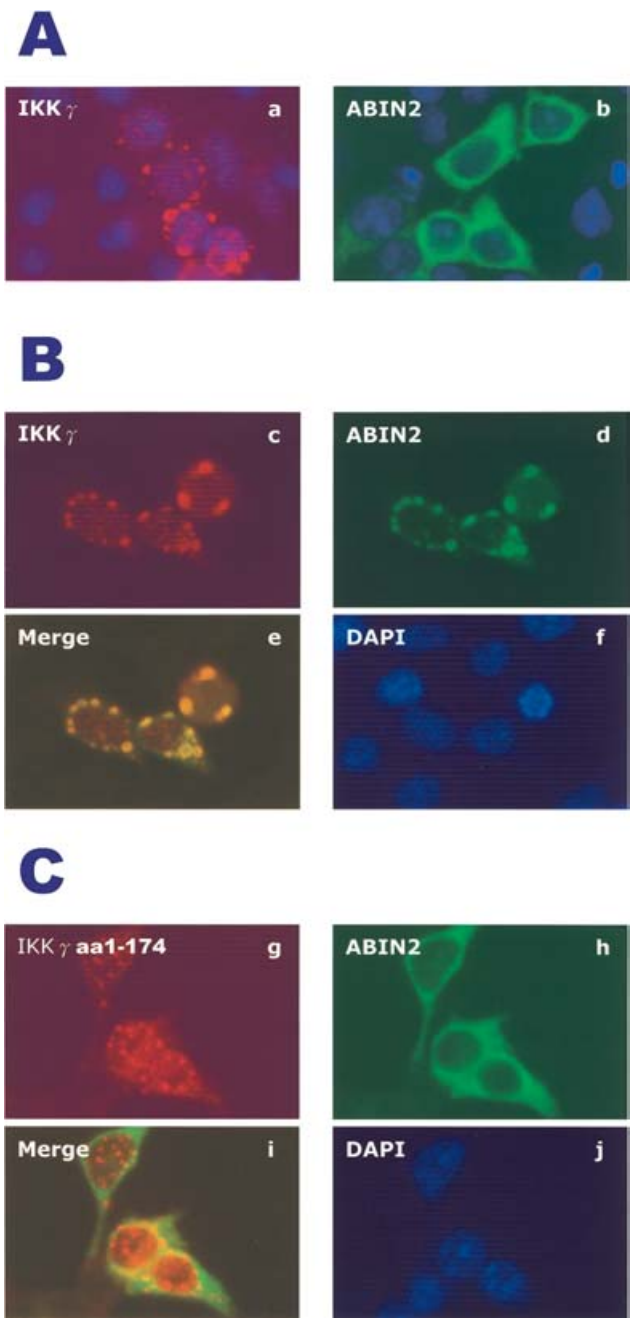


Figure 2 Co-localization of ABIN-2 and IKKγ in HEK-293T cells

(**A**) Expression of IKKγ or ABIN-2. The transiently expressed IKKγ or ABIN-2 was detected 24 h after transfection with pCMV-IKKγ and pFLAG-ABIN-2 respectively, by indirect immunofluorescence microscopy. Detection of expressed proteins was performed using either rabbit polyclonal antisera against IKKγ or mouse monoclonal antisera against FLAG, followed by immnofluorescent staining with Rhodamine-conjugated goat anti-rabbit IgG for IKKγ (a; red image) and FITC-conjugated goat anti-mouse IgG for ABIN-2 (b; green image). (**B**) Co-expression of IKKγ and ABIN-2. pCMV-IKKγ and pFLAG-ABIN-2 were co-expressed in HEK-293T cells and localization of proteins was detected as described in (**A**). IKKγ (c) and ABIN-2 (d) are shown to co-localize in punctate spherical structures when co-expressed. The merged images are shown (e). Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole) (f). (**C**) The non-ABIN-2-interacting IKKγ mutant, IKK$\gamma_{aa1-174}$, does not co-localize with ABIN-2. Plasmid constructs for IKK$\gamma_{aa1-174}$ and ABIN-2 expression were co-transfected into cells and localization of proteins was detected as described in (**A**). IKK$\gamma_{aa1-174}$ (g) and ABIN-2 (h) remain as distinct morphological features when co-expressed. The images are also compared in merged condition (i). Nuclei were stained with DAPI as indicated (j).

ABIN-2 binds to IKKγ through its coiled-coil domain

To define the region in ABIN-2 that was required for the interaction with IKKγ, we constructed and expressed a series of deletion mutants of ABIN-2 and examined their abilities to bind IKKγ (Figure 3A). The interaction was examined by both pull-down (Figure 3B) and co-immunoprecipitation (results not shown) assays. Sequential removal of the first three coiled-coil domains from the N-terminus of ABIN-2 did not affect the ability of ABIN-2 to interact with IKKγ (Figure 3). However, further deletion into the fourth coiled-coil domain completely abolished the binding of ABIN-2 to IKKγ. These results indicated that the region within the fourth coiled-coil domain of ABIN-2, particularly the area ranging from amino acids 253 to 346, was definitely important for IKKγ binding (Figure 3A).

ABIN-2 and RIP bind to the same region of IKKγ

Similarly, we created a series of deletion mutants of IKKγ in order to identify the regions in IKKγ that were required for ABIN-2 binding. Again, the interaction was examined by both pull-down and co-immunoprecipitation assays. As shown in Figure 4, only the mutant IKKγ proteins with the deletion encompassing amino acids 174–306 failed to interact with ABIN-2. Thus this region of IKKγ appeared to be important for ABIN-2 association, although further deletion of a coiled-coil sequence (CCS2; amino acids 260–281) within this region did not seem to have any effect on the binding activity (Figure 4).

It has been reported previously that IKKγ contains several protein domains that are responsible for the interaction with various effectors [11,26,27]. We compared the peptide sequences of ABIN-2-binding domain in IKKγ with those reported domains for various effectors and found that it overlapped with the domain identified as the RIP-interaction region [11]. RIP is known as one of the key effectors for the TNF signalling pathway and is essential for the TNF-induced NF-κB activation. In addition, the physical association of RIP with IKKγ has played a major role in the RIP-induced NF-κB activation [13,14,28]. Our results thus raise an interesting possibility that ABIN-2 and RIP interact with IKKγ at the same protein domain *in vivo*.

Since RIP and ABIN-2 may interact with IKKγ at the same region, we re-examined the sequences of ABIN-2 and RIP to find whether or not there is a common structural feature shared by these two proteins. Strikingly, a stretch of peptide of approx. 50 amino acids present in both ABIN-2 and RIP was found to have significant similarity (Figure 5). The similar region found in ABIN-2, amino acids 253–305, was indeed located within the domain mapped to be responsible for IKKγ binding (Figure 1). The similar region found in RIP, amino acids 278–327, on the other hand, was overlapping with a previously identified domain of RIP responsible for IKKγ binding [11]. We speculated that this region could define a novel structural domain of either ABIN-2 or RIP for the binding of IKKγ. We designated this domain as the core motif for binding (CMB).

As shown in Figure 6(A), the expression of wild-type ABIN-2 readily blocked the RIP-induced NF-κB activation in a dose-dependent manner. In contrast, the expression of ABIN-2 had little effect on the NF-κB activation induced by RelA (Figure 6B). To demonstrate that this effect was due to the competition between ABIN-2 and RIP, we took an approach by expressing RIP, IKKγ and various amounts of ABIN-2 together in HEK-293T cells. We then immunoprecipitated IKKγ and examined the protein level of RIP in the immunocomplexes in the presence of increasing amount of ABIN-2. By Western blotting, we showed that the decreasing amount of RIP bound to IKKγ was correlated with

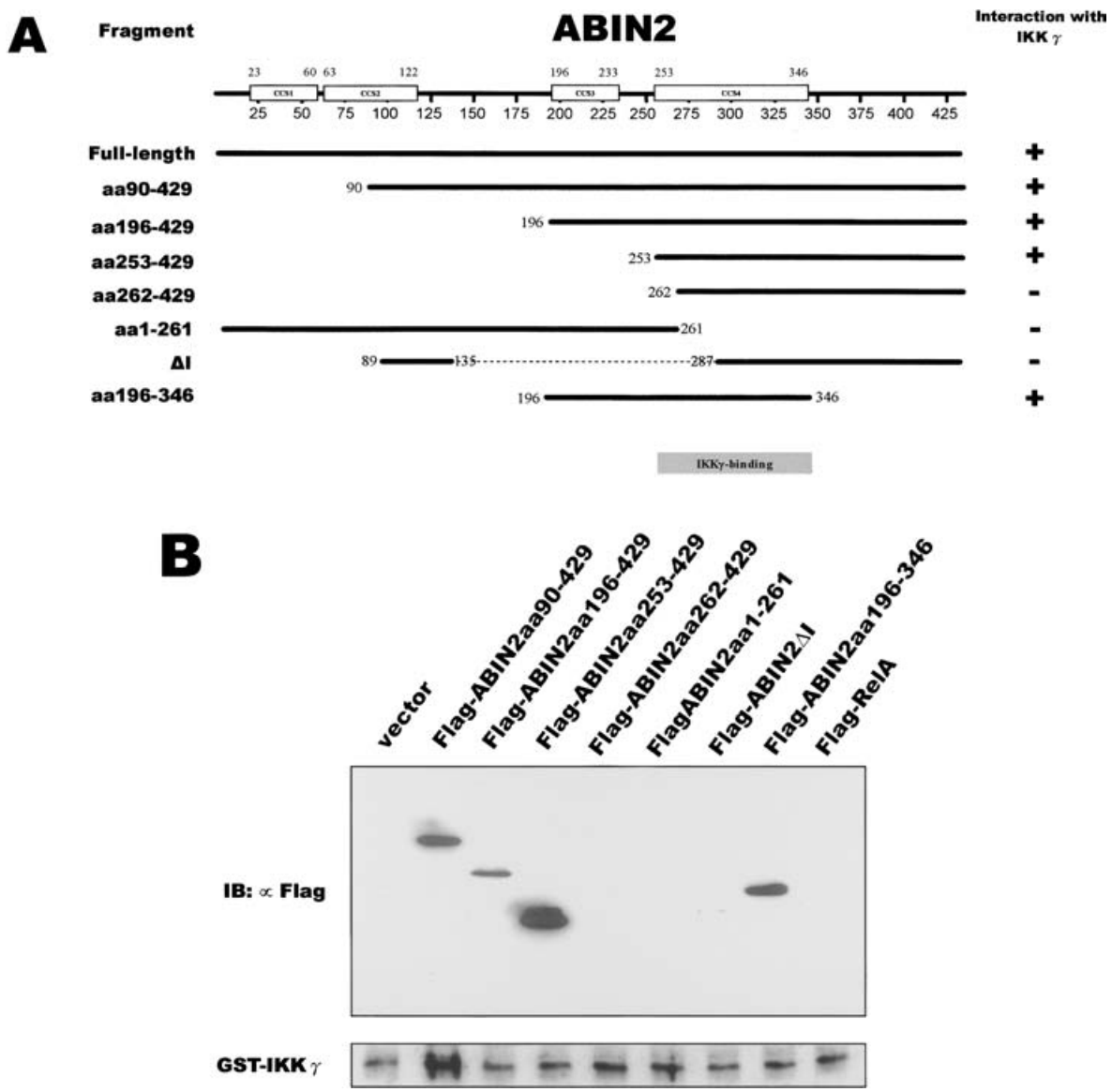


Figure 3 Analysis of the IKKγ-binding domains on ABIN-2

(**A**) The schematic diagram illustrates the regions of ABIN-2 deduced to be required for binding IKKγ. The hollow rectangles on ABIN-2 indicate the predicted coiled-coil structures (CCS), and each is marked with amino acid number above to show their exact locations in the protein. The relative positions of amino acids are also labelled underneath for the entire protein. Truncated peptides of ABIN-2 are labelled with amino acid number to show the location. The positive (+) or negative (−) result of the interaction is shown on the right for each pair of interactions. The broken line represents the deleted area. The shaded box at the bottom is the deduced area that is important for ABIN-2 to interact with IKKγ. (**B**) Verification of the interaction between IKKγ and ABIN-2 fragments by GST pull-down assay. The pull-down analysis was performed by using the immobilized GST–IKKγ to pull down various expressed FLAG-tagged ABIN-2s as indicated. Pulled-down proteins were then visualized by immunoblotting with an anti-FLAG (α Flag) antibody.

the increasing level of ABIN-2 (Figure 6C). Hence, ABIN-2 competed specifically with RIP to bind IKKγ and blocked NF-κB activation.

To address the question whether or not CMB played any role in the interaction with RIP and ABIN-2, we disrupted the CMB sequence from RIP and ABIN-2, and examined its effect on NF-κB activation, as well as its ability to interact with IKKγ as opposed to that of the wild-type. The CMB-deletion mutant of ABIN-2, ABIN-2ΔCMB, failed to associate with IKKγ and consequently could not inhibit the NF-κB activity induced by RIP (Figure 7A) or by TNFα (Figure 7B). More interestingly, the CMB-deletion mutant of ABIN-2 not only failed to interact with IKKγ, but also increased the basal level of NF-κB activity (Figure 7C). This was probably due to the loss of inhibitory function of ABIN-2ΔCMB in the NF-κB activation. Likewise, the RIP mutant, RIPΔCMB, diminished dramatically in its ability to activate NF-κB (Figure 8A). The Western blot analysis indicated that the mutant protein was not able to associate with IKKγ (Figure 8B). Taken together, we have provided the evidence that CMB in both ABIN-2 and RIP defines a novel protein structural domain important in the regulation of NF-κB activation.

ABIN-2 enhances the RIP-induced apoptosis

The identification of the protein domain of ABIN-2 responsible for the competition with RIP in order to block the activation of NF-κB provides a mechanism to explain the inhibitory function of ABIN-2. The way in which ABIN-2 acts to inhibit NF-κB by

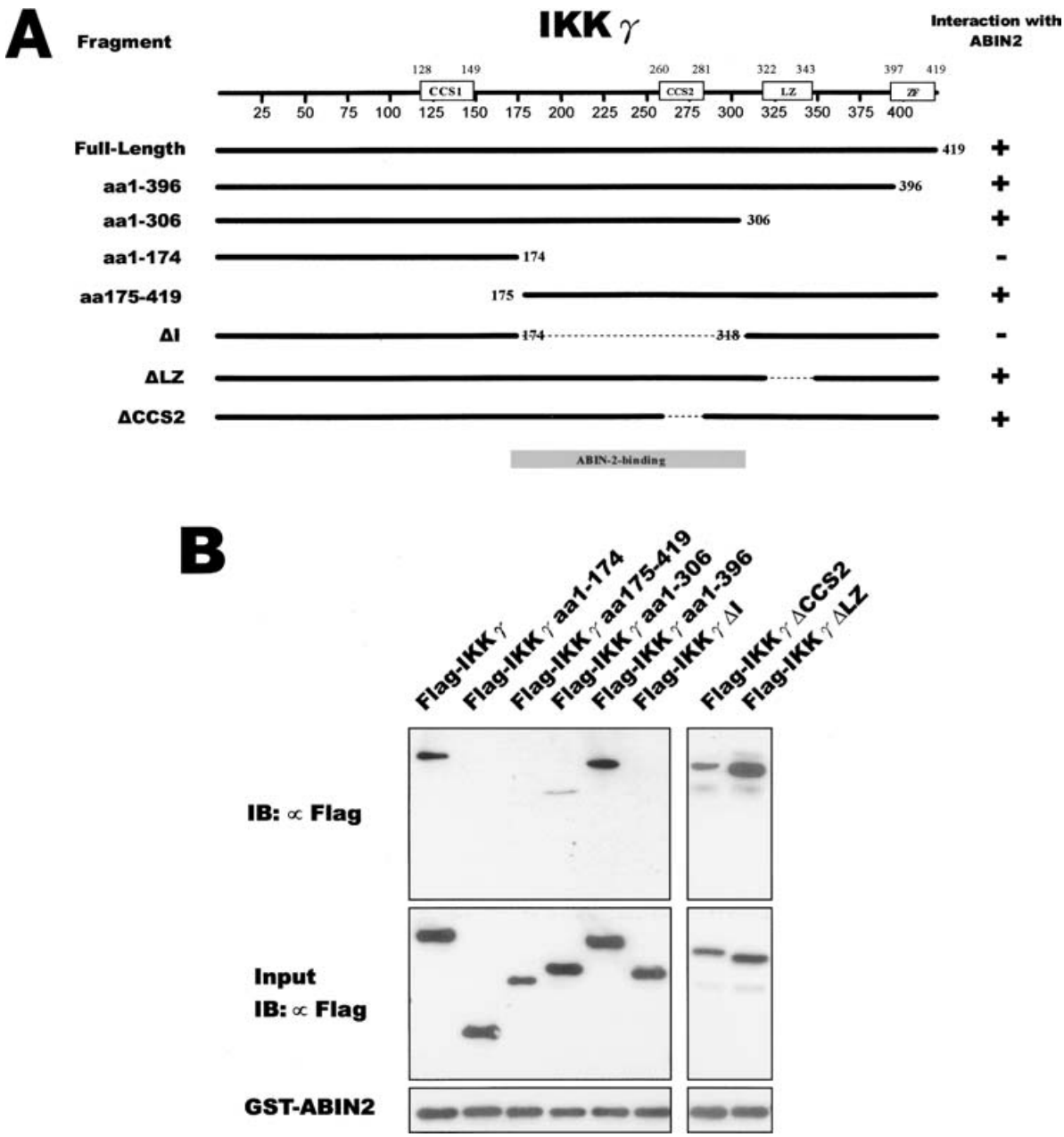


Figure 4 Analysis of the ABIN-2-binding domains on IKKγ

(**A**) The schematic diagram of IKKγ domains required for ABIN-2 binding. The hollow rectangles on IKKγ indicate the predicted coiled-coil structures (CCS), and each is marked with the amino acid number above to show their exact locations in the protein. Areas of sequences predicted to be leucine zipper (LZ) and zinc finger (ZF) are also indicated. The relative positions of amino acids are labelled underneath for the entire protein. Truncated peptides of IKKγ are labelled with the amino acid number to show the location. Broken lines represent the deleted areas. The positive (+) or negative (−) result of the interaction is shown on the right for each pair of interactions. The shaded box at the bottom is the deduced area that is important for IKKγ to interact with ABIN-2. (**B**) Verification of the interaction between ABIN-2 and IKKγ fragments by GST pull-down assay. The pull-down analysis was performed by using the immobilized GST–ABIN-2 to pull down various expressed FLAG-tagged IKKγs as indicated. Pulled-down proteins were then visualized by immunoblotting with an anti-FLAG (α Flag) antibody.

Figure 5 Defined sequences of the CMB

The sequences of ABIN-2 and RIP at CMB are aligned. Identical residues are highlighted in black boxes, and similar residues are in grey boxes. Amino acid numbers of the beginning and the end of each peptide are shown.

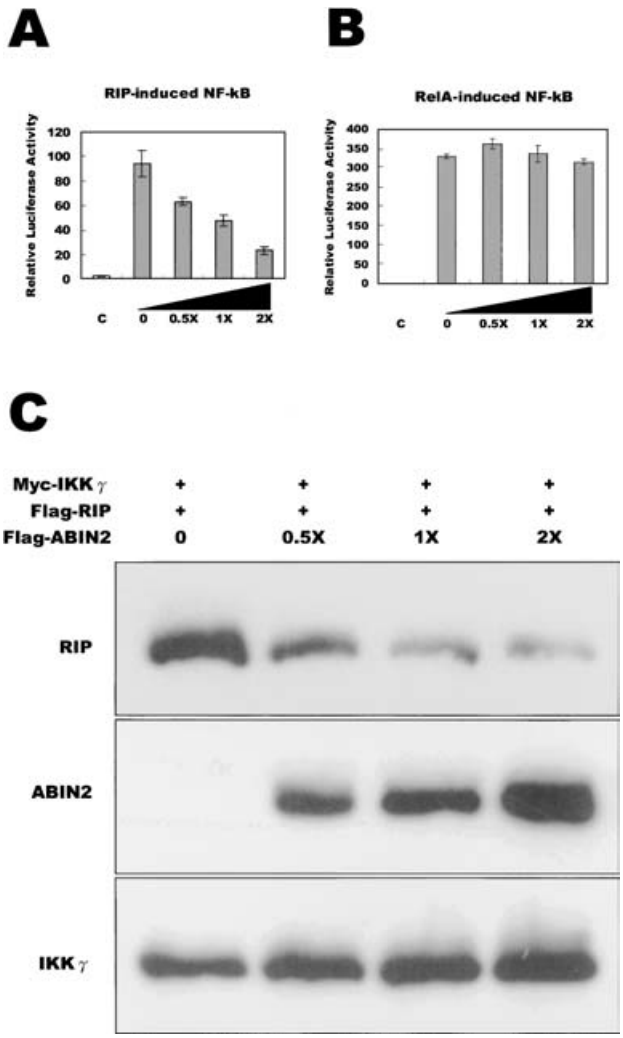


Figure 6 ABIN-2 modulation of NF-κB activity

(**A**) ABIN-2 inhibits the RIP-induced NF-κB activation in a dose-dependent manner. The experiment was performed by co-transfecting equal amounts of NF-κB-dependent luciferase reporter plasmid and RIP-expressing plasmid into HEK-293T cells, along with the increasing amounts of ABIN-2-expressing construct; 0, 0.5×, 1× and 2× indicate the amount of ABIN-2-expressing vector used in the experiment, which are equal to none, half, equal and twice the reporter plasmid used (1× = 300 ng) respectively. At 24 h after transfection, cell lysates were prepared, and the activities of luciferase were measured. (**B**) ABIN-2 has no effect on RelA-induced NF-κB activation. The measurement of NF-κB-dependent luciferase activity in HEK-293T cells was performed essentially the same as described in (**A**), except RelA-expressing construct was used to replace RIP vector. Results in (**A**) and (**B**) are mean relative luciferase activities for three separate experiments performed in duplicate. (**C**) ABIN-2 competes with RIP to bind IKKγ. Equal amounts of FLAG–RIP and Myc–IKKγ plasmids were transiently transfected along with various amounts of FLAG-tagged ABIN-2 into HEK-293T cells. The amounts of ABIN-2-expressing vector used in the experiment (0, 0.5×, 1× and 2×) are the same as described in (**A**). Cell lysates were prepared 24 h after transfection and immunoprecipitated with anti-Myc antisera. The associated RIP proteins were then detected by immunoblotting with anti-FLAG antisera.

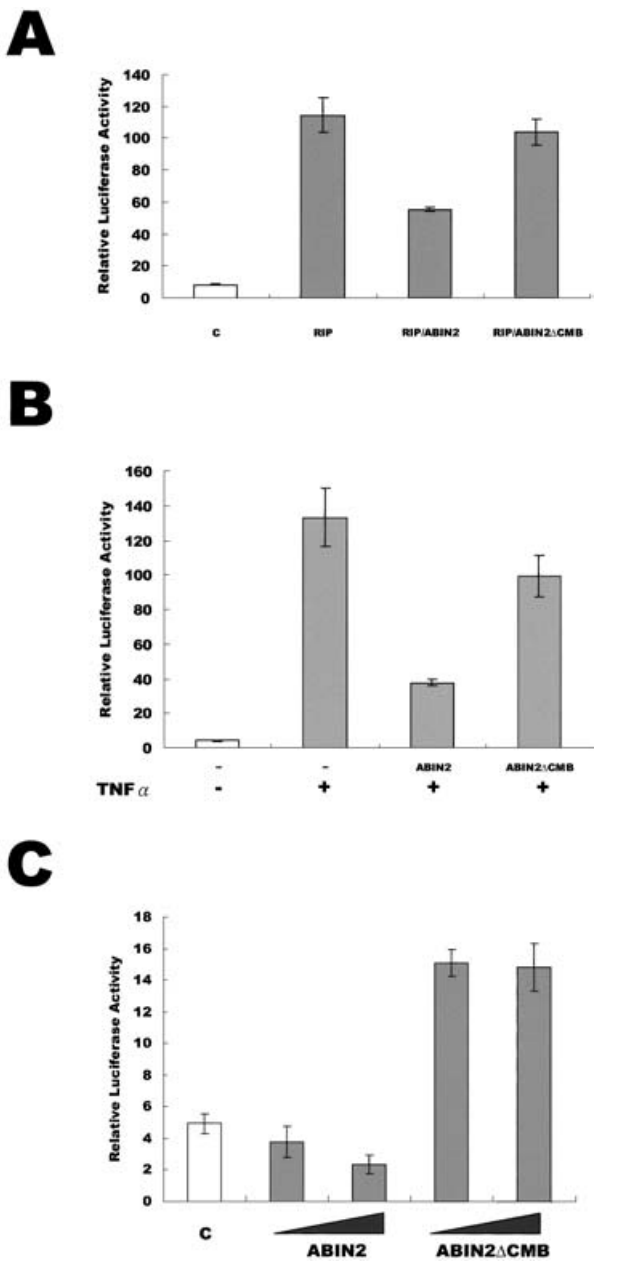


Figure 7 Nullification of CMB on ABIN-2 causes a dramatic effect on modulation of NF-κB activity

(**A**) The deletion of CMB on ABIN-2 fails to inhibit RIP-induced NF-κB activity. Cells were co-transfected with equal amounts of NF-κB-dependent luciferase reporter plasmid and RIP-expressing plasmid, along with either ABIN-2 or ABIN-2ΔCMB-expressing construct. The activity of luciferase was then measured. Cells without transfection (c) or transfected with RIP alone (RIP) were used as controls. (**B**) The deletion of CMB on ABIN-2 fails to inhibit TNFα-induced NF-κB activity. Cells were co-transfected with a fixed amount of NF-κB-dependent luciferase reporter plasmid along with an equal amount of either ABIN-2 or ABIN-2ΔCMB-expressing construct. At 24 h after transfection, cells were treated with 10 ng/ml TNF-α for 4 h and the luciferase activity was measured as described above. (**C**) The deletion of CMB on ABIN-2 has an impact on the basal level of NF-κB activity. Cells were co-transfected with a fixed amount of NF-κB-dependent luciferase reporter plasmid along with either ABIN-2 or ABIN-2ΔCMB-expressing plasmids, at an equal or twice amount. The measurement of reporter luciferase activity was carried out 24 h after transfection. Results are mean relative luciferase activities for three separate experiments performed in duplicate.

posing as a RIP-specific competitor for IKKγ binding is particularly interesting. Several lines of evidence have already suggested that key elements, such as RIP and IKKγ, also play a role in the determination of cellular pathway leading to NF-κB activation or apoptosis [25,29]. Overexpression of RIP not only activates NF-κB, but also induces apoptosis in a finely tuned regulatory manner [28,30]. Since ABIN-2 specifically blocked the RIP-induced NF-κB activity, we postulated that ABIN-2 could also have an effect on the RIP-induced apoptosis. To test the role of RIP and ABIN-2 in regulating apoptosis, we expressed RIP and ABIN-2 in HEK-293 cells. The expression of RIP in HEK-293 cells activated NF-κB with only slight induction of apoptosis (Figure 9, lane 2).

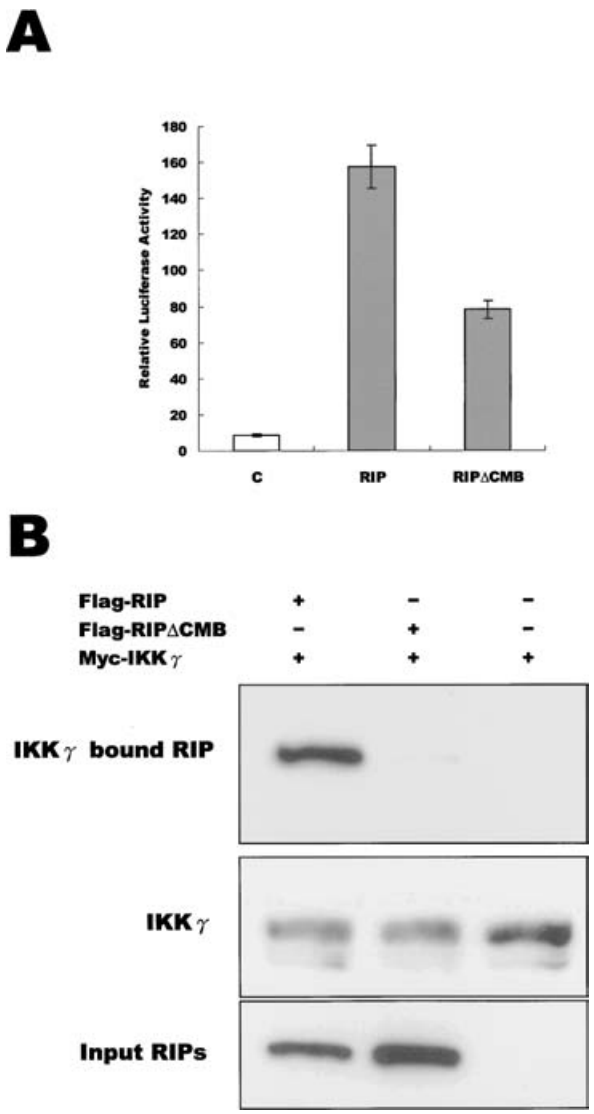


Figure 8 RIP requires CMB to induce NF-κB and to interact with IKKγ

(**A**) Deletion of CMB diminishes RIP-induced NF-κB activity. Cells were transfected with a fixed amount of NF-κB-dependent luciferase reporter plasmid along with an equal amount of wild-type RIP or RIPΔCMB-expressing construct. The reporter luciferase activity was measured as described in Figure 6. Results are mean relative luciferase activities for three separate experiments performed in duplicate. (**B**) The CMB-deleted RIP fails to interact with IKKγ. A fixed amount of Myc–IKKγ plasmid was transiently transfected along with an equal amount of FLAG-tagged RIP or RIPΔCMB into HEK-293T cells. Cell lysates were prepared 24 h after transfection and immunoprecipitated with anti-Myc antisera. The associated RIP proteins were then detected by immunoblotting with anti-FLAG antisera.

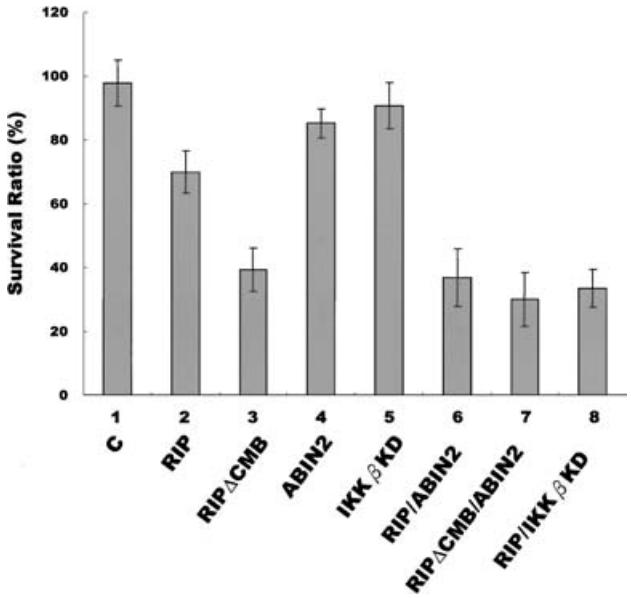


Figure 9 ABIN-2 affects RIP-induced apoptosis

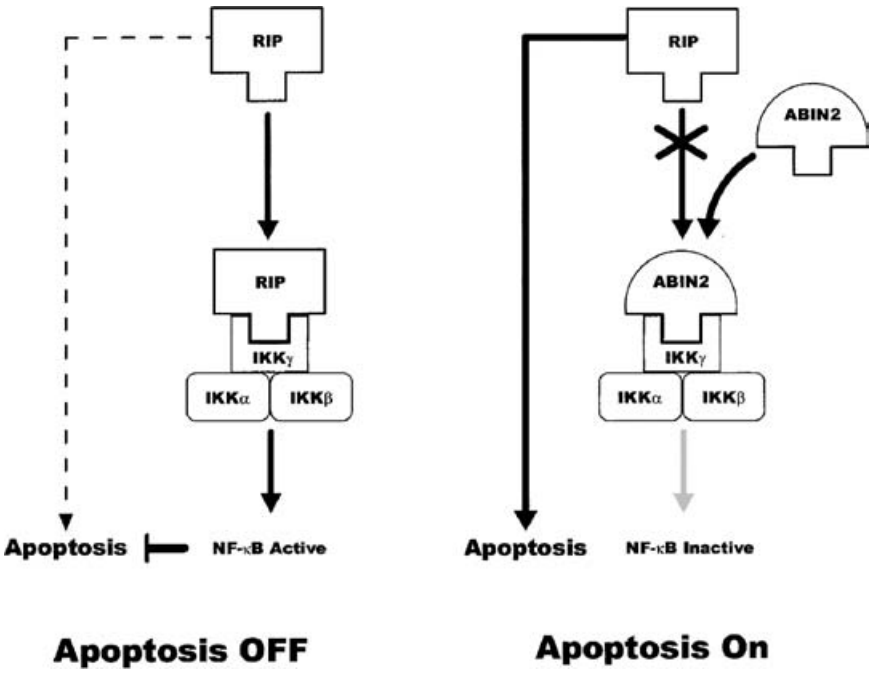
HEK-293 cells were transfected with various constructs as indicated. Living cells were monitored by the MTT assay 24 h after transfection as described in the Experimental section. The survival ratio in each condition was expressed relative to cells without transfection (C). Results are means for three independent experiments performed in triplicate.

Overexpression of ABIN-2 together with RIP had substantial effects on apoptosis of HEK-293 cells (Figure 9, lane 6), although the expression of ABIN-2 alone had little effect on cell viability (Figure 9, lane 4). In addition, the kinase-inactive mutant of IKKβ, which was able to block the NF-κB activation, also stimulated the RIP-induced apoptosis (Figure 9, lane 5). This was consistent with our prediction that blocking the NF-κB activation may promote the pathway leading to apoptosis. Finally, the deletion mutant of RIP, RIPΔCMB, which had weak activity to induce NF-κB, showed much stronger activity to induce apoptosis when expressed in HEK-293 cells compared with that of the wild-type RIP (Figure 9, lane 3). We propose that both RIP and ABIN-2 have an essential role in the determination of the signalling pathway leading to NF-κB activation or apoptosis by competing for binding to IKKγ (Scheme 1).

DISCUSSION

IKKγ is one of the components of IKK complex that plays an essential role in the signalling pathway leading to the activation of NF-κB in many cellular events [8–10,31,32]. One current model is that IKKγ might serve as a scaffolding protein to organize the formation of the multi-subunit IKK complex [12]. However, more recent studies have also revealed that IKKγ interacts with many other molecules, such as TANK (TNFR-associated factor family member-associated NF-κB activator), ASC [apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain)], CIKS (connection to IKK and stress-activated protein kinase/c-Jun N-terminal kinase), Act1 (NF-κB activator 1), NIK (NF-κB-inducing kinase), RIP, A20, CARDINAL (CARD inhibitor of NF-κB-activating ligands) and CSN3 [a component of the COP9 (constitutive photomorphogenesis 9) signalosome], from many cellular aspects in conducting the activation of NF-κB in a cell-type- and signal-specific manner [6,14,33–38]. Despite being in the midst of the already complex regulatory network of the NF-κB signalling pathway, IKKγ has apparently become the focus in recent studies to unravel the mechanisms of the pathway. By taking advantage of the study of an NF-κB inhibitor, ABIN-2, we have demonstrated in the present study a novel route of regulating NF-κB and apoptosis through IKKγ.

Using pull-down and immunoprecipitation assays, we reported that ABIN-2 specifically forms a stable complex with IKKγ. This association appears to be specific because other components in the signalling pathway of NF-κB activation could not be detected as associating with ABIN-2. Physical interaction of ABIN-2 and IKKγ has provided insight and elucidates an inhibitory mechanism for the NF-κB signalling pathway. In the present study, we showed that ABIN-2 exerts its inhibitory function by



Scheme 1 Model of ABIN-2 in the regulation of NF-κB and apoptosis

In the absence of ABIN-2, RIP is mainly recruited by IKKγ, which results in NF-κB activation. Meanwhile, RIP activates apoptotic pathway through an unknown mechanism. However, the active NF-κB is more pronounced, which promotes survival by preventing apoptosis from happening. When ABIN-2 is overexpressed, the binding of IKKγ by ABIN-2 would compete out RIP's chance to get into the IKK complex. As a result, NF-κB is not activated and that in turn stimulates RIP-induced apoptosis.

competing specifically for the binding site in IKKγ with RIP, thus blocking the RIP-induced NF-κB activation. It was shown that ABIN-2 abolished RIP's ability to bind IKKγ in a dose-dependent manner and inhibited the RIP-mediated NF-κB activation selectively, but not the activation pathways mediated by other molecules, such as RelA. We also demonstrated that ABIN-2 and RIP share a common structural feature of CMB that appears to be essential for IKKγ binding. Moreover, the ABIN-2 mutant that could not bind IKKγ due to the deletion of CMB also lost inhibitory effect towards the RIP-induced NF-κB activation. Similarly, RIP without CMB could not interact with IKKγ, nor induce NF-κB to a full extent.

By blocking the binding of RIP to IKKγ, ABIN-2 was able to co-operate with RIP to induce apoptosis in HEK-293 cells. Our results reinforce the idea that IKKγ is a pivotal point in regulating the cellular pathways leading to NF-κB activation or apoptosis. The novel function of ABIN-2 described in the present paper is reminiscent of a newly identified molecule CYLD (cylindromatosis), which serves as a potential tumour suppressor by acting as a NF-κB inhibitor [39,40]. The action of CYLD is also at IKKγ to block the activation of NF-κB. The failure of CYLD function results in the increase of NF-κB activity, and that, in turn, prevents apoptosis and leads to tumorigenesis [39–41]. In this regard, the failure of ABIN-2 would probably mimic the defect of CYLD and causes tumours.

The identification of an IKKγ-interacting domain, CMB, which is shared by both RIP and ABIN-2, is particularly significant. The sequence of CMB represents a novel peptide, which has never been defined before. The presence of this domain is important for the function of RIP and ABIN-2. It is noteworthy that a previous report has described that the cleavage of a death domain in RIP by caspase 8 prompts TNF-induced apoptosis [29]. The cleavage site by caspase 8 in RIP is located at the residue Asp^{324}, which is within the CMB sequence (amino acids 278–327). We hypothesize that the intact form of this domain is probably important for RIP to bind IKKγ efficiently. Once RIP is cleaved by caspase 8, it would hinder its ability to bind IKKγ, and hence results in the promotion of apoptosis. This possibility is currently under investigation. From initial analysis, CMB contains regions of two α-helices at both ends and a random coil structure in between. One interesting implication of this finding is to design peptideomimic chemicals or peptides with a similar structure to CMB and test their inhibitory effect on the NF-κB signalling pathway.

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