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Fas-associated Factor-1 Inhibits Nuclear Factor-κB (NF-κB) Activity by Interfering with Nuclear Translocation of the RelA (p65) Subunit of NF-κB*

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Fas-associated factor-1 (FAF1) is a Fas-binding pro-apoptotic protein that is a component of the death-inducing signaling complex in Fas-mediated apoptosis. Here, we show that FAF1 is involved in negative regulation of NF-κB activation. Overexpression of FAF1 decreased the basal level of NF-κB activity in 293 cells. NF-κB activation induced by tumor necrosis factor (TNF)-α, interleukin-1β, and lipopolysaccharide was also inhibited by FAF1 overexpression. Moreover, FAF1 suppressed NF-κB activation induced by transducers of diverse NF-κB-activating signals such as TNF receptor-associated factor-2 and -6, MEKK1, and IκB kinase-β as well as NF-κB p65, one of the end point molecules in the NF-κB activation pathway, suggesting that NF-κB p65 might be a target molecule upon which FAF1 acts. Subsequent study disclosed that FAF1 physically interacts with NF-κB p65 and that the binding domain of FAF1 is the death effector domain (DED)-interacting domain (amino acids 181–381), where DEDs of the Fas-associated death domain protein and caspase-8 interact. The NF-κB activity-modulating potential of FAF1 was also mapped to the DED-interacting domain. Finally, overexpression of FAF1 prevented translocation of NF-κB p65 into the nucleus and decreased its DNA-binding activity upon TNFα treatment. This study presents a novel function of FAF1, in addition to the previously known function as a component of the Fas death-inducing signaling complex, *i.e.* NF-κB activity suppressor by cytoplasmic retention of NF-κB p65 via physical interaction.

Fas-associated factor-1 (FAF1)[1] was identified by yeast two-hybrid assay using the cytoplasmic domain of Fas as bait (1). FAF1 is able to enhance Fas-induced apoptosis. It has also been demonstrated that FAF1 can initiate apoptosis in the absence of any extrinsic death signals when overexpressed (2). Recently, FAF1 has been demonstrated to be a component of the death-inducing signaling complex (DISC) in Fas-mediated apoptosis (3).

Although FAF1 has demonstrated its apoptotic potential, it does not contain typical death motifs such as the death domain, death effector domain (DED), and caspase recruitment domain. Instead, FAF1 has sequence motifs that are present in the proteins of the ubiquitination pathway (4). FAF1 has two ubiquitin-homologous domains in its N terminus and a domain (the UX domain) homologous to proteins involved in the ubiquitination pathway. The three-dimensional structure of the UX domain of FAF1 as determined by NMR analysis demonstrates its structural similarity to ubiquitin (5). Despite the amino acid sequence and structural similarities to ubiquitin, functions related to the ubiquitination pathway have not yet been assigned to FAF1.

Members of the tumor necrosis factor (TNF) receptor (TNFR) family such as Fas, TNFR1, and TNFR2 play critical roles in various cellular responses, including cell survival and apoptosis (6, 7). The initiation of signal transduction by these receptors requires adapter proteins to trigger cytoplasmic signal transduction cascades. The adapter proteins are not always specific to certain types of receptors. For example, the adapter molecule FADD is recruited not only to Fas, but also to TNFR1 and death receptor-3 (8). TRAF6 can act as an adapter for the interleukin-1 (IL-1) receptor as well as for TNFR2 (9, 10). Different signaling cascades initiate depending on the combination of adapter molecules recruited to the receptors. In TNFR1 signaling, the recruitment of the TRADD·FADD complex leads to the activation of the caspase cascade, resulting in apoptosis, whereas that of the TRADD·TRAF2 complex leads to the activation of NF-κB, resulting in survival (11, 12).

FLICE-associated huge protein (FLASH) was identified by yeast two-hybrid assay using the duplicated DEDs of caspase-8 as probes, and its DED-recruiting domain interacts with the DED of FADD (13). FLASH is recruited to the Fas DISC, which is composed of Fas, FADD, and caspase-8. Overexpression of FLASH could potentiate Fas-mediated apoptosis after stimulation, and FLASH has a role in TNF-mediated NF-κB activation via a TRAF2/NIK/IKK-dependent pathway (14).

FAF1 is similar to FLASH and FADD. Like FLASH, FAF1 binds to the DEDs of FADD and caspase-8 (3). FAF1 demonstrates similarity to FADD because both bind to Fas and

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[1] The abbreviations used are: FAF1, Fas-associated factor-1; DISC, death-inducing signaling complex; DED, death effector domain; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; FADD, Fas-associated death domain protein; TRAF, tumor necrosis factor receptor-associated factor; IL-1, interleukin-1; TRADD, tumor necrosis factor receptor family-associated death domain protein; FLASH, FLICE-associated huge protein; NIK, NF-κB-inducing kinase; IKK, IκB kinase; LPS, lipopolysaccharide; MEKK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase-1; CMV, cytomegalovirus; mAb, monoclonal antibody; pAb, polyclonal antibody; GST, glutathione *S*-transferase; DEDID, death effector domain-interacting domain; ASK1, apoptosis signal-regulating kinase-1.

caspase-8 directly and can enhance Fas-mediated apoptosis (15, 16). However, FADD contains the death domain and DED death motifs, whereas FAF1 does not. Both FLASH and FADD can induce NF-κB activation (13, 17). We are interested in whether FAF1 modulates the NF-κB pathway as do FLASH and FADD.

NF-κB is activated by various stimuli, including TNF, IL-1, and lipopolysaccharide (LPS) (18). NF-κB regulates the expression of genes involved in the immune response, inflammatory process, and apoptosis (19, 20). NF-κB signals subsequently activate two subunit kinases, IKKα and IKKβ (21–23). Phosphorylation of IκB proteins leads to their ubiquitination and subsequent degradation by the 26 S proteasome. After degradation of IκB, free NF-κB translocates from the cytoplasm to the nucleus and activates NF-κB-dependent genes (24).

In this study, we investigated whether FAF1 is involved in NF-κB activity modulation. We demonstrate that FAF1 can inhibit NF-κB activation by divergent signals, including TNFα, IL-1β, and LPS. This suppression of NF-κB activity is contributed by the protein interaction between FAF1 and NF-κB p65, leading to the inhibition of NF-κB activation by preventing the translocation of NF-κB p65 to the nucleus.

EXPERIMENTAL PROCEDURES

Plasmids—Human FAF1 and its deletion mutant expression vectors were described previously (2). pRK-TRAF2, pRK-TRAF6, pRK-TNFR1, pcDNA3.1-NF-κB-p65, the Ig-κB-*luc* plasmid, the pGL2-cIAP2−247-*luc* plasmid (25), and the mutant pGL2-cIAP2−247(mκB1,3)-*luc* plasmid (25) were kindly provided by Dr. Tae H. Lee (Yonsei University, Seoul, Korea). pFLAG-IKKβ was a gift from Dr. Hiroyasu Nakano (Juntendo University, Tokyo, Japan). The AP-1-*luc* plasmid and the pFC-MEKK1 expression vector were purchased from Stratagene (La Jolla, CA) (26). For construction of antisense FAF1, the primers used for PCR amplification were 5′-GAACTCGAGATGGCGTCCAACATGGAC-3′ (forward primer) and 5′-GCGCGAATTCTTACTCTTTTGCTTCAAGG-3′ (reverse primer). The amplified fragments were digested with XhoI and EcoRI and introduced into the pFLAG expression vector in an antisense orientation relative to that of the cytomegalovirus (CMV) promoter.

Cell Lines and Transfections—293, NIH3T3, and NIH3T3-p65$^{-/-}$ cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin (Invitrogen). Cells were maintained in 5% CO_2 at 37 °C. Cells were seeded at 1×10^6 cells/10-cm plate, allowed to attach overnight, and then transfected with the indicated constructs using the calcium phosphate precipitation method as described previously (2).

Luciferase Reporter Gene Assays—To determine whether FAF1 modulates NF-κB activation by various signals, 293 cells were transfected with 1 μg of test plasmid, 0.1 μg of Ig-κB-*luc* reporter plasmid, and 0.1 μg of CMV-β-galactosidase plasmid. To demonstrate that the action of FAF1 on NF-κB is specific, 0.1 μg of AP-1-*luc* plasmid, 0.5 μg of pGL2-cIAP2−247-*luc* plasmid, and 0.5 μg of pGL2-cIAP2−247(mκB1,3)-*luc* reporter plasmid were tested as controls. The total DNA concentration in each transfection was kept constant by adjusting with the empty vector. Cells were left untreated or were stimulated at 36 h post-transfection with TNFα (20 ng/ml), IL-1β (20 ng/ml), or LPS (100 ng/ml) for 6 h. The cell lysates were assayed for luciferase activities according to the manufacturer's protocol (Promega, Madison, WI). Transfection efficiencies were normalized by measuring β-galactosidase activities.

Immunoprecipitation and Antibodies—At 48 h post-transfection, cells were washed with phosphate-buffered saline, lysed in lysis buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 0.4 mM phenylmethylsulfonyl fluoride), and then sonicated using a Branson Model 450 sonifier. The cell lysates was centrifuged at 14,000 × *g* for 10 min, and the protein concentrations of the cell lysates were determined using a Bio-Rad protein assay kit. 100 μg of supernatant was incubated for 4 h with 1 μg of anti-FLAG monoclonal antibody (mAb) M2 (Sigma). The samples were further incubated with 30 μl of protein A/G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4 °C. The beads were washed five times with lysis buffer and separated by SDS-PAGE. Immunocomplexes were then transferred to nitrocellulose membranes and analyzed by Western blotting using the appropriate primary antibodies and secondary antibodies coupled to peroxidase. Proteins were visualized by enhanced chemiluminescence (Amersham Biosciences). The antibodies used were as follows: anti-FLAG mAb M2, anti-β-tubulin mAb, and anti-β-actin mAb (Sigma) and rabbit anti-p65 polyclonal antibody (pAb) H10 and polyclonal anti-goat and anti-mouse IgG (Santa Cruz Biotechnology). Mouse anti-FAF1 mAb was provided by Dr. Jong-Seok Lim (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea).

For detection of endogenous binding between FAF1 and NF-κB p65, 293, NIH3T3, and NIH3T3-p65$^{-/-}$ cells were treated with or without TNFα for 30 min. Cells were lysed in lysis buffer, and 100 μg of supernatant was incubated with anti-FAF1 mAb and mouse IgG_1, respectively, for 4 h at 4 °C. Immunocomplexes were collected by incubation with protein A/G Plus-agarose for 2 h at 4 °C and then analyzed by Western blotting as described above.

GST Pull-down Assay—293 cells were transfected with 1 μg of pcDNA3.1-NF-κB-p65 by the calcium phosphate precipitation method. At 48 h post-transfection, cells were harvested in lysis buffer as described above. GST-FAF1 deletion mutant fusion proteins were expressed in *Escherichia coli* BL21(DE3) cells and purified with glutathione-Sepharose 4B beads (Peptron, Daejeon) as specified by the manufacturer. For binding assay, 100 μg of cell lysate was incubated with 1 μg of either GST or GST-FAF1 deletion mutant fusion protein for 4 h at 4 °C in lysis buffer. Samples were washed four times with lysis buffer, and the bound proteins were separated by SDS-PAGE and detected by Western blotting with rabbit anti-NF-κB p65 pAb H10.

Electrophoretic Mobility Shift Assays—293 cells were transfected with pFLAG-FAF1 and harvested, and nuclear extracts were then prepared as described previously (27). Electrophoretic mobility shift assay was performed as described (26). Briefly, synthetic oligonucleotides were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and incubated with nuclear proteins (4 μg) at room temperature for 30 min in binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA (pH 7.5), 5% glycerol, 2 μg of poly(dI-dC), and 5% Nonidet P-40) for 20 min at room temperature, and the protein·DNA complexes were loaded onto a 5% native polyacrylamide gel and detected by autoradiography. To measure NF-κB activation, an end-labeled, double-stranded, NF-κB-specific oligonucleotide probe containing the two NF-κB tandem repeats derived from the human immunodeficiency virus long terminal repeat (5′-ATCAGGGACTTTCCGCTGGGGACTTTCCG-3′) was used (27). For control experiments, the region from bp −66 to −48 upstream of the surfactant protein A2 gene transcription initiation site, which contains the GT box (5′-TCTCAGGGGTGGGGAAGAA-3′), was used (28).

Cell Fractionation—Nuclear and cytosolic fractions were isolated by differential centrifugation as follows. To prepare the nucleus, 2×10^6 cells were lysed in lysis buffer for 15 min at 4 °C to swell the cells. The cells were passed 10–12 times through a 21-gauge needle, and cell lysates were centrifuged at 1000 × *g* for 10 min at 4 °C. The pellet was solubilized with 200 μl of lysis buffer and used as the nucleus. The supernatant was centrifuged at 100,000 × *g* for 1 h to separate the cytoplasmic fraction from the membrane fraction. The supernatant was immediately subjected to Western blot analysis.

RESULTS

Overexpression of FAF1 Inhibits NF-κB Activation—To test a possible involvement of FAF1 in the NF-κB activation pathway, we first examined whether overexpression of FAF1 can modulate NF-κB activation using a transient transfection system. As shown in Fig. 1*A*, overexpression of FAF1 inhibited TNFα-induced NF-κB activity as well as its basal transcriptional activity in 293 cells. Direct targeting of FAF1 expression using antisense FAF1 increased the basal level of NF-κB activation as well as NF-κB activation induced by TNFα. Inhibition of NF-κB by FAF1 was decreased in a dose-dependent manner (Fig. 1*B*). To test the specificity of FAF1 for NF-κB, we examined whether FAF1 can modulate the activity of other TNFα-induced transcription complexes such as AP-1. The experiment using the AP-1-*luc* reporter plasmid showed that FAF1 did not affect AP-1 activity in the presence or absence of TNFα (Fig. 1*C*, *left*). As an additional validation regarding the specificity of FAF1 for NF-κB, we experimented using the luciferase reporter containing the NF-κB-binding sites of the *cIAP2* promoter (pGL2-cIAP2−247-*luc*) and the luciferase reporter containing mutant functional NF-κB-binding elements (pGL2-cIAP2−247(mκB1,3)-*luc*) (25). The *cIAP2* gene is transcriptionally reg-

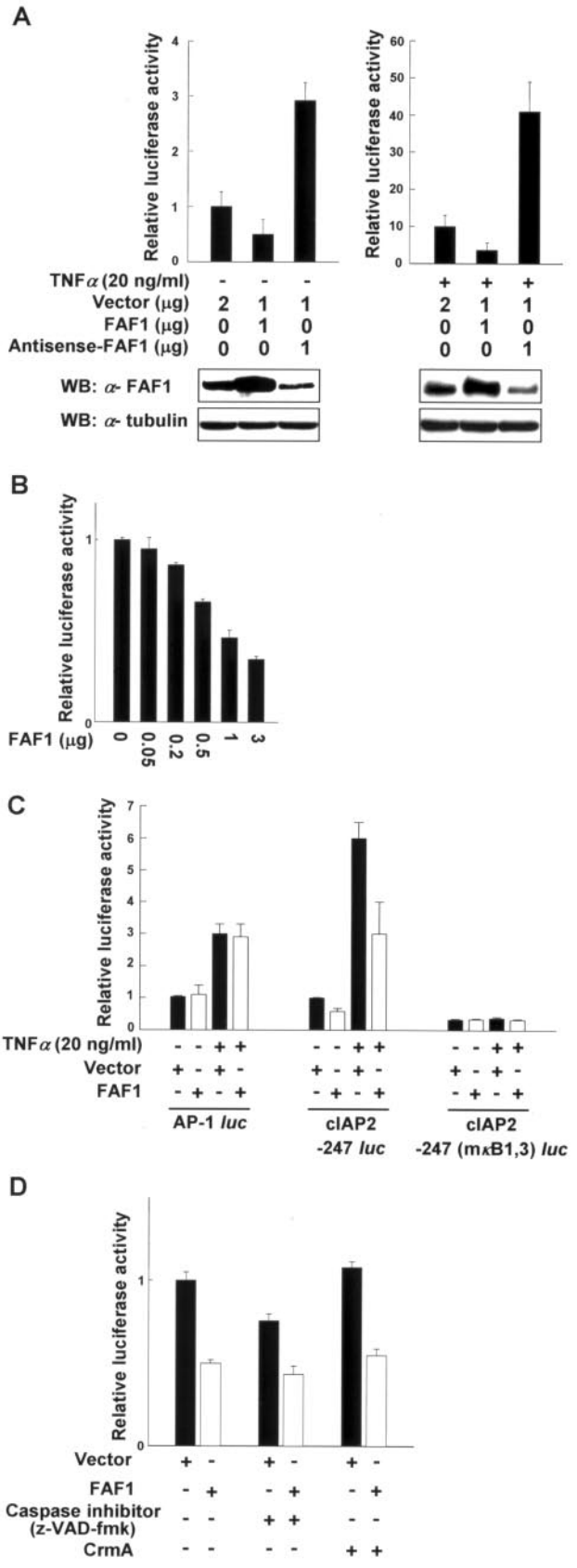

FIG. 1. **FAF1 inhibits NF-κB activation.** *A*, FAF1 inhibits basal and TNFα-stimulated NF-κB activation. 293 cells were cotransfected with 0.1 μg of Ig-κB-*luc* reporter plasmid and 0.1 μg of CMV-β-galactosidase plasmid together with 1 μg of pFLAG empty vector, pFLAG-FAF1, or pFLAG-antisense FAF1 as indicated. At 24 h post-transfection, cells were treated with TNFα (20 ng/ml) for 6 h or left untreated. Luciferase activity was then measured as described under "Experimental Procedures." Relative luciferase activity was normalized to β-galactosidase activity to correct for variability in transfection efficiency. To indicate the amounts of expressed proteins, the results of Western blotting (*WB*) of FAF1 and β-tubulin are shown at the bottom. *B*, FAF1 inhibits NF-κB activation in a dose-dependent manner. 0.1 μg of Ig-κB-*luc* reporter plasmid and 0.1 μg of CMV-β-galactosidase plasmid were transiently cotransfected with the indicated amounts (0.05, 0.2, 0.5, 1, and 3 μg) of pFLAG-FAF1. The total DNA amount was held at 3 μg by adjusting with the empty vector. *C*, FAF1 acts specifically upon NF-κB. 0.1 μg of AP-1-*luc* reporter plasmid and 0.1 μg of CMV-β-galactosidase plasmid were cotransfected together with 1 μg of pFLAG empty vector or pFLAG-FAF1 into 293 cells (*left*). Cells were transfected with 1 μg of pFLAG empty vector or pFLAG-FAF1 together with 0.5 μg of pGL2-cIAP2−247-*luc* reporter plasmid containing the NF-κB-binding sites of the *cIAP2* promoter (*middle*) or pGL2-cIAP2−247(mκB1,3)-*luc* reporter plasmid containing mutant functional NF-κB-binding sites (*right*) along with 0.1 μg of CMV-β-galactosidase plasmid. After 36 h, cells were treated with or without TNFα (20 ng/ml) for 6 h, and luciferase activity was measured. *D*, inhibition of FAF1 upon NF-κB activation occurs independently of caspase activation. 293 cells were cotransfected with 0.1 μg of Ig-κB-*luc* and 0.1 μg of CMV-β-galactosidase plasmid with 1 μg of pFLAG empty vector or pFLAG-FAF1 together with 1 μg of CrmA. At 12 h post-transfection, FAF1-transfected cells were treated with the pan-caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone (*z-VAD-fmk*; 20 μM). At 36 h post-transfection, cell lysates were prepared, and luciferase activity was measured.

ulated by TNFα and other stimuli activating NF-κB. As shown in Fig. 1*C* (*middle* and *right*), FAF1 down-regulated the transcription of pGL2-cIAP2−247-*luc*, but did not change that of pGL2-cIAP2−247(mκB1,3)-*luc*. These results verify the specificity of FAF1 for NF-κB. Next, we questioned whether the down-regulation of NF-κB activity is influenced by caspase activation. To address this question, NF-κB activation by overexpression of FAF1 was measured in the presence of a pan-caspase inhibitor (benzyloxycarbonyl-VAD-fluoromethyl ketone) and in cells in which FAF1 and CrmA, a poxvirus-derived specific inhibitor of caspase-8, were cotransfected (29). As shown in Fig. 1*D*, our data confirm that the down-regulation of NF-κB activity by FAF1 is independent of caspase activities.

FAF1 Suppresses NF-κB Activation via Multiple Pathways—We questioned whether FAF1 can exert its negative regulatory effect on diverse NF-κB-activating signals. When overexpressed, FAF1 inhibited TNFα-, IL-1β-, and LPS-mediated NF-κB activation (Fig. 2*A*). To further determine how FAF1 modulates NF-κB activity, we tested whether FAF1 inhibits NF-κB activation mediated via various signal transducers known to be NF-κB activators downstream of the TNFR superfamily and IL-1 receptor. As shown in Fig. 2*B*, when overexpressed, FAF1 inhibited TRAF2-, TRAF6-, and MEKK1-mediated NF-κB activation, suggesting that FAF1 might function at a point where divergent signals merge in the NF-κB activation pathways. Certainly, FAF1 inhibited NF-κB activation by overexpression of IKKβ, a component of the IKK complex where divergent NF-κB-activating signals converge, suggesting that FAF1 might function in the IKK complex and/or its downstream components in the NF-κB activation pathway. Indeed, FAF1 inhibited NF-κB activation by overexpression of NF-κB p65, one of the end point molecules in the NF-κB activation pathway. This suggests that NF-κB p65 might be the target molecule upon which FAF1 acts.

FAF1 Interacts with NF-κB p65—Based on the fact that FAF1 inhibits NF-κB activation by overexpression of NF-κB p65, we investigated whether FAF1 can physically interact with NF-κB p65. 293 cells were transfected with pcDNA3.1-NF-κB-p65 with or without pFLAG-FAF1. At 48 h post-transfection, cell lysates were immunoprecipitated using anti-FLAG mAb. Fig. 3*A* illustrates that FAF1 can interact with NF-κB p65 directly in 293 cells. To investigate whether FAF1 and NF-κB p65 are endogenously associated, we performed co-immunoprecipitation experiments using total cell lysates. As

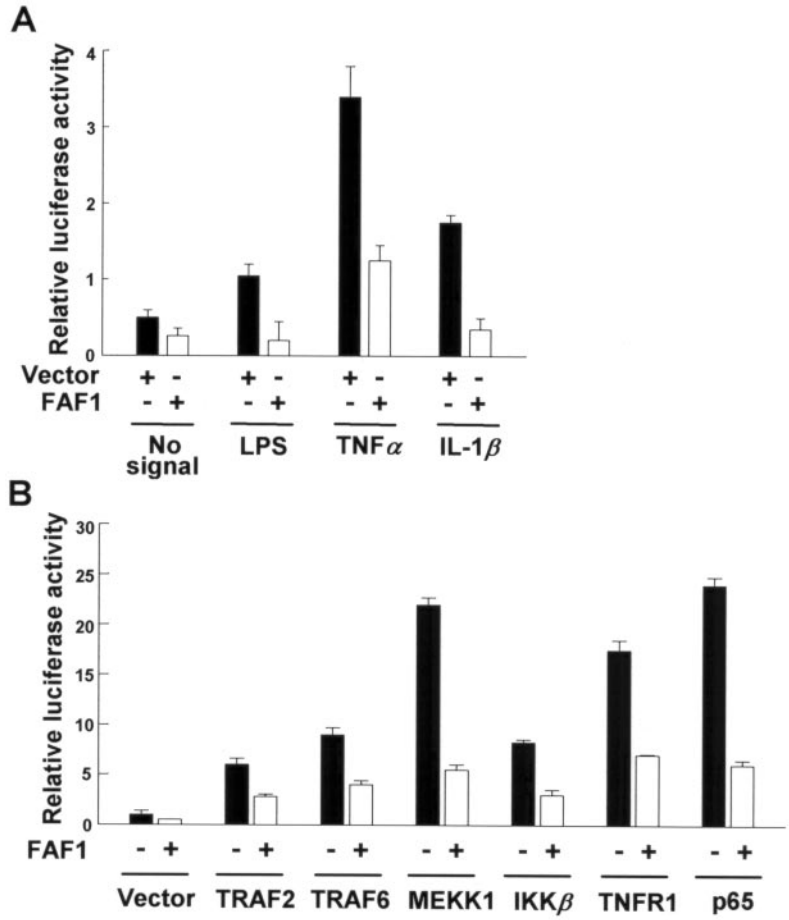

FIG. 2. **FAF1 inhibits NF-κB activation induced by TNFα, IL-1β, LPS, and various signal transducers involved in NF-κB signaling.** *A*, FAF1 inhibits TNFα-, IL-1β-, and LPS-induced NF-κB activation. Reporter genes were transiently cotransfected with 1 μg of pFLAG empty vector or pFLAG-FAF1. At 24 h post-transfection, cells were treated with TNFα (20 ng/ml), IL-1β (20 ng/ml), or LPS (100 ng/ml) for 6 h or left untreated. *B*, FAF1 inhibits NF-κB activation by various signal transducers. 293 cells were transfected either with 1 μg of empty vector or with 1 μg of expression plasmid encoding TRAF2, TRAF6, MEKK1, IKKβ, TNFR1, or NF-κB p65 along with 0.1 μg of Ig-κB-*luc* plasmid and 0.1 μg of CMV-β-galactosidase plasmid. At 36 h post-transfection, cell lysates were prepared, and luciferase activity was measured.

shown in Fig. 3*B*, endogenous NF-κB p65 was co-immunoprecipitated with FAF1 from 293 cell lysates induced by TNFα for 30 min. However, anti-FAF1 antibody did not co-immunoprecipitate NF-κB p65 from the lysates of untreated 293 cells. The same experiment was performed with NIH3T3 cells, and equivalent results were obtained. However, no immunoprecipitation of FAF1 was detected in NIH3T3-p65$^{-/-}$ cells. These results suggest that the TNFα signal might have facilitated FAF1 and NF-κB p65 interaction in the cell.

The DED-interacting Domain (DEDID) of FAF1 Interacts with NF-κB p65—To determine the FAF1 region responsible for the interaction with NF-κB p65, GST pull-down assay was performed using GST-FAF1 fusion proteins. The extracts of 293 cells transfected with NF-κB p65 were incubated with either GST or GST-FAF1 deletion mutant proteins (Fig. 4*A*). GST-FAF1 DEDID (amino acids 181–381) and GST-FAF1 bound to NF-κB p65 (Fig. 4*B*), implying that the DEDID is sufficient for binding. It is worth mentioning that the DEDID is the region where the DEDs of FADD and caspase-8 also bind. To determine whether the binding affects NF-κB activity, pFLAG-FAF1 and pFLAG-FAF1-DEDID were transfected, and NF-κB activity was measured. When overexpressed, the FAF1 DEDID and FAF1 demonstrated NF-κB activity-inhibiting potential (Fig. 4*C*).

FAF1 Inhibits Nuclear Translocation and Decreases the DNA-binding Activity of NF-κB—To determine how FAF1/NF-κB p65 interaction modulates NF-κB activity, we examined the influence of FAF1 on NF-κB activity in gel shift experiments (Fig. 5*A*). An inducible protein·DNA complex was observed in the nuclear extracts upon TNFα treatment for 20 min. As expected, overexpression of FAF1 decreased significantly the DNA-binding activity, whereas FAF1 had no effect on GT box binding to DNA with or without TNFα treatment (Fig. 5*B*). We further confirmed the effects of FAF1 on the translocation of NF-κB p65 into the nucleus of 293 cells with or without TNFα stimulation by Western blot analysis (Fig. 5*C*). TNFα stimulation increased the protein levels of NF-κB p65 in the nucleus. However, FAF1 overexpression decreased significantly the nuclear translocation of NF-κB p65, suggesting that FAF1 interferes with NF-κB activity by preventing the nuclear translocation of NF-κB p65 from the cytoplasm.

DISCUSSION

Our previous study showed that FAF1 functions as a component of the Fas DISC in Jurkat cells (3). In this study, we carried out experiments to examine whether FAF1 proteins can modulate the signal transduction pathway leading to NF-κB activation, which is required for anti-apoptotic signals. Our study discloses a new function of FAF1. FAF1 negatively regulates NF-κB activation by various extracellular stimuli. This is caused by the cytoplasmic retention of NF-κB p65 by FAF1 via physical interaction.

The modulation of NF-κB activation by death-inducing molecules has been reported. FADD, caspase-8, and FLASH were reported not only to induce cell death, but also to activate NF-κB (14, 17, 30–32). However, pro-apoptotic molecules such as ASK1 and prostate apoptosis response 4 negatively regulate NF-κB activation (33–35). Because a pro-apoptotic FAF1 protein inhibits NF-κB activation as do the ASK-1 and PAR-4 proteins, FAF1 can be added to the list of NF-κB-down-regulating proteins with apoptotic potential.

There are other proteins that interact with NF-κB p65. Wu *et al.* (36) reported that the promyelocytic leukemia protein PML, a pro-apoptotic protein, represses NF-κB transactivation by

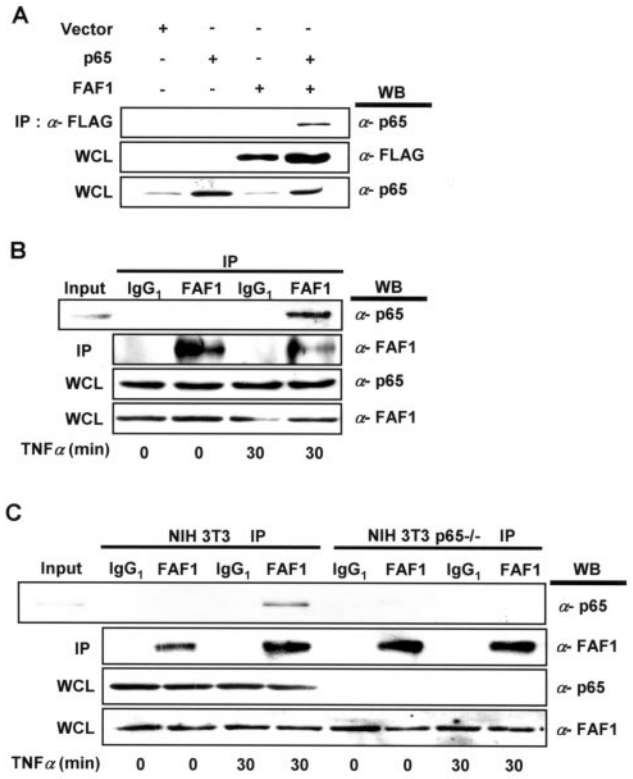

FIG. 3. **FAF1 interacts with NF-κB p65.** *A*, FAF1 physically interacts with NF-κB p65 *in vivo*. 293 cells were transfected with 1 μg of empty vector, pcDNA3.1-NF-κB-p65, or pFLAG-FAF1. At 36 h post-transfection, cell lysates were immunoprecipitated (*IP*) with anti-FLAG mAb and immunoblotted with anti-p65 pAb H10. *B* and *C*, endogenous FAF1 interacts with NF-κB p65 upon TNFα stimulation. 293, NIH3T3 and NIH3T3-p65$^{-/-}$ cells were treated TNFα (20 ng/ml) for 30 min or left untreated. Cells were lysed and then immunoprecipitated with anti-FAF1 mAb or anti-mouse IgG_1. Western blotting (*WB*) was performed using anti-p65 pAb H10 or anti-FAF1 mAb. 5 μg of the untreated cell lysate was loaded as input (which denotes the position of NF-κB p65). *WCL*, whole cell lysate.

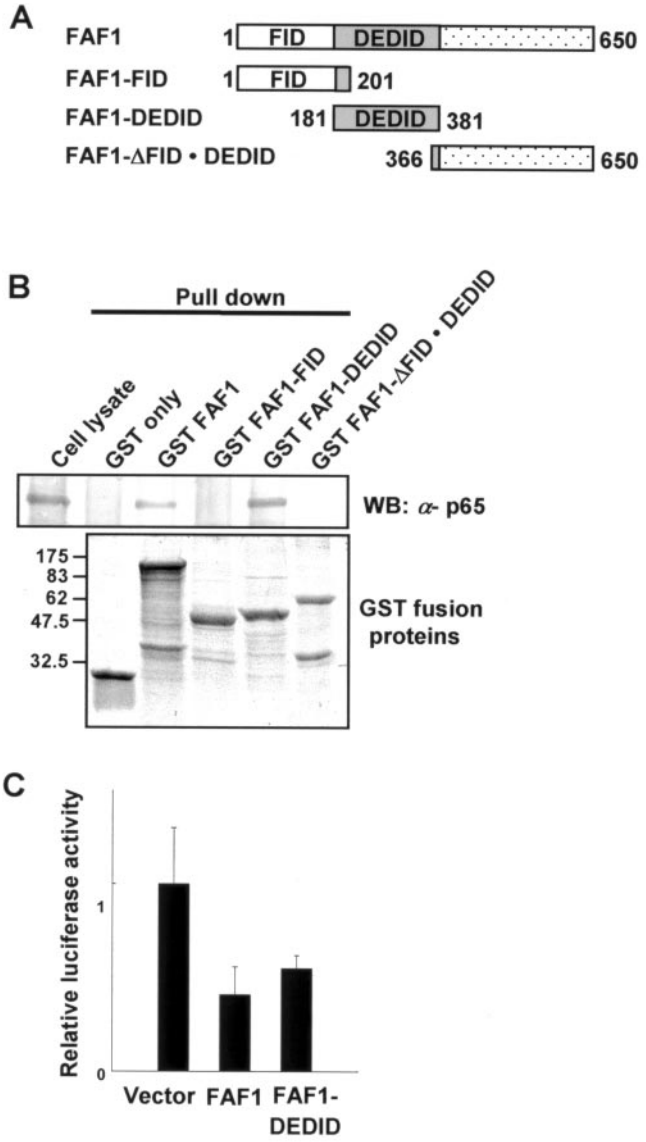

FIG. 4. **The FAF1 DEDID (amino acids 181–381) modulates NF-κB activation.** *A*, schematic representation of FAF1 deletion mutants. The locations of the Fas-interacting domain (*FID*) and DEDID are indicated. *B*, the DEDID of FAF1 binds to NF-κB p65 *in vitro*. 293 cells were transfected with pcDNA3.1-NF-κB-p65. At 36 h post-transfection, total cell lysates (100 μg/lane) were incubation for 4 h with GST or GST-FAF1 deletion mutant fusion proteins purified from *E. coli*. Bound proteins were precipitated by glutathione beads and detected by Western blotting (*WB*) using anti-NF-κB p65 antibody. *C*, the FAF1 DEDID can suppress NF-κB activation. 293 cells were transfected with 1 μg of pFLAG empty vector, pFLAG-FAF1, or pFLAG-FAF1-DEDID along with 0.1 μg of Ig-κB-*luc* plasmid and 0.1 μg of CMV-β-galactosidase plasmid. At 36 h post-transfection, cell lysates were prepared, and luciferase activities were then measured.

interacting with NF-κB p65 in the nucleus. In addition, histone deacetylase-1 and p202 also negatively regulate the transcriptional activity of NF-κB by interacting with NF-κB p65 (37, 38). Those are nuclear proteins. In this respect, FAF1 presents a new regulatory mechanism for inhibition of NF-κB activation, *i.e.* preventing nuclear translocation of NF-κB p65 from the cytoplasm.

The interaction between FAF1 and NF-κB p65 has been confirmed in two different cell types, *i.e.* 293 and NIH3T3 cells. The interaction occurred in TNFα-treated cells, but barely in untreated cells, showing that FAF1/NF-κB p65 interaction is tightly regulated and also stressing the physiological importance of this interaction. It is well known that the TNFα signal degrades IκB and frees NF-κB. Thus, it is conceivable that IκB inhibits FAF1/NF-κB p65 binding and that degradation of IκB by the TNFα signal would free NF-κB to bind to FAF1.

The DED is a pro-apoptotic signaling domain that mediates NF-κB activation (17, 30). DED-containing proteins such as caspase-8, caspase-10, and Casper can activate NF-κB. One regulatory mechanism of the DED in the NF-κB pathway occurs via the physical interactions of DED with NF-κB pathway components. Caspase-8 interacts with multiple proteins in the NF-κB pathway, such as receptor-interacting protein, NIK, IKKα, and IKKβ (30), and activates NF-κB. The FAF1 DEDID interacts with the DEDs of FADD and caspase-8 (3). Binding of FAF1 to caspase-8 might prevent caspase-8 from binding to the NF-κB pathway components. If FAF1/caspase-8 binding inhibits the interactions of caspase-8 with NF-κB pathway components, FAF1/caspase-8 binding would also reduce the positive influence of caspase-8 on NF-κB activation. Thus, in addition to FAF1/NF-κB p65 binding, FAF1/caspase-8 binding might have additional negative effects on NF-κB activation.

FAF1 down-regulates NF-κB activation and promotes apoptosis as a component of the Fas DISC. Even though both activities will drive cells in a pro-apoptotic direction, the relationship between these two activities and whether they converge in the same apoptotic effector molecule are not clear. However, these two activities seem to occur independently based on the fact that down-regulation of NF-κB occurred in the presence of caspase inhibitors. This study shows that FAF1

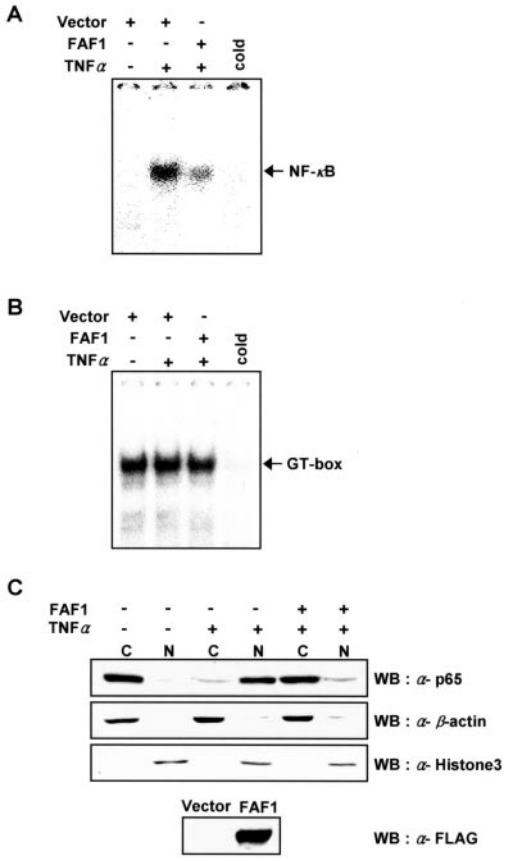

FIG. 5. **FAF1 inhibits the translocation of NF-κB p65 from the cytosol to the nucleus.** *A*, FAF1 inhibits TNFα-induced NF-κB DNA-binding activity. 293 cells were transfected with 1 μg of pFLAG-FAF1 or empty vector (pFLAG-CMV2). At 30 h post-transfection, cells were treated for 20 min with 30 ng/ml TNFα as indicated. Nuclear extracts (4 g) were assessed by electrophoretic mobility shift assay as described under "Experimental Procedures." The complex was efficiently competed by unlabeled oligomer (*cold*). *B*, FAF1 does not affect GT box DNA-binding activity. To control for loading amounts of nuclear extracts, the same preparations of nuclear extracts used in *A* were incubated with radiolabeled oligonucleotide containing the GT box and analyzed by electrophoretic mobility shift assay. *C*, FAF1 inhibits the translocation of NF-κB p65 from the cytosol to the nucleus. FAF1-transfected 293 cells were treated for 20 min with 30 ng/ml TNFα. Cytoplasmic (*C*) and nuclear (*N*) extracts were assessed for translocation of NF-κB p65. Immunoblotting with anti-histone-3 and anti-β-actin mAbs served as positive controls for the nuclear and cytoplasmic fractions, respectively. *WB*, Western blot.

is involved in dual signaling mechanisms, one via the Fas DISC and the other via down-regulation of NF-κB. Thus, the combined action of the FAF1 protein may enhance the efficacy of receptor-mediated cell death.

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Fas-associated Factor-1 Inhibits Nuclear Factor-κB (NF-κB) Activity by Interfering with Nuclear Translocation of the RelA (p65) Subunit of NF- κB

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