FAF1 Suppresses IκB Kinase (IKK) Activation by Disrupting the IKK Complex Assembly

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This study presents a molecular inhibitory mechanism by Fas-associated factor 1 (FAF1) on IκB kinase (IKK) activation, where divergent NF-κB-activating stimuli converge. FAF1 interacts with IκKB in response to proinflammatory stimuli (such as tumor necrosis factor-α, interleukin-1β, and lipopolysaccharide) and suppresses IKK activation. Interaction of the leucine-zipper domain of IκKB with FAF1 affected the IKK heterocomplex (IKKα/β) and homocomplex (IKKa/α, IKKβ/β) formations and attenuated IKK-y recruitment to IKKβ. Overexpression of FAF1 reduced the level of IKKβ activity, whereas FAF1 depletion increased the activity. These results indicate that FAF1 inhibits IKK activation and its downstream signaling by interrupting the IKK complex assembly through physical interaction with IKKβ. Taken together, FAF1 robustly suppresses NF-κB activation through the inhibition of IKK activation in combination with previously reported cytoplasmic retention of NF-κB p65 (Park, M. Y., Jang, H. D., Lee, S. Y., Lee, K. J., and Kim, E. (2004) J. Biol. Chem. 279, 2544–2549). Such redundant suppression would prevent inadvertent activation of the NF-κB pathway.

Fas-associated factor 1 (FAF1) is evolutionarily conserved from flies to mammals (1–4) and is involved in various key biological processes. FAF1 potentiates the Fas pathway as a member of the Fas death-inducing signaling complex and mediates chemotherapeutic-induced cell death (5, 6). FAF1 also functions as an integral regulatory component of the transient receptor potential vanilloid type 1 (TRPV1) signaling pathway (7). FAF1 is involved in the ubiquitination pathway and interacts with ubiquitin and valosin-containing protein, which is a mult ubiquitin chain-targeting factor (8). In addition, FAF1 inhibits the chaperone activities of the heat-shock proteins Hsc70 and Hsp70 (9).

FAF1 is also involved in the nuclear factor-κB (NF-κB) signaling pathway. FAF1 inhibits NF-κB activation in HEK 293 cells by binding to NF-κB p65 (10). NF-κB inhibition by FAF1 has also been reported in Drosophila (4), Caspar, a fly homolog of human FAF1, selectively suppresses the immune deficiency (imd) pathway. Loss-of-function caspar mutants constitutively expressed antibacterial genes in the absence of bacterial infections, indicating that caspar is an endogenous suppressor of the Imd pathway. Selective involvement of IκB kinase (IKK) complex in the Imd pathway led us to examine the regulatory mechanism, if any, between FAF1 and the IKK complex.

The IKK complex is mainly composed of two catalytic subunits, IKKα/IKK1 and IKKβ/IKK2, and a regulatory subunit, IKKγ/NF-κB essential modulator (NEMO)/IKKAP1. Knockout mice studies have demonstrated that IKKβ has a dominant role in NF-κB activation induced by proinflammatory cytokines, whereas IKKα is essential for morphogenic signaling (11). Although IKKγ lacks the catalytic function, IKKγ is essential for activation of the IKK complex. Both IKKα and IKKβ contain an N-terminal kinase domain, a central leucine-zipper (LZ) domain, and a C-terminal helix-loop-helix (HLH) domain (12–14). Homo- and hetero-oligomerizations between IKKα and IKKβ occur through their LZ domains, and the HLH domains mediate recruitment of IKKγ to the IKK complex.

This study reveals a novel function of FAF1: as an endogenous suppressor of IKK activation. FAF1 disrupts IKK complex assembly by physical interaction with the LZ domain of IKKβ. Association between FAF1 and IKKβ was induced by proinflammatory stimuli. Such an induced interaction indicates that FAF1 is an NF-κB pathway suppressor with a unique mode of action.

**EXPERIMENTAL PROCEDURES**

Reagents and Constructs—Recombinant tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) (R&D Systems Inc.,

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‖ The abbreviations used are: FAF1, Fas-associated factor 1; siFAF1, FAF1-specific siRNA; siRNA, small interfering RNA; NF-κB, nuclear factor-κB; Imd, immune deficiency; IKK, IκB kinase; LZ, leucine-zipper; HLH, helix-loop-helix; TNF, tumor necrosis factor; IL-1, interleukin-1; LPS, lipopolysaccharide; ChIP, chromatin immunoprecipitation; FID, Fas-interacting domain; DEDID, death effector domain-interacting domain; EGS, ethylene glycol-bis-succinimidyl succinate; GST, glutathione S-transferase; GFP, green fluorescent protein.
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The HEK 293 cells were untreated or treated with various stimuli. The stimulation conditions were TNFα (20 ng/ml), IL-1β (10 ng/ml), and LPS (100 ng/ml) for 10 min unless otherwise indicated. The whole-cell lysates (WCLs) were immunoprecipitated (IP) with anti-FAF1 antibody or anti-mouse IgG1 antibody and then immunoblotted with anti-IKKβ antibody. The whole-cell lysates were immunoblotted with the indicated antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. 8 and C, in GST pulldown assays, 35S-labeled proteins or cell lysates that were pulled down by GST fusion proteins were analyzed by autoradiography or immunoblot assays with the indicated antibodies. In B and C, the bottom panels show the Coomassie Blue staining of the GST fusion proteins. In B, the upper panel shows a schematic representation of FAF1 deletions. Lower left panel, 35S-labeled IKKβ was pulled down by GST fusion proteins of the truncated FAF1 was analyzed by autoradiography. Lower right panel, HEK 293 cells were transfected with pRK5-FLAG-IKKβ. 36 h after transfection, the whole-cell lysates were analyzed by GST pulldown assays. In C, the upper panel shows a schematic representation of IKKβ deletions. Lower panel, 35S-labeled FAF1 was pulled down by GST fusion proteins of the truncated IKKβ was analyzed by autoradiography. WB, Western blot; KD, kinase domain; ULD, ubiquitin-like domain.

Luciferase Assay, GST Pulldown Assay, and in Vitro Kinase Assay—Luciferase assay (10, 17), GST pulldown assays (3, 10), and in vitro kinase assays (18) were performed as described previously.

Chromatin Immunoprecipitation (ChIP)—The HEK 293 cells were lysed in SDS lysis buffer (19), and equal amounts of DNA were immunoprecipitated with 1 μg of anti-p65 antibody. Immunoprecipitated DNA was eluted and PCR-amplified. PCR products were separated by agarose gel electrophoresis and stained.
with ethidium bromide. ChIP primers for the IkBa gene (accession number NM_020529) were: IkBa promoter, sense, 5'-GAGGACCCCAATTCAAATCG-3', and antisense, 5'-TCAGGCTCGGGAATTTCCTC-3'.

RESULTS

Interaction between FAF1 and IKKβ Is Induced by Proinflammatory Stimuli—Endogenous association of FAF1 and IKKβ was induced in response to proinflammatory stimuli such as TNFα, IL-1β, and LPS (Fig. 1A). GST pulldown assays using various GST fusion proteins of the truncated FAF1 showed that the N-terminal half of FAF1 (amino acids 1–381) interact with IKKβ (Fig. 1B). Similarly, GST pulldown assays between various domains of IKKβ and FAF1 demonstrated that the LZ domain of IKKβ strongly binds to FAF1 (Fig. 1C). The kinase domain of IKKβ also showed weak binding to FAF1.

FAF1 Inhibits IKK Complex Formation—Considering that the LZ domain of IKKβ is responsible for interaction with FAF1, we examined oligomerization of IKKα and IKKβ leading to IKK complex assembly (20). Levels of endogenous IKK complex (Fig. 2A) and homo- (IKKα/α, IKKβ/β) and hetero-complex (IKKα/β) (supplemental Fig. S1A) formations were increased when FAF1 was depleted by FAF1-specific siRNA (siFAF1) expression. IKK complex formations were decreased when FAF1 expression was rescued by the transfection of siRNA-resistant FAF1 construct, FAF1-res (Fig. 2A and supplemental Fig. S1C), indicating that FAF1 suppress IKK complex formation.

We then examined which FAF1 domain had the potential to suppress IKK complex formation. Transfection of FAF1-FID reduced IKK complex formation, but those of FAF1-DEDID (death effector domain-interacting domain) and FAF1-ΔFID-DEDID did not (supplemental Fig. S1B). Expression of siRNA-resistant FAF1-FID reduced the endogenous IKKα/β/γ oligomerization (Fig. 2A). This indicates that FAF1-FID has the potential for IKK complex disruption.

We asked whether FAF1 affected IKKβ recruitment to the tetrameric IKKγ, which is the essential regulatory component that facilitates the trans-autophosphorylation of IKKβ neces-
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FAF1 Inhibits the Catalytic Activity of IKKβ—Next, the catalytic activity of IKKβ in the disrupted IKK complex by FAF1 was measured. FAF1 overexpression reduced the TNFα-induced catalytic activities of IKKβ in a dose-dependent manner (Fig. 2A, upper right panel). However, introduction of FAF1-specific siRNA increased the level of IKKβ activity (Fig. 2A, upper left panel). Similarly, FAF1 overexpression attenuated IL-1β- or LPS-induced IKKβ activations (Fig. 2B, lower panels). Consistent with these in vivo results, the addition of GST-FAF1 also inhibited the IKK catalytic activity in vitro in a dose-dependent manner (Fig. 2B, right panel). To further confirm the inhibitory function of FAF1, the level of IκBα, which is an endogenous marker of IKK activity, was examined. After TNFα treatment, the level of IκBα in mock-transfected cells was more rapidly reduced than the level in FAF1-transfected cells, indicating that FAF1 inhibits IκBα phosphorylation (Fig. 2C). The level of IKKβ activity was inversely correlated with the FAF1 expression level, indicating that FAF1 negatively regulates the IKKβ activity. When IKKα-specific siRNA was transfected, FAF1 inhibited IKKβ activation (supplemental Fig. S3). This indicates that FAF1-mediated IKKβ activity suppression occurred independently of IKKα. Considering that fly homologs of mammalian IKKβ and IKKγ, but not IKKα, participate in the fly Imd pathway (22), inhibition of the fly Imd pathway by FAF1 correlates well with the IKKα-independent IKKβ suppression in mammalian cells shown here.

FAF1-FID Is Crucial for Suppression of IKKβ Activation—Both the FID and the DEDID of FAF1 interact with IKKβ (Fig. 1B); therefore, we investigated which domain is responsible for suppression of IKKβ activation. Transfection of full-length FAF1 and FAF1-FID led to significant suppression of IKKβ catalytic activity, whereas FAF1-DEDID and FAF1ΔFID-DEDID did not (Fig. 2A, left panel). Consistent with these results, the addition of siFAF1-DEDID increased the level of active IKK complex by FAF1 was measured. FAF1 overexpression reduced the TNFα-induced catalytic activities of IKKβ in a dose-dependent manner (Fig. 2A, upper right panel). However, introduction of FAF1-specific siRNA increased the level of IKKβ activity (Fig. 2A, upper left panel). Similarly, FAF1 overexpression attenuated IL-1β- or LPS-induced IKKβ activations (Fig. 2B, lower panels). Consistent with these in vivo results, the addition of GST-FAF1 also inhibited the IKK catalytic activity in vitro in a dose-dependent manner (Fig. 2B, right panel). To further confirm the inhibitory function of FAF1, the level of IκBα, which is an endogenous marker of IKK activity, was examined. After TNFα treatment, the level of IκBα in mock-transfected cells was more rapidly reduced than the level in FAF1-transfected cells, indicating that FAF1 inhibits IκBα phosphorylation (Fig. 2C). The level of IKKβ activity was inversely correlated with the FAF1 expression level, indicating that FAF1 negatively regulates the IKKβ activity. When IKKα-specific siRNA was transfected, FAF1 inhibited IKKβ activation (supplemental Fig. S3). This indicates that FAF1-mediated IKKβ activity suppression occurred independently of IKKα. Considering that fly homologs of mammalian IKKβ and IKKγ, but not IKKα, participate in the fly Imd pathway (22), inhibition of the fly Imd pathway by FAF1 correlates well with the IKKα-independent IKKβ suppression in mammalian cells shown here.

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This study demonstrates that FAF1 has a novel function as a suppressor of IKK complex activation. The FAF1-FID domain selectively inhibits IKK activation by disrupting IKK complex assembly. Together, previous reports of cytoplasmic retention of NF-κB p65 and the Imd pathway suppression, and this study on suppression of IKKβ activation, show that FAF1 is a genuine suppressor of the NF-κB pathway.

Suppressors of the IKK activation have been identified (11). Phosphatases (protein phosphatase 2A and protein phosphatase 2C), heat-shock proteins (Hsp70 and Hsp27), and deubiquitinating enzymes (CYLD and A20) interact with IKKs and suppress their activation (18, 23–30). However, FAF1 differs from the suppressors that bind to IKKs in quiescent cells. By contrast, interaction of FAF1 with IKK is induced by IKK-activating signals. Such a binding pattern would be useful to prevent over-activation of a pathway that requires tight regulation.

The detailed mechanism underlying induced binding of FAF1-
IKKβ is yet to be determined. Phosphorylation of FAF1 has been reported (31); however, the phosphorylation status of FAF1 remained unchanged despite stimulation (data not shown). In addition, in vitro binding studies have shown that phosphorylation is not a prerequisite for the binding, indicating that phosphorylation of FAF1 might not be the determining factor. Other types of modification in FAF1 or in FAF1-interacting partners that occur in response to stimuli might facilitate the FAF1-IKK interaction. Proteomic analysis of FAF1-interacting proteins in response to NF-κB-activating stimuli would help us to investigate this issue.

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