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FLASH Coordinates NF-κB Activity via TRAF2*

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FLASH is a protein recently shown to interact with the death effector domain of caspase-8 and is likely to be a component of the death-inducing signaling complex in receptor-mediated apoptosis. Here we show that antisense oligonucleotide-induced inhibition of FLASH expression abolished TNF-α-induced activation of NF-κB in HEK293 cells, as determined by luciferase reporter gene expression driven by a NF-κB responsive promoter. Conversely, overexpression of FLASH dose-dependently activated NF-κB, an effect suppressed by dominant negative mutants of TRAF2, NIK, and IKKα, and partially by those of TRAF5 and TRAF6. TRAF2 was co-immunoprecipitated with FLASH from the cell extracts of HEK293 cells or HeLa cells stably expressing exogenous FLASH (HeLa/HA-FLASH). Furthermore, serial deletion mapping demonstrated that a domain spanning the residues 856–1191 of FLASH activated NF-κB as efficiently as the full-length and could directly bind to TRAF2 *in vitro* and in the transfected cells. Taken together, these results suggest that FLASH coordinates downstream NF-κB activity via a TRAF2-dependent pathway in the TNF-α signaling.

TNF-α[1] is a pleiotropic cytokine associated with various cellular defense responses, with lethal effects such as septic shock with inflammation, and with apoptosis in susceptible cells (1, 2). TNF-α signaling is transduced through its receptor, TNF-R, to simultaneously elicit two opposing effects: apoptosis and activation of an anti-apoptotic transcription factor NF-κB (3–5). During initiation of apoptosis, FADD is complexed with activated TNF-R1 and TRADD via the death domain (4, 6, 7) and recruits caspase-8 to the resultant death-inducing signaling complex (DISC), which leads to apoptosis via activation of a caspase cascade (4, 8–10). Similarly, Fas (CD95/APO-1) recruits FADD to its activated receptor to induce apoptosis (11).

In contrast, with respect to stress signaling and immune response, TNF-R interacts with TRAFs and RIP, leading to the activation of NF-κB. Whereas overexpression of the wild type TRAF2, -5, or -6 activates NF-κB, their truncated versions lacking zinc-binding domains inhibit NF-κB activation induced by various stimuli (12–17). Whereas TRAF2 transduces TNF-α-mediated activation of NF-κB, TRAF6 is associated with interleukin-1 and CARD4 signaling (17, 18), indicating that TRAFs are common mediators for NF-κB activation and display an ability to stimulate signal-specific NF-κB activation. Subsequent activation of NIK, a member of the mitogen-activated protein kinase family (4, 19, 20), and the downstream kinases, IKKα and IKKβ, leads to the phosphorylation of IκBs for degradation and the activation of NF-κB (20–25).

It has recently been reported that FLASH is likely to be a component of DISC involved in Fas- and TNF-mediated apoptosis (26). FLASH contains a death-effector-domain-recruiting domain (DRD) in the C-terminal region, which interacts with the death effector domain (DED) of caspase-8 or FADD (26). Still, transient overexpression of FLASH marginally affects apoptosis (26, 27), making its precise function with respect to receptor-mediated signaling (*e.g.* via TNF-R) unclear. In addition, the finding that caspase-8 and FADD may be involved in the signaling to NF-κB activation as well as to apoptosis (28, 29) suggests that FLASH may function to coordinate stress responses, a possibility that prompted us to investigate the role of FLASH in NF-κB activation. In this report, we used an antisense oligonucleotide (AS) and overexpression analysis to show that FLASH transduces the TNF-α signal, leading to the activation of NF-κB via a TRAF2-NIK-IKK-dependent pathway.

EXPERIMENTAL PROCEDURES

Reagents—Anti-IκBα and anti-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Crutz, CA) and Sigma, respectively. Anti-HA antibody and anti-TRAF2 antibody (SC-7346) were from Roche Molecular Biochemicals (Mannheim, Germany) and Santa Cruz Biotechnology, respectively. TNF-α and all other molecular biology grade materials were from Sigma or New England Biolabs (Hertfordshire, UK).

Construction of Recombinant Expression Plasmids—pME18S-FLAG and pME18S-FLAG-FLASH were kindly provided by Dr. Yonehara (University of Kyoto, Japan). pHA-FLASH was generated by subcloning the FLASH cDNA into the *Eco*RI/*Xba*I sites of pcDNA-HA plasmid. FLASH deletion constructs were assembled by polymerase chain reaction (PCR) using the following synthetic oligonucleotides as primers: 5′-CCGGAATTCATGGCAGATGATGACAATGGT-3′ and 5′-ATAAGAATGCGGCCGCCTAGCTCTCCATGCTAACAACT-3′ for pME18S-FLAG-ΔA-(1–858) (pFL-ΔA-FLASH); 5′-CCGGAATTCATGGAGAGCTCATGTGCAATT-3′ and 5′-ACCGGGCCCCTATCCAGTTCTAGGCAAAGA-3′ for pcDNA-HA-ΔB-(856–1552) (pHA-ΔB-FLASH); 5′-CCGGAATTCATGGCAGATGATGACAATGGT-3′ and 5′-ATAAGAATGCGGCCGCCTACAGTGAAGATTTAAAATTC-3′ for pME18S-FLAG-ΔC-

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[1] The abbreviations used are: TNF-α, tumor necrosis factor-α; FLASH, FLICE-associated huge protein; AS, antisense oligonucleotide; DED, death effector domain; DRD, DED-recruiting domain; NAD, NF-κB-activating domain; DISC, death-inducing signaling complex; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; β-gal, β-galactosidase; HEK, human embryonic kidney cells; GST, glutathione *S*-transferase; TRAF, TNF receptor-associated factor; NIK, NF-κB-inducing kinase; Ikk, IκB kinase.

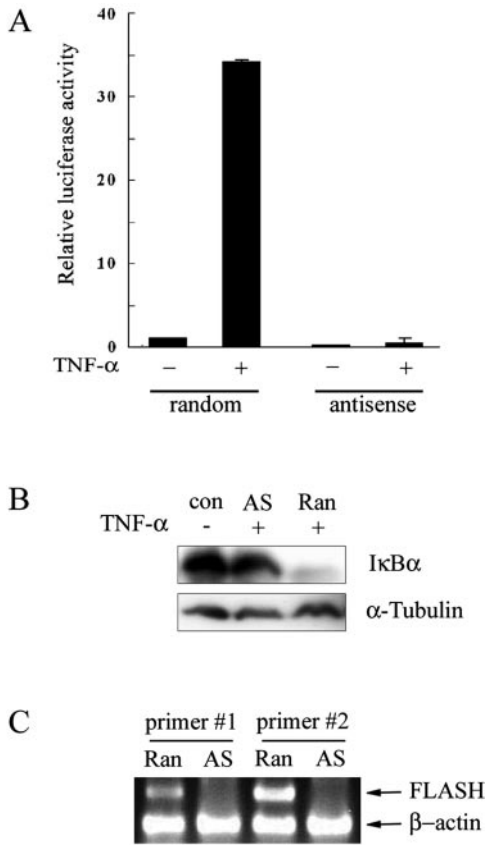

FIG. 1. **Suppression of TNF-α-induced activation of NF-κB using a FLASH antisense oligonucleotide.** HEK293 cells were co-transfected for 36 h with NF-κB-luciferase reporter plasmid (pNF-κB-luc), pCMV-β-gal, and either 5 μM FLASH AS-2 or a scrambled oligonucleotide (random), and were then incubated with TNF-α (30 ng/ml) for an additional 26 h. *A*, activity of luciferase reporter genes was normalized to that of β-galactosidase, which served as an internal control. *Bars* represent mean ± S.D. from at least four independent experiments. *B,* HEK293 cells were lysed, and Western blotting was performed with anti-IκBα antibody. For an internal control, the same extracts were probed with antibody to α-tubulin. *C*, reverse transcription of RNA isolated from cells treated with scrambled (*Ran*) or AS-2 (*AS*) oligonucleotides. The PCR reaction was carried out with two different sets of FLASH primers. PCR of β-actin was performed to normalize FLASH expression.

(1–1191) (pFL-ΔC-FLASH); 5′-CCGGAATTCATGGAGAGCTGCTCATGTGCAATT-3′ and 5′-CATTTAGGTGACACTA-3′ for pME18S-FLAG-ΔD-(1553–1962) (pFL-ΔD-FLASH); 5′-CCGGAATTCATGGAGAGCTCATGTGCAATT-3′ and 5′-CATTTAGGTGACACTA-3′ for pME18S-FLAG-ΔE-(856–1962) (pFL-ΔE-FLASH). The PCR products were then inserted into the *Eco*RI/*Not*I sites of pME18S-FLAG (ΔA, ΔC, ΔD, and ΔE), the *Eco*RI/*Apa*I sites of pcDNA3-HA (pHA-ΔB-FLASH), or the *Eco*RI/*Not*I sites of pcDNA3-HA (pHA-ΔD-FLASH). GST-NAD-FLASH and GST-DRD-FLASH fusion proteins were generated by subcloning PCR products amplified by 5′-CGCGGATCCCCTAGAGTTTCTGCTGAA-3′ and 5′-CCGCTCGAGTTACAGTGAAGATTTAAATT-3′ for pGST-NAD-FLASH and 5′-CGCGGATCCGATAAGAGTAAACTAACTC-3′ and 5′-CCGCTCGAGTTATTCACAGGAGCCAGGAGA-3′ for pGST-DRD-FLASH into the *Bam*HI/*Xho*I sites of pGEX4T-3 (Amersham Pharmacia Biotech.). All PCR products were confirmed by DNA sequencing. pTRAF2, pTRAF5, pTRAF6, pFL-NIK, pCR3.1-IKKα, dominant negative forms of TRAF2, TRAF5, TRAF6, NIK, and IKKα, pNF-κB-luc, and pCasp8 were described previously (30, 37).

Cell Culture, Stable Cells, and DNA Transfection—HEK293 and Jurkat cells were cultured in Dulbecco's modified Eagle's medium and RPMI 1640, respectively, supplemented with 10% fetal bovine serum (BIOFLUIDS). Cells were subcultured to a density of 2×10^5 cells/well in 6-well dishes and allowed to stabilize for 1 day. Cells were then typically transfected with 600 ng of NF-κB-luciferase reporter plasmid (pNF-κB-luc), 200 ng of pCMV-β-gal, and 1 μg of vector or the indicated expression plasmid using LipofectAMINE according to the manufacturer's instructions (Life Technologies, Inc.). The total amount of transfected plasmid DNA was kept constant within individual experiments by adding appropriate amounts of pcDNA or pME18S. HeLa cells stably expressing HA-FLASH (HeLa/HA-FLASH) were generated as described by Chung *et al.* (38).

Antisense Oligonucleotide Treatment—AS-2 (5′-ATTCAGCAACTTACTTGC-3′) is an antisense oligonucleotide complementary to human FLASH mRNA and corresponds to a location around the stop codon, 5942–5959 bp downstream of the translation initiation site. Comparison of this oligonucleotide sequence with the database detected the only homology to the FLASH sequence. The following scrambled sequence was used as a control: (5′-GCTACTAGTAGCAGCTAC-3′). Cells (3×10^6 per well) were continuously treated with 5 μM FLASH antisense or the scrambled oligonucleotide for 48 h in culture medium containing LipofectAMINE reagent.

RNA Isolation and RT-PCR—Total RNA was isolated from HEK293 cells using TRIzol reagent (Life Technologies, Inc.). RT-PCR was performed for quantification of FLASH mRNA using β-actin mRNA as a control. Two sets of oligonucleotides were designed; 5′-TAGGTGCTTTTATTGACTTGACACAA-3′ (sense) and 5′-CAGGAATTCAGCAACTTATCTGCAT-3′ (antisense) (predicted product length: 725 bp) for FLASH primer 1 and 5′-GAAGGTAATCATCCTGCATTAGCTGT-3′ (sense) and 5′-GAGCTTCATTAGCTGCTGGAATCTT-3′ (antisense) (predicted product length: 714 bp) for FLASH primer 2. The nucleotide sequences of the β-actin primers were 5′-CAACCGCGAGAAGATGACCC-3′ (sense) and 5′-GAAGGAAGGCTGGAAGAGTG-3′ (antisense) (predicted product length: 457 base pairs). The PCR products were confirmed by DNA sequencing.

Luciferase and β-Galactosidase Assays—Cells were harvested 24 h after transfection, and luciferase activities in the cell extracts were determined using a luciferase assay system (Promega). To measure β-galactosidase activity, the cell extracts were mixed with equal amounts of β-galactosidase assay buffer (2×) containing 200 mM sodium phosphate (pH 7.3), 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 mg/ml *O*-nitrophenyl-β-D-galactopyranoside and incubated at 37 °C for 30 min. The absorbance at 420 nm was then measured using an ELISA reader (Molecular Device, Sunnyvale, CA).

Generation of Anti-FLASH Antibody and Western Blot Analysis—GST-DRD-FLASH fusion proteins were expressed in BL21(DE3) by addition of 0.2 mM isopropyl-β-D-thiogalactoside, purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) and administered into a rabbit in a series of four injections. Anti-FLASH antibody was purified from the serum by antigen-affinity chromatography. For Western blot analysis, cell lysates were prepared, and protein concentrations were determined using a DC protein assay kit (Bio-Rad). Western blotting was then carried out as previously described (31); proteins were visualized using an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

In Vitro Binding Assay—The expression of GST fusion proteins in BL21 (DE3) harboring pGEX-4T, pGEX-DRD-FLASH, or pGEX-NAD-FLASH was induced with 0.2 mM isopropyl-β-D-thiogalactoside during exponential growth. Harvested cells were resuspended and lysed by sonication in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM dithiothreitol, 0.5 mM EDTA, and 10% (v/v) glycerol. The supernatant lysates were incubated with glutathione-Sepharose 4B. TRAF2, or caspase-8 labeled with [^{35}S]methionine using the TNT system (Promega) were then added to GST fusion proteins (20 μg each) coupled to glutathione-Sepharose 4B in a final volume of 500 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 0.5 mM EDTA, 0.01% Triton X-100, 0.5 mg/ml bovine serum albumin, and 10% (v/v) glycerol). After being incubated at 4 °C for 2 h with gentle mixing, the beads were washed three times with the binding buffer, separated by 12% SDS-PAGE, and detected by autoradiography.

Immunoprecipitation—HEK293 cells were transfected with pHA-FLASH, pHA-ΔB-FLASH, and pTRAF2 plasmids and lysed in radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, and 1 mM NaF). FLASH was immunoprecipitated from cell lysates after incubation with anti-FLASH and anti-HA antibodies and protein-A-coupled-Sepharose CL-4B (Amersham Pharmacia Biotech.) at 4 °C for 2 h. TRAF2, HA-FLASH, and HA-ΔB-FLASH were then detected by Western blot analysis using anti-TRAF2 and anti-HA monoclonal antibodies, respectively.

RESULTS

Suppression of TNF-α-induced NF-κB Activation by a FLASH Antisense Oligonucleotide—To identify a role for

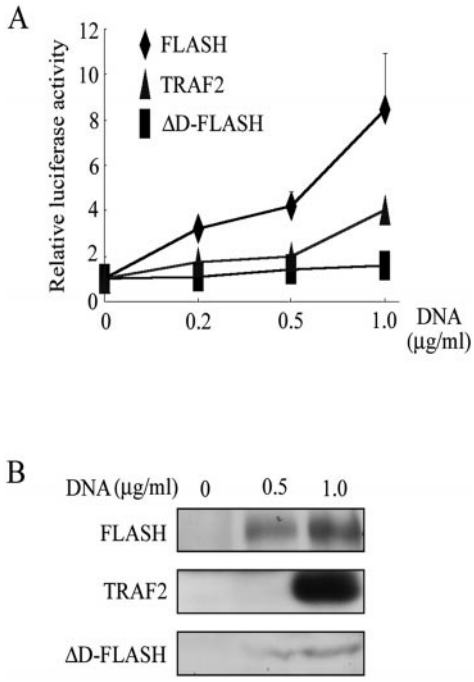

FIG. 2. **NF-κB activation induced by FLASH expression.** HEK293 cells were transfected for 36 h with pNF-κB-luc, pCMV-β-gal and the indicated amounts of pFLASH, pTRAF2, or pHA-ΔD-FLASH. *A*, relative NF-κB-driven luciferase activities in transfectants expressing FLASH, TRAF2, or ΔD-FLASH; activity in the control cells was arbitrarily set to a value of 1. *B*, Western blots showing the expression levels of exogenous FLASH (anti-FLASH antibody), ΔD-FLASH (anti-HA antibody), and TRAF2 (anti-TRAF2 antibody).

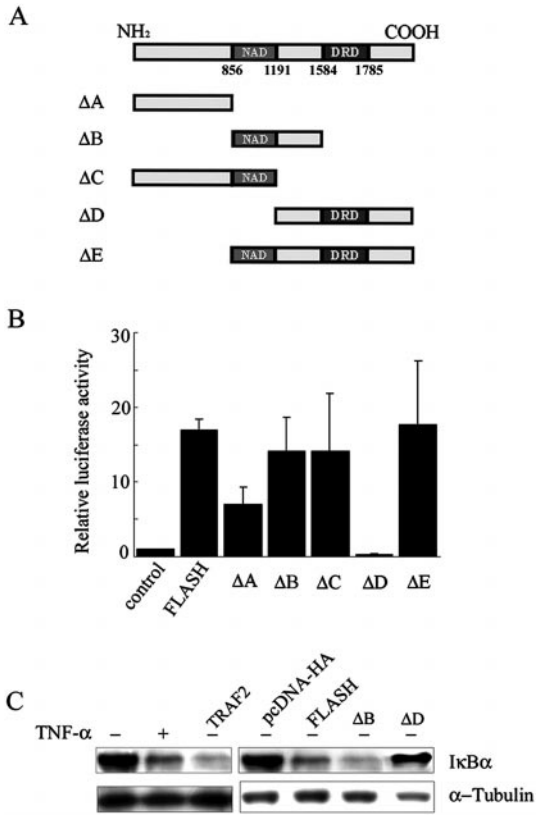

FIG. 3. **Mapping of the FLASH domain responsible for NF-κB activation.** *A*, schematic diagrams of full-length FLASH and its deletion (Δ) constructs. The death-effector domain-recruiting domain (*DRD*) and a putative NF-κB-activating domain (*NAD*) are indicated. *B*, HEK293 cells were co-transfected with pNF-κB-luc and either pcDNA (*control*), full-length FLASH, or deletion constructs. One day later, luciferase reporter gene assays were performed and for each construct, luciferase activities were adjusted so that the control was 1. *Bars* indicate mean ± S.D. of the induction of NF-κB activity relative to the control from at least four independent experiments. *C*, HEK293 cells were left untreated, treated with TNF-α for 1 h, or transfected with pcDNA-HA, pHA-FLASH, pHA-ΔB-FLASH, pFL-ΔD-FLASH, or pTRAF2. After 1 day, cell extracts were prepared and analyzed by Western blotting with anti-IκBα antibody.

FLASH in TNF-α signaling, we initially examined its contribution to NF-κB activation by directly targeting FLASH expression using an AS. Four different ASs were synthesized based on the nucleotide sequence of human FLASH and when examined, they showed essentially similar effects on TNF-α-signaling (data not shown). Treating HEK293 cells with AS-2, but not with a scrambled oligonucleotide (as a negative control), abolished TNF-α-induced activation of NF-κB, as assessed by luciferase reporter gene expression driven by a NF-κB responsive promoter (Fig. 1*A*), and also suppressed TNF-α-induced degradation of IκBα (Fig. 1*B*).

Because we could detect exogenous FLASH expression (Fig. 2*B*) but failed to detect expression of endogenous FLASH with Western blot analysis using anti-FLASH antibody, the effects of AS-2 or the scrambled oligonucleotide on FLASH expression were assessed by RT-PCR (Fig. 1*C*). RT-PCR and Northern blot analysis have both been used to examine the effects of antisense on gene expression (32). FLASH mRNA was undetectable in HEK293 cells treated with AS-2, whereas tubulin expression was unaffected, and readily detectable in cells treated with the scrambled oligonucleotide, indicating that FLASH expression was reduced by AS-2 treatment. These results suggest that FLASH is involved in TNF-α-induced activation of NF-κB.

Activation of NF-κB by FLASH Expression and Domain Mapping for NF-κB Activation—To more directly assess the role of FLASH in the activation of NF-κB signaling, we examined an effect of its overexpression on NF-κB activity in HEK293 cells. We found that, indeed, NF-κB activity was dose-dependently related to FLASH expression (Fig. 2*A*). The relative levels of expression of FLASH and TRAF2 were confirmed by Western blot analysis (Fig. 2*B*).

FLASH contains DRD at its C terminus and a putative oligomerization domain at its N terminus (Fig. 3*A*) (26). To ascertain which of these mediates induction of NF-κB activity, effects of several FLASH deletion mutants (Fig. 3*A*, Δ*A*–Δ*E*) were examined. Expression of these deletions was confirmed in the transfected HEK293 cells by Western blot analysis using anti-HA or anti-FLAG antibodies (data not shown). Determination of NF-κB activity following the respective expression of each of these constructs showed that the truncation in the ΔB-, ΔC-, and ΔE-FLASH constructs had no effect on the ability of FLASH to activate NF-κB (Fig. 3*B*). The ΔD-FLASH, by contrast, completely abolished NF-κB activation, whereas the ΔA-FLASH partially induced NF-κB activity (Fig. 3*B*). Because the ΔB-, ΔC-, and ΔE-FLASH contain a common region including a putative oligomerization domain, the region responsible for the activation of NF-κB apparently spans most of the oligomerization domain and part of the ΔA-FLASH domain (Fig. 3*A*). We designate the common region in the ΔB-, ΔC-, and ΔE-FLASH spanning residues 856–1191 as the NF-κB activation domain (NAD) of FLASH.

Because it is known that TNF-α treatment of cells leads to the activation of NF-κB through the phosphorylation and degradation of IκBα (33, 34), exposure of HEK293 cells to TNF-α resulted in the degradation of IκBα (Fig. 3*C, left panel*). Transient expression of FLASH, TRAF2, or ΔB-FLASH led to the degradation of IκBα, whereas overexpression of the ΔD-FLASH did not affect the degradation of IκBα (Fig. 3*C, right panel*), consistent with the results of NF-κB activity assays in Fig. 3*B*.

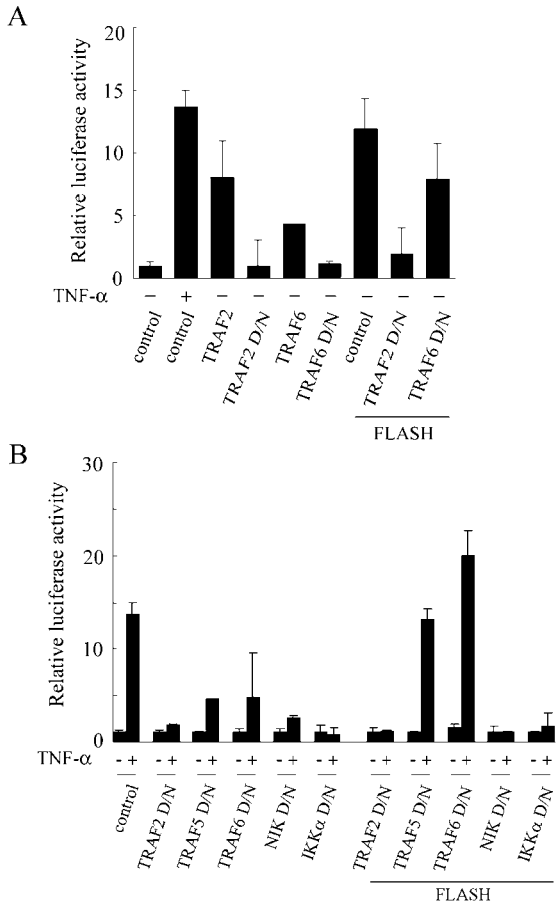

FIG. 4. **Effects of various dominant negative mutants on FLASH-induced activation of NF-κB.** *A*, HEK293 cells were left untreated or treated with TNF-α after co-transfection with pNF-κB-luc and either pcDNA (*control*) or expression plasmids encoding the wild type or dominant negative mutants (*D*/*N*) of the indicated mediators in the presence or absence of pHA-FLASH. One day later, luciferase activities were measured and normalized to that of β-galactosidase. *B*, HEK293 cells were co-transfected with pNF-κB-luc and the indicated dominant negative mutants. The cells were then exposed to TNF-α (30 ng/ml) for 6 h, after which luciferase activities were measured.

These results demonstrate and confirm that FLASH expression through NAD in ΔB-FLASH induces NF-κB activation by the degradation of IκBα.

FLASH Signals NF-κB Activation through TRAF2-NIK-IKKs—It has previously been shown that various signaling proteins downstream of TNF-R, including TRADD, TRAFs, RIP, NIK, and IKKs, are involved in TNF-α-induced activation of NF-κB. We therefore examined the respective roles of these proteins in FLASH-induced activation of NF-κB. Expression of dominant negative mutants of TRAF2-(87–501), NIK (K429A,K430A), or IKKα (K44A) inhibited NF-κB activation induced by FLASH (Fig. 4*A*) or TNF-α (Fig. 4*B*). On the other hand, although dominant negative mutants of TRAF5-(205–558) and TRAF6-(289–530) partially suppressed TNF-α- or FLASH-induced activation of NF-κB (Fig. 4*B*), they showed little effect on both TNF-α- and FLASH-induced activation of NF-κB (Fig. 4, *A* and *B*), which thus appears to be mainly mediated via a TRAF2-NIK-IKK-pathway.

FLASH Interacts with TRAF2 in Vitro and in Vivo through NAD—That FLASH interacts with TRAF2 was then examined with *in vitro* binding assay and immunoprecipitation. GST pull-down assay showed that GST-NAD fusion protein could bind to TRAF2, whereas GST-DRD interacted only with caspase-8, indicating that NAD of FLASH specifically interacts with TRAF2 *in vitro* (Fig. 5). Immunoprecipitation assays were then performed to examine cellular interaction of FLASH and

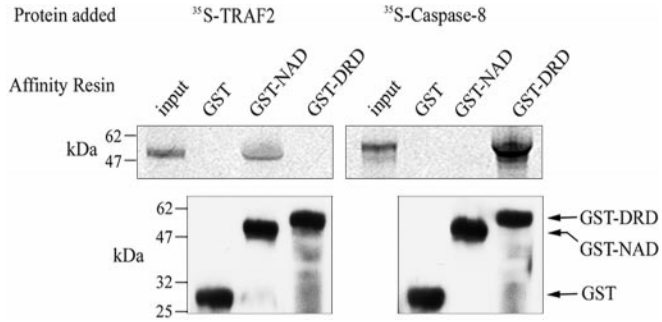

FIG. 5. ***In vitro* binding of NAD to TRAF2.** GST, GST-NAD, and GST-DRD fusion proteins were purified from *Escherichia coli*. Proteins bound to their affinity resins (each equivalent to 20 μg of protein) were incubated with TRAF2 or caspase-8 labeled with [35S]methionine as described under "Experimental Procedures." After separation by 12% SDS-PAGE, the bound proteins were detected by autoradiography (*upper panels*), and resin-coupled proteins were visualized by Western blotting with anti-GST polyclonal antibody (*lower panels*).

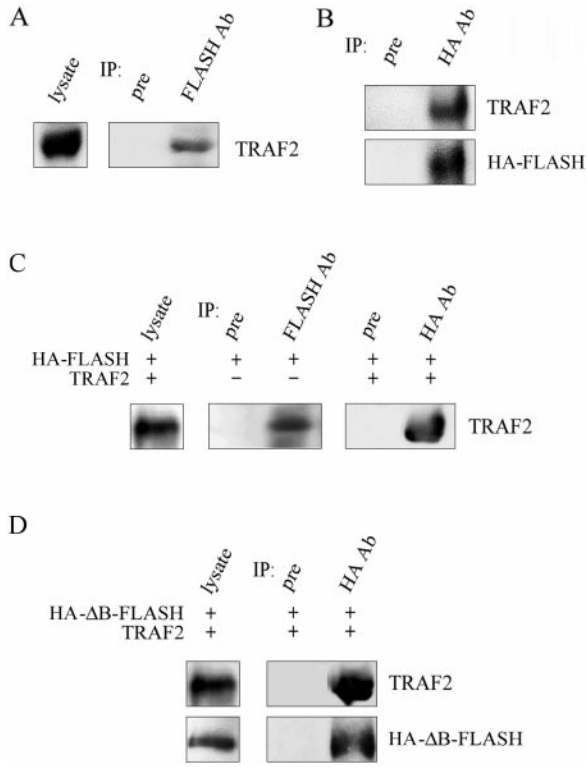

FIG. 6. **Cellular interaction of FLASH with TRAF2.** *A*, proteins from Jurkat cell extracts were immunoprecipitated (*IP*), separated by SDS-PAGE, and immunoblotted with preimmune (*pre*) or anti-TRAF2 monoclonal antibody. *B*, proteins from HeLa cell permanently expressing HA-FLASH (HeLa/HA-FLASH) were immunoprecipitated with anti-HA antibody and probed with anti-TRAF2 antibody (*upper panel*) or anti-HA antibody (*lower panel*). *C*, HEK293 cells were transiently transfected with pHA-FLASH in the presence or absence of pTRAF2. One day later, proteins were immunoprecipitated with anti-FLASH antibody or anti-HA antibody and immunoblotted with anti-TRAF2 antibody. *D*, HEK293 cells were co-transfected with pHA-ΔB-FLASH and pTRAF2, immunoprecipitated with anti-HA antibody, and immunoblotted with anti-TRAF2 (*upper panels*) or anti-HA antibody (*lower panels*).

TRAF2 in Jurkat, HEK 293, and HeLa cells. Immunoprecipitation with anti-FLASH antibody and subsequent Western blot analysis using anti-TRAF2 antibody revealed that endogenous TRAF2 was co-precipitated with endogenous FLASH, but not by preimmune serum (Fig. 6*A*). Endogenous FLASH could not be detected with Western blot analysis using anti-FLASH antibody in the immunoprecipitates probably because of its low expression level in Jurkat cells. We have then generated HeLa

cells stably expressing exogenous FLASH tagged with HA (HeLa/HA-FLASH) and further examined the intracellular interactions. Immunoprecipitation with anti-HA antibody followed by Western blotting using anti-TRAF2 antibody or anti-HA antibody showed the presence of TRAF2 and HA-FLASH in the immunoprecipitates (Fig. 6*B*). These results indicate that FLASH interacts with TRAF2 in the cells.

We have then examined cellular interaction of NAD of FLASH with TRAF2. HEK293 cells were transfected with pHA-FLASH (Fig. 6*C*) or pHA-ΔB-FLASH (Fig. 6*D*) in the presence or absence of TRAF2. Immunoprecipitation and Western blot analysis showed that endogenous (Fig. 6*C*, *middle panel*) or exogenous TRAF2 (Fig. 6*C*, *right panel*) was co-precipitated with FLASH by anti-FLASH antibody or anti-HA antibody. As expected from *in vitro* binding assays, ΔB-FLASH was co-immunoprecipitated with TRAF2 by anti-HA antibody from lysates of HEK293 cells transfected with both pHA-ΔB-FLASH and pTRAF2 (Fig. 6*D*). These results suggest that FLASH may directly interact with TRAF2 through its NAD.

DISCUSSION

Previous studies have suggested that TNF-R-mediated apoptosis and NF-κB activation pathways pass through TRADD, a death domain-containing adaptor protein interacting with TNF-R in a TNF-dependent process; TRADD-FADD-caspase-8 and TRADD-TRAF2-RIP-NIK-IKKs cascades lead to the induction of apoptosis and activation of an anti-apoptotic transcription factor NF-κB, respectively. Whereas FLASH may be required for the activation of caspase-8 during Fas- and TNF-R-mediated apoptosis (26, 27), the data presented here provide the first evidence for involvement of FLASH in NF-κB activation by TNF-R. The physical interaction of FLASH with TRAF2, demonstrated here *in vitro* and *in vivo* (Figs. 5 and 6), lends further support to the idea that FLASH transduces TNF-α signals via a TRAF2-dependent pathway of NF-κB activation. Though dominant negative mutants of TRAF5 and 6 also partially suppressed FLASH- and TNF-α-mediated activation of NF-κB (Fig. 4, *A* and *B*), complex formation of FLASH with additional signal mediators leading to NF-κB signaling such as other TRAFs, TRADD, or RIP remains to be elucidated.[2]

FLASH as a component of apoptotic signaling complexes is likely to mediate apoptosis signals probably triggered by cell surface receptors. However, the fact that FLASH activates NF-κB may explain the observation that in many cell types, TNF treatment did not induce apoptosis in the absence of gene expression. With respect to the role of FLASH as an activator interacting with caspase-8 and FADD, the lack of a significant increase of apoptosis following overexpression of both FLASH and caspase-8 or the inability of TNF-α to induce apoptosis in a subset of tumor cells may be attributed to FLASH-mediated activation of NF-κB. NAD-mediated activation of NF-κB may antagonize DRD-mediated apoptotic signals by encoding inhibitory proteins such as IAPs and IEX-1L (5, 35, 36). Moreover, recent reports that FADD and caspase-8 may be required for cell survival and proliferation during heart and thymus development may be explained by our observations of FLASH-mediated activation of NF-κB. This speculation is reinforced by our observation that FLASH transduced NF-κB signaling evoked by caspase-8 (data not shown).

FLASH seems to be an upstream component of various receptor-mediated signals including TNF-α and most likely has a dual function in apoptosis and NF-κB signaling. We have additional evidences that FLASH is also an indispensable component in receptor-mediated apoptosis. As a component of DISC during apoptosis and also of the protein complex including TRAFs leading to NF-κB signaling, FLASH needs to be further characterized for the stoichiometry of protein-protein interactions and for a fine-tuning activity balancing survival and apoptosis.

[2] Y. H. Choi, K. B. Kim, B. J. Kim, and Y. K. Jung, manuscript in preparation.

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FLASH Coordinates NF-κB Activity via TRAF2

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