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Activation of NF-κB by FADD, Casper, and Caspase-8*

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Fas-associated death domain protein (FADD), caspase-8-related protein (Casper), and caspase-8 are components of the tumor necrosis factor receptor type 1 (TNF-R1) and Fas signaling complexes that are involved in TNF-R1- and Fas-induced apoptosis. Here we show that overexpression of FADD and Casper potently activates NF-κB. In the presence of caspase inhibitors, overexpression of caspase-8 also activates NF-κB. A caspase-inactive point mutant, caspase-8(C360S), activates NF-κB as potently as wild-type caspase-8, suggesting that caspase-8-induced apoptosis and NF-κB activation are uncoupled. NF-κB activation by FADD and Casper is inhibited by the caspase-specific inhibitors crmA and BD-fmk, suggesting that FADD- and Casper-induced NF-κB activation is mediated by caspase-8. FADD, Casper, and caspase-8-induced NF-κB activation are inhibited by dominant negative mutants of TRAF2, NIK, IκB kinase α, and IκB kinase β. A dominant negative mutant of RIP inhibits FADD- and caspase-8-induced but not Casper-induced NF-κB activation. A mutant of Casper and the caspase-specific inhibitors crmA and BD-fmk partially inhibit TNF-R1-, TRADD, and TNF-induced NF-κB activation, suggesting that FADD, Casper, and caspase-8 function downstream of TRADD and contribute to TNF-R1-induced NF-κB activation. Moreover, activation of caspase-8 results in proteolytic processing of NIK, which is inhibited by crmA. When overexpressed, the processed fragments of NIK do not activate NF-κB, and the processed C-terminal fragment inhibits TNF-R1-induced NF-κB activation. These data indicate that FADD, Casper, and pro-caspase-8 are parts of the TNF-R1-induced NF-κB activation pathways, whereas activated caspase-8 can negatively regulate TNF-R1-induced NF-κB activation by proteolytically inactivating NIK.

Tumor necrosis factor receptor 1 (TNF-R1)[1] is a prototypical member of the TNF receptor family. Stimulation of TNF-R1 can simultaneously induce two opposite effects: apoptosis and activation of the anti-apoptotic transcription factor NF-κB (1, 2). TNF-R1 contains a death domain, which interacts with the cytoplasmic death domain-containing protein TRADD in a TNF-dependent process (2, 3). Once TRADD is recruited to TNF-R1, it functions as an adapter protein to recruit several structurally and functionally divergent proteins, including FADD, RIP, TRAF2, and the cellular inhibitor of apoptosis protein (2–4). The interaction of TRADD with FADD leads to apoptosis through the activation of a caspase cascade, which is initiated by the interaction of FADD with caspase-8 (2, 5, 6). The interaction of TRADD with TRAF2 and RIP activates NIK, a member of the mitogen-activated protein kinase kinase kinase family (2, 7–10). Once activated, NIK further activates two downstream kinases, IKKα and IKKβ, which form either homodimer or heterodimer complexes and directly phosphorylate IκBs (8, 11–15). Phosphorylation of IκBs leads to their degradation and subsequent activation of NF-κB. Recent gene knockout experiments suggest that IKKβ, but not IKKα, is required for TNF-induced NF-κB activation (16–19). These early studies indicate that two independent TNF-R1 signaling pathways, leading to either apoptosis or NF-κB activation, bifurcate at TRADD.

Recently, others and we cloned a FADD- and caspase-8-related molecule, called Casper (cFLIP/CASH/I-FLICE/CLARP) (20–24). Casper contains two death effector domains (DEDs) and a caspase-like domain. Casper, however, is not a caspase because it does not contain a conserved cysteine residue found in all caspases. Casper interacts with FADD and caspase-8 through their respective DEDs (20–24). The caspase-like domain of Casper interacts with caspase-3 and TRAF2 (20). Overexpression of Casper induces apoptosis (20, 22, 24), which is inhibited by the cowpox viral protein crmA and by a dominant negative mutant of caspase-8 (20), suggesting that Casper-induced apoptosis is mediated by caspase-8. A Casper deletion mutant lacking its C-terminal 45 amino acids protects TNF-R1-, Fas-, TRADD-, and FADD-induced apoptosis, suggesting that Casper functions downstream of FADD and is involved in TNF-R1- and Fas-induced apoptosis pathway (20, 24). Other reports, however, have suggested that Casper inhibits apoptosis induced by TNF receptor family members (21, 22, 23). The reasons for this discrepancy are not clear.

In this report, we show that FADD, Casper, and caspase-8 can activate the transcription factor NF-κB via a TRAF2-NIK-IKKs-dependent pathway, which contributes to TNF-R1-induced NF-κB activation. In addition, we show that activation of caspase-8 can also negatively regulate TNF-R1-induced NF-κB activation by proteolytically inactivating NIK.

EXPERIMENTAL PROCEDURES

Reagents—The recombinant human TNF and IL1 (R&D Systems Inc., Minneapolis, MN), the monoclonal antibodies against the Flag epitope (Sigma), the Myc epitope (Santa Cruz Biotechnology, Santa Cruz, CA), the rabbit polyclonal antibody against the C-terminal domain (aa 700–947) of NIK (Santa Cruz Biotechnology), and the caspase inhibitor BD-fmk (Enzyme Systems, Livermore, CA) were purchased

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[1] The abbreviations used are: TNF-R, tumor necrosis factor receptor; TNF, tumor necrosis factor; Casper, caspase-8-related protein; NIK, NF-κB-inducing kinase; RIP, receptor-interacting protein; IKK, IκB kinase; FADD, Fas-associated death domain protein; TRAF2, TNF receptor-associated factor 2; TRADD, TNF receptor-associated death domain protein; DED, death effector domain; aa, amino acid(s).

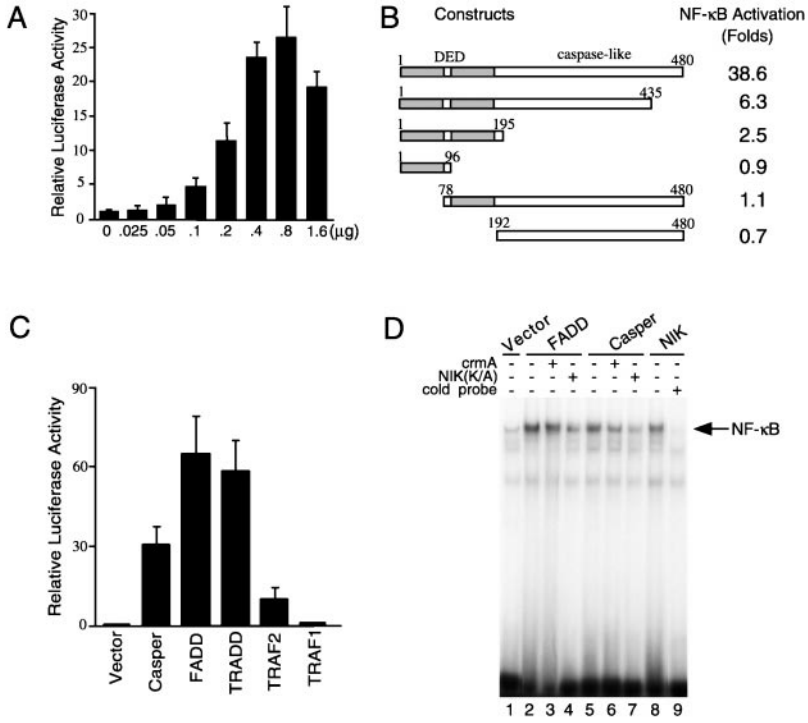

FIG. 1. **Activation of NF-κB by Casper and FADD.** *A,* Casper activates NF-κB in a dose-dependent manner. *B,* domain mapping of Casper-induced NF-κB activation. *C,* activation of NF-κB by FADD. 293 cells (2×10^5) were transfected with the indicated amounts of Casper expression plasmid (*A*), 2 μg of the expression plasmids for Casper or its mutants (*B*), or 2 μg of the indicated plasmids (*C*). Luciferase reporter gene assays were performed 14 h after transfection. Data shown are relative luciferase activities compared with a control empty vector transfection. *D,* gel shift analysis of NF-κB activation by Casper and FADD. 293 cells (8×10^5) were transfected with 5 μg of expression plasmid for FADD, Casper, or NIK, in the presence or absence of 5 μg of expression plasmid for crmA or NIK(KK429/430AA). 14 h after transfection, nuclear extracts were prepared, and electrophoretic mobility shift assays were performed with a radiolabeled double-strand NF-κB probe.

from the indicated manufacturers. The human 293 embryonic kidney cells and HeLa cells were provided by Dr. Zhaodan Cao (Tularik Inc., South San Francisco, CA) and Dr. David Riches (National Jewish Center, Denver, CO), respectively.

Constructs—The NF-κB luciferase reporter construct was provided by Dr. Gary Johnson (National Jewish Center). Mammalian expression vectors encoding Casper and its mutants (20), FADD (25), caspase-8 (20), TRADD (1), TRADD(296S) (26), TRAF2 and TRAF2(87–501) (27), RIP (559–671) (4), NIK and NIK(KK429/430AA) (10), IKKα(K44A) and IKKβ(K44A) (14), and RSV-β-galactosidase (28) have been described previously.

Mammalian expression vectors for Myc-tagged NIK and its deletion mutants, and Flag-tagged caspase-8(C360S) were constructed by polymerase chain reaction amplification of the corresponding cDNA fragments and subsequently cloning into a CMV promoter-based vector.

Cell Transfection and Reporter Gene Assays—The human embryonic kidney 293 and HeLa cell lines were maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Inc.). For reporter gene assays, $\sim 2 \times 10^5$ cells/well were seeded on 6-well (35 mm) dishes. Cells were transfected the following day by the standard calcium phosphate precipitation method (29) with 0.5 μg of NF-κB-luciferase reporter construct and various amounts of testing plasmids. Within the same experiment, each transfection was performed in triplicate, and where necessary, enough amount of empty control plasmid was added to keep each transfection receiving the same amount of total DNA. To normalize for transfection efficiency and protein amount, 0.5 μg of RSV-β-galactosidase plasmid was added to all transfections. Luciferase reporter assays were performed using a luciferase assay kit (Pharmingen, San Diego, CA) following the manufacture's protocols. β-Galactosidase activity was measured using the Galacto-Light chemiluminescent kit (TROPIX Inc., Medford, MA). Luciferase activities were normalized on the basis of β-galactosidase expression levels. Data shown are averages and standard deviations from one of the representative experiments in which each transfection was performed in triplicate.

Western Analysis—Cells were lysed in 1 ml of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis. Western blotting analyses were performed as described (20).

Electrophoretic Mobility Shift Assays—293 cells (8×10^5) were transfected with 5 μg of expression plasmid for FADD, Casper, or NIK, in the presence or absence of 5 μg of expression plasmid for crmA or NIK(KK429/430AA). Where necessary, enough amount of empty control plasmid was added to keep each transfection receiving the same amount of total DNA (10 μg). 14 h after transfection, cells were harvested, and nuclear extracts were prepared as described (30). Aliquots of the nuclear extracts (20 μg) were incubated with 0.4 ng of a radiolabeled double-stranded oligonucleotide containing the following NF-κB binding sequence: 5′ GGGGACTTTCCC 3′. Each reaction was supplemented with 0.8 μg of poly(dI-dC) for blocking nonspecific binding. Nucleoprotein complexes were resolved by electrophoresis on a 6% nondenaturing acrylamide gel in Tris-Borate-EDTA buffer.

RESULTS

Activation of NF-κB by Casper and FADD—Because Casper interacts with TRAF2 (20), a signaling protein involved in NF-κB activation by TNF-R1 and several other members of the TNF receptor family (2, 31), we determined whether Casper was capable of inducing NF-κB in luciferase reporter gene assays. We performed luciferase assays ~14 h after transfection, a time point at which most transfected cells did not die. As shown in Fig. 1*A,* overexpression of Casper activated NF-κB in a dose-dependent manner.

Casper contains two DEDs at its N terminus and a caspase-like domain at its C terminus (Fig. 1*B*). Previously, it has been shown that the C-terminal caspase-like domain of Casper is responsible for its apoptotic activity (20). To determine which domain is required for Casper-induced NF-κB activation, we examined various Casper deletion mutants for their ability in activating NF-κB in reporter gene assays. As shown in Fig. 1*B,* Casper(78–480) and Casper(192–480), two Casper deletion mutants lacking N-terminal 77 amino acids (the first DED) or 191 amino acids (both the first and second DEDs) respectively and which have been shown to be capable of inducing apoptosis (20), did not activate NF-κB. Casper(1–435), a deletion mutant lacking C-terminal 45 amino acids that has been shown to function as a dominant negative mutant for TNF-R1- and Fas-induced apoptosis (20, 24), weakly activated NF-κB (Fig. 1*B*). These data suggest that both the DEDs and the caspase-like domain of Casper are needed for its full NF-κB-inducing activity.

We next determined whether FADD, which interacts with Casper and functions upstream of Casper in TNF-R1- and Fas-induced apoptosis pathways (20, 24), could also activate NF-κB. We found that overexpression of FADD potently acti-

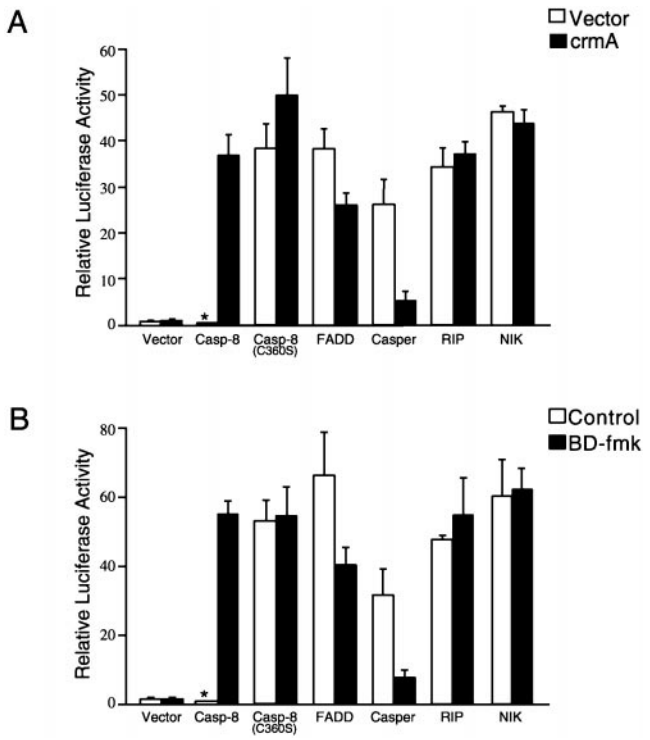

FIG. 2. **Activation of NF-κB by caspase-8 and the effects of caspase inhibitors on FADD-, Casper-, and caspase-8-induced NF-κB activation.** *A,* activation of NF-κB by caspase-8, caspase-8(C360S), FADD, and Casper, in the absence (*white bars*) or presence (*black bars*) of crmA. 293 cells (2×10^5) were transfected with 1 μg of the indicated expression plasmids, together with 2 μg of expression plasmid for crmA (*black bars*) or an empty control vector (*white bars*). Luciferase reporter gene assays were performed 14 h after transfection. *B,* BD-fmk inhibits FADD- and Casper-induced but not RIP- and NIK-induced NF-κB activation. 293 cells (2×10^5) were transfected with 1 μg of the indicated expression plasmids. Immediately after transfection, cells were treated with 20 μM BD-fmk or left untreated. 14 h after transfection, luciferase reporter gene assays were performed. Data shown are relative luciferase activities compared with the control empty vector transfection.

vated NF-κB in reporter gene assays (Fig. 1*C*). The induction folds of NF-κB activity by Casper and FADD were comparable with those induced by TRADD and TRAF2, two signaling proteins previously shown to be involved in TNF-R1-induced NF-κB activation (Fig. 1*C*). An empty control plasmid or overexpression of TRAF1 did not activate NF-κB (Fig. 1*C*). Gel shift experiments with nuclear extracts from Casper- and FADD-transfected cells further confirmed that Casper and FADD could activate NF-κB (Fig. 1*D*).

Activation of NF-κB by Caspase-8—Because caspase-8 interacts with FADD and Casper and functions downstream of FADD in TNF-R1- and Fas-induced apoptosis pathways, we determined whether caspase-8 could also activate NF-κB. When overexpressed, caspase-8 potently and rapidly induces apoptosis of transfected cells, and this makes it impossible to measure potential NF-κB activation by caspase-8 (Ref. 20 and data not shown). Previously, we have shown that the cowpox viral protein crmA can block caspase-8-induced apoptosis by physically interacting with and inhibiting processing of caspase-8 precursor (20). In the presence of crmA, overexpression of caspase-8 potently activated NF-κB (Fig. 2*A*). In this experiment, crmA alone did not activate NF-κB (Fig. 2*A*). These data suggest that caspase-8 can activate NF-κB, and this activation is independent of its proteolytic activity. To further confirm this conclusion, we determined whether a caspase-inactive mutant could activate NF-κB. Previously, it has been shown that a point mutation of caspase-8, C360S, abolishes the apoptotic activity of caspase-8 and functions as a dominant negative mutant for TNF-R1- and Fas-induced apoptosis (5). We constructed such a mutant, caspase-8(C360S), and found that it had no detectable apoptotic activity when overexpressed in 293 or HeLa cells (data not shown). When transfected alone, however, caspase-8(C360S) could activate NF-κB to the same degree as that induced by wild-type caspase-8 in the presence of crmA. Taken together, these data suggest that caspase-8 can activate NF-κB as an unprocessed and caspase-inactive precursor.

Inhibition of FADD- and Casper-induced NF-κB Activation by Specific Caspase Inhibitors—FADD and Casper induce apoptosis through activation of a downstream caspase cascade initiated at caspase-8 (5, 6, 20, 22, 24). It has been shown that crmA interacts directly with caspase-8 but not with caspase-3 and that overexpression of crmA inhibits caspase-8-induced apoptosis (20). CrmA does not interact with FADD and Casper but can inhibit FADD- and Casper-induced apoptosis (2, 5, 6, 20, 32, 33). To determine whether caspase-8 is involved in FADD- and Casper-induced NF-κB activation, we tested whether crmA could block FADD- and Casper-induced NF-κB activation. We found that both FADD- and Casper-induced NF-κB activation was inhibited by crmA in reporter gene assays (Fig. 2*A*) and in gel shift experiments (Fig. 1*D*). Moreover, activation of NF-κB by FADD and Casper, but not by RIP and NIK, was also inhibited by the caspase inhibitor BD-fmk (Fig. 2*B*). These data suggest that caspase-8 is involved in FADD- and Casper-induced NF-κB activation.

Effects of Dominant Negative Mutants of TRAF2, RIP, NIK, and IKKs on NF-κB Activation Induced by FADD, Casper, and Caspase-8—Previously, it has been shown that the downstream signaling proteins of TNF-R1, including TRADD, TRAF2, RIP, NIK, IKKα, and IKKβ, are involved in TNF-R1-induced NF-κB activation (1–4, 8, 11–19, 34). Because FADD, Casper, and caspase-8 are also components of the TNF-R1 signaling complex, we determined whether they activate NF-κB through the classic NF-κB activation pathway mediated by TNF-R1. As shown in Fig. 3, TRAF2(87–501) (31), NIK(KK429/430AA) (10), IKKα(K44A) (13), and IKKβ(K44A) (14), dominant negative mutants of their respective wild-type counterparts, inhibited FADD-, Casper-, and caspase-8(C360S)-induced NF-κB activation. Inhibition of Casper-induced NF-κB activation by NIK(KK429/430AA) was further confirmed by gel shift experiments (Fig. 1*D*). RIP(559–671), a dominant negative mutant of RIP that inhibits TNF-R1-induced NF-κB activation (4), potently inhibited FADD- and caspase-8(C360S)-induced but not Casper-induced NF-κB activation (Fig. 3). Moreover, FADD-, Casper-, and caspase-8-induced NF-κB activation was completely blocked by IκBα(SS/AA) (Fig. 3), an IκBα mutant of which the two phosphorylation sites have been mutated to alanines. TRADD(296S), a dominant negative mutant of TRADD, which has been shown to inhibit TNF-R1-induced NF-κB activation (26, 35), did not inhibit Casper- and caspase-8-induced NF-κB activation but partially inhibited FADD-induced NF-κB activation (Fig. 3).

Inhibition of TNF-R1-induced NF-κB Activation by a Casper Mutant and by Caspase Inhibitors—To determine whether FADD, Casper, and caspase-8 are involved in TNF-R1-induced NF-κB activation, we examined whether a Casper deletion mutant and the caspase-specific inhibitors crmA and BD-fmk could inhibit TNF-R1-induced NF-κB activation. Previously, it has been shown that Casper (192-480Y/F), a Casper mutant containing aa 192–480 and in which tyrosine 360 is changed to phenylalanine, has little apoptotic activity (20). This mutant did not activate NF-κB in reporter gene assays (data not shown). As shown in Fig. 4*A,* Casper(192-480Y/F) partially

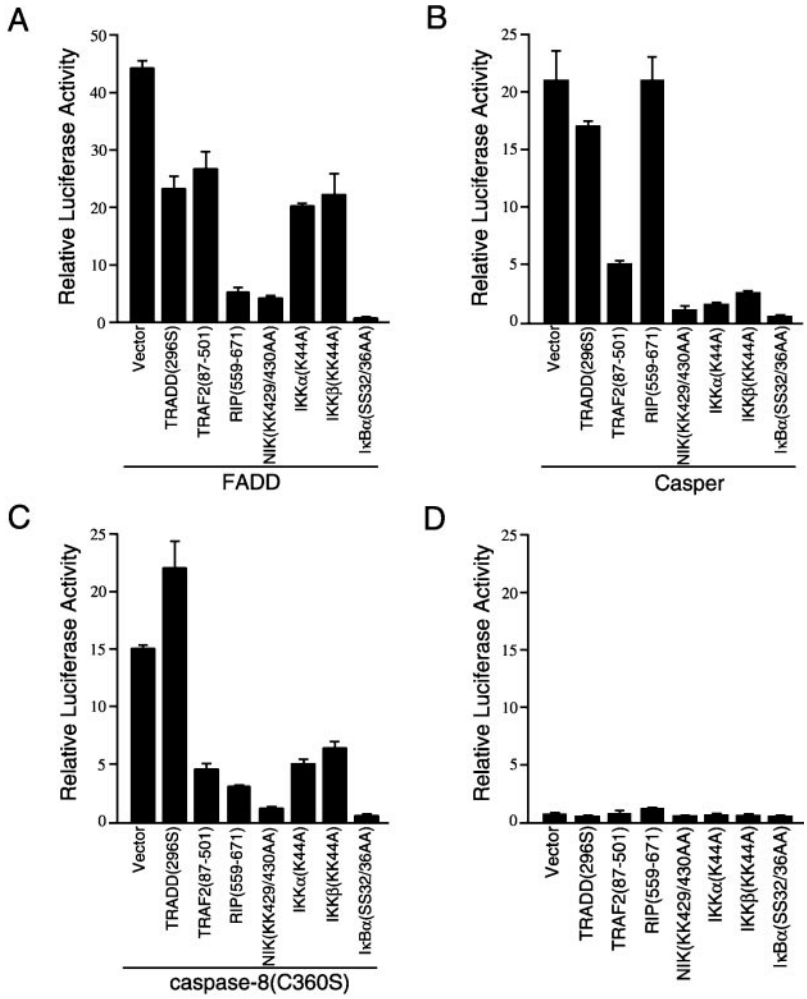

FIG. 3. **Effects of various dominant negative mutants on FADD-, Casper-, and caspase-8(C360S)-induced NF-κB activation.** 293 cells (2×10^5) were transfected with 1 μg of the mammalian expression plasmid for FADD (*A*), Casper (*B*), caspase-8(C360S), or a control empty vector (*D*), together with 2 μg of the indicated expression plasmids for various dominant negative mutants. Luciferase reporter gene assays were performed 14 h after transfection. Data shown are relative luciferase activities compared with the empty control plasmid transfection.

inhibited TNF-R1- and TRADD-induced but not NIK-induced NF-κB activation. In addition, crmA and BD-fmk also partially inhibited TNF-R1- and TRADD-induced but not NIK-induced NF-κB activation (Fig. 4*A*). Moreover, we found that BD-fmk partially inhibited TNF-, but not IL1-induced NF-κB activation in 293 and HeLa cells (Fig. 4, *B* and *C*). Taken together, these data suggest that FADD, Casper, and caspase-8 contribute to TNF-R1-induced NF-κB activation.

Proteolytic Processing of NIK by Caspase-8—Recently, it has been shown that RIP, a protein kinase critically involved in TNF-induced NF-κB activation, is cleaved by activated caspase-8 (36). This process results in inhibition of TNF-induced NF-κB activation (36). Because FADD, Casper, and caspase-8 activate NF-κB through the RIP-NIK-IKKs kinase cascade, we examined whether NIK and IKKβ are also processed by activated caspase-8. To do this, we co-transfected 293 cells with expression plasmids for N-terminal Myc epitope-tagged NIK or Flag epitope-tagged IKKβ in the presence or absence of expression plasmid for caspase-8. 11 h after transfection, Western blot analysis was performed with antibodies against the Myc epitope, Flag epitope, or the C-terminal domain (aa 700–947) of NIK. As shown in Fig. 5*A,* in the absence of caspase-8, NIK was expressed as a single ~110-kDa band. In the presence of caspase-8, NIK was processed into at least three fragments. These include a ~40-kDa fragment containing the N-terminal domain (detected by the anti-Myc antibody), a ~35-kDa fragment containing the C-terminal domain (detected by the antibody against the C-terminal domain of NIK), and, by deduction, a ~30-kDa fragment containing the intermediate kinase domain (Fig. 5*A*). A ~65-kDa fragment was also detected (Fig. 5*A*) by the antibody against the C-terminal domain of NIK. This fragment may be an intermediate containing the kinase domain and the C-terminal domain. The proteolytic processing of NIK by caspase-8 was inhibited by overexpression of crmA (Fig. 5*A*), further confirming that NIK was processed by caspase-8. In the same experiment, caspase-8 did not process IKKβ (Fig. 5*B*), suggesting that IKKβ is not a substrate of caspase-8.

To determine the effect of NIK processing on its ability to activate NF-κB, mammalian expression vectors for NIK's N-terminal domain (aa 1–366), kinase domain (aa 366–653), and kinase plus C-terminal domains (aa 366–947) were constructed. As shown in Fig. 6, overexpression of NIK(1–366), NIK(366–653), and NIK(366–947), either separately or with various combinations, failed to activate NF-κB in reporter gene assays. In the same experiments, wild-type NIK strongly activated NF-κB. The expression levels of the NIK mutants are similar to that of wild-type NIK as shown by Western blot analysis (data not shown), excluding the possibility that the NIK mutants do not activate NF-κB due to failure of expression.

To determine the effects of processed NIK fragments on TNF-R1-induced NF-κB activation, expression vectors for TNF-R1 and the NIK mutants were co-transfected into 293 cells, and luciferase reporter gene assays were performed. As shown in Fig. 6, NIK(1–366) had no effect on TNF-R1-induced NF-κB activation. NIK(366–653) partially inhibited TNF-R1-induced NF-κB activation. NIK(366–947), either alone or together with NIK(1–366) and NIK(366–653), potently inhibited TNF-R1-induced NF-κB activation. Previously, it has also been

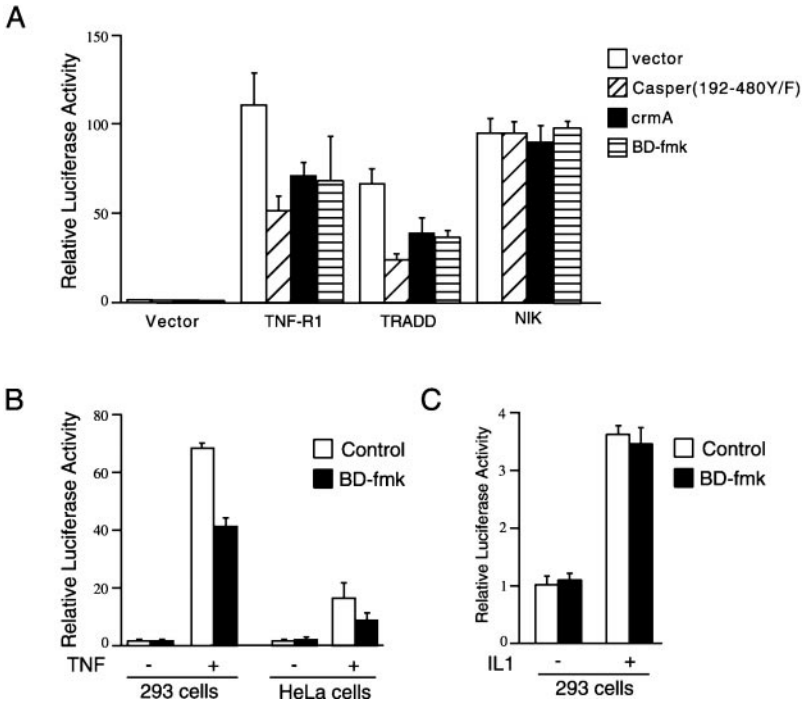

FIG. 4. **Inhibition of TNF-R1-, TRADD-, and TNF-induced NF-κB activation by a Casper mutant and two caspase inhibitors.** *A,* inhibition of TNF-R1- and TRADD-induced but not NIK-induced NF-κB activation by Casper(192-480Y/F), crmA, and BD-fmk. 293 cells (2×10^5) were transfected with 1 μg of the indicated expression plasmid, together with 2 μg of expression plasmid for Casper(192-480Y/F) (▨) or crmA (■) or an empty control plasmid (□). Immediately after transfection, cells were treated with 20 μM BD-fmk (▤) or left untreated as indicated. Luciferase reporter gene assays were performed 14 h after transfection. *B,* inhibition of TNF-induced NF-κB activation by BD-fmk. 293 cells or HeLa cells (2×10^5) were transfected with 0.5 μg of NF-κB-Luciferase reporter plasmid and 0.5 μg of RSV-β-galactosidase plasmid. 12 h after transfection, cells were left untreated (*white bars*) or treated with BD-fmk (20 μM) (*black bars*). 5 min after addition of BD-fmk, cells were further treated with TNF (20 ng/ml) (+) or left untreated (−) for 6 h. Luciferase reporter gene assays were then performed. *C,* IL-1-induced NF-κB activation is not inhibited by BD-fmk. Experiments were performed as in *B,* except that TNF was replaced by IL-1. Data shown are relative luciferase activities compared with the empty control plasmid transfection without any treatment.

shown that the C-terminal domain of NIK (aa 624–947) can functions as a dominant negative mutant to TNF-induced NF-κB activation (8, 10, 35). Taken together, these data suggest that caspase-8-mediated cleavage of NIK abolishes its ability to activate NF-κB and the cleaved products can inhibit TNF-R1-induced NF-κB activation.

DISCUSSION

Previous studies suggested that the TNF-R1-mediated apoptosis and NF-κB activation pathways bifurcate at TRADD, a death domain-containing adapter protein interacting with TNF-R1 in a TNF-dependent process (2, 3). It has also been suggested that the TRADD-FADD-Casper-caspase-8 cascade leads to induction of apoptosis, whereas the TRADD-TRAF2-RIP-NIK-IKKs cascade leads to activation of the anti-apoptotic transcription factor NF-κB (1–8, 37). These studies suggest that two independent signaling pathways are responsible for the two opposite effects induced by TNF-R1. In this study, we found that the death-inducing proteins FADD, Casper, and caspase-8 could also activate NF-κB.

FADD- and Casper-induced NF-κB activation was inhibited by crmA and the caspase-specific inhibitor BD-fmk (Fig. 2), suggesting that FADD- and Casper-induced NF-κB activation is mediated by caspase-8. In the presence of the caspase inhibitors crmA or BD-fmk, caspase-8 strongly activates NF-κB. The caspase-inactive point mutant of caspase-8, caspase-8(C360S), also activates NF-κB (Fig. 2). These data indicate that procaspase-8 can activate NF-κB, which is independent of the proteolytic processing and caspase activity of caspase-8. Previous studies have shown that apoptosis induced by caspase-8 requires its proteolytic processing and activation. Therefore, caspase-8-induced apoptosis and NF-κB activation are uncoupled.

As discussed above, the caspase-specific inhibitors crmA and BD-fmk had distinct effects on FADD-, Casper-, and caspase-8-induced NF-κB activation. One of the possible explanations is that crmA and BD-fmk, which are serphin inhibitors of caspase-8, may block the signal transduction from FADD and Casper to caspase-8, but not from caspase-8 to downstream NF-κB activation pathway.

Activation of NF-κB by FADD, Casper, and caspase-8 was inhibited by dominant negative mutants of TRAF2, NIK, and IKKs (Fig. 3), suggesting that FADD, Casper, and caspase-8 activate NF-κB through TRAF2-, NIK-, and IKK-dependent pathways. A dominant negative mutant of RIP inhibits FADD- and caspase-8-induced but not Casper-induced NF-κB activation, suggesting that RIP is required for FADD- and caspase-8-induced but is dispensable for Casper-induced NF-κB activation. The mechanism behind this difference is currently unknown.

The caspase-specific inhibitors crmA and BD-fmk, which inhibit FADD- and Casper-induced NF-κB activation (Fig. 2), also partially inhibit TNF-R1-, TRADD-, and TNF-induced NF-κB activation (Fig. 4). In addition, a Casper mutant, Casper(192-480Y/F), partially inhibits TNF-R1-, TRADD-, and TNF-induced NF-κB activation (Fig. 4). Based on these data, we hypothesize that two parallel cascades bifurcating at TRADD are responsible for the full NF-κB activation induced by TNF-R1. The primary cascade is activated through direct interaction of TRADD with TRAF2 and RIP. In the second cascade, TRAF2 and RIP are activated by FADD, Casper, and caspase-8.

Our data are consistent with the observation that Fas, another death receptor that recruits FADD, Casper, and

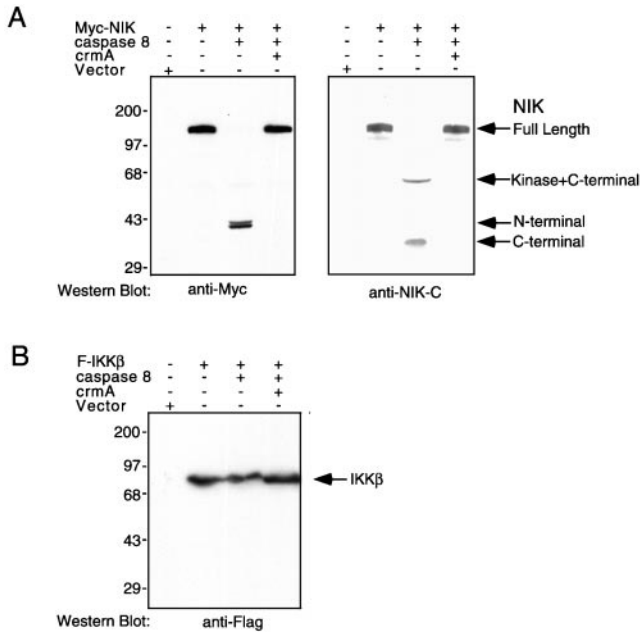

FIG. 5. **NIK, but not IKK*β*, is processed by caspase-8.** 293 cells (2×10^5) were transfected with expression vectors for N-terminal Myc epitope-tagged NIK (*A*) or N-terminal Flag epitope-tagged IKK*β* (*B*), together with various combinations of plasmids as indicated at the *top*. 11 h after transfection, cells were lysed, and Western blotting experiments were performed with an anti-Myc antibody (*A, left panel*), a rabbit polyclonal antibody against the C-terminal domain (aa 700–947) of NIK (*A, right panel*), or an anti-Flag antibody (*B*).

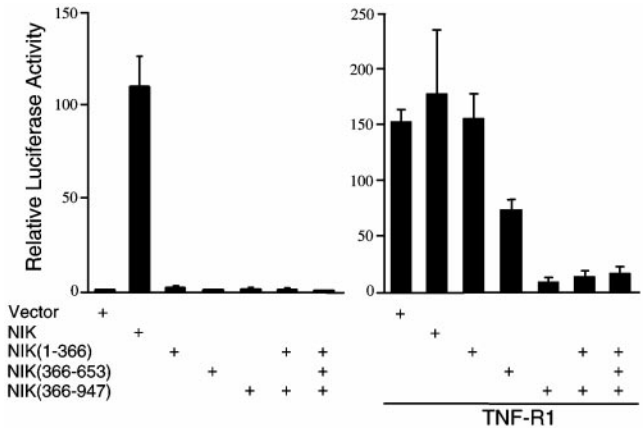

FIG. 6. **Inhibition of TNF-R1-induced NF-κB activation by the processed NIK fragments.** 293 cells (2×10^5) were transfected with 2 μg of the indicated plasmids (+), together with 1 μg of an empty control plasmid (*left panel*) or an expression plasmid for TNF-R1 (*right panel*). Luciferase reporter gene assays were performed 14 h after transfection. Data shown are relative luciferase activities compared with the empty control plasmid.

caspase-8 but not TRADD, TRAF2, or RIP to its signaling complex (2, 6, 7, 20, 38, 32), can activate NF-κB (39, 40).

Casper can induce apoptosis or, in other cases, prevent cells from apoptosis induced by TNF receptor family members (20–24). A similar observation has been made for FADD. Although it has been well documented that FADD is required for TNF-R1-, Fas-, and TRAIL-R1(DR4)-induced apoptosis (2, 32, 33, 35, 41, 42), other studies suggest that FADD is necessary for T cell receptor-mediated proliferation of T lymphocytes (42). Recently, it has been shown that caspase activation is also required for T cell proliferation (43). Gene knockout experiments indicate that both FADD and caspase-8 are required for the normal development of heart (41, 42, 44). One of the explanations for this observation is that FADD and caspase-8 may protect cells from apoptosis or may be required for cell survival and proliferation during heart development. These apparent discrepancies on the functions of FADD, Casper, and caspase-8 may be explained by the observations that these proteins can induce both apoptosis and the anti-apoptotic transcription factor NF-κB. Recently, it has been proposed that NF-κB activation may protect cells from apoptosis, probably through transcriptional induction of apoptosis inhibitors (45–50). In this context, FADD, Casper, and caspase-8 may inhibit apoptosis or promoting cell survival and proliferation by activating the anti-apoptotic transcription factor NF-κB.

The fact that FADD, Casper, and caspase-8 can activate NF-κB may explain the observation that in most cell types, TNF family members do not induce apoptosis in the absence of transcription or translation inhibitors. It is possible that inhibitory proteins induced by NF-κB may antagonize the apoptotic effect induced by the FADD-Casper-caspase-8 cascade and that the fate of a cell following FADD, Casper, and caspase-8 activation is determined by the relative signaling levels leading to apoptosis and NF-κB activation. Our findings imply that apoptosis signaling by the FADD-Casper-caspase 8 cascade is continuously orchestrated by a negative feedback control mechanism through its intrinsic ability to activate the anti-apoptotic transcription factor NF-κB.

Our findings also indicate that caspase-8 can cleave NIK into three fragments: the N-terminal domain, the intermediate kinase domain, and the C-terminal domain. We further show that the processed fragments of NIK can not activate NF-κB. Instead, NIK(366–947) potently inhibited TNF-R1-induced NF-κB activation. Previously, it has been shown that NIK(624–947) can also function as a dominant negative mutant for TNF-induced NF-κB activation (8, 10, 51). A recent study indicates that RIP, a protein kinase critically involved in TNF-R1-induced NF-κB activation, is also cleaved by activated caspase-8 (36). Furthermore, the cleavage of RIP results in the blockage of TNF-induced NF-κB activation (36). Taken together, these observations suggest that the caspase-8 precursor can activate NF-κB, whereas the activated caspase-8 can negatively regulate TNF-R1-induced NF-κB activation through inactivating RIP and NIK.

Based on our data, we propose the following working model. Once FADD, Casper, and caspase-8 precursor are recruited to TNF-R1 complex, they signal NF-κB activation through TRAF2-, RIP-, NIK-, and IKK-dependent pathways. Recruitment of caspase-8 precursor to TNF-R1 signaling complex can also subsequently result in proteolytic processing and activation of caspase-8. The activated caspase-8 can then cleave RIP and NIK, a process resulting in negative regulation of TNF-R1-induced NF-κB activation.

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