The C-terminal Domain of the Long Form of Cellular FLICE-inhibitory Protein (c-FLIP_L) Inhibits the Interaction of the Caspase 8 Prodomain with the Receptor-interacting Protein 1 (RIP1) Death Domain and Regulates Caspase 8-dependent Nuclear Factor κB (NF-κB) Activation

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Background: Caspase 8 and c-FLIP_L regulate NF-κB activation via RIP1.
Results: The caspase 8 prodomain mediates NF-κB activation, and its interaction with the RIP1 death domain is inhibited by c-FLIP_L but not c-FLIP(p43).
Conclusion: The C-terminal domain of c-FLIP_L inhibits the interaction of caspase 8 with the RIP1 death domain and caspase 8-dependent NF-κB activation.
Significance: This study provides a novel molecular mechanism by which c-FLIP_L cleavage is required for NF-κB activation.

Caspase 8 plays an essential role in the regulation of apoptotic and non-apoptotic signaling pathways. The long form of cellular FLICE-inhibitory protein (c-FLIP_L) has been shown previously to regulate caspase 8-dependent nuclear factor κB (NF-κB) activation by receptor-interacting protein 1 (RIP1) and TNF receptor-associated factor 2 (TRAF2). In this study, the molecular mechanism by which c-FLIP_L regulates caspase 8-dependent NF-κB activation was further explored in the human embryonic kidney cell line HEK 293 and variant cells barely expressing caspase 8. The caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone greatly diminished caspase 8-dependent NF-κB activation. The Caspase 8 prodomain mediates NF-κB activation and, thereby, regulates caspase 8-dependent NF-κB activation...
in response to Fas or antigen receptors, caspase 8 can trigger the activation of the transcription factor nuclear factor κB (NF-κB) that drives many target genes, including antiapoptotic proteins (19–22).

Cellular FLICE-inhibitory protein (c-FLIP) is a crucial modulator of caspase 8, existing in the various splicing forms c-FLIPL, c-FLIPs, and c-FLIPS (23, 24). The full-length c-FLIPL shows overall homology to caspase 8 and contains two N-terminal DEDs followed by the protease domain that lacks the catalytic cysteine, whereas c-FLIPS and c-FLIPs contain only two DEDs and a short C-terminal tail (25, 26). Upon heterodimerization with caspase 8 in the DISC, c-FLIPL is processed at Asp-376 into an N-terminal p43 fragment designated c-FLIP(p43) and modulates caspase 8 catalytic activity (27, 28). c-FLIP is known to play a regulatory role in the apoptotic and non-apoptotic signaling pathways (25, 26, 29–31). c-FLIPL inhibits death receptor-mediated apoptosis by preventing the homodimerization of caspase 8 in the DISC, but it can also exert proapoptotic activity under limited conditions (32). c-FLIP possesses the ability to induce the activation of the NF-κB signaling pathway (33–39), and we have shown previously that c-FLIP(p43) is able to induce NF-κB activation by interaction with TNF receptor-associated factor 2 (TRAF2) (36). c-FLIP(p22), another c-FLIP fragment cleaved by caspase 8 at Asp-196, has also been shown to mediate NF-κB activation by binding to the inhibitor of NF-κB (IκB) kinase complex (38). By contrast, it has been shown that c-FLIPL and c-FLIPS inhibit NF-κB activation induced by FasL (19, 20, 40). These seemingly contradictory findings may be explained partly by the notion that c-FLIPL and its isoforms can promote or inhibit Fas-mediated NF-κB activation, depending on their expression levels and cellular context (39).

RIP1 is known to be critical to induce NF-κB activation in response to death receptors and other stimuli (41, 42). We have shown that caspase 8 promotes the interaction between RIP1 and c-FLIP(p43) (37). It has also been demonstrated that c-FLIP(p43) interacts with the IκB kinase complex upon Fas stimulation (39). Thus, in addition to the specific interaction between c-FLIP(p43) and TRAF2 (36), these studies collectively indicate the importance of c-FLIPL processing in the activation of the NF-κB signaling pathway. In this study, we have further explored the molecular mechanism by which c-FLIPL processing regulates NF-κB activation. Our results demonstrate that the C-terminal domain of c-FLIPL inhibits the interaction between the caspase 8 prodomain and the RIP1 DD and, thereby, regulates caspase 8-dependent NF-κB activation.

**EXPERIMENTAL PROCEDURES**

**Cells**—HEK293 cells (ICR9068) were provided by the Health Science Research Resources Bank (Osaka, Japan). HEK293 cells and HEK293C8L cells (HEK293 cells expressing caspase 8 at very low levels) (36, 43) were maintained in DMEM (Invitrogen) supplemented with 10% (v/v) heat-inactivated FCS (Nichirei Biosciences, Tokyo, Japan) and penicillin/streptomycin/gentamicin mixture (Nacalai Tesque, Kyoto, Japan). **Antibodies**—Antibodies to β-actin (AC-15, Sigma-Aldrich, St. Louis, MO), human caspase 8 (5D3 and 5F7, Medical & Biological Laboratories, Nagoya, Japan), FADD (clone 1, BD Biosciences), Fas (B-10, Santa Cruz Biotechnology, Santa Cruz, CA), FLAG (M2, Sigma-Aldrich), c-FLIP (Dave-2, Alexis, Lausen, Switzerland), RIP1 (clone 38, BD Biosciences, and H-207, Santa Cruz Biotechnology), TNF receptor 1 (H-5, Santa Cruz Biotechnology), TNF receptor 1-associated death domain protein (TRADD) (C-20, Santa Cruz Biotechnology), TRAF2 (C-20, Santa Cruz Biotechnology), and vesicular stomatitis virus (VSV) (PSD4, Sigma-Aldrich) were obtained commercially. **Reagents**—Benzoyloxy carbonyl-Val-Ala-Asp(Ome)-fluoromethyl ketone (zVAD-fmk) was purchased from the Peptide Institute (Osaka, Japan). Recombinant human TNF-α was provided by Dainippon Pharmaceutical (Osaka, Japan). FLAG-tagged human soluble FasL (139–281) was produced in supernatants of HEK293T cells and concentrated by Ultracell®-10K (Merck Millipore, Darmstadt, Germany). FLAG-tagged FasL was further purified with agarose beads conjugated to anti-FLAG antibody M2 (Sigma-Aldrich). FLAG-tagged FasL in a purified form or concentrated supernatants by ultrafiltration were cross-linked with anti-FLAG antibody (M2, 0.5 μg/ml) and used for experiments.

**Expression Vectors**—pCR3 expression vectors containing either a FLAG tag or VSV tag for human c-FLIPL, human c-FLIP(p43), human c-FLIPS, human c-FLIPL(D376A), human caspase 8, caspase 8(p43), mouse catalytically inactive caspase 8(C362G), and human RIP1 have been described previously (36). Human caspase 8 deletion mutants (1–178, 1–216, 217–274, 217–479, 375–479, and 385–479), human caspase 8(C362G), and human RIP1 deletion mutants (1–324, 325–671, 1–558, 325–558, and 559–671) were generated by PCR amplification and subcloned into pcCR3-based expression vectors containing a FLAG tag or VSV tag. The human DED family of proteins was generated by PCR amplification using cDNA libraries prepared from human cancer cell lines (A549 cells for DED-containing DNA-binding protein 2 (DEDD2), phosphoprotein enriched in astrocytes 15 kDa (DEDD2), phosphoprotein 1–558, 325–558, and 559–671) were generated by PCR amplification and subcloned into pcCR3-based expression vectors containing a FLAG tag. **Assay for Apoptosis**—Cells were stained with Hoechst 33342 (10 μM, Merck Millipore) for the last 30 min of incubation. The stained cells were washed with PBS and observed for nuclear morphology under a fluorescence light microscope (Axiovert 200 M, Carl Zeiss, Jena, Germany). Apoptosis (percent) was calculated as (condensed nuclei / total nuclei) × 100.

**Reporter Assay**—Cells were seeded in 24-well culture plates the day before transfection. The cells were transfected with various expression vectors together with reporter vectors encoding an NF-κB-responsive firefly luciferase gene (80–300 ng/well), a CMV promoter-driven Renilla luciferase gene (20–100 ng/well), or a β-galactosidase gene (200–400 ng/well) by calcium phosphate method or Lipofofectamine™ 2000 (Invitrogen). The NF-κB-responsive firefly luciferase gene was controlled by two copies of κB sequences from the Igκ enhancer (37). The transfected cells were solubilized in digitonin lysis buffer (10 mM Hepes-KOH (pH 7.2), 100 μM digitonin, 250 mM sucrose, 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, and 1 mM DTT) and centrifuged (15,000 × g, 5 min) to sediment insoluble
c-FLIP<sub>L</sub> Inhibits Interaction between Caspase 8 and RIP1

materials. Aliquots of supernatants were mixed with either firefly luciferase assay solution (25 mM Tris-phosphate (pH 7.8), 10 mM MgCl₂, 15% glycerol, 1% Triton X-100, 1% bovine serum albumin, 0.25 mM luciferin, 1 mM ATP, and 1 mM DTT) or Renilla luciferase assay solution (100 mM potassium phosphate (pH 7.5), 500 mM NaCl, 1 mM EDTA, and 24 μM coelenterazine). Relative light units were immediately measured with a Lumitester K-100 luminometer (Hamamatsu, Japan). β-Galactosidase activity was determined by incubation of aliquots of supernatants with Z buffer (100 mM sodium phosphate (pH 7.5), 10 mM KCl, 1 mM MgSO₄, and 40 mM β-galactosidase) at 37 °C until a yellow color developed. Absorbance at 415 nm was measured with a Model 680 microplate reader (Bio-Rad). Values of Renilla luciferase activity and β-galactosidase activity were used to normalize transfection efficiency.

Immunoprecipitation—Cells were seeded in 60-mm culture dishes the day before transfection. The cells were transfected with various expression vectors, including FLAG-tagged expression vectors (total 2 to 4 μg DNA), by the calcium phosphate method. The cells were harvested, washed with PBS, and solubilized in Nonidet P-40 lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 2 mM sodium vanadate) containing the protease inhibitor mixture Complete™ (Roche Diagnostics). After repeated centrifugation (15,000 × g, 5 min), postnuclear lysates were precleared with Sepharose 6B (Sigma-Aldrich) for 1 h and then immunoprecipitated with anti-FLAG M2 affinity gel (Sigma-Aldrich) for 3 h. The affinity gel was washed several times with Nonidet P-40 lysis buffer.

Western Blotting—Cells were washed with PBS and solubilized in Triton X-100 lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Triton X-100, and 2 mM dithiothreitol) containing the protease inhibitor mixture Complete™ (Roche Diagnostics). Postnuclear lysates were collected as supernatants by centrifugation (15,000 × g, 5 min). Protein samples (30 μg/lane) were separated by SDS-PAGE and transferred onto Hybond-ECL nitrocellulose membranes (GE Healthcare). The membranes were incubated with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Blots of either β-actin or equally transfected proteins for immunoprecipitation were used for loading controls. Protein bands were detected by ECL Western blotting detection reagent (GE Healthcare) and detected by exposure to Hyperfilm™ ECL (GE Healthcare) or ImageQuant LAS 4000 Mini (GE Healthcare). ImageQuant LAS 4000 Mini was used to quantify the intensity of the bands.

Statistical Analysis—Statistical significance was assessed by one-way analysis of variance followed by the Tukey’s test for multiple comparisons. Differences of p < 0.05 were considered to be statistically significant.

RESULTS

Caspase 8 Is Required for FasL-induced NF-κB Activation—HEK293 cells expressed Fas and TNF-R1 as well as caspase 8, FADD, TNF receptor 1-associated death domain protein, RIP1, and TRAF2, whereas HEK293C8L cells expressed caspase 8 at very low levels while expressing the other proteins at levels similar to HEK293 cells (Fig. 1A). c-FLIP<sub>L</sub> and c-FLIP<sub>p43</sub> were only weakly expressed in HEK293 and HEK293C8L cells (Fig. 1B and data not shown). Upon FasL stimulation, ~35% of HEK293 cells underwent apoptosis within 6 h, whereas HEK293C8L cells did not (Fig. 1C). In HEK293C8L cells, apoptosis was increased in response to FasL stimulation when wild-type caspase 8, but not catalytically inactive caspase 8, was transiently expressed (Fig. 1D). NF-κB activation was induced by FasL stimulation in HEK293 cells at levels several times lower than TNF-α stimulation (Fig. 1E). In HEK293C8L cells, FasL was unable to induce NF-κB activation (Fig. 1F). When catalytically inactive caspase 8 was transiently expressed in HEK293C8L cells, NF-κB activation was induced by FasL (Fig. 1F). Thus, these results indicate that caspase 8 is required for FasL-induced NF-κB activation in HEK293C8L cells.

Augmented NF-κB Activation by c-FLIP<sub>L</sub>, but Not c-FLIP(p43), Requires Caspase Activity—To examine the effect of c-FLIP on FasL-induced NF-κB activation, HEK293 cells were transiently transfected with c-FLIP<sub>L</sub> or its cleaved form, c-FLIP(p43), and then stimulated with FasL. c-FLIP<sub>L</sub> and c-FLIP(p43) alone increased NF-κB activation at high concentrations in HEK293 cells (Fig. 2, A and B). Nevertheless, FasL was able to augment NF-κB activation in HEK293 cells transfected with c-FLIP<sub>L</sub> or c-FLIP(p43) (Fig. 2, A and B). The stimulation index of FasL-induced NF-κB activation became smaller when c-FLIP<sub>L</sub> or c-FLIP(p43) augmented NF-κB activation by themselves (Fig. 2, A and B). The caspase inhibitor zVAD-fmk blocked NF-κB activation induced by c-FLIP<sub>L</sub>, whereas it only marginally affected that induced by c-FLIP(p43) or RIP1 in HEK293 cells (Fig. 2C). This is consistent with our previous observation with HEK293T cells that expressed much higher amounts of protein products (36). To examine the effect of c-FLIP<sub>L</sub> processing on NF-κB activation induced by FasL, HEK293 cells were transiently transfected with c-FLIP<sub>L</sub> or c-FLIP(p43) and then stimulated with FasL in the presence or absence of zVAD-fmk. In mock-transfected HEK293 cells, zVAD-fmk did not obviously affect FasL-induced NF-κB activation (Fig. 2D). However, interestingly, zVAD-fmk markedly inhibited FasL-induced NF-κB activation in HEK293 cells transiently transfected with c-FLIP<sub>L</sub> (Fig. 2D). By contrast, FasL-induced NF-κB activation was relatively insensitive to zVAD-fmk in c-FLIP(p43)-transfected cells (Fig. 2D). In mock-transfected HEK293 cells, FasL stimulation induced the cleavage of caspase 8 from p55/p53 into p43/p41 and p18 (Fig. 2, E–G). In HEK293 cells transfected with c-FLIP<sub>L</sub>, FasL stimulation increased the cleavage of c-FLIP<sub>L</sub> into c-FLIP(p43) (Fig. 2F). zVAD-fmk efficiently inhibited the processing of c-FLIP<sub>L</sub> and caspase 8 (Fig. 2, E–G). By contrast, the FasL-induced cleavage of caspase 8 into its p43/p41 and p18 fragments was not decreased by expression of c-FLIP<sub>L</sub> or c-FLIP(p43) (Fig. 2, E–G). This might be due to the fact that the dose of FasL was high and overcame the effect of c-FLIP<sub>L</sub> or c-FLIP(p43) or that the time point was sufficiently long after FasL stimulation to allow substantial caspase 8 cleavage to proceed even with c-FLIP<sub>L</sub> or c-FLIP(p43). It might be also possible that c-FLIP<sub>L</sub> or c-FLIP(p43) was expressed between the levels that promote or inhibit the processing of caspase 8 because it
has been shown that c-FLIPL can act as both an activator and an inhibitor of caspase 8 (44–46). Overall, these results suggest that augmented NF-κB activity by c-FLIPL, but not c-FLIPL(p43), requires caspase activity in HEK293 cells.

Caspase 8 Interacts with the RIP1 DD—RIP1 is composed of a kinase domain, an intermediate domain, a RIP homotypic interaction motif, and a C-terminal DD (Fig. 3A). RIP1(559–671) contains only a DD and serves as a dominant-negative form that blocks RIP1-mediated NF-κB activation. In our previous study, caspase 8 was found to interact with endogenous RIP1 but not TRAF2 (36, 37). To identify the RIP1 region responsible for interacting with caspase 8, FLAG-tagged RIP1 deletion mutants were transiently coexpressed with caspase 8, FLAG-tagged RIP1 deletion mutants were transiently coexpressed with caspase 8 (dark gray bar) or an empty vector (light gray bar) in 16 h and then incubated with or without −FasL (200 ng/ml) for 6 h (D). Apoptosis (percent) was measured by Hoechst 33342 staining. Results represent the mean ± S.D. of triplicates. **, p < 0.01 compared with FasL (−). Data are representative of at least three independent experiments.

The Caspase 8 Prodomain Interacts with the RIP1 DD—Caspase 8 is composed of two DEDs and a caspase domain consisting of large (p18) and small (p10) subunits (Fig. 4A). To identify the region of caspase 8 responsible for the interaction with RIP1(559–671), RIP1(559–671) was transiently coexpressed with FLAG-tagged caspase 8 deletion mutants in HEK293C8L cells (Fig. 4B). RIP1(559–671) was immunoprecipitated with caspase 8, caspase 8(p43), caspase 8(1–216), and caspase 8(1–178) in a dose-dependent manner, caspase 8(1–216), and caspase 8(1–178) were weaker pulled down RIP1 but not other RIP family members that lack a DD (i.e. RIP2, RIP3, and RIP4) (Fig. 3E).

The Caspase 8 Prodomain Is Sufficient to Induce NF-κB Activation by FasL or c-FLIP(p43)—NF-κB activation was induced by overexpression of caspase 8, caspase 8(p43), caspase 8(1–216), and caspase 8(1–178) in a dose-dependent manner, although caspase 8(1–216) and caspase 8(1–178) were weaker
c-FLIP<sub>L</sub> Inhibits Interaction between Caspase 8 and RIP1

![Graphs and images showing the inhibition of NF-κB activation by c-FLIP<sub>L</sub> and c-FLIP(p43) in various conditions involving FasL and zVAD-fmk.](image-url)
to induce NF-κB activation compared with caspase 8 and caspase 8(p43) (Fig. 5A and data not shown). By contrast, neither caspase 8(217–374), caspase 8(217–479), or caspase 8(375–479), nor caspase 8(385–479), induced NF-κB activation in HEK293C8L cells (Fig. 5A). FasL was able to induce NF-κB activation in HEK293C8L cells transfected with caspase 8(1–178) (Fig. 5B). These data indicate that the caspase 8 prodomain is sufficient to mediate FasL-induced NF-κB activation.

c-FLIP(p43) was able to induce NF-κB activation in HEK293C8L cells when catalytically inactive caspase 8 was coexpressed (Ref. 36 and data not shown). In addition to full-length caspase 8, c-FLIP(p43) was found to induce NF-κB activation markedly when HEK293C8L cells were cotransfected with caspase 8(1–178) and caspase 8(1–216) (Fig. 5, C and D). NF-κB activation induced by c-FLIP(p43) plus caspase 8(1–178) or caspase 8(1–216) was inhibited by RIP1(559–671) (Fig. 5, E and F). Thus, these data indicate that the caspase 8 prodomain is sufficient to mediate c-FLIP(p43)-induced NF-κB activation in a RIP1-dependent manner.

The C-terminal Region of c-FLIPL Inhibits the Interaction between RIP1 DD and Caspase 8—We further investigated whether c-FLIPL regulates the interaction between RIP1 and caspase 8. Endogenous RIP1 was pulled down efficiently with caspase 8 and caspase 8(p43) but barely with c-FLIP(p43) and c-FLIP(p43) in HEK293C8L cells (37). These data indicate that RIP1 interacts with caspase 8 or caspase 8(p43) more preferentially than c-FLIP(p43) or c-FLIP(p43). Moreover, because caspase 8, but not c-FLIP(p43), interacted with the RIP1 DD (Fig. 4C), we hypothesized that c-FLIP(p43) may inhibit the interaction between caspase 8 and the RIP1 DD. To address whether c-FLIP(p43) inhibits the binding of the RIP1 DD to caspase 8, RIP1(559–671) was immunoprecipitated with caspase 8 in the presence of c-FLIP(p43) in HEK293C8L cells. zVAD-fmk was included to prevent the processing of c-FLIP(p43) into c-FLIP(p43). c-FLIP(p43) and c-FLIP(p43) were immunoprecipitated with caspase 8 with similar efficacy (Fig. 6A). The interaction of RIP1(559–671) with caspase 8 was decreased markedly when c-FLIP(p43) was coexpressed (Fig. 6A). By contrast, c-FLIP(p43) allowed the interaction between caspase 8 and RIP1(559–671) (Fig. 6A). Thus, these data indicate that the C-terminal region of c-FLIP(p43) inhibits the interaction between the RIP1 DD and caspase 8.

In HEK293 cells, c-FLIPL or its uncleavable form, c-FLIPL(D376A), induced spontaneous NF-κB activation that was only weakly inhibited by RIP1(559–671) (Fig. 6, B and C). In addition, FasL was able to induce NF-κB activation in the presence of c-FLIP(p43) but exerted weaker NF-κB activation in the presence of c-FLIP(p43)(D376A) (Fig. 6, B and C). FasL-induced NF-κB activation in the presence of c-FLIP(p43), but not c-FLIP(p43)(D376A), was inhibited by RIP1(559–671) (Fig. 6C). RIP1(559–671) also strongly blocked FasL-induced and spontaneous NF-κB activation in the presence of c-FLIP(p43) (Fig. 6C). These results support the view that c-FLIPL regulates a RIP1-dependent and a RIP1-independent pathway to NF-κB activation, whereas c-FLIP(p43) regulates only a RIP1-dependent pathway, and c-FLIPL(D376A) regulates only a RIP1-independent pathway. Overall, these findings are consistent with a model in which caspase 8 activity is necessary to cleave c-FLIP(p43) to c-FLIP(p43) to promote a RIP1-dependent pathway of NF-κB activation, which may be the main c-FLIP pathway at normal physiological levels.

**DISCUSSION**

Caspase 8 and its modulator, c-FLIP(p43), were reported to regulate NF-κB activation in response to FasL stimulation. We found that FasL-induced NF-κB activation was diminished by zVAD-fmk only in the presence of c-FLIP(p43) but not c-FLIP(p43). The caspase 8 prodomain was identified to interact with the RIP1 DD and to be sufficient to mediate NF-κB activation induced by FasL or c-FLIP(p43). Moreover, c-FLIPL, but not c-FLIP(p43), inhibited the interaction between the RIP1 DD and caspase 8. Thus, these results reveal that the C-terminal domain of c-FLIPL specifically inhibits the interaction between the caspase 8 prodomain and the RIP1 DD and, thereby, regulates caspase 8-dependent NF-κB activation in response to FasL stimulation.

Caspase 8 is essential to induce NF-κB activation in response to FasL stimulation (19, 20). By contrast, c-FLIPL and c-FLIPS have been reported to inhibit FasL-induced NF-κB activation (19, 20, 30). Recently, it has been shown that subtle differences in concentrations of c-FLIP isoforms determine apoptosis induction as well as NF-κB activation in the Fas signaling pathway (32, 39). Thus, it is mostly likely that c-FLIP isoforms are able to regulate FasL-induced NF-κB activation positively or negatively under various cellular conditions. Even though the amount of c-FLIP isoforms is much smaller than that of caspase 8, they are efficiently recruited to the DISC because of their higher affinity to the DISC compared with caspase 8 (47). Because c-FLIPL and c-FLIPS inhibit the recruitment of caspase 8 to the Fas-DISC (40, 48), it is possible that the inhibition of FasL-induced NF-κB activation by c-FLIPL or c-FLIPS is ascribed largely to the inhibition of caspase 8 recruitment to the Fas signaling complex. Thus, these findings are consistent with a model in which caspase 8 activity is necessary to cleave c-FLIP(p43) to c-FLIP(p43) to promote a RIP1-dependent pathway of NF-κB activation, which may be the main c-FLIP pathway at normal physiological levels.
**FIGURE 3. Caspase 8 interacts with RIP1 DD.**

**A**, schematic of RIP1 constructs showing that RIP1 is composed of a kinase domain (KD), an intermediate domain (ID), a RIP homotypic interaction motif (RHIM), and a DD. The arrow indicates the cleavage site by caspase 8. B and C, HEK293C8L cells were transfected with expression vectors for FLAG-tagged RIP1 (1–671) or its deletion mutants or an empty vector (control) together with expression vectors for VSV-tagged caspase 8 (8) or VSV-tagged caspase 8(p43) (C) for 24 h. Cell lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates (IP) were analyzed by Western blotting using anti-caspase 8 antibody. Data are representative of at least three independent experiments.

**D**, HEK293C8L cells were transfected with expression vectors for FLAG-tagged caspase 8, FLAG-tagged caspase 8(p43), or an empty vector (control) together with an expression vector for VSV-tagged RIP1(559–671) for 24 h. Cell lysates were analyzed by Western blotting using anti-FLAG antibody for caspase 8 and anti-VSV antibody for RIP1(559–671). Data are representative of three independent experiments.

**E**, HEK293C8L cells were transfected with (+/H11001) or without (−/H11002) expression vectors for FLAG-tagged caspase 8 and the VSV-tagged RIP family of proteins for 24 h. Cell lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were analyzed by Western blotting using anti-VSV antibody for the RIP family of proteins. Cell lysates were analyzed by Western blotting using anti-FLAG antibody for caspase 8 and anti-VSV antibody for the RIP family of proteins. Data are representative of two independent experiments.
Nevertheless, it has been shown that c-FLIPL does not inhibit caspase 8 recruitment but, rather, promotes the formation of the caspase 8(p43)-c-FLIP(p43) heterodimers in the Fas-DISC (32, 35). Moreover, c-FLIPL was found to increase the recruitment of RIP1 and TRAF2 to the Fas DISC (35). Thus, c-FLIPL is most likely to mediate NF-κB activation in response to FasL stimulation only when c-FLIPL does not inhibit the recruitment of caspase 8 to the Fas DISC but inhibits the full activation of caspase 8, which leads to the formation of the active heterotetramer.

We have shown that FasL-induced NF-κB activation is regulated by c-FLIP L at the step of caspase 8-dependent cleavage in the DISC. c-FLIP L is processed by caspase 8 into N-terminal c-FLIP(p43) and C-terminal c-FLIP(p12) in the DISC. The caspase 8 complex associated with either c-FLIP L or c-FLIP(p43) exerts enzymatic activity (27, 28). It has been shown recently that the enzymatic activity of the caspase 8-c-FLIP complex inhibits RIP1/RIP3-dependent necrosis (17). By contrast, the enzymatic activity of the caspase 8-c-FLIP(p43) complex is dispensable for FasL-induced NF-κB activation because zVAD-fmk did not inhibit FasL-induced NF-κB activation in the presence of c-FLIP(p43). However, zVAD-fmk markedly inhibited NF-κB activation induced by FasL stimulation only in the presence of c-FLIPL. Consistent with these results, it has been shown previously that zVAD-fmk inhibits caspase 8-dependent NF-κB activation induced by overexpression of c-FLIPL (34, 36, 38). These studies indicate that c-FLIPL processing to c-FLIP(p43) by caspase 8 is indispensable for FasL-induced NF-κB activation. In response to FasL stimulation, it is possible that caspase inhibitors such as zVAD-fmk promote NF-κB activation by suppressing caspase 8-dependent apoptosis in Fas-susceptible cells expressing no c-FLIP isoforms. However, as shown in this paper, caspase inhibitors are also likely to block NF-κB activation by suppressing the cleavage of c-FLIPL into c-FLIP(p43) in Fas-resistant cells expressing a sufficient amount of c-FLIPL.

As discussed above, caspase 8 activity is required for c-FLIPL cleavage into c-FLIP(p43) but not for downstream molecular events in the FasL-induced NF-κB signaling pathway. We showed previously that c-FLIPL, but not c-FLIP L, specifically interacts with TRAF2 (36). It has also been reported that c-FLIP(p43) interacts with the IkB kinase complex upon Fas stimulation (39). These studies collectively underscore the importance of c-FLIP L cleavage for FasL-induced NF-κB activation. Upon TNF-α stimulation, TNF receptor 1 recruits TNF receptor 1-associated death domain protein, which binds TRAF2 and RIP1, and in the membrane-associated complex both proteins undergo polyubiquitination, which recruits and activates the TGF-β-activated kinase (TAK1) complex and theIkB kinase complex (49). In agreement with this study, it has been shown that RIP1 plays an essential role in FasL-induced NF-κB activation (20). However, in contrast with TNF receptor 1, the caspase 8-c-FLIP(p43) heterodimers might be used as a

\[ c-FLIP_L \text{ Inhibits Interaction between Caspase 8 and RIP1} \]

**FIGURE 4. The caspase-8 prodomain interacts with RIP1 DD.** A, schematic of caspase 8 constructs showing that caspase 8 is composed of two DEDs and a caspase domain consisting of large (p18) and small (p10) subunits. The arrows indicate the cleavage sites by caspase 8, B and C, HEK293C8L cells were transfected with expression vectors for FLAG-tagged caspase 8 and its deletion mutants or an empty vector (control) together with an expression vector for VSV-tagged RIP1(559–671) for 24 h (B). HEK293C8L cells were transfected with expression vectors for the FLAG-tagged DED family of proteins or an empty vector together with an expression vector for VSV-tagged RIP1(559–671) for 24 h (C). Cell lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates (IP) were analyzed by Western blotting using anti-RIP1 antibody (B) and anti-VSV antibody (C) for RIP1(559–671). Cell lysates were analyzed by Western blotting using anti-FLAG antibody for caspase 8 (B) and the DED family of proteins (C) and anti-VSV antibody for RIP1(559–671). Data are representative of three (B) and two (C) independent experiments.
platform that recruits TRAF2 and RIP1 and induces NF-κB activation upon FasL stimulation.

RIP1 interacts with multiple proteins, including death receptors, adaptor proteins, and caspases (41, 42). Consistent with previous studies (33, 50), we have shown that RIP1 interacts with caspase 8. However, partly different from these studies (33, 50), we have found that the RIP1 DD is responsible for interaction with the caspase 8 prodomain. Other RIP family members that did not contain a DD (including RIP3) were not immunoprecipitated with caspase 8. Unlike transfected RIP1, which has been shown to interact with the c-FLIPL in HEK293T cells overexpressing both proteins (33), endogenous RIP1 was immunoprecipitated efficiently with c-FLIP (p43).

**FIGURE 5.** The caspase 8 prodomain is sufficient to mediate NF-κB activation induced by FasL or c-FLIP (p43). A, HEK293C8L cells were transfected with expression vectors for caspase 8 and its deletion mutants (1000 ng/well) together with reporter vectors encoding an NF-κB-responsive luciferase gene for 16 h. NF-κB activation (fold) is shown as mean ± S.D. of triplicates. **, *p < 0.01 compared with the control. Data are representative of four independent experiments. B, HEK293C8L cells were transfected with indicated concentrations (nanograms per well) of caspase 8(1–178) together with reporter vectors encoding an NF-κB-responsive luciferase gene for 16 h and then incubated with (black bars) or without (gray bars) FasL (200 ng/ml) for 6 h. NF-κB activation (fold) is shown as mean ± S.D. of triplicates. **, *p < 0.01 compared with the control at each concentration of caspase 8(1–178). Data are representative of three independent experiments. C and D, HEK293C8L cells were transfected with the indicated concentrations (nanograms per well) of expression vectors for caspase 8(1–216) (D) either with (black bars) or without (gray bars) expression vectors for c-FLIP (p43) (300 ng/well) together with reporter vectors encoding an NF-κB-responsive luciferase gene for 16 h. NF-κB activation (fold) is shown as mean ± S.D. of triplicates. **, *p < 0.01 compared with the control at each concentration of caspase 8(1–178) or caspase 8(1–216). Data are representative of three independent experiments. E and F, HEK293C8L cells were transfected with the indicated concentrations (nanograms per well) of an expression vector for RIP1 (559–671) either with (+) or without (−) expression vectors for caspase 8(1–178) (E) or caspase 8(1–216) (F) (100 ng/well) plus c-FLIP (p43) (300 ng/well) together with reporter vectors encoding an NF-κB-responsive luciferase gene for 16 h. NF-κB activation (fold) is shown as mean ± S.D. of triplicates. **, *p < 0.01 compared with the control at each concentration of caspase 8(1–178) or caspase 8(1–216) plus c-FLIP (p43). Data are representative of two independent experiments.
c-FLIP<sub>L</sub> Inhibits Interaction between Caspase 8 and RIP1

Caspase 8 and caspase 8(p43) but not c-FLIP<sub>L</sub>. Thus, it is most likely that RIP1 primarily binds caspase 8 via the DD-DED interaction in the caspase 8-c-FLIP(p43) heterodimers.

Ripoptosome is a large, 2-MDa intracellular complex that contains RIP1, FADD, caspase 8, caspase 10, and c-FLIP isoforms and regulates the cell death decision between caspase-dependent apoptosis and RIP1-dependent necrosis (52, 53). In the absence of cellular inhibitor of apoptosis proteins (c-IAPs), c-FLIP<sub>L</sub> inhibits the formation of Ripoptosome and blocks apoptosis and necrosis, whereas c-FLIP<sub>S</sub> promotes Ripoptosome formation and necrosis (52). The interaction of RIP1 with caspase 8 in the Ripoptosome is decreased by c-FLIP<sub>L</sub> but increased by c-FLIP<sub>S</sub> (52). This is in line with this study showing that the C-terminal domain of c-FLIP<sub>L</sub> inhibits the binding of RIP1-DD to caspase 8. However, in contrast to c-FLIP<sub>L</sub> being cleaved efficiently to c-FLIP(p43) by caspase 8 in the Fas-DISC, it is possible that the caspase 8-c-FLIP<sub>L</sub> heterodimer is not efficiently converted to the caspase 8-c-FLIP(p43) heterodimer in the Ripoptosome and that the caspase 8-c-FLIP<sub>L</sub> heterodimer is most likely to either interfere with RIP1 recruitment or mediate RIP1 cleavage and, thereby, prevent RIP1/RIP3-dependent necrosis.

Overexpression of c-FLIP<sub>L</sub> or its uncleavable form, c-FLIP<sub>L</sub>(D376A), induced spontaneous NF-κB activation that was not inhibited by RIP1(559–671). By contrast, FasL-induced NF-κB activation in the presence of c-FLIP<sub>L</sub> or c-FLIP(p43) was inhibited by RIP1(559–671). Thus, it is possible that c-FLIP<sub>L</sub> regulates a RIP1-dependent as well as a RIP1-independent NF-κB pathway, whereas c-FLIP(p43) regulates only a RIP1-dependent NF-κB pathway, and c-FLIP(D376A) regulates only a RIP1-independent NF-κB pathway. We have reported previously that a dominant-negative TRAF2 inhibits NF-κB activation induced by c-FLIP(p43) but not that induced by c-FLIP<sub>L</sub>(D376A) (36). Moreover, we have shown that TRAF2 interacts specifically with c-FLIP(p43) but not c-FLIP<sub>L</sub> (36). Thus, it is most likely that c-FLIP(p43) mediates NF-κB activation in a TRAF2/RIP1-independent manner, whereas c-FLIP(D376A) mediates NF-κB activation in a TRAF2/RIP1-independent manner. The molecular mechanism of the NF-κB signaling pathway induced by c-FLIP<sub>L</sub>(D376A) remains to be clarified.

How does c-FLIP<sub>L</sub> or c-FLIP(p43) regulate the interaction of caspase-8 and RIP1? In this study, we have shown that the C-terminal domain of c-FLIP<sub>L</sub> inhibits the interaction between caspase 8 and the RIP1 DD. We propose a working model for NF-κB activation regulated by caspase 8 and c-FLIP<sub>L</sub> (Fig. 7). When c-FLIP isoforms are not sufficiently expressed, caspase 8 undergoes homodimerization and autoprocessing in response to FasL stimulation, leading to the formation of active heterotetramers and the induction of apoptosis. In the presence of c-FLIP<sub>L</sub>, caspase 8 is able to heterodimerize predominantly with c-FLIP<sub>L</sub> in the Fas-DISC and, subsequently, cleaves c-FLIP<sub>L</sub> at Asp-376 to produce N-terminal c-FLIP<sub>L</sub>(p43) and C-terminal c-FLIP(p12). The C-terminal domain of c-FLIP<sub>L</sub> has an ability to inhibit the interaction between the caspase 8 DED and RIP1 DD by a currently unknown mechanism. The removal of c-FLIP(p12) allows caspase 8 to interact with RIP1. In addition, TRAF2 is specifically interacted with c-FLIP(p43) (36). The caspase 8-c-FLIP(p43) heterodimer subsequently promotes NF-κB activation via RIP1 and TRAF2 in a catalytic

**FIGURE 6. The C-terminal domain of c-FLIP<sub>L</sub> inhibits the interaction between the RIP1 DD and caspase 8. A, HEK293CBL cells were transfected with (+) or without (−) expression vectors for FLAG-tagged caspase 8, VSV-tagged c-FLIP<sub>L</sub>, VSV-tagged c-FLIP(p43), or VSV-tagged RIP1(559–671) for 24 h. zVAD-fmk (30 μM) was included to prevent the cleavage of c-FLIP<sub>L</sub> by caspase 8. Cell lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates (IP) were analyzed by Western blotting using anti-VSV antibody for both c-FLIP and RIP1(559–671) and anti-FLAG antibody. Cell lysates were analyzed by Western blotting using anti-VSV antibody for caspase 8 and anti-VSV antibody for both c-FLIP and RIP1(559–671). The asterisk indicates the heavy chain of mouse IgG. Data are representative of three independent experiments. B and C, HEK293 cells were transfected with expression vectors (100 ng/well) for c-FLIP<sub>L</sub>, c-FLIP(p43), c-FLIP(D376A), or an empty vector (control) and with (+) or without (−) RIP1(559–671) (200 ng/well) together with reporter vectors encoding an NF-κB-responsive luciferase gene for 16 h and then incubated with (+) or without (−) FasL (200 ng/ml) for 6 h. NF-κB activation (fold) is shown as mean ± S.D. of triplicates (B). Data are representative of at least three independent experiments. **, p < 0.01 compared with FasL (−) and RIP1(559–671) (−) in each c-FLIP transfection (C). NF-κB activation (percent) is shown as mean ± S.E. of five independent experiments. *p < 0.05; **, p < 0.01 compared with FasL (+).
c-FLIP<sub>L</sub> Inhibits Interaction between Caspase 8 and RIP1

![Image of interaction diagram](image-url)

**FIGURE 7. Schematic of NF-κB activation regulated by caspase 8 and c-FLIP<sub>L</sub>.** Caspase 8 is composed of two DEDs and a caspase domain. c-FLIP<sub>L</sub> is composed of two DEDs and a caspase-like domain that lacks a catalytic cysteine in the active site. RIP1 contains a kinase domain (KD) and a DD. Upon FasL stimulation, caspase 8 undergoes homodimerization and autoprocessing at three Asp residues, leading to the formation of active heterotetramers consisting of large (p18) and small (p10) caspase subunits that ultimately induce apoptosis. In the presence of c-FLIP<sub>L</sub>, caspase 8 forms a heterodimer with c-FLIP<sub>L</sub>, and then cleaves c-FLIP<sub>L</sub> at Asp376 to produce N-terminal c-FLIP<sub>L</sub>(p43) and C-terminal c-FLIP<sub>L</sub>(p12). The C-terminal domain of c-FLIP<sub>L</sub> inhibits the interaction between the caspase 8 DD and RIP1 DD in the caspase 8-c-FLIP<sub>L</sub> heterodimer. After c-FLIP<sub>L</sub>(p12) is removed, caspase 8 and c-FLIP<sub>L</sub>(p43) are allowed to interact with RIP1 and TRAF2, respectively, and thereby, induce NF-κB activation.

Activity-independent manner. It has been proposed that the caspase 8-c-FLIP<sub>L</sub> heterodimer inhibits RIP1/RIP3 activity to prevent necrosis in a catalytic activity-dependent manner (54). In several cell lines, the full-length RIP1 was detectable in the Fas DISC and associated with the caspase 8-c-FLIP<sub>L</sub>(p43) complex (35, 37, 51). Therefore, it is possible that RIP1 is not necessarily cleaved in the Fas DISC and plays an essential role in the promotion of NF-κB activation within the caspase 8-c-FLIP<sub>L</sub>(p43) complex.

In conclusion, we have shown that the C-terminal domain of c-FLIP<sub>L</sub> blocks FasL-induced NF-κB activation by inhibiting the interaction between RIP1 and caspase 8. Thus far, it is well known that c-FLIP<sub>L</sub> modulates the recruitment of caspase 8 to the DISC as well as the catalytic activity of caspase 8 by heterodimerization. Our current findings provide a novel molecular mechanism by which the caspase 8-dependent c-FLIP<sub>L</sub> cleavage regulates FasL-induced NF-κB activation and may partly explain previously controversial studies on a physiological role of c-FLIP<sub>L</sub> for FasL-induced NF-κB activation.

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