Selective Knockdown of the Long Variant of Cellular FLICE Inhibitory Protein Augments Death Receptor-mediated Caspase-8 Activation and Apoptosis*

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Death receptors trigger apoptosis by activating the apical cysteine proteases caspase-8 and -10 within a death-inducing signaling complex (DISC). c-FLIP (cellular FLICE inhibitory protein) is an enzymatically inactive relative of caspase-8 and -10 that binds to the DISC. Two major c-FLIP variants result from alternative mRNAs splicing: a short, 26-kDa protein (c-FLIPS) and a long, 55-kDa form (c-FLIPL). The role of c-FLIPS as an inhibitor of death receptor-mediated apoptosis is well established; however, the function of c-FLIPL remains controversial. Although overexpression of transfected c-FLIPL inhibits apoptosis, ectopic expression at lower levels supports caspase-8 activation and cell death. Simultaneous ablation of both c-FLIP variants augments death receptor-mediated apoptosis, but the impact of selective depletion of c-FLIPL on caspase-8 activation and subsequent apoptosis is not well defined. To investigate this, we developed small interfering RNAs that specifically knock down expression of c-FLIPL in several cancer cell lines and studied their effect on apoptosis initiation by Apo2L/TRAIL (Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand). Knockdown of c-FLIPL augmented DISC recruitment, activation, processing, and release of caspase-8, thereby enhancing effector-caspase stimulation and apoptosis. Thus, endogenous c-FLIPL functions primarily as an inhibitor of death receptor-mediated apoptosis.

Apoptosis is essential for development, tissue homeostasis, and immune function (1). The key mediators of apoptosis are caspases, a family of cysteine proteases that cleave a critical set of cellular proteins near specific aspartic acid residues (2). Apoptosis-inducing members of the tumor necrosis factor superfamily, such as Fas ligand (FasL) and Apo2L/TRAIL, activate caspases through the cell extrinsic apoptosis signaling pathway by engaging their respective “death” receptors: Fas and DR4 or DR5, leading to assembly of the DISC (3). Upon ligand stimulation, the adapter protein FADD (Fas-associated death domain) binds to the receptor through homophilic death domain interactions. FADD then recruits the apoptosis initiator proteases caspase-8 and -10, through homophilic death effector domain (DED) interactions. The proximity of caspase molecules in the DISC facilitates their dimerization and thereby stimulates their proteolytic activity (4–6). Activation of caspase-8 is followed by two self-processing events; the first cleaves a 10-kDa fragment (p10) from the full-length caspase, leaving an intermediate fragment (p43/41) at the DISC, and the second cleaves an 18-kDa fragment (p18) from the p43/41 intermediate, allowing formation of an active caspase-8 (p18/p10)2 heterotetramer, which is released into the cytosol. In turn, cytosolic caspase-8 catalyzes the cleavage and activation of downstream effector caspases, such as caspase-3 and -7, which execute the apoptotic death program.

c-FLIP (cellular FLICE inhibitory protein) is structurally related to procaspase-8 and -10 but lacks enzymatic activity (7, 8). At least 10 splice variants of c-FLIP exist on the mRNA level, but only two c-FLIP proteins are detected: a 26-kDa short form (c-FLIPS) and a 55-kDa long form (c-FLIPL) (9, 10). c-FLIPS resembles its viral counterpart, v-FLIP, consisting of two DEDs and a short C-terminal tail but entirely lacking a caspase-like domain (11, 12). In contrast, c-FLIPL contains two N-terminal DEDs and a C-terminal caspase-like domain, similar to caspase-8 and -10. However, c-FLIPL is proteolytically inactive, because it lacks a specific cysteine residue that is critical for caspase activity.

Both c-FLIP variants are capable of binding to the Fas DISC (7, 8). The presence of c-FLIPS or c-FLIPL in the Fas DISC does not preclude caspase-8 recruitment; rather, DISC-associated caspase-8c-FLIP complexes are formed (9, 13–15). Because c-FLIPS lacks caspase cleavage sites, it is not processed by apical caspases in the DISC. In contrast, c-FLIPL is cleaved between its large (p20) and small (p12) subunits by apical caspases in the DISC; however, a second cleavage between the large subunit and the N-terminal DEDs has not been detected, probably because of the lack of a conserved cleavage site (9). Interaction of caspase-8 with c-FLIPS does not support caspase-8 activation; therefore, binding of c-FLIPS to the Fas DISC inhibits Fas-mediated apoptosis (7, 8). Indeed, ectopic overexpression of c-FLIPS inhibits apoptosis induction by FasL or Apo2L/TRAIL (7, 8, 16). Conversely, it has been shown that ectopically expressed c-FLIPL at the Fas DISC can support caspase-8 activation (14, 15). Furthermore, recent studies with purified components show that c-FLIPL activates caspase-8 or -10 through heterodimerization (17). However, caspase activity within heterodimers with c-FLIPL permits only partial processing of caspase-8, resulting in cleavage between the large and small subunit, but not between the DEDs and the large subunit, similar to the cleavage of c-FLIPS described above (13). The partial processing of active caspase-8 in caspase-8/c-FLIPL...
hetereodimers retains active caspases-8 at the DISC, preventing its release into the cytosol (18).

Although the role of c-FLIP<sub>L</sub> as an inhibitor of death receptor-mediated apoptosis is well understood and resembles the activity of v-FLIP proteins (7, 8), the specific involvement of c-FLIP<sub>L</sub> in death receptor modulation remains controversial. Early experiments suggested that c-FLIP<sub>L</sub> promotes apoptosis, because massive overexpression of this variant by transient transfection spontaneously induced apoptosis in mammalian cells (10, 19–21). Yet subsequent studies found that ectopic overexpression of c-FLIP<sub>L</sub> inhibited Fas-mediated apoptosis (9, 11, 13, 19, 22, 23). Furthermore, stable overexpression of c-FLIP in tumor cells promoted tumor growth in vivo, consistent with anti-apoptotic action (24, 25). However, more recent work showed that ectopic c-FLIP<sub>L</sub> expression at levels that are roughly equivalent to those of endogenous c-FLIP<sub>L</sub> in HeLa and MCF7 cells augmented the induction of caspase-8 processing and apoptosis by FasL (15).

Embryonic fibroblasts from c-FLIP<sub>L</sub> gene knockout mice, which are deficient in both c-FLIP<sub>L</sub> variants, displayed increased sensitivity to death ligands (26, 27). Similarly, small interfering RNA (siRNA) knockdown of endogenous c-FLIP<sub>L</sub> and c-FLIPS enhanced apoptosis induction by FasL (28, 29) or Apo2L/TRAIL (30–34). To date, loss-of-function studies that specifically interrogate the role of c-FLIP<sub>L</sub> in modulating caspase-8 activation at the DISC have not been reported. To examine the involvement of endogenous c-FLIP<sub>L</sub> in caspase-8 activation, we developed siRNA oligonucleotides that selectively knock down the expression of either c-FLIP<sub>L</sub> or c-FLIPS in several cancer cell lines and studied the effect of these siRNAs on apoptosis initiation by Apo2L/TRAIL. Knockdown of c-FLIP<sub>L</sub> enhanced the recruitment, activation, and processing of caspase-8 by the DISC, leading to greater effector caspase stimulation and apoptosis. Thus, despite the ability of c-FLIP<sub>L</sub> to support caspase-8 activation upon heterodimerization, the dominant role of endogenous c-FLIP<sub>L</sub> in the modulation of death receptor-mediated apoptosis initiation appears to be inhibitory.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Antibodies, and Reagents—**A549 and H460 human lung carcinoma cells (catalog numbers CCI-185 and HTB-177, respectively) were obtained from ATCC and cultured in 50% Dulbecco’s modified Eagle’s medium, 50% F-12 medium supplemented with 10% FBS, and antibiotics (penicillin/streptomycin). HeLa human cervical carcinoma cells (catalog number CCL-2) and 293 human embryonic kidney cells (catalog number CRL-1573) were obtained from ATCC and cultured in 100% Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and antibiotics (penicillin/streptomycin). U2OS human osteosarcoma cells were a gift from Ellen Filvaroff (Genentech, Inc., South San Francisco, CA) and were maintained in McCoy’s F12 medium supplemented with 10% FBS and antibiotics (penicillin/streptomycin). Anti-c-FLIP<sub>L</sub> antibody (NP6) (catalog number APO-20A-070-C050) was purchased from Alexis Biochemicals, (San Diego, CA). Anti-caspase-8 antibodies (1C12 (catalog number 9746) and 5C7 (catalog number 05-447)) were from Cell Signaling Technology (Beverly, MA) and Upstate Cell Signaling Solutions (Lake Placid, NY), respectively. Anti-caspase-3 antibody (catalog number SA-320) was from BioMol (Plymouth Meeting, PA). Anti-actin antibody (catalog number 69100) was from ICN Biomedicals (Aurora, OH). Anti-Apo2L (2E11) was generated at Genentech, Inc. Anti-DR4 (3G1 and 4G7) and anti-DR5 (3H3 and 5C7) monoclonal antibodies were generated at Genentech, Inc. using receptor-Fc fusion proteins as antigens. Anti-DR4 (4G7) and anti-DR5 (5C7) monoclonal antibodies, used to immunoprecipitate the Apo2L/TRAIL DISC, were conjugated to agarose using the ImmunoPure Protein G IgG Plus orientation kit (catalog number 44990) from Pierce. The anti-DR4 (3G1) and anti-DR5 (3H3) monoclonal antibodies, used for immunodetection of DR4/5 in DISC immunoprecipitations, were biotinylated using EZ-link Sulfo-NHS-LC biotinulation kit (catalog number 21217) from Pierce. As secondary reagents, we used horseradish peroxidase-conjugated goat anti-mouse IgG1 (catalog number 559626) from BD PharMingen (San Diego, CA), horseradish peroxidase-conjugated goat anti-mouse IgG2b (catalog number 1090-02) from Southern Biotechnology Associates (Birmingham, AL), or horse radish peroxidase-conjugated streptavidin from Amersham Biosciences. As substrates for immunodetection we used either ECL from Amersham Biosciences or SuperSignal West Dura Extended Duration Substrate from Pierce. Nontagged soluble Apo2L/TRAIL was prepared as described (35). FLAG-tagged Apo2L/TRAIL was prepared and cross-linked with anti-FLAG antibody M2 (Sigma) as described (36). FLAG-tagged FasL was prepared by cloning amino acids 130–281 of human FasL into pCMV FLAG (Sigma), followed by transient expression in Chinese hamster ovary cells and purification by affinity chromatography.

**Immunodetection—**The cell lysates were prepared as described (36) and quantitated using the BCA protein assay from Pierce. Between 15 and 50 μg of protein/lane was loaded onto a 10% (c-FLIP, caspase-8) or 4–12% (caspase-3) SDS-polyacrylamide gel, electrophoresed, and electrophorograms were transferred to nitrocellulose following manufacturer’s instructions (Invitrogen). The membranes were rinsed in phosphate-buffered saline containing 0.5% Tween 20 (PBS-T) and blocked with 5% milk in PBS-T overnight. The concentration of all primary antibodies was 1–2 μg/ml, secondary antibodies were used at 1:1,000–1:4,000 dilution, and horseradish peroxidase-streptavidin was used at a 1:250 dilution. The membranes were incubated with primary antibodies for 1–3 h, washed with PBS-T (3×), and incubated with secondary antibodies for 1 h, washed three times for 10 min in PBS-T, and exposed to either ECL or SuperSignal.

**Immunoprecipitation and DISC Analysis—**These experiments were done as previously described for Apo2L/TRAIL–FLAG + anti-FLAG DISC analysis (36). The DR4/5 DISC immunoprecipitation experiments also were performed as described, except that anti-DR4 (4G7) and anti-DR5 (5C7) monoclonal antibodies were directly conjugated to agarose for the immunoprecipitation.

**siRNA Preparation and Transfection—**siRNA oligonucleotides against c-FLIP<sub>L</sub> and c-FLIPS were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using their custom SMARTpool and siDESIGN technology. siCONTROL Non-Targeting siRNA pool control (catalog number D-001260-15-20) was also from Dharmacon Research, Inc. siRNA oligonucleotides were resuspended to a concentration of 20 μM. The cells were seeded for transfection in medium without antibiotics 1 day before transfection so that they were 85–95% confluent on the day of transfection. For transfection, regular medium was replaced with low serum media (0–10% FBS, depending on cell type) without antibiotics. The cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) at a ratio of 1:1 to 1:3 siRNA (μg)/siRNA complexes (nl), with a final concentration of 90–120 μM. The cells were incubated with the siRNA-Lipofectamine 2000 complexes overnight, and then low serum medium was replaced with normal media (10% FBS) without antibiotics and incubated for a total of 48–60 h before further analysis.

**Apoptosis Assays—**To analyze cells for the percentage of apoptosis, we quantitated the period of subdiploid DNA content stained with propidium iodide after ethanol fixation and RNase treatment and then analyzed by flow cytometry as previously described (37), using CellQuest Pro software with a FACSCalibur (BD Biosciences).

**Caspase Activity Assays in Whole Cell Extracts—**The caspase-8 activity assay was performed using the BD ApoAlert caspase fluorescent assay kit (catalog number K2028-2), according to the manufacturer’s instructions. siRNA-transfected cells were harvested, counted, and aliquoted at equal numbers for each treatment. The cells were treated with 100 ng/ml FLAG-tagged Apo2L/TRAIL for varying amounts of time at 37 °C and then lysed in manufacturer-provided cell lysis buffer. The cell supernatants were transferred to a 96-well plate and 2× reaction buffer/dithiothreitol and the fluorescent caspase-8 substrate (IETD-AFC) were added and incubated for 1–3 h at 37 °C before reading in a fluorometer at 400/505 nm. The caspase 3/7 assay was performed using Apo-One homogenous caspase-3/7 assay (Promega) according to the manufacturer’s instructions. siRNA-transfected cells were harvested, counted, and aliquoted at equal numbers for each treatment. The cells were treated with FLAG-tagged Apo2L/TRAIL at varying doses for 4 h at 37 °C and then lysed in manufacturer-provided homogenous caspase-3/7 substrate (containing the caspase-3/7 substrate Z-DEVD-R110). The lysates were incubated at room temperature for 1–3 h before reading in a fluorometer at 485/530 nm.

**Caspase Activity Assays at the Apo2L/TRAIL DISC—**The caspase-8 activity assay was performed using the Caspase-Glo 8 assay kit from Promega (catalog number G8201), according to the manufacturer’s instructions, with the following modifications: siRNA-transfected cells were harvested, counted, and aliquoted at equal numbers (1 × 10<sup>7</sup>)
cells) for each treatment. The cells were treated with 333 ng/ml FLAG-tagged Apo2L/TRAIL + anti-FLAG M2 antibody for varying amounts of time at 37 °C and then lysed. The cell lysates were transferred to Immunopure Immobilized protein A/G beads (Pierce; catalog number 20421) and immunoprecipitated overnight at 4 °C. Protein A/G beads were washed four times in lysis buffer, resuspended in 100 μl of phosphate-buffered saline (not eluted), and transferred to a white-walled 96-well plate. Equal volume (100 μl) Caspase-8 Glo reagent (Z-LETD-aminoluciferin) was added and incubated for 30 min to 2 h at room temperature before reading in a luminometer.

Affinity Precipitation of Active Caspase-8—siRNA-transfected cells were harvested, counted, and aliquoted at equal numbers (1–2 × 10⁵) cells for each treatment. The cells were treated with 333 ng/ml FLAG-tagged Apo2L/TRAIl + anti-FLAG M2 for varying amounts of time at 37 °C and then lysed. The cell lysates were incubated with 10 μM Biotin-IETD-FMK at 37 °C for 30 min to label active sites and transferred to immunopure immobilized streptavidin beads (Pierce; catalog number 20349) for precipitation overnight at 4 °C. Streptavidin beads were washed five times in lysis buffer and eluted in sample buffer for immunoblot analysis.

RESULTS

Knockdown of Endogenous c-FLIP, Enhances Apoptosis Induction by Apo2L/TRAIl—Taking advantage of the different C-terminal ends of the c-FLIP variants, we designed siRNA oligonucleotides (38) expected to knock down either c-FLIP₁ (P5), c-FLIPₛ (s1), or both variants together (P1, P3, P6). We transfected human A549 lung carcinoma cells with these siRNAs, incubated the cells for 48 h, and examined the levels of c-FLIP proteins by immunoblot analysis (Fig. 1A). The results confirmed the ability of oligonucleotides P5 and s1 to knock down expression of c-FLIP₁ and c-FLIPₛ, respectively, whereas P1 and P3 knocked down both c-FLIP variants, and P6 was ineffective at knocking down c-FLIPₛ. Apoptosis analysis (Fig. 1C) indicated that knockdown of c-FLIPₛ increased the level of spontaneous cell death to ~10% as compared with transfection of the control siRNA, whereas knockdown of c-FLIP₁ had a minimal effect. Notably, depletion of both c-FLIP variants resulted in 33–35% of the cells undergoing spontaneous apoptosis. This observation suggests that the two c-FLIP variants may act cooperatively in A549 cells to block apoptosis signals that may spontaneously emanate from death receptors. We therefore focused our subsequent experiments on knocking down either c-FLIP₁ or c-FLIPₛ but not both.

Given the high degree of sequence homology between c-FLIP and caspase-8 and -10, we examined the effect of c-FLIP siRNA transfection and consequent protein reduction on caspase-8 and -10 protein levels. None of the c-FLIP siRNA oligonucleotides showed a significant effect on the amounts of caspase-8 and -10 (Fig. 1B). Transfection with the P1 and P3 siRNA oligonucleotides was associated with some degree of spontaneous processing of caspase-8, as indicated by the presence of a p43/41 band, consistent with increased apoptosis in cells with both c-FLIP variants reduced (Fig. 1C). These results verify that the c-FLIP siRNA oligonucleotides specifically knock down c-FLIP₁, c-FLIPₛ, or both but not caspase-8 or -10.

To assess the effect of c-FLIP knockdown on death receptor-induced apoptosis, we transfected the A549 cells with control or c-FLIP variant-specific siRNA, incubated them for 48 h, added increasing doses of Apo2L/TRAIl for another 4 h, and determined the resulting amount of apoptosis, c-FLIP variant knockdown was confirmed (Fig. 2A). Knockdown of c-FLIP₁ modestly increased the level of apoptosis in the absence of added ligand and substantially enhanced apoptosis induction by Apo2L/TRAIl at all doses (Fig. 2A); depletion of c-FLIPₛ also augmented apoptosis sensitivity, particularly at higher doses of Apo2L/TRAIl. Next, we examined the effect of c-FLIP variant knockdown on the time course of Apo2L/TRAIl-induced apoptosis. To this end, we transfected A549 cells with control or c-FLIP variant-specific siRNA and treated with an intermediate dose of Apo2L/TRAIl (33 ng/ml) for increasing amounts of time. c-FLIP variant knockdown was confirmed (Fig. 2B). c-FLIPₛ-depleted A549 cells were more sensitive to Apo2L/TRAIl than control cells (Fig. 2B). Knockdown of c-FLIPₛ also increased Apo2L/TRAIl-induced apoptosis much earlier than control cells (Fig. 2B). Knockdown of c-FLIPₛ had a minimal effect when compared with control cells (Fig. 2B). Thus, both the dose response and the kinetic analysis data indicate that depletion of c-FLIPₛ, and to a lesser extent c-FLIP₁, sensitizes A549 cells to Apo2L/TRAIl-induced apoptosis.

Having established that knockdown of either c-FLIPₛ or c-FLIP₁, increased sensitivity to Apo2L/TRAIl-induced apoptosis, we examined the effect of c-FLIP knockdown on apoptosis induction by another death ligand, FasL. In similar dose-response experiments, knockdown of c-FLIPₛ also enhanced FasL-induced apoptosis over a wide range of doses (Fig. 2C). Likewise, reduction of c-FLIPₛ increased sensitivity to FasL but only at higher ligand doses (Fig. 2C). Therefore, knockdown of either c-FLIPₛ or c-FLIP₁ in A549 cells enhances apoptosis induction by Apo2L/TRAIl as well as FasL.

To verify that the effect of c-FLIP variant knockdown was not unique to A549 cells, we extended our study to include...
several cancer cell lines that display differential sensitivity to apoptosis induction by Apo2L/TRAIL (Fig. 3). The total amount of c-FLIP, as well as the relative level of c-FLIP\(_L\) and c-FLIP\(_S\), varied between cell types (Fig. 3A). As in the previous dose-response experiments using A549 cells, we transfected the cells with control or variant siRNA followed by treatment with Apo2L/TRAIL for 4 h before analyzing them for apoptosis (Fig. 3, B–E). In each experiment, we confirmed the extent of c-FLIP variant depletion by immunoblot analysis of cell extracts 48 h after siRNA transfection (Fig. 3, B–E, insets). In human H460 lung carcinoma cells, knockdown of c-FLIP\(_L\) induced a small increase in basal apoptosis and enhanced Apo2L/TRAIL-induced apoptosis, while knockdown of c-FLIP\(_S\) also augmented apoptosis, particularly at ligand doses of 3–100 ng/ml (Fig. 3B). The sensitization by c-FLIP\(_L\) or c-FLIP\(_S\) knockdown was less pronounced in H460 than A549 cells, perhaps because H460 cells have less endogenous c-FLIP (Fig. 3A). (Because of greater sensitivity of H460 cells to Apo2L/TRAIL, we used the trimeric, rather than the more potent cross-linked form of the ligand, to induce apoptosis in this cell line.) In human HeLa cervical carcinoma cells, knockdown of c-FLIP\(_L\) also increased basal apoptosis (Fig. 3C), and depletion of either c-FLIP\(_L\) or c-FLIP\(_S\) substantially enhanced ligand-induced apoptosis. In human U2OS osteosarcoma cells, knockdown of c-FLIP\(_L\) had a minimal effect, whereas depletion of c-FLIP\(_S\) markedly enhanced Apo2L/TRAIL sensitivity (Fig. 3D). Notably, in these cells knockdown of c-FLIP\(_S\) increased the basal amount of cell death to a greater extent than depletion of c-FLIP\(_L\). As in U2OS cells, knockdown of c-FLIP\(_L\) in human 293 embryonic kidney cells enhanced Apo2L/TRAIL-induced apoptosis to a greater extent than c-FLIP\(_L\) knockdown (Fig. 3E). Thus, notwithstanding some variation between cell lines, the knockdown of either c-FLIP\(_L\) or c-FLIP\(_S\) consistently increases sensitivity to Apo2L/TRAIL-induced apoptosis.

**Knockdown of Endogenous c-FLIP\(_L\) Enhances Effector-Caspase Activation by Apo2L/TRAIL**—To interrogate the mechanisms underlying sensitization to Apo2L/TRAIL, we further characterized the effect of c-FLIP variant knockdown on apoptotic events in A549 cells, beginning with the activation of effector caspases. We transfected the cells with control or c-FLIP variant-specific siRNA and incubated them for 48 h. We then treated the cells with Apo2L/TRAIL for 4 h at various concentrations (Fig. 4A) or for different amounts of time at 330 ng/ml (Fig. 4B) and assessed the level of caspase-3 processing by immunoblot analysis. Caspase-3 processing could be detected by loss in the procaspase-3 protein band (solid arrow) and gain in its processed fragments (open arrows) (Fig. 4, A and B). As compared with control, c-FLIP\(_L\) knockdown resulted in greater processing of caspase-3 in response to lower concentrations of Apo2L/TRAIL, whereas depletion of c-FLIP\(_S\) had an intermediate effect (Fig. 4A). Kinetic analysis showed that knockdown of c-FLIP\(_L\) or c-FLIP\(_S\) as compared with controls resulted in more complete caspase-3 processing in response to Apo2L/TRAIL, particularly at the 2- and 4-h time points (Fig. 4B). Alternatively, we measured caspase-3/7 activity in a substrate conversion assay (Fig. 4C). In this assay, cleavage of the DEVD peptides from the substrate Z-DEVD-R110 releases the fluorescent R110 group, which represents caspase activity measured by relative fluorescence units. Because this assay was performed in whole cell lysates, which may contain a complex mixture of caspases, it should be noted that although DEVD is preferentially cleaved by caspase-3 and -7, it could also be cleaved by other caspases. Depletion of c-FLIP\(_L\) significantly enhanced the stimulation of Z-DEVD-R110 cleavage by Apo2L/TRAIL, whereas knockdown of c-FLIP\(_S\) also increased effector-caspase activation, albeit to a lesser extent (Fig. 4C). Given the increased processing of caspase-3 (Fig. 4, A and B), it is likely that this activated caspase contributes to the increased cleavage of Z-DEVD-R110 after depletion of c-FLIP\(_L\) or c-FLIP\(_S\). Thus, consistent with the apoptosis results described above, knockdown of endogenous c-FLIP\(_L\) or c-FLIP\(_S\) in A549 cells increases effector-caspase activation in response to Apo2L/TRAIL.

**Knockdown of Endogenous c-FLIP\(_L\) Enhances Caspase-8 Recruitment to the Apo2L/TRAIL DISC**—To examine how depletion of c-FLIP\(_L\) might enhance the stimulation of effector-caspases, we turned our attention to caspase-8, which is a key mediator of effector-caspase activation in the cell extrinsic apoptosis pathway. Both c-FLIP\(_L\) and c-FLIP\(_S\) are recruited to the Apo2L/TRAIL DISC upon stimulation (32, 39, 40) (Fig. 5B). Therefore, we assessed the effect of c-FLIP variant knockdown on the recruitment of caspase-8 to the Apo2L/TRAIL DISC. In these experiments, we stimulated A549 cells with FLAG-epitope-tagged Apo2L/TRAIL that was cross-linked with anti-FLAG antibody to enhance its activity and immunoprecipitated the ligand-associated DISC through the FLAG epitope (Fig. 5A). Alternatively, we treated the cells with non-cross-linked Apo2L/TRAIL, which acts less potently on this cell line, and
immunoprecipitated the DISC with combined antibodies to the Apo2L/TRAIL receptors DR4 and DR5 (Fig. 5B). Apo2L/TRAIL recruited larger amounts of full-length caspase-8 (p55/53) to the DISC in cells depleted of c-FLIPL as compared with controls (Fig. 5). Increased procaspase-8 (p55/53) levels were particularly apparent at 10 and 30 min of stimulation with cross-linked Apo2L/TRAIL, and at 10, 30, and 120 min of exposure to non-cross-linked Apo2L/TRAIL, in c-FLIPL-depleted cells. Depletion of c-FLIPS did not significantly enhance caspase-8 recruitment, in keeping with the relatively smaller effect of c-FLIPS knockdown on apoptosis induction in A549 cells (Fig. 2).

Direct immunoblot analysis of cell lysates from both experiments confirmed that c-FLIPL and c-FLIPS were selectively and effectively knocked down (Fig. 5, A and B, right panels).

Because of cross-reactivity between the anti-FLAG antibody and c-FLIP (not shown), we were unable to examine the presence of c-FLIP in the anti-FLAG DISC immunoprecipitates. However, immunoblot analysis of the anti-DR4/5 immunoprecipitates with c-FLIP antibody indicated that full-length c-FLIPL was associated with the DISC (Fig. 5B, FLIP panel, top arrow), migrating electrophoretically just above the immunoglobulin heavy chain of the anti-DR4/5 antibodies (asterisk); indeed, this band was present in control or c-FLIPS-depleted cells, but not in c-FLIPL-depleted cells, confirming its identity as c-FLIPL. Furthermore, c-FLIPL appeared to be processed by the Apo2L/TRAIL DISC, as indicated by the presence of a p43 fragment in the corresponding lanes of the c-FLIP immunoblot (Fig. 5B, p43). In control or c-FLIPL-depleted, but not c-FLIPS-depleted cells, c-FLIPS was also detected in association with the Apo2L/TRAIL DISC (Fig. 5B, FLIP panel, bottom arrow). These results suggest that each c-FLIP variant is capable of binding to the DISC, where it may compete for caspase-8 binding.

Knockdown of Endogenous c-FLIPL Enhances Caspase-8 Activation by Apo2L/TRAIL—An unexpected finding in the experiments depicted in Fig. 5 was the diminished amount of the caspase-8 p43/41 intermediate in the DISC isolated from c-FLIPL-depleted cells as compared with controls. Given that c-FLIPL knockdown increased the recruitment of full-length procaspase-8 to the DISC, one might have expected to see more of the processed caspase-8 p43/41 intermediate as well. Nevertheless, after 10 or 30 min of Apo2L/TRAIL treatment, p43/41 was substantially less abundant in the DISC of c-FLIPL-depleted cells as compared with control or c-FLIPS-depleted cells (Fig. 5). By 120 min of stimulation with cross-linked Apo2L/TRAIL, the p43/41 level appeared similar in all siRNA-transfected cells (Fig. 5A). In contrast, in c-FLIPS-depleted cells stimulated with non-cross-linked Apo2L/TRAIL, reduced levels of p43/41 persisted in the DISC at 120 min (Fig. 5B). This temporal difference between the two experiments may be related to the greater potency and hence faster kinetics of apoptosis induction in A549 cells by cross-linked versus non-cross-linked Apo2L/TRAIL.

The apparently paradoxical decrease in caspase-8 p43/41 levels could potentially be explained by one of two mechanisms. Knockdown of c-FLIPL may attenuate caspase-8 activation and
A detect by immunoblot analysis with the same caspase-3 antibody as in ng/ml) for 0–4 h, as indicated in the figure. Caspase-3 processing was protease activity as indicated by DEVD-AFC cleavage. The cell lysates containing equal cell numbers were assayed in triplicate for enzymatic activity was measured using a substrate conversion assay. With FLAG-Apo2L/TRAIL (330 ng/ml) antibody to detect both full-length and cleaved caspase-3. In assessing was detected by immunoblot analysis using a caspase-3-specific antibody to detect both full-length and cleaved caspase-3. In the DISC and prevents fully processed active caspase-8 from being released to the cytosol. This latter alternative would be consistent with our data indicating that the knockdown of c-FLIP<sub>L</sub> increases effector-caspase stimulation and apoptosis (Figs. 2–4).

To address these possibilities, we examined the effect of c-FLIP<sub>L</sub> variant knockdown on caspase-8 activation. First, we used a biotinylated form of the caspase-8 inhibitor IETD-FMK, which binds selectively and irreversibly to the activated form of the protease, and streptavidin, which binds with high affinity to biotin, to precipitate the stimulated caspase from c-FLIP depleted cells. It should be noted that the commonly named “caspase-8 inhibitors” probably cannot distinguish between the closely related caspase-8 and -10; therefore we focused on caspase-8 specifically by using a caspase-8 antibody to detect activated caspase-8 in the streptavidin precipitates. Immunoblot analysis showed that within 30 min of stimulation with Apo2L/TRAIL, significantly larger amounts of full-length (p55/53) active caspase-8 were present in c-FLIP<sub>L</sub>-depleted cells as compared with control or c-FLIP<sub>R</sub>-depleted cells (Fig. 6A). The full-length active caspase-8 likely was associated with the stimulated DISC. By 60 min of Apo2L/TRAIL stimulation, the p43/41 intermediate and p18 subunit of caspase-8 began to appear in c-FLIP<sub>L</sub>-depleted cells, but not in control or c-FLIP<sub>R</sub>-depleted cells (Fig. 6A). By 2 h, c-FLIP<sub>R</sub>-depleted cells showed less active caspase-8 in the full-length form and more in the p43/41 and p18 forms, suggesting further processing of the active protease. The same caspase-8 processing events appeared to occur more slowly in control or c-FLIP<sub>R</sub>-depleted cells stimulated with Apo2L/TRAIL. Although indirect, these results suggested that in c-FLIP<sub>R</sub>-depleted cells, Apo2L/TRAIL stimulates greater DISC recruitment and activation of caspase-8 homodimers, augmenting its consequent processing to the fully cleaved (p18) form. A potential caveat of this experiment is that it is possible that the IETD biotin-probe does not bind to caspase-8 complexed with c-FLIP<sub>L</sub> (heterodimers) as well as to caspase-8 homodimers. However, if this were true, it might point to the possibility that activated caspase-8 may have different cellular targets depending on its presence in caspase-8/c-FLIP<sub>L</sub> heterodimers versus caspase-8 homodimers. Moreover, the affinity of the IETD-biotin-probe for the cleaved p18 subunit of activated caspase-8 should be similar regardless of the presence of caspase-8/c-FLIP<sub>L</sub> heterodimers.

To examine DISC activation of caspase-8 more directly, we adapted a novel substrate conversion assay to DISC analysis. A549 cells were transfected with siRNA, treated with Apo2L/TRAIL, and harvested at various time points for DISC immunoprecipitation as in Fig. 5. Rather than elute the immunoprecipitated DISC from the beads, we analyzed the activity of its associated caspase-8 by monitoring the processing of a specific synthetic substrate (z-LETD-aminoluciferin) that emits light upon cleavage. At 10 min after Apo2L/TRAIL addition, DISC-associated caspase-8 activity was reproducibly higher in c-FLIP<sub>L</sub>-depleted cells as compared with control cells (Fig. 6B). This caspase-8 activity peaked at 30–60 min and then decreased, in agreement with the kinetics of caspase-8 activation seen in Fig. 6A. The eventual decrease in DISC-associated caspase-8 activity probably was caused by the subsequent cleavage of the N-terminal DEDs, allowing release of the activated caspase (p18 and p10 subunits) from the DISC to the cytoplasm. The effect of knocking down c-FLIP<sub>R</sub> was less pronounced than the depletion of c-FLIP<sub>L</sub> (Fig. 6B). Together, these experiments provide direct support for the conclusion that knockdown of c-FLIP<sub>L</sub> and, to a lesser degree in these cells, c-FLIP<sub>R</sub> enhances caspase-8 activation at the DISC in response to Apo2L/TRAIL.

To verify the increase in caspase-8 stimulation, we analyzed apical caspase activity in whole cell lysates, by monitoring cleavage of a specific synthetic fluorescent substrate (IETD-AFC). Because IETD is preferentially cleaved by caspase-8, cleavage of the IETD peptides from IETD-AFC, which releases the fluorescent AFC group, is likely representative of caspase-8 activity (relative fluorescence units). However, because this assay is performed in whole cell lysates, which may contain a complex mixture of caspases, it is possible that other caspases...
Knockdown of endogenous c-FLIP_{L} enhances caspase-8 recruitment to the Apo2L/TRAIL DISC. FLAG-Apo2L/TRAIL (330 ng/ml) plus anti-FLAG M2 antibody (660 ng/ml) (A) or nontagged Apo2L/TRAIL (1 μg/ml) (B) were added to siRNA-transfected A549 cells either after lysis (0 min) or for the indicated stimulation time (10–120 min) before cell lysis. The left panels show Apo2L/TRAIL DISC analysis; the right panels depict immunoblots that verify c-FLIP variant knockdown. The proteins (full-length and cleaved) are detected by c-FLIP, caspase-8, DR4/5, and Apo2L/TRAIL-specific antibodies as indicated by arrows. The asterisk indicates immunoglobulin heavy chain. Lane C, control siRNA; FL, c-FLIP_{L} siRNA; FS, c-FLIP_{S} siRNA; IP, immunoprecipitation.

Knockdown of c-FLIP_{L} increases DISC recruitment, activation, processing, and release of caspase-8 in response to Apo2L/TRAIL. A549 cells were transfected with c-FLIP_{L}, c-FLIP_{S}, or nontargeting control siRNA oligonucleotides. After 48 h, equal cell numbers were treated with 100 ng/ml or 333 ng/ml FLAG-Apo2L/TRAIL + anti-FLAG M2 for 0–4 h. A, precipitation of active caspase-8 and detection by immunoblot. The cell lysates were incubated with biotin-IETD-FMK to label active caspase-8, precipitated with streptavidin beads, followed by caspase-8 immunodetection. B, DISC-bound caspase-8 activity was measured using the Caspase-Glo 8 Assay kit (Promega). Treated cell extracts were immunoprecipitated in a standard DISC immunoprecipitation, and noneluted beads were transferred to 96-well plates for detection of caspase-8 enzymatic activity using a luminescent (Z-LETD-aminoluciferin) substrate. C, caspase-8 enzymatic activity in whole cell lysates was measured using cleavage of fluorescent IETD-AFC as a read-out. Whole cell lysates containing equal numbers of cells were assayed in triplicate for caspase-8 activity. Multiple independent experiments have been performed with similar results. The error bars represent the standard deviations. Lane C, control siRNA (●); FL, c-FLIP_{L} siRNA (□); FS, c-FLIP_{S} siRNA (●).
could also cleave the IETD peptide. By 2 h of Apo2L/TRAIL stimulation, IETD-AFC cleavage was markedly increased in cells with either c-FLIP\(_L\) or c-FLIP\(_S\) knockdown as compared with controls (Fig. 6C). Taken together with the increased activation and complete processing of caspase-8 at the DISC (Fig. 6, A and B), these results suggest that c-FLIP\(_L\) knockdown augments caspase-8 activation by Apo2L/TRAIL.

**DISCUSSION**

The two major variants of c-FLIP are capable of binding to the DED region of the FADD adaptor protein, as do caspase-8 and -10. It is well established that c-FLIP\(_S\), which consists essentially of two tandem DEDs, prevents apical caspase activation and consequent apoptosis in the cell extrinsic pathway, similar to its structurally related c-FLIP variants (7–9, 11, 12). In contrast to c-FLIP\(_L\), c-FLIP\(_L\) contains not only two DEDs, but also a catalytically inactive caspase-like domain at its C-terminal end. Based on several overexpression studies, c-FLIP\(_L\) was believed to act similarly to c-FLIPS as an inhibitor of death receptor-mediated apoptosis. Furthermore, our findings indicate that endogenous c-FLIPS acts as an inhibitor of death receptor-mediated apoptosis inhibition (14, 15). Moreover, purified caspase-8 was shown to heterodimerize with c-FLIP\(_L\), and this heterodimer had proteolytic activity (17). Given that the levels of c-FLIP\(_L\) can vary widely between cell types (7, 8), it is difficult to ascertain how well in vitro experiments or ectopic expression of this protein at any level represents its endogenous function in a given cellular context. This question may be better addressed by loss-of-function studies.

The gene knockout and siRNA knockdown studies of c-FLIP reported to date show that reduction of both c-FLIP variants sensitizes cells to death receptor-induced apoptosis (26–34). In the present study, we assessed the specific function of endogenous c-FLIP\(_L\) as compared with c-FLIP\(_S\) by using a selective siRNA knockdown approach. Our results reinforce the conclusion that endogenous c-FLIP\(_L\) acts as an inhibitor of death receptor-mediated apoptosis. Furthermore, our findings indicate that c-FLIP\(_L\) functions primarily as an inhibitor, rather than a promoter, of death receptor-mediated apoptosis. We found that depletion of endogenous c-FLIP\(_L\) augments apoptosis induction by Apo2L/TRAIL in several cell lines. In A549, H460, and HeLa cells, siRNA knockdown of c-FLIP\(_L\) resulted in greater sensitization to apoptosis than did depletion of c-FLIP\(_S\), whereas in U2OS and 293 cells, we observed the inverse relationship. This differential magnitude may reflect the variation in endogenous amounts of c-FLIP\(_L\) and c-FLIP\(_S\) between these cell lines. Our experiments with the A549 cell line as a model system showed that knockdown of c-FLIP\(_L\) enables Apo2L/TRAIL to activate effector caspases more robustly, consistent with the observed augmentation in apoptosis induction. Better effector-caspase activation was evident from the increased Apo2L/TRAIL stimulation of caspase-3 processing and caspase-3/7 activity in c-FLIP\(_L\)-depleted cells. In the cell extrinsic pathway, caspase-8 plays a key role in the processing and activation of effector caspasess (3). In c-FLIP\(_L\)-depleted cells, Apo2L/TRAIL recruited more caspase-8 to the DISC, suggesting that endogenous c-FLIP\(_L\) competes with caspase-8 for DISC association. paradoxically, less of the intermediate p43/41 product of caspase-8 processing was found in the Apo2L/TRAIL DISC of c-FLIP\(_L\)-depleted cells. Further analysis revealed, however, that knockdown of c-FLIP\(_L\) increases the amount of caspase-8 activated at the DISC. This probably results in more complete processing of the caspase and hence faster turnover of the p43/41 intermediate and consequent release of the (p10/p18)-activated caspase from the DISC to the cytosol. Affinity-based precipitation of active caspase-8 through a biotinylated, non-cleavable substrate analog verified the enhancement in caspase-8 stimulation, as did enzymatic activity analysis of the immunoprecipitated DISC and of cell lysates.

In summary, the loss-of-function experiments described here suggest that like c-FLIP\(_S\), c-FLIP\(_L\) exerts primarily an inhibitory effect on death receptor-mediated apoptosis induction. Although earlier work showed that ectopically expressed c-FLIP\(_L\) can support caspase-8 activation, the link between caspase-8/c-FLIP\(_L\) heterodimerization-induced caspase-8 activity at the DISC and subsequent apoptosis events was not clear (14, 15, 17). Whereas some reports suggested that ectopic expression of c-FLIP\(_L\) increased caspase-8 activity at the Fas DISC and enhanced FasL-mediated apoptosis, others suggested that the active DISC-bound caspase-8/c-FLIP\(_L\) heterodimers may remain membrane-associated and hence unable to induce apoptosis (14, 15, 17). It is conceivable that the increased caspase activity of DISC-bound caspase-8/c-FLIP\(_L\) heterodimers does not support apoptosis but rather modulates a different function that is directed toward distinct substrates. Miechau et al. (14) suggested for example that the caspase-8/c-FLIP\(_L\) heterodimers cleave the local substrate RIP rather than being released to the cytosol to process caspase-3. Indeed, caspase-8 and c-FLIP have been implicated in additional cellular functions besides apoptosis, namely, proliferation (41–45), NF-κB activation (46, 47), and mitogen-activated protein kinase signaling (48). Furthermore, the DISC-restricted p43 fragment of c-FLIP\(_L\) may provide a neo-epitope for protein-protein interactions. In support of this notion, Kataoka and Tschopp (47) reported that the p43 cleavage product c-FLIP\(_L\) interacts with TRAF2 to induce NF-κB signaling. The possibility that, in some situations, active caspase-8/c-FLIP\(_L\) heterodimers may link death receptors to apoptotic signaling pathways warrants further study.

Regardless, we have shown here that siRNA knockdown of endogenous c-FLIP\(_L\) enhances DISC recruitment and activation of caspase-8. This leads to better processing and release of mature caspase-8 into the cytoplasm and hence to stronger stimulation of effector caspases and apoptosis. Thus, even though caspase-8/c-FLIP\(_L\) heterodimers possess proteolytic activity, the primary role of endogenous c-FLIP\(_L\) in the control of the cell extrinsic apoptosis pathway is inhibitory.

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