c-FLIP$_L$ is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis

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Activation of the caspase cascade is a pivotal step in apoptosis and can occur via death adaptor-mediated homo-oligomerization of initiator procaspases. Here we show that c-FLIP$_L$, a protease-deficient caspase homolog widely regarded as an apoptosis inhibitor, is enriched in the CD95 death-inducing signaling complex (DISC) and potently promotes procaspase-8 activation through hetero-dimerization. c-FLIP$_L$ exerts its effect through its protease-like domain, which associates efficiently with the procaspase-8 protease domain and induces the enzymatic activity of the zymogen. Ectopic expression of c-FLIP$_L$ at physiologically relevant levels enhances procaspase-8 processing in the CD95 DISC and promotes apoptosis, while a decrease of c-FLIP$_L$ expression results in inhibition of apoptosis. c-FLIP$_L$ acts as an apoptosis inhibitor only at high ectopic expression levels. Thus, c-FLIP$_L$ defines a novel type of caspase regulator, distinct from the death adaptors, that can either promote or inhibit apoptosis.
Keywords: apoptosis/caspase activation/caspase-8/CD95 (Fas/APO-1)/c-FLIP

Introduction

The key mediators of apoptosis are a group of cysteinyl, aspartate-specific proteases known as caspases (Chang and Yang, 2000). Produced as latent precursors or procaspases, these proteases become activated during apoptosis through proteolytic processing at critical aspartate residues. The activation often occurs sequentially in a cascade, with an initiator procaspase being activated first, which then cleaves and activates executioner procaspases. Active executioner caspases cleave a critical set of cellular proteins to dismantle a cell. Activation of the initiator procaspases is a key regulatory step in apoptosis, and to date all proteins known to promote this activation directly are death adaptors, including the mammalian proteins FADD and Apaf-1 and the *Caenorhabditis elegans* protein CED-4. During apoptosis, these adaptors bind to the N-terminal prodomain region of procaspases, and facilitate the oligomerization of the C-terminal protease domain.

CD95 (APO-1/Fas) is a cell surface death receptor belonging to the TNFR (tumor necrosis factor receptor) superfamily (Peter *et al.*, 1998). Upon binding to the trimeric CD95 ligand or agonistic antibodies, CD95 recruits FADD/MORT1 and procaspase-8 (FLICE/MACH/Mch5) to form the death-inducing signaling complex (DISC), in which procaspase-8 becomes activated (Kischkel *et al.*, 1995; Medema *et al.*, 1997). The precise mechanism of procaspase-8 activation in the DISC is not well understood, although homo-oligomerization of procaspase-8 alone can induce its activation (Martin *et al.*, 1998; Muzio *et al.*, 1998; Yang *et al.*, 1998a). Of particular interest is the role of a procaspase-8-like, protease-deficient protein c-FLIP (Goltsev *et al.*, 1997; Han *et al.*, 1997; Hu *et al.*, 1997; Inohara *et al.*, 1997; Irmler *et al.*, 1997; Shu *et al.*, 1997; Srinivasula *et al.*, 1997; Rasper *et al.*, 1998). c-FLIP is expressed mainly in a long (c-FLIP$_L$) and a short (c-FLIP$_S$) splice form. The latter contains only two tandem repeats of DEDs and inhibits procaspase-8 activation in the DISC (Krueger *et al.*, 2001a). In contrast, c-FLIP$_L$ shares extensive homology with procaspase-8, with a C-terminal domain that is highly homologous to the procaspase-8 protease domain yet enzymatically inactive due to the lack of key active site residues.

To date, the role of c-FLIP$_L$ in apoptosis remains controversial. In most reports, c-FLIP$_L$ has been described as anti-apoptotic, largely due to its ability to inhibit apoptosis at high levels of ectopic expression (reviewed in Krueger *et al.*, 2001b). However, in cell lines that have been examined quantitatively, the level of endogenous c-FLIP$_L$ is merely 1% of that of endogenous procaspase-8 (Scaffidi *et al.*, 1999). It is unclear what role c-FLIP$_L$ plays in these cells. Mice deficient in c-FLIP (which lack both c-FLIP$_L$ and c-FLIP$_S$) were generated recently. Embryonic fibroblasts (MEFs) derived from the mice (through an *in vitro* selection process for cell growth) were shown to be more sensitive to CD95-induced apoptosis than the wild-type MEFs. However, inconsistent with these results, c-FLIP$^{-/-}$ mice showed developmental defects that strikingly resembled those of caspase-8$^{-/-}$ or FADD$^{-/-}$ mice (Yeh *et al.*, 2000). These mice all died between E10.5 and E11.5 with a failure in heart formation and extreme hemorrhage, suggesting a function for c-FLIP$_L$ that is similar to that of caspase-8 and FADD. Furthermore, transient overexpression of c-FLIP$_L$ could induce as well as inhibit apoptosis, and this pro-apoptotic function required the c-FLIP$_L$ protease-like domain (Goltsev *et al.*, 1997; Han *et al.*, 1997; Inohara *et al.*, 1997; Shu *et al.*, 1997). However, it remains undetermined whether
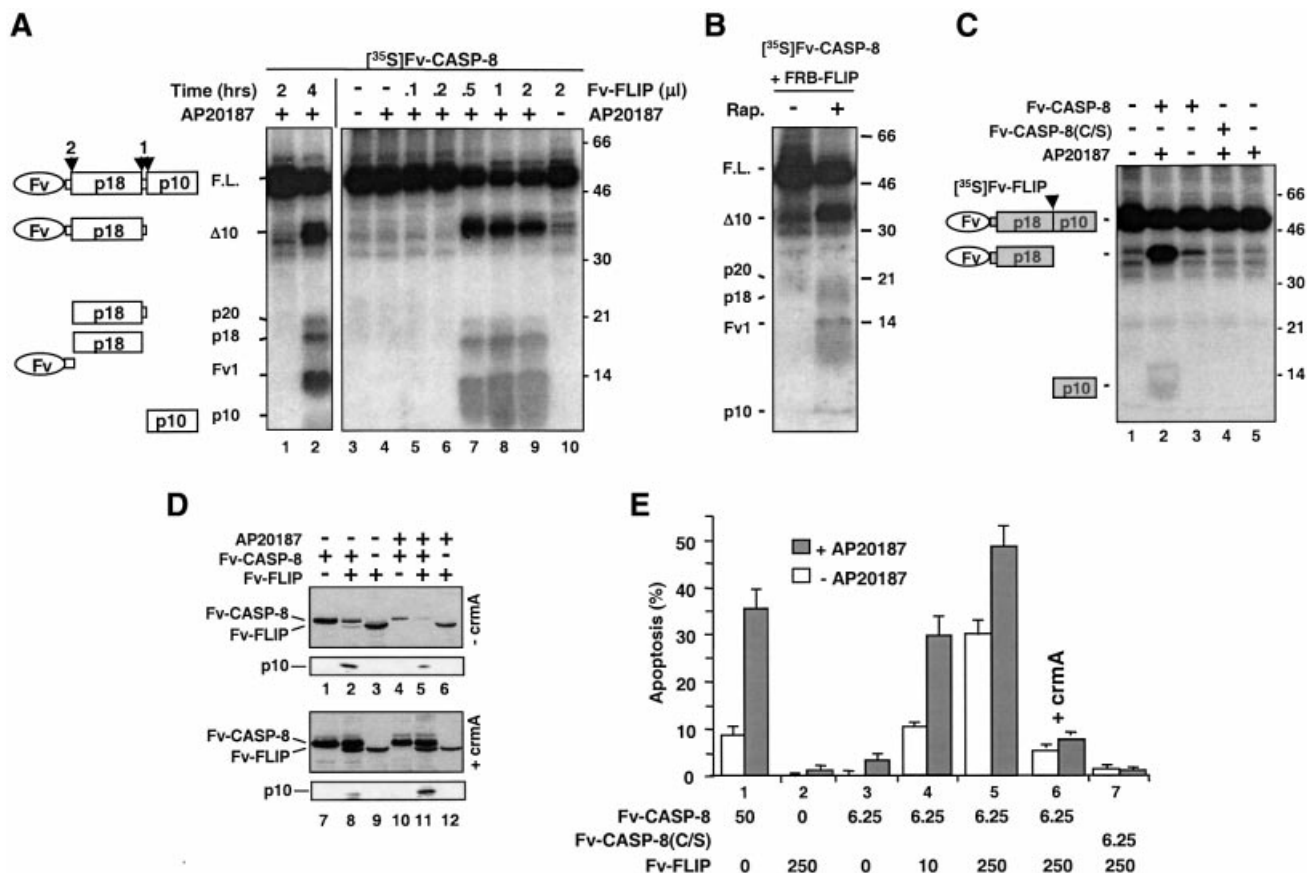
Fig. 1. c-FLIP_L enhances caspase-8 activation upon induced proximity. (A) The c-FLIP_L protease-like domain enhances caspase-8 processing. A 1 µl aliquot of *in vitro* translated, [35S]-labeled Fv-CASP-8 was treated with 100 nM AP20187 for the indicated time (lanes 1 and 2), or treated with vehicle (–) or 100 nM AP20187 (+) for 2 h in the presence of the indicated amounts of *in vitro* translated, non-radioisotope-labeled Fv-FLIP (lanes 3–10). The reaction mixtures were then resolved by SDS–PAGE, and [35S]-labeled products were visualized by autoradiography. The deduced domain structures of the marked bands are shown on the left and the order of processing is marked above. Molecular weight standards (in kDa) are shown on the right. (B) Enhancement of caspase-8 processing by c-FLIP_L is due to their hetero-dimerization. A 1 µl aliquot of [35S]Fv-CASP-8 was treated with 1 mM rapamycin (Rap.) for 8 h in the presence of 1 µl of unlabeled FRB-FLIP. The products were analyzed as in (A). No processing was observed in the absence of FRB-FLIP (not shown). Domain structures are shown on the left and molecular weight standards on the right. (C) Dimerization induces processing of Fv-FLIP by caspase-8. A mixture of 1 µl of [35S]Fv-FLIP and 1 µl of unlabeled Fv-CASP-8 or Fv-CASP-8(C360S) was treated with or without AP20187 for 2 h and analyzed as in (A). The deduced domain structures are shown on the left. (D) Caspase-8 processing in transfected cells. 293 cells were transfected with the indicated combinations of Fv-CASP-8 (1 µg), Fv-FLIP (1 µg) and pRK5-CrmA (2 µg). At 13 h after transfection, cells were treated with vehicle (–) or 50 nM AP20187 (+) for 5 h. Cell extracts were analyzed by immunoblotting analysis using an anti-FLAG antibody. (E) Fv-FLIP enhances the cell death activity of caspase-8 in mammalian cells. HeLa cells were transfected with the indicated combinations of Fv constructs (in ng) and pRK5-crmA (1.5 µg, lane 6), together with pCMV-lacZ. At 6 h after transfection, cells were incubated with AP20187 (final concentration 125 nM), FK506 (200 nM) and z-DEVD (5 µM) for 10 h and scored for apoptosis.

c-FLIP_L can promote apoptosis at endogenous expression levels and how this might be possible without a protease activity. In the current study, we show that expression of c-FLIP_L at levels comparable with the endogenous protein enhances procaspase-8 activation in the DISC and CD95-mediated apoptosis through hetero-dimerization with the protease domain of caspase-8 and enrichment at the DISC, whereas it inhibits apoptosis at high levels of expression. c-FLIP_L is therefore a dual-function regulator for caspase-8 activation and apoptosis dependent on its level of expression.

Results

c-FLIP_L potently enhances procaspase-8 activation upon their hetero-dimerization

During CD95-mediated apoptosis, both c-FLIP_L and procaspase-8 are recruited to the DISC (Scaffidi *et al.*,

1999). To assess the effect of c-FLIP_L on procaspase-8 activation, we first mimicked their interaction in the DISC using an inducible dimerization system based on FK506-binding protein (FKBP) and its divalent ligand. We fused the protease domain of procaspase-8 to a derivative of FKBP called Fv (Clackson *et al.*, 1998) (Fv-CASP-8, Figure 6A). After 4 h of treatment with a divalent Fv ligand AP20187, *in vitro*-translated, [35S]methionine-labeled Fv-CASP-8 underwent autoproteolytic processing (Figure 1A, lane 2). The activation of procaspase-8 in this system closely resembled that which occurred in the DISC (Medema *et al.*, 1997), because it not only yielded the same final products but also proceeded with the same order of events (D.W.Chang, Z.Xing, V.L.Capacio, M.E.Peter and X.Yang, submitted for publication). Interestingly, when Fv-FLIP was included in the reaction, the processing of Fv-CASP-8 was markedly enhanced; >50% of Fv-CASP-8 was cleaved after only 2 h of AP20187
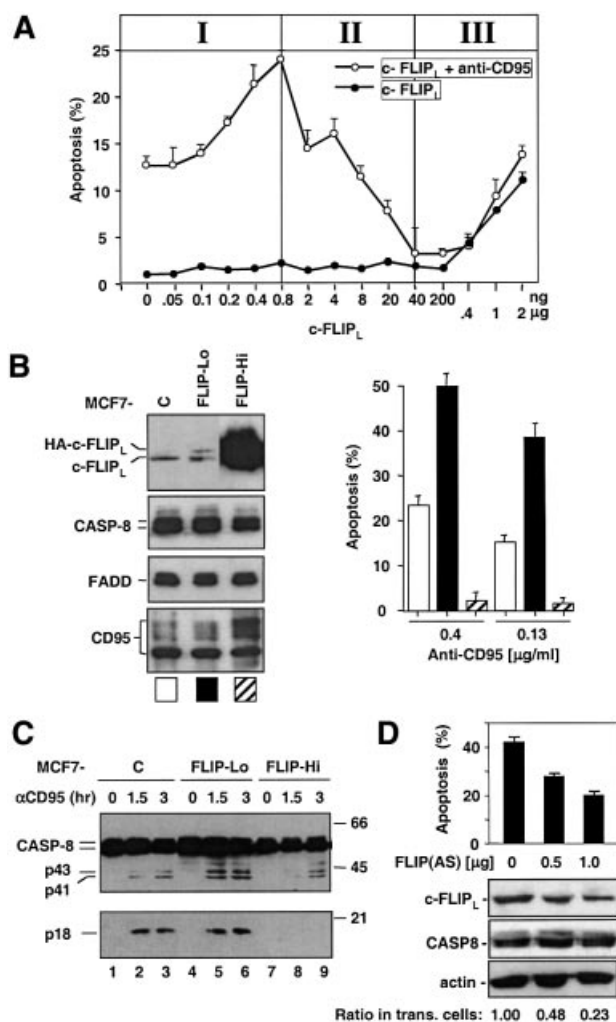
treatment (Figure 1A, lanes 7–9), whereas in the absence of Fv-FLIP virtually no zymogen cleavage was observed at this time point (Figure 1A, lanes 1 and 4). AP20187 could induce not only the Fv-FLIP:Fv-CASP-8 hetero-dimerization but also the homo-dimerization of each of these two Fv fusion proteins. To confirm that the increase in procaspase-8 processing was indeed due to the pro-caspase-8:c-FLIP$_L$ hetero-interaction, we took advantage of rapamycin-induced hetero-dimerization of FKBP and FRB (the rapamycin-binding region of the FKBP–rapamycin-associated protein; Chen *et al.*, 1995). As shown in Figure 1B, forced hetero-dimerization of Fv-CASP-8 with FRB–FLIP (Figure 6A) resulted in the processing of procaspase-8. Interestingly, AP20187-induced procaspase-8:c-FLIP$_L$ dimerization also led to the cleavage of c-FLIP$_L$, resulting in the release of its small subunit (Figure 1C, lane 2), and this processing was similar to that which occurred in the DISC (see below).

Activation of procaspase-8 by c-FLIP$_L$ and processing of c-FLIP$_L$ by caspase-8 were not *in vitro* phenomena since co-expression of Fv-CASP-8 and Fv-FLIP in 293 cells also led to the processing of both proteins, generating the signature 10 kDa fragment p10 (Figure 1D, lane 2 versus lanes 1 and 3). The low level of processing seen here in the absence of AP20187 might represent the basal tendency of these fusion proteins to engage each other upon overexpression (see below). Addition of AP20187 further decreased the levels of transfected proteins (Figure 1D, lane 5). Because cell death among transfected cells was increased by AP20187 treatment (see below),

which might cause the decrease in protein levels, we co-transfected these two proteins together with crmA, a poxviral caspase inhibitor that blocks caspase-8-induced apoptosis but not the generation of p10 (Medema *et al.*, 1997; Yang *et al.*, 1998a). When apoptosis was prevented by crmA, the amount of p10 was increased upon AP20187 treatment (Figure 1D, lane 11 versus lane 8). Taken together, these results show that c-FLIP$_L$ catalyzes autoprocessing of procaspase-8 upon their hetero-dimerization.

To examine the effect of c-FLIP$_L$ on caspase-8 cyto-toxic activity, we transfected Fv-CASP-8 and Fv-FLIP individually or in combination into HeLa cells and quantified apoptosis in these cells. In this experiment, small amounts of Fv-CASP-8 caused minimal cell death even in the presence of AP20187 (Figure 1E, column 3). However, co-transfection with increasing amounts of Fv-FLIP caused a dose-dependent enhancement of apoptosis in >30% of transfected cells. (Figure 1E, columns 4 and 5, open bars). Cell death was increased further by addition of AP20187 (Figure 1E, columns 4 and 5, closed bars) and inhibited by the monomeric FKBP ligand, FK506 (data not shown). In addition, apoptosis was diminished by the caspase inhibitors crmA (Figure 1E, column 6) and zDEVD-fmk (data not shown), and could not be induced by the active site mutant of Fv-CASP-8 [Fv-CASP-8

Fig. 2. c-FLIP$_L$ functions as an activator for CD95-mediated apoptosis. (**A**) Dosage-dependent effect of c-FLIP$_L$ on CD95-mediated apoptosis. HeLa cells seeded in 6-well plates were transfected with the indicated amounts of c-FLIP$_L$/pcDNA3 construct plus the vector DNA to make the total amount of DNA constant. After 16 h, cells were treated with CH11 (75 ng/ml) plus cycloheximide (1 µg/ml) for 6 h and scored for apoptosis. (**B**) CD95-mediated apoptosis in MCF7-CD95 cells stably expressing c-FLIP$_L$. Left: expression of c-FLIP$_L$, caspase-8, FADD and CD95 in the MCF7-CD95 cells expressing no exogenous c-FLIP$_L$ (MCF7-C) or stably expressing low (MCF7-FLIP-Lo) or high levels (MCF7-FLIP-Hi) of c-FLIP$_L$. The exogenous c-FLIP$_L$ (HA-c-FLIP$_L$) migrated more slowly than the endogenous c-FLIP$_L$ on SDS–PAGE due to the tags. Right: MCF7-CD95 cells were treated with the indicated concentrations of anti-APO-1 plus protein A (5 ng/ml) for 3 h and scored for apoptosis. These cells were similarly sensitive to stauro-sporine-induced apoptosis (data not shown). (**C**) Activation of overall caspase-8 in the MCF7-CD95 cells expressing different levels of c-FLIP$_L$. Different MCF7 cells were treated with anti-APO-1 as in (B) for the indicated times, and whole-cell lysates were analyzed by immunoblotting with the anti-caspase-8 mAb C15, which recognized a region in the large subunit. No p18 band was detected in MCF7-FLIP-Hi cells even after a much longer exposure. (**D**) Endogenous c-FLIP$_L$ is required for CD95-mediated apoptosis. HeLa cells cultured in 12-well plates were transfected with the indicated amount of the c-FLIP$_L$ anti-sense plasmid plus 0.15 µg of pEGFP-N3. The total amount of DNA used for transfection was made constant using vector DNA. Top panel: 24 h after transfection, cells were treated with anti-APO-1 (0.4 ng/ml) plus protein A (5 ng/ml) for 4 h, and green fluorescent protein (GFP)-positive cells were scored for apoptosis. Without CD95 stimulation, the background cell death in each condition was approximately the same and <5%. Bottom panels: expression levels of endogenous c-FLIP$_L$, caspase-8 and actin detected at 24 h post-transfection. The ratio of c-FLIP proteins in different lanes was determined using a Bio-Rad Densitometer GS-700, which was then adjusted according to the transfection efficiency (~70% as judged by EGFP expression) to obtain the ratio of c-FLIP$_L$ in transfected (trans.) cells.

(C/S), Figure 1E, column 7]. Similar results were observed when 293 cells were used for transfection (data not shown). This experiment also showed that transfection of ~8-fold more procaspase-8 DNA was required to induce cell death equivalent to that observed with co-transfection of Fv-CASP-8 and Fv-FLIP (Figure 1E, column 1 versus columns 4 and 5). Therefore, c-FLIP$_L$ is a potent activator of caspase-8-mediated killing.

Ectopic expression of c-FLIP$_L$ at physiologically relevant levels enhances CD95-mediated apoptosis

To test the effect of c-FLIP$_L$ on CD95-dependent and -independent apoptosis, we introduced increasing amounts of c-FLIP$_L$ DNA into HeLa cells. The effect of c-FLIP$_L$ was dependent on its expression levels, which can be functionally grouped into three distinct concentration ranges. Very small amounts of c-FLIP$_L$ sensitized these cells to CD95-mediated apoptosis (region I in Figure 2A). The pro-apoptotic function of c-FLIP$_L$ required the caspase-like domain, as a c-FLIP$_L$ mutant lacking the large subunit (c-FLIP$_L$Δ, see Figure 6A) did not show this activity (data not shown). These results raised the possibility that the function of endogenous c-FLIP$_L$ in HeLa cells is pro-apoptotic. Consistent with published reports, high levels of ectopic c-FLIP$_L$ expression led to decreased CD95 sensitivity (region II in Figure 2A), while at very high expression levels c-FLIP$_L$ induced apoptosis on its own (region III in Figure 2A). Similar dosage-dependent effects of c-FLIP$_L$ were also observed in another CD95-sensitive cell line MCF7-CD95 (data not shown). At very high expression levels achievable by transient expression, c-FLIP$_L$ may associate with and activate procaspase-8 outside of the DISC through both their DED domains and their protease/protease-like domain, which explains c-FLIP$_L$-enhanced apoptosis independent of the DISC.

To further analyze the effect of c-FLIP$_L$ on CD95-mediated apoptosis, we generated MCF7-CD95 cells that stably expressed exogenous c-FLIP$_L$. In the clones that expressed exogenous c-FLIP$_L$ at levels comparable with the endogenous protein, sensitivity to CD95-mediated apoptosis increased >2-fold, whereas, in clones that had a much higher expression level of c-FLIP$_L$, apoptosis was almost completely blocked (Figure 2B). The effect of c-FLIP$_L$ on apoptosis correlated with overall procaspase-8 activation in these cells (Figure 2C). In the cells expressing low levels of exogenous c-FLIP$_L$, overall processing of procaspase-8 was enhanced compared with control cells. In contrast, in cells expressing high levels of c-FLIP$_L$, no mature caspase-8 subunit p18 was detected even though generation of the processing intermediates p43/p41 was evident (Figure 2C). This latter result confirmed a recent report that c-FLIP$_L$ at high expression levels blocks the full activation of procaspase-8 (Krueger *et al.*, 2001a). To eliminate effects that could be caused by clonal variation, we also constructed MCF7-CD95 cells that express exogenous c-FLIP$_L$ under the control of an inducible metallothionein promotor. Again, maximal apoptosis sensitivity in these inducible cells was observed when the exogenous c-FLIP$_L$ level was near that of the endogenous protein, whereas c-FLIP$_L$ inhibited apoptosis at higher concentrations (data not shown). Taken together, our results suggest that in both HeLa and MCF7 cells,
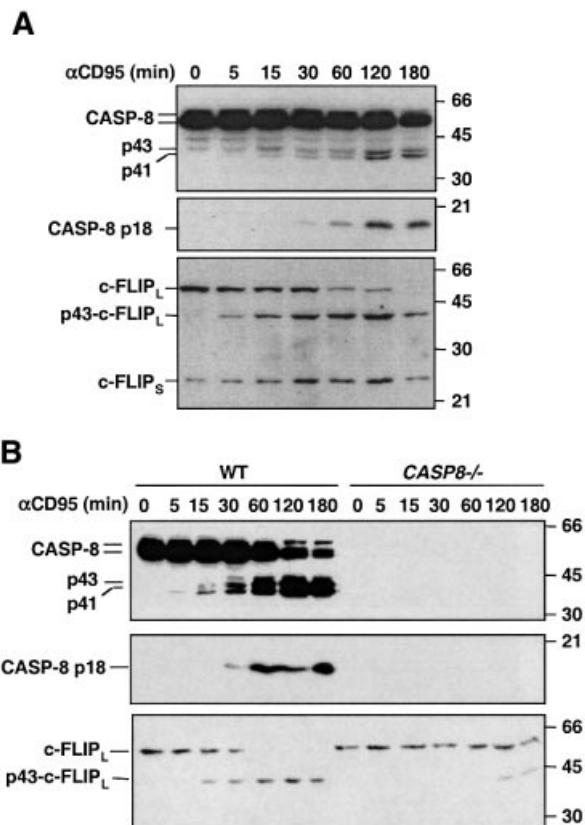

Fig. 3. Processing of procaspase-8 and c-FLIP$_L$ during CD95-mediated apoptosis. (A) Processing of caspase-8 and c-FLIP$_L$ in MCF7-CD95 cells. MCF7-CD95 cells were treated with anti-APO-1 (0.5 μg/ml) plus protein A (10 ng/ml) for the indicated times. Whole-cell lysates were analyzed by immunoblotting with anti-caspase-8 (top and middle) or anti-c-FLIP (bottom) antibody. Molecular weight standards are shown on the right. (B) Processing of c-FLIP$_L$ requires caspase-8 activity. Wild-type or caspase-8 deficient Jurkat cells were treated with anti-APO-1 and the cell lysates were analyzed as in (A). The small amount of c-FLIP$_L$ processing after prolonged treatment was probably caused by caspase-10, which is also recruited to the DISC (Figure 5C).

c-FLIP$_L$ at endogenous levels promotes CD95-mediated apoptosis.

To test directly for a pro-apoptotic role for endogenous c-FLIP$_L$, we downregulated endogenous c-FLIP$_L$ in HeLa cells using an antisense c-FLIP$_L$ construct. A 77% reduction in the expression level of endogenous c-FLIP$_L$ with no change in expression of caspase-8 resulted in a decrease of CD95 apoptosis sensitivity by ~50% (Figure 2D). This inhibition occurred in spite of a concurrent reduction of the anti-apoptotic splice variant c-FLIP$_S$ (data not shown). This result indicates that in HeLa cells, c-FLIP$_L$ is required for maximal apoptosis induced by CD95.

Processing of c-FLIP$_L$ depends on caspase-8 and occurs very early during CD95-mediated apoptosis

In the DISC of most cells, only the processed p43 fragment of c-FLIP$_L$ can be detected, suggesting that most of the processed c-FLIP$_L$ is cleaved at the DISC. A comparison of the cleavage kinetics between c-FLIP$_L$ and procaspase-8 revealed that in MCF7-CD95 cells, processing of c-FLIP$_L$ preceded that of procaspase-8 during CD95-mediated apoptosis (Figure 3A). In contrast, c-FLIP$_S$ was not
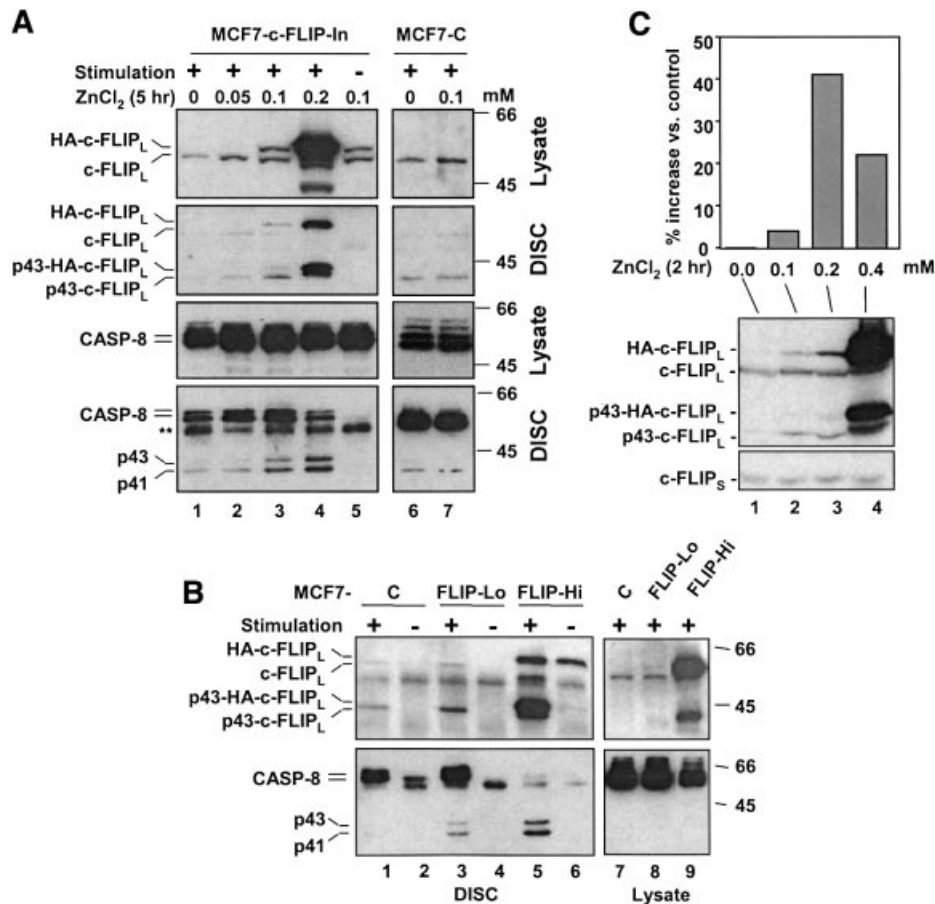
Fig. 4. c-FLIP$_L$ enhances caspase-8 activation in the DISC. (**A**) Effects of c-FLIP$_L$ on caspase-8 processing in the DISC in MCF7-CD95 cells inducibly expressing c-FLIP$_L$. MCF7-FLIP-In cells were treated with the indicated concentration of ZnCl$_2$ for 5 h. Cells were then stimulated with anti-APO-1 (+) or left untreated (–), and DISC complexes were isolated. Cell lysates and DISC were subjected to immunoblotting analysis using anti-c-FLIP (top two panels) or anti-caspase-8 antibody (bottom two panels). **IgG heavy chain. (**B**) Caspase-8 processing in MCF7 transfectants stably expressing c-FLIP$_L$. Different MCF7 cells were stimulated with anti-APO-1 or left untreated. Cell lysates and DISC were analyzed by immunoblotting with anti-c-FLIP (top panels) or anti-caspase-8 (bottom panels) antibody. (**C**) c-FLIP$_L$ increases caspase-8 activity in the DISC. A total of 2×10^7 MCF7-c-FLIP-In cells were treated with ZnCl$_2$ for 2 h, washed and kept without ZnCl$_2$ for another 4 h at 37°C. The DISC was isolated and the caspase-8 activity in the DISC was assayed using IETD-AFC. The data shown are representative of three independent experiments.

cleaved (Figure 3A). The cleavage of c-FLIP$_L$ was dependent mainly on caspase-8 because it was severely delayed in Jurkat cells lacking functional caspase-8 (Juo *et al.*, 1998; Figure 3B). Taken together, these results are consistent with the notion that c-FLIP$_L$ is involved in procaspase-8 activation in the DISC at the initiation step of CD95-mediated apoptosis.

c-FLIP$_L$ enhances procaspase-8 activation in the DISC

To study directly c-FLIP$_L$'s activity in the DISC in living cells, we analyzed the activation of procaspase-8 at the DISC in cells expressing exogenous c-FLIP$_L$ either stably or inducibly under the control of the metallothionein promotor. Similar to the Fv-mediated procaspase-8 activation *in vitro* (Figure 1A), the activation of procaspase-8 in the DISC occurs in two proteolytic steps, with the first cleavage separating the large and small subunits and the second step separating the large subunit and the DISC-binding prodomain. This second step leads to the release of the mature caspase into the cytosol. When higher levels of exogenous c-FLIP$_L$ were expressed in the inducible cells, more c-FLIP$_L$ was recruited subsequently to the DISC, and

concurrently more procaspase-8 was partially processed (Figure 4A, lanes 3 and 4 versus lane 2). Similar results were observed in the c-FLIP$_L$ stable transfectants; in cells stably expressing high levels of c-FLIP$_L$, the DISC complex predominantly contained partially processed caspase-8 (Figure 4B).

To confirm that the processed caspase-8 was enzymatically active, we directly assayed the activity of caspase-8 in the DISC using a fluorogenic caspase-8 substrate IETD-AFC. Caspase-8 activity in the DISC was enhanced in cells expressing exogenous c-FLIP$_L$, with the highest activity observed when exogenous c-FLIP$_L$ was expressed at a level similar to the endogenous c-FLIP$_L$ (Figure 4C, lane 3). However, even high expression levels of c-FLIP$_L$ did not completely block the caspase activity of the DISC, and the activity of the c-FLIP$_L$-containing DISC was still higher when compared with the DISC of cells expressing only endogenous c-FLIP$_L$ (Figure 4C, lane 4 versus lane 1). This discrepancy was probably due to c-FLIP$_L$'s ability to enhance the protease activity of the zymogen form of caspase-8 (see below). Furthermore, in cells stably expressing high levels of c-FLIP$_L$, the amount of caspase-8 in the DISC was only reduced by ~50% (Figure 4B, lane 5

versus lane 1). This modest decrease in the steady-state level of caspase-8 in the DISC did not appear to account for the nearly complete inhibition of apoptosis in these cells (Figure 2B), but rather the lack of generation of mature caspase-8 subunits in these cells upon CD95 treatment did (Figure 2C), consistent with previous studies by us and others (Scaffidi *et al.*, 1999; Krueger *et al.*, 2001a). Therefore, high levels of c-FLIP$_L$, although promoting the first cleavage of procaspase-8 in the DISC, prevent recruitment of additional cytosolic procaspase-8 to the DISC, thereby inhibiting apoptosis. In contrast, low levels of c-FLIP$_L$ expression enhance procaspase-8 activation in the DISC and allow processed caspase-8 to be released from the DISC, resulting in enhanced activation of cytosolic procaspase-8 and apoptosis (Figures 2 and 4).

c-FLIP$_L$ is a specific activator for procaspases engaged by death receptors

To determine whether c-FLIP$_L$ affects the activation of other initiator procaspases, we tested its effect on the processing of procaspase-9 and -10. Procaspase-9 is linked to mitochondria-mediated apoptosis, while procaspase-10 is another tandem DED-containing caspase implicated in death receptor signaling. Similar to procaspase-8, dimerization of either procaspase-9 or -10 led to their self-processing (Figure 5A and B; D.W.Chang, Z.Xing, V.L.Capacio, M.E.Peter and X.Yang, submitted for publication). However, c-FLIP$_L$ enhanced procaspase-10 but not procaspase-9 activation. These data suggested that caspase-10 might be involved in CD95-mediated apoptosis as a DISC component. We indeed found procaspase-10, together with c-FLIP$_L$, to be part of the endogenous DISC of type I cells that we have characterized previously (Scaffidi *et al.*, 1998; Figure 5C), which is consistent with recent observations (Kischkel *et al.*, 2001; Wang *et al.*, 2001). Interestingly, even in these cell lines which are highly sensitive to CD95-mediated apoptosis, the DISC also contained only the p43 fragment of c-FLIP$_L$ but not the full-length c-FLIP$_L$, consistent with the notion that c-FLIP$_L$ is an important activator for CD95-mediated apoptosis. Together, these results show that c-FLIP$_L$ could be a specific regulator of the two procaspases directly engaged by death receptors.

An intact protease-like domain is required for c-FLIP$_L$ function

To study the mechanism by which c-FLIP$_L$ enhances caspase activation, we first examined which region(s) of the c-FLIP$_L$ caspase-like domain, which shares 60% similarity with the procaspase-8 protease domain, is responsible for its activity. We deleted either the large or the small subunit of Fv-FLIP (Figure 6A). Neither deletion mutant induced procaspase-8 processing *in vitro* or its cell death activity when transfected into HeLa cells (Figure 6B, lane 4 and 5, and C, columns 2 and 3), suggesting that both subunits of c-FLIP$_L$ are required to engage procaspase-8. In addition, despite their high homology, replacement of either the large or the small subunit of c-FLIP$_L$ with the corresponding subunit of procaspase-8 (Figure 6A) rendered c-FLIP$_L$ inactive (Figure 6B, lanes 7–9, and C, columns 5 and 6). These results indicate that c-FLIP$_L$ is not simply an active site mutant of tandem DED pro-
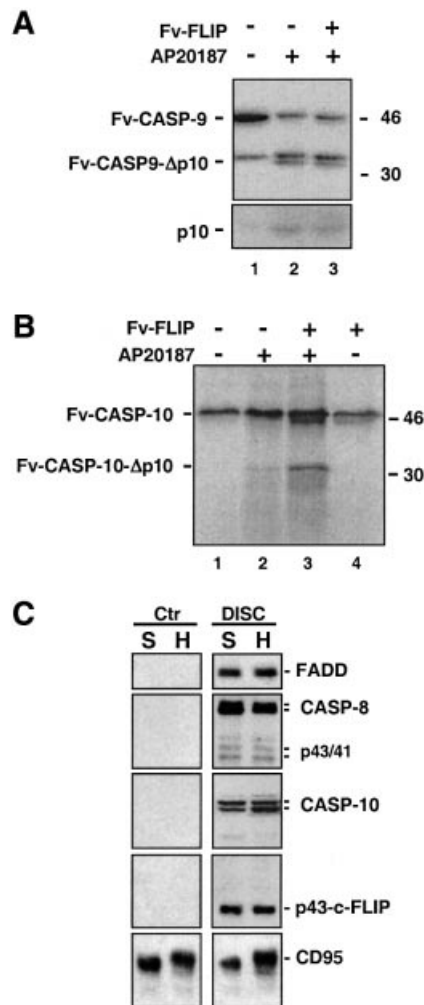

Fig. 5. Specific effect of c-FLIP$_L$ on caspase-8 and -10 and identification of caspase-10 as a DISC component. (**A** and **B**) Fv-FLIP enhances caspase-10 but not caspase-9 processing. A 1 μl aliquot of [^{35}S]Fv-CASP-9 (A) or [^{35}S]Fv-CASP-10 (B) was treated with 100 nM AP20187, in the presence or absence of 1 μl of unlabeled Fv-FLIP. The reaction mix was analyzed as in Figure 1A. Domain structures are shown on the left, and molecular weight standards on the right. (**C**) Identification of caspase-10 as a DISC component. CD95 was immunoprecipitated from stimulated (DISC) or unstimulated (Ctr) SKW6.4 (S) and H9 (H) cells. CD95 and the associated proteins were analyzed by immunoblotting using appropriate monoclonal antibodies.

caspases. Its active subunit-like domains, instead, have a specific and relevant function.

To determine the importance of c-FLIP$_L$ cleavage for procaspase-8 activation, we mutated the cleavage site Asp376 to glutamate (D/E) (Figure 6A). Both the *in vitro* procaspase-8 processing assay and the *in vivo* apoptosis assay showed that the mutation also impaired the proapoptotic function of c-FLIP$_L$ (Figure 6B, lane 6, and C, column 4). Thus, many aspects of the c-FLIP$_L$ caspase-like domain appear to be designed specifically to mediate its catalysis of procaspase-8 activation.

c-FLIP$_L$ is recruited preferentially to the DISC

Previous studies showed that initiator procaspases are activated by induced dimerization of the zymogen molecules. We recently found that this activation requires
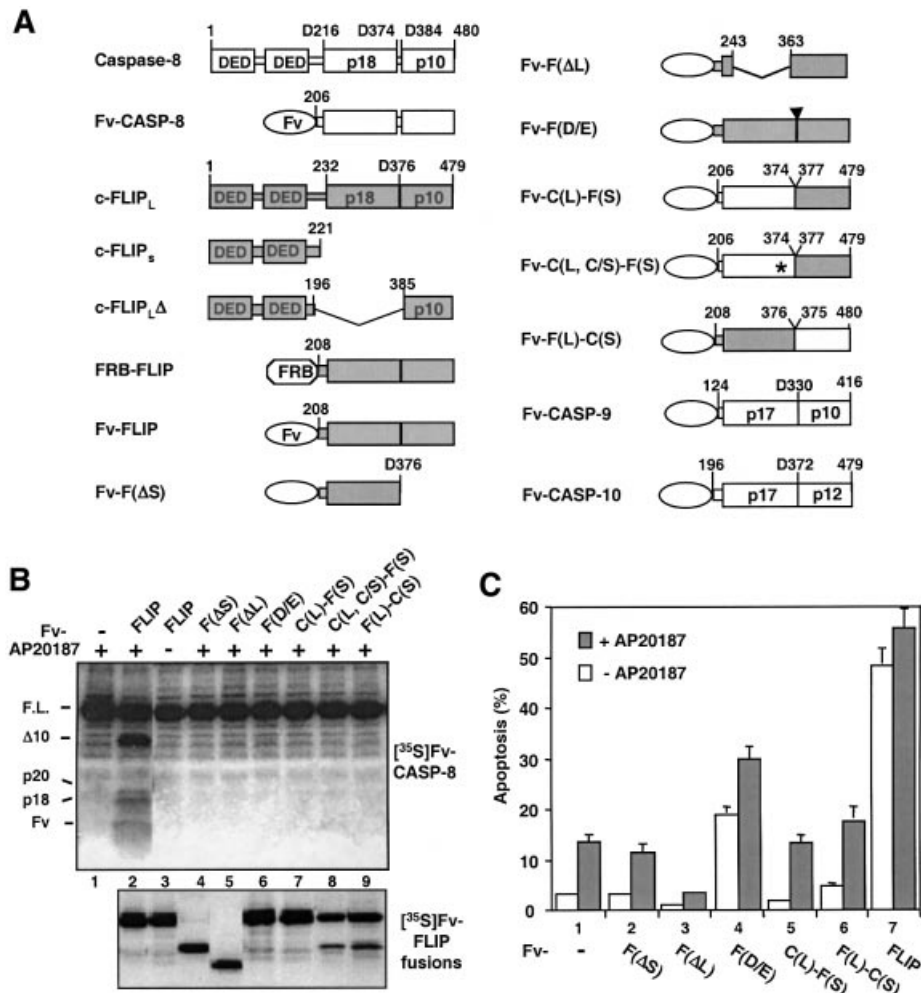
Fig. 6. The entire protease-like domain is required for c-FLIP$_L$ function. (**A**) Schematic structures of caspase-8, c-FLIP$_L$, c-FLIP$_S$ and various constructs used in this study. DED, death effector domain. Amino acids present in each construct are labeled. Regions of c-FLIP$_L$ and caspase-8 in the chimeric constructs are indicated by shaded and open boxes, respectively. The domains that form the mature caspases as well as the corresponding domain on c-FLIP$_L$ (p10, p18, etc.) are marked. Fv, a derivative of FK506-binding protein (Clackson *et al.*, 1998); FRB, the minimal rapamycin-binding domain of the FKBP–rapamycin-associated protein (FRAP) (Chen *et al.*, 1995); asterisk, caspase-8 active site Cys360 to serine mutation; arrowhead, c-FLIP$_L$ processing site Asp376 to alanine mutation. (**B**) Capase-8 processing induced by c-FLIP$_L$ mutants and c-FLIP$_L$:caspase-8 chimeras. Top panel: 1 μl of [^{35}S]Fv-CASP-8 was treated with or without AP20187, in the presence of 1 μl of the indicated unlabeled Fv fusion proteins. Deduced domain structures are shown on the left. Bottom panel: the Fv fusion proteins used in the top panel were labeled with [^{35}S]methionine to confirm equal expression of protein. (**C**) Effects of c-FLIP$_L$ mutants and c-FLIP$_L$:caspase-8 chimeras on caspase-8-induced apoptosis. HeLa cells were transfected with Fv-CASP-8 (25 ng) and the indicated Fv-FLIP mutants (500 ng), and apoptosis was determined as in Figure 1E.

the formation of a structural intermediate, comprised of two stably associated procaspase molecules, that possesses caspase activity (D.W.Chang, Z.Xing, V.L.Capacio, M.E.Peter and X.Yang, submitted for publication). This mechanism does not require both dimeric partners to possess enzymatic activity, and thus would allow a proteolytically inactive caspase homolog like c-FLIP$_L$ to promote procaspase activation allosterically. We thus compared the heterophilic interaction between the c-FLIP$_L$ protease domain and the procaspase-8 protease domain with the homophilic interaction of the procaspase-8 protease domain. The procaspase-8 protease domain or the c-FLIP$_L$ protease-like domain tagged with a hemagglutinin (HA) epitope was co-expressed in 293 cells with the procaspase-8 protease domain tagged with FLAG epitope (the caspase-8 active site mutant was used to prevent caspase activation). Cell lysates were immunoprecipitated with an anti-FLAG antibody and the pre-

cipitates were then subjected to immunoblotting analysis using an anti-HA antibody. Interestingly, the heterophilic c-FLIP$_L$:caspase-8 interaction was significantly stronger than the homophilic caspase-8:caspase-8 interaction (Figure 7A). A similar result was observed when *in vitro*-translated proteins were used for the co-immunoprecipitation assay (data not shown).

The expression of endogenous c-FLIP$_L$ in many cell types is ~1% of that of procaspase-8 (Scaffidi *et al.*, 1999). A confounding question is how such a small amount of c-FLIP$_L$ could make a significant difference in death receptor-mediated apoptosis. Assuming that c-FLIP$_L$ binds to procaspase-8 with higher affinity than procaspase-8 does itself, the c-FLIP$_L$ concentration after recruitment to the DISC may be higher than that of procaspase-8 when compared with their cytoplasmic concentration. We therefore quantified the amounts of c-FLIP$_L$ and procaspase-8 in the cytosol and in the DISC of MCF7-CD95
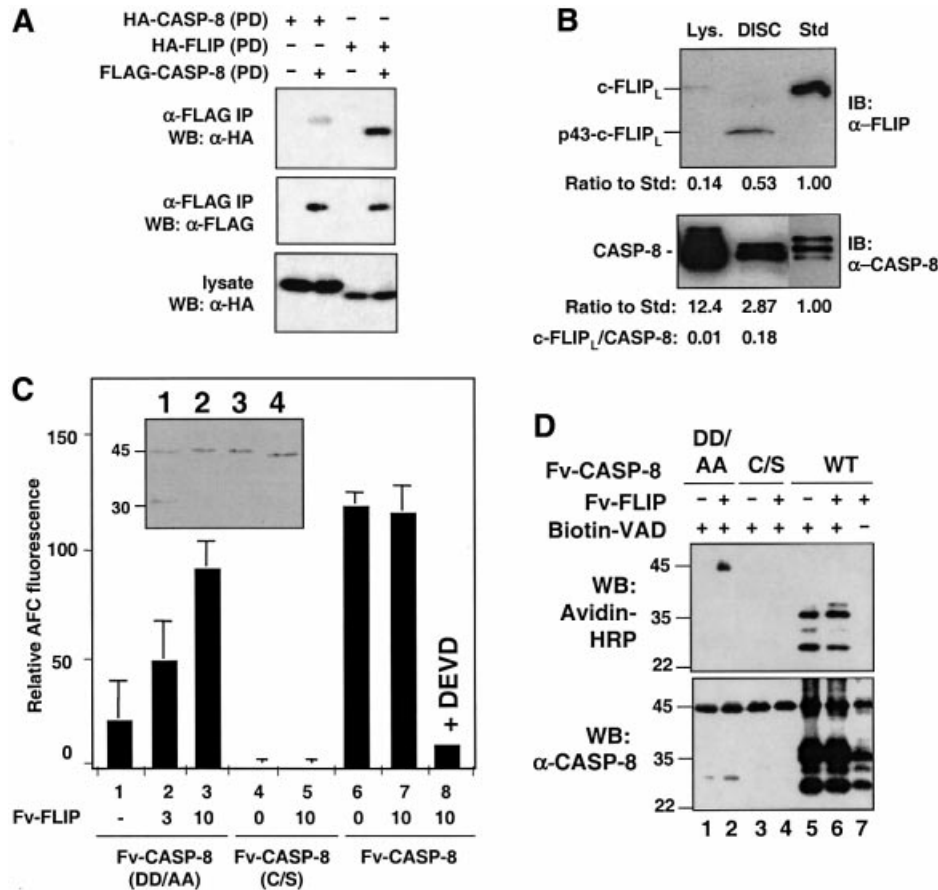
Fig. 7. The c-FLIP$_L$ protease-like domain induces the enzymatic activity of the caspase-8 zymogen. (**A**) The c-FLIP$_L$ protease-like domain interacts with the caspase-8 protease domain more efficiently than the caspase-8 protease domain does with itself. 293 cells were transfected with HA-tagged caspase-8 protease domain (PD) or HA-tagged c-FLIP$_L$ protease-like domain together with FLAG-tagged caspase-8-PD. The cell lysates were immunoprecipitated with an anti-FLAG antibody. The immunoprecipitated proteins and the lysates were analyzed by immunoblotting as indicated. (**B**) Ratios of c-FLIP$_L$ versus caspase-8 in cell lysate and in the DISC. The DISC complexes and whole-cell lysates (lys.) were made from 3×10^6 MCF7-CD95 cells. Thirty percent of these DISC complexes and 0.3% of the lysate (lys.) were resolved by SDS–PAGE together with a c-FLIP$_L$ (top) or a caspase-8 (bottom) standard (Std), and analyzed by immunoblotting (IB) with the indicated antibodies. For making protein standards, HA-tagged c-FLIP$_L$ and caspase-8 proteins were generated by *in vitro* translation. The relative concentration of these two proteins was determined by anti-HA immunoblotting, and equal amounts of these two proteins were used. The ratio of c-FLIP$_L$/caspase-8 in the DISC was higher when the expression level of c-FLIP$_L$ increased. In the MCF7-CD95 cells stably expressing low and high levels of c-FLIP$_L$, the ratio is ~1:2 and 10:1, respectively (Figure 4B, lanes 3 and 5). (C and D) Fv-FLIP enhances the caspase activity of non-cleavable Fv-CASP-8(DD/AA). (**C**) Recombinant Fv-CASP-8 fusion proteins (100 ng) were incubated with the indicated amount of Fv-FLIP protein (in ng). Caspase-8 activity was measured using IETD-AFC. The data shown were representative of three independent experiments done in duplicate or triplicate. The insert shows analysis of recombinant Fv-CASP-8 (1), Fv-CASP-8(DD/AA) (2), Fv-CASP(C/S) (3) and Fv-FLIP (4) purified from bacteria. About 100 ng of each protein was resolved by SDS–PAGE and visualized by Coomassie Blue staining. (**D**) Fv-CASP-8 proteins were labeled with biotin-DEVD in the presence or absence of Fv-FLIP. The mixes were then resolved by SDS–PAGE and immunoblotted with either avidin–HRP (top) or anti-caspase-8 mAb C15 (bottom).

cells. This analysis revealed that c-FLIP$_L$ indeed was recruited more efficiently to the DISC upon CD95 stimulation than procaspase-8; c-FLIP$_L$ was enriched 18-fold in the DISC when compared with procaspase-8. As a result, the ratio of c-FLIP$_L$ to procaspase-8 in the DISC reached ~1:5 (Figure 7B).

The protease-like domain of c-FLIP$_L$ induces the activity of the caspase-8 zymogen

The correlation between the binding efficiency of c-FLIP$_L$ to the procaspase-8 protease domain and its ability to promote procaspase-8 activation suggested that c-FLIP$_L$ may enhance procaspase-8 activity by adopting a structure that is more efficient in inducing the activity of caspase-8 zymogen than the procaspase-8 protease domain itself. To test whether c-FLIP$_L$ could induce the activity of procaspase-8 without causing its cleavage, we

expressed recombinant Fv-FLIP and a non-processable form of Fv-CASP-8 [Fv-CASP-8(DD/AA)], along with Fv-CASP-8 and Fv-CASP-8(C/S) from bacteria and purified them to homogeneity. As expected, the wild-type Fv-CASP-8 was partially processed, while Fv-CASP-8(DD/AA) and Fv-CASP-8(C/S) were in the precursor form (insert of Figure 7C). The protease activity of Fv-CASP-8(DD/AA) was significantly induced by Fv-FLIP in a dose-dependent manner (Figure 7C). This enhancement was found to be AP20187 independent, due to the very high binding efficiency of two proteins at the concentrations used (data not shown). No activity was detected when the catalytically inactive mutant of caspase-8 was used (Figure 7C, column 5), and c-FLIP could not enhance the activity of already processed Fv-CASP-8, suggesting that it only acts on the caspase-8 proform (Figure 7C, column 7).

To confirm that the measured caspase-8 activity originated from the full-length caspase-8 protein and not from aberrant processing products, we used a biotinylated, irreversible inhibitor of caspases (biotin-VAD-fmk) that covalently modifies the active site, to allow direct visualization of the activation of unprocessed caspase-8 by c-FLIP$_L$. Upon incubation with Fv-FLIP$_L$, full-length Fv-CASP-8(DD/AA) protein was found to be affinity labeled with this inhibitor (Figure 7D, lane 2). As a negative control, Fv-FLIP had no effect on the catalytically inactive Fv-CASP-8(C/S) (Figure 7D, lane 4). Again Fv-FLIP did not enhance the activity of Fv-CASP-8, which represented mostly processed caspase-8 in the experiment (Figure 7D, lane 6). Taken together, these results suggest that c-FLIP$_L$ promotes caspase-8 activation through induction of the enzymatic activity of the zymogen but not of the caspase-8 active subunits.

Discussion

c-FLIP has been widely regarded as an inhibitor of death receptor-mediated apoptosis due to its ability to inhibit apoptosis at high levels of expression (Krueger *et al.*, 2001b). By examining the effect of c-FLIP$_L$ on procaspase-8 activation in a controlled caspase activation system that mimics the DISC, in combination with direct analyses of cells expressing c-FLIP$_L$ at physiologically relevant levels and of cell lines with decreased c-FLIP$_L$ expression, we now show that c-FLIP$_L$ can also function as a novel type of caspase activator. Unlike death adaptors that associate with the N-terminal prodomain of procaspases to facilitate oligomerization of the C-terminal protease domain, c-FLIP$_L$ promotes procaspase-8 activation by acting directly on the protease domain.

We found that the effect of c-FLIP$_L$ on cells depends on its expression levels. Three different concentration-dependent effects could be distinguished, allowing us to propose the following model that could explain the contradictory findings in the literature on the function of c-FLIP$_L$. At low expression levels (region I in Figure 2A), which are probably found in most cells, c-FLIP$_L$ enhances the DISC activity. At intermediate expression levels (region II in Figure 2A), possibly found in some cell types such as monocytes/macrophages and certain tumors (Irmler *et al.*, 1997; Griffith *et al.*, 1998; Perlman *et al.*, 1999) and achieved in c-FLIP$_L$ transgenic mice (Medema *et al.*, 1999; Van Parijs *et al.*, 1999), c-FLIP$_L$ acts as an inhibitor of the DISC. Only at very high non-physiological concentrations achieved by transient overexpression (region III in Figure 2A) is c-FLIP$_L$ cytotoxic by itself without the need for stimulation of CD95, probably through a direct association with caspase-8 outside the DISC. In contrast to c-FLIP$_L$, c-FLIP$_S$ has been shown to be a dedicated apoptosis inhibitor and it prevents the first cleavage of procaspase-8 activation in the DISC (Krueger *et al.*, 2001a). Interestingly, stimulation of CD95-sensitive T cells through either the T-cell receptor or CD28 was shown to result in a specific upregulation of only c-FLIP$_S$, demonstrating that c-FLIP$_L$ and c-FLIP$_S$ are not always co-regulated (Kirchhoff *et al.*, 2000).

c-FLIP$^{-/-}$ MEFs were reported to show increased sensitivity to death receptor-mediated apoptosis. This result, which seems to contradict our study, might be reconciled by the possibility that the MEFs express levels of c-FLIP$_L$ high enough to inhibit death receptor-mediated apoptosis. An alternative but not mutually exclusive possibility is that c-FLIP$_S$ may play a major role in the inhibition of CD95-mediated apoptosis in these cells. Remarkably, mice deficient for either c-FLIP, FADD or caspase-8 all share strikingly similar developmental defects (Yeh *et al.*, 2000). This observation on the level of whole animals clearly places these three DISC components on the same functional axis, and could be explained by c-FLIP$_L$ playing an essential role as a caspase-8 activator during development.

The two main apoptosis pathways are started by two large prodomain initiator caspases. Caspase-8 initiates the extrinsic and caspase-9 the intrinsic pathway. The intrinsic pathway is regulated by the ying:yang of pro-apoptotic proteins released from mitochondria, such as cytochrome *c* or Smac/Diablo-like proteins, and the expression levels of IAP proteins that regulate the activity of the caspases downstream of mitochondria. In comparison, without c-FLIP, the extrinsic pathway would appear to be regulated only by the intensity of the stimulus received through death receptors. Neither FADD nor procaspase-8 is significantly regulated on the transcriptional level and, at present, no inhibitor of the IAP class has been found that would act on caspase-8. The identification of c-FLIP$_L$ as a dual functional regulator for this pathway reveals a previously unexpected complexity of regulation that may be adapted to the specific functions of caspase-8. The modulation of apoptosis sensitivity by a protease-inactive caspase homolog like c-FLIP$_L$, instead of a caspase itself, also represents a highly effective and safe mechanism for the regulation of apoptosis. It greatly decreases the risk of basal toxicity associated with high levels of caspases since c-FLIP$_L$ only acts on DISC-bound caspases. In addition, it allows a fine-tuned and versatile regulation of caspase activation in response to different stimuli. To our knowledge, c-FLIP$_L$ represents the first example of an inactive protease analog that acts as an activator of proteases.

Unlike caspase-8 or FADD, the expression of c-FLIP$_L$ appears to be highly regulated, for example by the mitogen-activated protein kinase pathway and the NF-κB pathway (Kreuz *et al.*, 2001; Micheau *et al.*, 2001). Upregulation of c-FLIP$_L$ was also reported in certain tumor cells, and these tumor cells may escape apoptosis induced through death receptors (Irmler *et al.*, 1997; Griffith *et al.*, 1998). The expression level of c-FLIP$_L$ is thus a focal point for apoptosis regulation, and it can not only determine whether the apoptosis pathway is turned on or off but also seems to allow the cell to switch between apoptosis and survival pathways. c-FLIP$_L$ may therefore represent an attractive target for therapeutic intervention with death receptor signaling.

Materials and methods

Cell lines and reagents

HeLa, 293, SKW6.4 (B lymphoblastoid) and H9 (T-cell leukemia) cells were obtained from ATCC. MCF7 cells stably expressing CD95 (MCF7-CD95) and the wild-type and caspase-8-deficient Jurkat cells were gifts from Drs E.Alnemri and J.Blenis, respectively. The following reagents were obtained from the indicated sources: AP20187 (ARIAD Pharmaceuticals); z-DEVD (z-Asp-Glu-Val-Asp) and biotin-z-VAD (Enzyme System Products); anti-CD95 CH11 (MBL Co.); anti-FLAG

antibody and anti-CD95 (C20, for western blotting; Santa Cruz Biotech); protein A (Pierce); anti-caspase-10 4C1 (PanVer); anti-FADD A36620 (Transduction Lab); and anti-actin, cycloheximide, rapamycin and ZnCl$_2$ (Sigma). Monoclonal antibodies specific for caspase-8 (C15) and c-FLIP (NF-6) (Scaffidi *et al.*, 1999), and anti-APO-1 (Kischkel *et al.*, 1995) have been described previously.

To generate c-FLIP$_L$ stable and inducible cell lines, c-FLIP$_L$/pRK5 and c-FLIP$_L$/pMT (see below) were transfected into MCF7-CD95 cells together with pBabe-Puro using SuperFect-mediated gene transfer (Qiagen). Cells were selected in Dulbecco's modified Eagle's medium/ fetal bovine serum (DMEM/FBS) with 2 µg/ml of puromycin and screened for exogenous c-FLIP$_L$ expression by immunoblotting. Two independent MCF7-FLIP-Lo cell lines, several MCF7-FLIP-Hi cell lines and three MCF7-FLIP-In cell lines were obtained. Similar results were obtained with the different clones.

Expression constructs

pRK5-crmA (Dr D.Goeddel) and c-FLIP$_L$/pcDNA3 (Dr S.Hu) were obtained from the indicated sources. c-FLIP$_L$ Δ/pcDNA3 expression plasmid was generated by deleting the c-FLIP$_L$ sequence between the *Bam*HI and *Eco*RI sites. The full-length c-FLIP$_L$ was cloned into pRK5 and the plasmid pMT (containing the metallothionein promoter) with an HA tag at the N-terminus and a FLAG tag at the C-terminus. The antisense c-FLIP$_L$ construct, with the entire open reading frame expressed in the reversed orientation, was made in pRK7. The Fv and FRB fusion constructs were made in pRK5 similarly to the Fkp fusion constructs (Yang *et al.*, 1998a). Fragments for various mutations and chimeras were generated by PCR. The expression constructs for the procaspase-8 protease domain (amino acids 206–480) and the c-FLIP$_L$ protease-like (amino acids 208–479) were made in pRK5 with a C-terminal HA or FLAG tag.

In vitro cleavage of caspases

In vitro transcription and translation were carried out with the TNT reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine or non-radioisotope-labeled methionine. A 1 µl aliquot of [^{35}S]methionine-labeled product was mixed with the indicated amounts of unlabeled product. The total volume of *in vitro* translated products was kept constant by adding *in vitro* translation reaction mix containing the parental plasmid. *In vitro* translated products were then mixed with an equal volume of 2× CED3 buffer (Yang *et al.*, 1998a) containing AP20187, and incubated at 30°C for the indicated time.

Apoptosis assay

Cell death was measured as described, with modifications (Yang *et al.*, 1998a). Anti-APO-1 (in combination with protein A) and CH11 (in combination with cycloheximide) were used to induce apoptosis through CD95. Transfection was performed using the calcium phosphate precipitation method. After transfection and/or treatment, cells were fixed and, for cells co-transfected with pCMV-lacZ, also stained with X-gal. Apoptosis was scored based on characteristic morphological changes including membrane blebbing, cell shrinkage and detachment from the plates. Data (mean ± SD) were obtained from three or more independent experiments, and in each experiment >300 cells/transfected cells were examined.

DISC analysis

The DISC analysis was performed essentially as described (Kischkel *et al.*, 1995). A total of 3 × 10^6 MCF7-CD95 cells and 1 × 10^7 H9 and SKW6.4 cells were treated with 2 µg/ml anti-APO-1 for 20 min (MCF7-CD95 cells) or 5 min (H9 and SKW6.4 cells) at 37°C and then lysed in lysis buffer (Kischkel *et al.*, 1995) (stimulated condition), or the cells were first lysed and then supplemented with 2 µg/ml of anti-CD95 (unstimulated condition). CD95 was then immunoprecipitated for 2 h at 4°C with protein A–Sepharose beads. To detect caspase-8 activity associated with the receptor, the DISC was incubated in cleavage buffer [20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% CHAPS, 10% sucrose] containing 40 µM caspase-8-specific fluorogenic substrate IETD-AFC for 1 h at 37°C. Caspase activity was determined using a fluorescence plate reader with a 400 nm excitation filter and 508 nm emission filter. Values of immunoprecipitated CD95 from unstimulated cells were taken as background and subtracted from those obtained with stimulated cells.

Assay of caspase-8 activity in vitro

Purification of bacterially expressed Fv-CASP-8 fusions was described elsewhere (D.W.Chang, Z.Xing, V.L.Capacio, M.E.Peter and X.Yang,

submitted for publication), and Fv-FLIP was purified similarly by Ni-NTA and DEAE columns. Fv-caspase-8 protein was mixed with Fv-FLIP in 150 µl of CED3 buffer containing 7 µM IETD-AFC. After incubation at 37°C for 15 min, the reaction mix was measured for fluorescence in a Bio-Rad Versaflor fluorometer. Alternatively, Fv-CASP-8 and Fv-FLIP proteins were mixed and incubated in MDB buffer (10 mM HEPES pH 7.0, 50 mM NaCl, 2 mM MgCl$_2$, 5 mM EDTA, 1 mM DTT) containing 100 µM biotinylated VAD-fmk at 37°C for 45 min. The proteins were then separated by SDS–PAGE, and biotinylated products were detected by immunoblotting with horseradish peroxidase (HRP)–avidin.

Co-immunoprecipitation assay

A total of 1 × 10^6 293 cells were transiently transfected with 3 µg of the indicated HA-tagged and FLAG-tagged protein expression constructs. After 16 h, cells were lysed with 1 ml of IP-lysis buffer (Yang *et al.*, 1998b) containing a protease inhibitor cocktail. The lysates were immunoprecipitated with 20 µl of anti-FLAG M2 antibody conjugated to agarose beads (Sigma) for 3 h. The beads were then washed three times with lysis buffer and resuspended in loading buffer for SDS–PAGE and immunoblotting analyses.

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