Cellular FLICE-inhibitory Protein Splice Variants Inhibit Different Steps of Caspase-8 Activation at the CD95 Death-inducing Signaling Complex*

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Andreas Krueger, Ingo Schmitz, Sven Baumann, Peter H. Krammer, and Sabine Kirchhoff‡

From the Tumor Immunology Program, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Upon stimulation, CD95 (APO-1/Fas) recruits the adapter molecule FADD/MORT1, procaspase-8, and the cellular FLICE-inhibitory proteins (c-FLIP) into the death-inducing signaling complex (DISC). According to the induced proximity model, procaspase-8 is activated in the DISC in an autoproteolytic manner by two subsequent cleavage steps. c-FLIP proteins exist as a long (c-FLIPL) and a short (c-FLIPS) splice variant, both of them capable of protecting cells from death receptor-mediated apoptosis. In stably transfected BJAB cells, both c-FLIPLN and c-FLIPLP block procaspase-8 activation at the DISC. However, cleavage is blocked at different steps. c-FLIPL allows the first cleavage step of procaspase-8, leading to the generation of the p10 subunit. In contrast, c-FLIPS completely inhibits cleavage of procaspase-8. Interestingly, p43-c-FLIPL, lacking the p12 subunit also prevents cleavage of procaspase-8. In contrast, a nonprocessable mutant of c-FLIPS allows the first cleavage of procaspase-8. In conclusion, both c-FLIP proteins prevent caspase-8 activation at different levels of procaspase-8 processing at the DISC. Our results indicate that c-FLIPLN induces a conformation of procaspase-8 that allows partial but not complete proteolytical processing, whereas in contrast c-FLIPS even prevents partial procaspase-8 activation at the DISC.

Apoptosis plays an important role in tissue homeostasis. In the immune system, apoptosis is used for negative and positive selection of T and B cells in the thymus and bone marrow, respectively, and to maintain immune homeostasis (1). Apoptosis can be induced by death receptors, a subgroup of the TNF1/nerve growth factor receptor superfamily (2). The best characterized member of the death receptor subfamily is CD95, also known as APO-1 or Fas. Stimulation of CD95 with its cognate ligand, CD95L, leads to clustering of the monomeric receptor or, as recently suggested, to conformational changes of preformed receptor complexes (3). This enables binding of the adapter molecule FADD/MORT1 (4, 5) and of procaspase-8 (FLICE, MACH, Mch5) (6–8) to CD95 via homophilic death domain and death effector domain (DED) interactions, respectively, forming the death-inducing signaling complex (DISC) (9). Recruitment of procaspase-8 to the DISC leads to its proteolytic activation through several cleavage steps. Free p18 and p10 subunits form the active caspase-8 heterotrimer. Active caspase-8 then initiates a cascade of caspase activation finally leading to cell death (10).

Sensitivity toward CD95-mediated apoptosis can be modulated at different levels in the CD95 signaling pathways (e.g. by the viral caspase inhibitors CrmA or p35 (11–14) or in certain cells (type II cells)) by Bcl-2/Bcl-xL, overexpression inhibiting mitochondrial changes during apoptosis (15). Viral FLICE-inhibitory proteins (v-FLIP), which are components of the class of γ-herpesviruses, form another family of apoptosis-inhibitory molecules. These molecules are composed of two death effector domains, a structure resembling the N-terminal half of procaspase-8. Via DED-DED-interaction, v-FLIP proteins are recruited to the CD95-DISC, preventing procaspase-8 recruitment and processing and thereby CD95-induced apoptosis (16).

A human homolog of v-FLIP is called c-FLIP/FLAME-1/c-FLICE/Casper/CASH/MIRT/CLARP/Usturpin (17–24). On the mRNA level, several c-FLIP splice variants exist. On the protein level, however, only two endogenous forms, c-FLIL and c-FLISP, could be detected so far (17, 20, 24, 25). c-FLIL is structurally similar to procaspase-8, since it contains two death effector domains and a caspase-like domain. However, this domain lacks residues that are important for the catalytic activity of caspase-8, most notably the cysteine within the active site. The short form of c-FLIP, c-FLISP, structurally resembles v-FLIP.

Despite the analysis of mice deficient for c-FLIP, which indicates a role of c-FLIP in cardiac development (26), the definitive physiological role of this molecule still remains controversial. Recent reports show that high expression of FLIP promotes tumor growth and facilitates immune escape of tumors (27, 28). In addition, mouse embryonic fibroblasts deficient of c-FLIP clearly display an increased sensitivity toward death receptor-mediated apoptosis (26).

Several reports suggest an involvement of c-FLIP in the modulation of the immune response (17, 29–33). We recently demonstrated a potential physiological role for c-FLIPS and found that it is up-regulated upon restimulation of the T cell receptor in primary human T cells. This finding correlates with rescue of these cells from activation-induced cell death (34). In addition, we demonstrated up-regulation of c-FLIPS after CD3/CD28 costimulation, which might contribute to protection toward activation-induced cell death (35).
The mechanism of c-FLIP<sub>L</sub> function has only been partially elucidated (25). Both procaspase-8 and c-FLIP<sub>L</sub> are recruited to the DISC. After initial cleavage of both molecules, the cleavage intermediates remain bound to the DISC and can no longer be replaced by procaspase-8. This prevents activation of the cytoplasmic procaspase-8 pool and renders the cell resistant to CD95-induced apoptosis (25). So far, nothing is known about the mechanism of c-FLIP<sub>S</sub> function.

In a previous study, we found that c-FLIP<sub>S</sub> but not c-FLIP<sub>L</sub> contributes to escape from activation-induced cell death in T cells (34, 35). Therefore, we further clarify the mechanisms of apoptosis inhibition mediated by c-FLIP<sub>S</sub> to detect possible differences compared with c-FLIP<sub>L</sub> action. To investigate this issue, we generated BJAB clones stably expressing c-FLIP<sub>S</sub> or c-FLIP<sub>L</sub> or both. Overexpression of either c-FLIP<sub>S</sub> or c-FLIP<sub>L</sub> results in resistance toward death receptor-mediated cell death. The two splice variants, however, cause this effect in a distinct fashion. Thus, procaspase-8 activation is inhibited at two different cleavage steps at the DISC by c-FLIP<sub>S</sub> and c-FLIP<sub>L</sub>, respectively, the two splice variants of c-FLIP. A detailed analysis of the domains of c-FLIP<sub>S</sub> revealed that full-length c-FLIP<sub>S</sub> but not a mutant lacking the p12 subunit, contributes to the first cleavage step of caspase-8.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The B lymphoblastoid cell line BJAB and the T cell line H9 were maintained in RPMI 1640 (Life Technologies, Inc.), 10 mM HEPES (Life Technologies), 2 mg/ml Gentamycin (Life Technologies), 10% fetal calf serum (Life Technologies) in 5% CO<sub>2</sub>.

**Antibodies and Reagents**—Monoclonal antibodies against FADD (mouse IgG1) and the FLAG epitope (MDYKDDDKK, clone M2, mouse IgG1) were purchased from Transduction Laboratories (Lexington, KY) and Sigma, respectively. The C15 monoclonal antibody (mouse IgG2b) recognizes the p18 subunit of caspase-8 (36), the C5 monoclonal antibody (mouse IgG1) was from Transduction Laboratories (Lexington, KY) and Sigma, respectively. The C15 monoclonal antibody (mouse IgG2b, Sigma) recognizes the p18 subunit of caspase-8 (36), the anti-c-FLIP monoclonal antibody NF6 (mouse IgG1) was described in (25), and anti-APO-1 is an agonistic monoclonal antibody (IgG3, σ) recognizing an epitope on the extracellular part of CD95 (APO-1/Fas) (37). The horseradish peroxidase-conjugated goat anti-mouse IgG1 and IgG2b were from Southern Biotechnology Associates (Birmingham, AL). Antisera against the caspase-9 and FADD, respectively, were used along with the other antibodies as described (38). TNF<sub>a</sub> was purchased from Biomol (Plymouth Meeting, PA). All other chemicals used were of analytical grade and purchased from Merck or Sigma.

**Isolation of the c-FLIP<sub>S</sub> Coding Region**—The coding sequence of c-FLIP<sub>S</sub> was isolated after total RNA isolation of H9 cells, reverse transcriptase-polymerase chain reaction, and subsequent polymerase chain reaction, using the following primer pair: c-FLIP sense, 5'-CTTGGAGGTGCTTCTCGTGAAGTCATCCATCCAGG-3'; c-FLIP anti-sense, 5'-gggcatCTACATGGAACAAACTTTCCAG-3'. The sequences shown in capital letters are homologous to the coding sequence shown). To analyze the antiapoptotic function of the trans-
manner as compared with cells expressing c-FLIP L or c-FLIPS alone (Fig. 1D). TRAIL- and TNFα-mediated apoptosis were completely inhibited. We conclude from these results that the CD95 DISC was not saturated with c-FLIP proteins in the single transfected cells, and therefore an additive effect in cells expressing both c-FLIP proteins was observed. In contrast, c-FLIP expression levels in the single transfected BJAB cells were sufficient for blocking of TRAIL- and TNFα-mediated apoptosis in BJAB cells, which might be due to lower expression levels of receptors for TRAIL and TNFα as compared with CD95.

c-FLIPS Completely Blocks Cleavage of Procaspase-8—To investigate the molecular mechanisms of c-FLIPS-mediated apoptosis inhibition, we determined activation of procaspase-8 as one of the first detectable events upon CD95 triggering. In vector-transfected cells, procaspase-8 was completely processed, and the p10 and p18 active subunits were detectable after 10–30 min (Fig. 2B, lane 3; for the procaspase-8 cleavage pattern, also compare Fig. 2A). In contrast, the generation of active subunits was completely inhibited in cells overexpressing both c-FLIP proteins. Neither the p18 nor the p10 subunits were detectable, and uncleaved procaspase-8 was detectable over a period of up to 3 h (Fig. 2B, lane 18). In cells expressing c-FLIP L, the generation of the p18 subunit was blocked, and only small
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**FIG. 2.** Different procaspase-8 cleavage products upon CD95 triggering in the presence of c-FLIP<sub>S</sub> or c-FLIP<sub>L</sub>. A, a simplified model for procaspase-8 processing. Procaspase-8 processing occurs in two consecutive steps: the generation of the p10 and the p43/41 subunits (1) and processing of the p43/41 cleavage product into the prodomain and the p18 subunit (2). B, time course of procaspase-8 and c-FLIP<sub>p</sub> processing in BJAB cells stably expressing FLAG-tagged c-FLIPS (lanes 13–18) or FLAG-tagged c-FLIP<sub>L</sub> (lanes 7–12) demonstrated in Fig. 1B. Control, BJAB cells transfected with empty vector (lanes 1–6).

amounts of the p10 subunit of procaspase-8 were generated (Fig. 2B, lanes 7–12). In the latter case, c-FLIP<sub>L</sub> only allowed the p43/41 cleavage product of caspase-8 to be generated. The p43/41 cleavage product stays at the DISC, thereby preventing recruitment and activation of cytosolic procaspase-8. Similar to procaspase-8, c-FLIP<sub>L</sub> is also cleaved at the DISC by either procaspase-8 or its p43/41 cleavage product (Fig. 2B, lanes 8–12). Thus, both c-FLIP proteins block the generation of active caspase-8. Our results suggest that c-FLIP<sub>L</sub> completely prevents procaspase-8 cleavage, whereas c-FLIP<sub>L</sub> allows the first cleavage step of procaspase-8.

**c-FLIP<sub>S</sub> Completely Blocks DISC Activity**—Since both procaspase-8 and c-FLIP<sub>S</sub> are recruited into the DISC (25), we assumed that in the presence of c-FLIP<sub>S</sub> procaspase-8 cleavage is blocked at the DISC or that its recruitment is completely inhibited. Therefore, we performed a DISC analysis by immunoprecipitation of either CD95-unstimulated or -stimulated BJAB cells that were either transfected with c-FLIP or vector alone, and we determined DISC-associated caspase-8 cleavage products by Western blot analysis. In cells overexpressing c-FLIP<sub>S</sub>, only unprocessed procaspase-8 was detectable in the DISC (Fig. 3, lane 6). In addition, cleavage of c-FLIP<sub>S</sub> was also significantly reduced (Fig. 3, lane 6). That was also found in cellular lysates (compare Fig. 2B). In contrast, the presence of c-FLIP<sub>L</sub> did not prevent the generation of the p43/41 cleavage product of caspase-8. This finding explains the detection of the p10 caspase-8 subunit in cellular lysates. FADD recruitment to the DISC was not modulated in the presence of c-FLIP<sub>S</sub> or c-FLIP<sub>L</sub>. In summary, c-FLIP<sub>S</sub> blocks cleavage of procaspase-8 at the DISC, whereas c-FLIP<sub>L</sub> prevents further processing of the p43/41 cleavage product.

The First Cleavage Step of Procaspase-8 Activation Is Driven by Full-length c-FLIP<sub>L</sub>—The fact that the p43 cleavage form of c-FLIP<sub>L</sub> is the predominant form at the DISC upon CD95 triggering (see Ref. 25 and Fig. 3), suggested that cleavage of c-FLIP<sub>L</sub> is important for blocking caspase-8 activation. To analyze the mechanism of procaspase-8 cleavage inhibition by c-FLIP<sub>L</sub> in more detail, we generated expression constructs coding for N-terminally FLAG-tagged mutants of c-FLIP<sub>L</sub> which either resemble the p43 cleavage form lacking the p12 subunit of c-FLIP<sub>L</sub> entirely (FLAG-p43<sub>mu</sub>) or contain a defective cleavage site (FLAG-D376N) (20) (compare Fig. 4A and scheme in Fig. 6). BJAB cells stably transfected with these expression constructs or empty vector were identified by Western blotting (Fig. 4B), and the sensitivity toward CD95-mediated apoptosis was investigated. Overexpression of either FLAG-tagged p43-c-FLIP<sub>L</sub> (p43<sub>mu</sub>) or FLAG-tagged uncleavable c-FLIP<sub>L</sub> (D376N) resulted in cells resistant toward LZ-CD95L-induced apoptosis (Fig. 4C). Therefore, both the full-length and the p43 cleavage form of c-FLIP<sub>L</sub> act in an antiapoptotic manner and block CD95-mediated apoptosis. Thus, cleavage of c-FLIP<sub>L</sub> is not required for its antiapoptotic function.

To determine the step in apoptosis signaling at which the mutated c-FLIP<sub>L</sub> molecules inhibit cleavage of procaspase-8, we looked for cleavage products after CD95 triggering. In vector (control)-transfected cells, procaspase-8 was completely processed, and the p10 and p18 active subunits were detectable (Fig. 4D, lanes 1–6). In cells transfected with an expression construct coding for the FLAG-tagged p43 cleavage product of c-FLIP<sub>L</sub> (p43<sub>mu</sub>), the generation of the p10 subunit was delayed and not detectable after 10 min (Fig. 4D, lane 4). In contrast, in cells overexpressing FLAG-tagged uncleavable c-FLIP<sub>L</sub> (D376N), procaspase-8 was cleaved into its p43/41 and p10 subunits within the first 10 min of stimulation (Fig. 4D, compare lanes 8 and 14). The p18 subunit was not generated in the presence of both c-FLIP<sub>L</sub> mutants (Fig. 4D). Thus, blocking of procaspase-8 cleavage by p43<sub>mu</sub> and the D376N mutant oc-
curred at different steps. To study this phenomenon in more detail, we performed DISC analysis of vector- or c-FLIP mutant-transfected BJAB cells and tested for associated caspase-8 cleavage products by Western blot analysis. In vector-transfected cells, both the full-length caspase-8 and the p43/p41-caspase-8 form were detectable in the DISC. In contrast, in D376N-expressing cells, only the p43/p41 cleavage products of caspase-8 could be detected, thus resembling the composition of the DISC in the presence of high amounts of wild-type c-FLIP. The presence of p43 mu inhibited the generation of the active caspase-8 subunits, which explains our findings presented in Fig. 4D and resembles the DISC in c-FLIP S high expressing cells. These results indicate that the presence of full-length c-FLIP but not cleavage of c-FLIP is necessary for the initial

**Fig. 4. Full-length c-FLIP is required for procaspase-8 processing.** A, overview of FLAG-tagged c-FLIP expression constructs containing the sequence of unprocessable c-FLIP (D376N) or the truncated c-FLIP (p43 mu) used for stable expression in BJAB cells. The numbers indicate the amino acids in the resulting proteins. B, Western blot analysis of lysates of BJAB cells stably transfected with expression vectors coding for the indicated FLIP proteins. Lane 1, vector-transfected cells (Control); lane 2, FLAG-tagged p43-c-FLIP (p43 mu); lane 3, FLAG-tagged unprocessable c-FLIP (D376N). C, BJAB cells described for B were incubated with the indicated concentrations of LZ-CD95L. Apoptosis was measured by the amount of DNA degradation. D, time course of procaspase-8 and c-FLIP processing in BJAB cells stably expressing D376N (lanes 7–12) or p43 mu (lanes 13–18) demonstrated in A and B. Control, BJAB cells transfected with empty vector (lanes 1–6). E, DISC analysis of BJAB cells shown in A–D triggered with 10 μg of LZ-CD95L (+) or left untriggered (−).
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DISCUSSION

The apoptosis-inducing signal transduction pathways of the CD95 system have been well characterized (39). In contrast, much less is known about mechanisms of inhibition of CD95-mediated apoptosis. One protein that has an inhibitory function on the DISC by reducing the generation of active caspase-8 is c-FLIP L. However, its mechanism of inhibition is not well understood. A second cellular splice variant of c-FLIP, c-FLIP S, also has antiapoptotic effects. Both c-FLIP proteins contain two DEDs and are recruited into the DISC and thereby block death receptor-mediated apoptosis. In this report, we provide important insight into the molecular mechanisms of c-FLIP-mediated apoptosis inhibition. BJAB cells that express high amounts of either c-FLIP L or c-FLIP S are protected against death receptor-mediated apoptosis. The presence of c-FLIP L prevents the initial cleavage step of procaspase-8, and therefore, its full-length form can be detected at the DISC. In contrast, c-FLIP S allows the initial cleavage step but blocks further processing and thus the generation of the p18 subunit. The difference in processing of procaspase-8 in the presence of the two c-FLIP splice variants is also reflected in cell lysates and sheds new light on caspase-8 activation at the DISC.

From these results, we suggest the following model of c-FLIP proteins mediated inhibition of death receptor initiated apoptosis. In the presence of low concentrations of c-FLIP proteins, procaspase-8 represents the majority of tandem DED-containing proteins at the DISC and is activated by trans- and autocatalytical cleavage due to the close proximity of several procaspase-8 molecules (Fig. 6B; Ref 40). c-FLIP L, that is recruited into the DISC is cleaved by caspase-8. High amounts of c-FLIP L in the DISC abolish the close proximity of procaspase-8 molecules, instead leading to proximity of c-FLIP S and procaspase-8, resulting in the first but not the second step of procaspase-8 processing. In this conformation, only the p10 subunit of caspase-8 and the p12 subunit of c-FLIP L are generated (Fig. 6C). Since c-FLIP L itself has no intrinsic catalytic activity, the generation of the p10 subunit of caspase-8 proceeds autocatalytically, whereas the generation of the p18 subunit would require transtrial activity (Fig. 6D). High amounts of c-FLIP S in the DISC totally prevent procaspase-8 cleavage (Fig. 6D). This indicates that c-FLIP S, in contrast to c-FLIP L, still induces a conformation of the DISC that leads to autocatalytic activity of procaspase-8 and the first cleavage step. This hypothesis is supported by the cleavage pattern of procaspase-8 in the presence of c-FLIP L mutants. The uncleavable c-FLIP L mutant, like the wild-type c-FLIP L, allows the generation of the p10 subunit of caspase-8. Therefore the full-length protein of c-FLIP L, but not its cleavage, facilitates the first cleavage step of procaspase-8 (Fig. 6F). Our results are further supported by the observation that the deletion mutant of c-FLIP L that does not contain the p12 subunit (p43 mut) prevents the first cleavage step of procaspase-8, similar to c-FLIP S (Fig. 6E). Both the p43 cleavage product of c-FLIP L and the uncleaved form block caspase-8 activation and, therefore, inhibit apoptosis. Given that procaspase-8 interacts with c-FLIP proteins in the DISC in a dimeric manner, our results suggest that the generation of the p10 subunit of caspase-8 occurs autocatalytically. In contrast, the second cleavage step leading to release of the p18 subunit requires transtrial activity. However, our results do not exclude interactions of dimers with dimers due to the multimeric nature of death receptor complexes.

High expression of c-FLIP L also prevents cleavage of c-FLIP L at the DISC. This phenomenon was also detected in restimulated primary T cells (34). It may be due to spatial interference of c-FLIP L with the interaction of procaspase-8 and c-FLIP S. Another possibility is that initial cleavage of procaspase-8, blocked by c-FLIP S, is required for its transtrial activity and, thus, c-FLIP L is not cleaved at the DISC upon high expression of c-FLIP S.

Transfectants expressing FLAG-tagged c-FLIP L and Myc-tagged c-FLIP S showed an additive effect with respect to protection against CD95-mediated apoptosis. This indicates that DISCs in single transfectants are not saturated for c-FLIP binding. In addition, p43/41-caspase-8 could be detected in the DISC, which was not the case in c-FLIP S single transfectants. This could be explained by either a dominant function of c-FLIP L over c-FLIP S or, alternatively, by caspase-8 cleavage in DISC species containing c-FLIP L alone.

Since both c-FLIP splice variants act as antiapoptotic pro-
teins, the question arises why certain physiological stimuli exclusively induce either c-FLIP_L or c-FLIP_S, like BCR/CD40 (32, 33) or TCR/CD28 (34, 35), respectively. Therefore, the biological function of different procaspase-8 cleavage patterns remains to be elucidated. Since c-FLIPS totally prevents cleavage of both caspase-8 and c-FLIP_L, one might speculate that the generation of these cleavage products is blocked because they are necessary for the recruitment of yet to be identified molecules into the DISC or, alternatively, they prevent the association of unidentified molecules.

Recently, it was reported that certain, but not all, viral and cellular FLIP proteins enhance activation of the NF-κB and AP-1 pathways upon stimulation of death receptors, possibly via recruitment of RIP, TRAF1, and TRAF2 into the DISC (41–43). However, one study also shows suppressive effects of DED-containing proteins, such as c-FLIP, on the NF-κB pathway (44). Therefore, the role of c-FLIP in activation of NF-κB by death receptors needs to be addressed in more detail.

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Fig. 6. Model for c-FLIP mediated inhibition of procaspase-8 processing at the DISC. A, triggering of CD95 leads to the recruitment of FADD, procaspase-8, and c-FLIP proteins into the DISC. Binding of procaspase-8 results in its activation by auto- and transproteolytic cleavage. The remaining caspase-8 prodomain is replaced by uncleaved procaspase-8, which is processed in the same manner as described above. B–F, depending on the ratio of caspase-8 and c-FLIP proteins at the DISC (gray box), different products are released from the DISC upon receptor triggering. B, low amounts of c-FLIP proteins allow processing of procaspase-8, leading to formation of the active caspase-8 heterotetramer composed of the p18 and p10 subunits. C, in the presence of high amounts of c-FLIP_L, procaspase-8 is recruited into the DISC, and cleavage is blocked after the generation of the p43 cleavage products of both caspase-8 and c-FLIP_L. D, in the presence of high amounts of c-FLIP_S, procaspase-8 is recruited into the DISC but remains unprocessed. In each case, modulation of caspase-8 cleavage renders cells resistant to CD95-mediated cell death. E, high amounts of truncated c-FLIP_L-p43mu prevent procaspase-8 processing completely. F, expression of unprocessable c-FLIP_L-D376N allows initial cleavage of procaspase-8 but prevents further processing, leading to accumulation of p43/41-caspase-8 in the DISC.
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