The Role of c-FLIP in Modulation of CD95-induced Apoptosis*

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Upon stimulation, CD95 (APO-1/Fas) recruits the adapter molecule Fas-associated death domain protein (FADD/MORT1 and caspase-8 (FADD-like interleukin-1β-converting enzyme (FLICE)/MACH/MCH5) into the death-inducing signaling complex (DISC). Recently, a molecule with sequence homology to caspase-8 was identified, termed cellular FLICE-inhibitory protein (c-FLIP). c-FLIP has been controversially reported to possess apoptosis-promoting and -inhibiting functions. Using c-FLIP-specific monoclonal antibodies, we now show that c-FLIP is expressed in two isoforms, both of which, like FADD and caspase-8, are recruited to the CD95 DISC in a stimulation-dependent fashion. In stably transfected BJAB cells, c-FLIP blocks caspase-8 activation at the DISC and thereby inhibits CD95-mediated apoptosis. During this process, both caspase-8 and c-FLIP undergo cleavage between the p18 and p10 subunits, generating two stable intermediates of 43 kDa that stay bound to the DISC. c-FLIP has been suggested to play a role in protecting activated peripheral T cells from CD95-mediated apoptosis (Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steinr, V., Bodmer, J. L., Schroeter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997) Nature 388, 190–195). In contrast to this hypothesis, neither caspase-8 nor c-FLIP were cleaved in these cells, ruling out c-FLIP as the main factor regulating DISC activity. Moreover, recruitment of FADD, caspase-8, and c-FLIP to the DISC was strongly reduced in the apoptosis-resistant but readily detectable in the apoptosis-sensitive T cells.

Apoptosis is a form of programmed cell death that plays an important role in tissue homeostasis. In the immune system, apoptosis is used for negative selection in the thymus and bone marrow and to eliminate unwanted lymphocytes following an immune response (1, 2). A subgroup of the TNFα/nervous growth factor-receptor superfamily, the death receptors, has been shown to induce apoptosis after triggering with ligand or agonistic antibodies (3). The best characterized member of the death receptor subfamily is CD95, also known as APO-1 or Fas. Stimulation of CD95 with agonistic antibody leads to clustering of the receptor. This enables the adapter molecule FADD/MORT1 (4, 5) and the death protease caspase-8 (FLICE, MACH, MCH5) (6–8), to bind to the receptor via homophilic death domain and death effector domain (DED) interactions, respectively, forming the death-inducing signaling complex (DISC) (9). Recruitment of caspase-8 to the DISC leads to its proteolytic activation, which initiates a cascade of caspases, leading to apoptosis (10).

Stimulation of resting peripheral T cells (in the following referred to as day 0 T cells) during an immune response leads to their activation (day 1 T cells) resulting in increased expression of several genes including IL-2 and CD95 (1). However, besides high CD95 surface expression, day 1 T cells are resistant to CD95-mediated apoptosis (11, 12). Repeated stimulation of previously activated T cells results in activation-induced cell death, which has been shown to involve the CD95 receptor/ligand system (13–16). After prolonged culture in the presence of IL-2, activated T cells develop an apoptosis-sensitive phenotype (day 6 T cells) (11, 12). Since CD95 surface expression does not significantly change between day 1 and day 6 T cells, the apoptosis-resistant phenotype of day 1 T cells must be caused by a block in CD95 signal transduction. We have previously shown that activation of caspase-8 at the DISC is inhibited in these resistant T cells, whereas Bcl-xL is highly up-regulated (17).

Sensitivity toward CD95-mediated apoptosis can be modulated e.g. by the viral caspase inhibitors CrmA or p35 (18–21) or in certain cells (type II cells) by Bcl-2/Bcl-xL overexpression inhibiting mitochondrial changes during apoptosis (22). A new class of virus-encoded apoptosis-inhibitory molecules, designated viral FLICE-inhibitory proteins (v-FLIPs), has been described (23–25). These molecules are composed of two death effector domains, a structure resembling the N-terminal half of caspase-8. Via DED-DED interaction, v-FLIPs are recruited to the CD95 DISC (23), preventing caspase-8 recruitment and processing and thereby CD95-induced apoptosis.

Recently, a cellular homologue of v-FLIP was identified by different groups and termed c-FLIP (26), CASH (27), Casper (28), CLARP (29), FLAME (30), I-FLICE (31), MRIT (32), and Usurpin (33). On the mRNA level, c-FLIP seems to exist as multiple splice variants, but on the protein level only two endogenous forms, c-FLIP and c-FLIPshort, could be detected (26, 28, 33). c-FLIP is structurally similar to caspase-8, since it contains two death effector domains and a caspase-like domain. However, this domain lacks residues that are important for its catalytic activity, most notably the cysteine within the active site. The short form of c-FLIP structurally resembles v-FLIP. The role of c-FLIP in apoptosis signaling is controversially discussed. Some reports have described it as a proapoptotic molecule (27–29, 32) and others as an antiapoptotic molecule (26, 27, 30, 31, 33). In addition, whether c-FLIP interacts with FADD and/or caspase-8 is not clear. Some groups have reported that c-FLIP can interact with both FADD and caspase-8 (26–
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28, 30, 32), while others could only detect an interaction between c-FLIP and caspase-8 (29, 31, 33). All of these studies were done in vitro or by overexpressing c-FLIP. The in vivo situation of the endogenous protein remains unclear. However, it was suggested that in day 1 T cells, the apoptosis-resistant phenotype correlates with high c-FLIP expression, which was decreased in the sensitive day 6 T cells (26).

To elucidate the role of c-FLIP in the CD95 pathway, we generated BJAB cells stably overexpressing c-FLIP and monoclonal antibodies against this protein. Using these tools, we now show that c-FLIP is predominantly found as a 55-kDa isoform in most cell lines independent of their resistant state toward CD95-mediated apoptosis. A minor c-FLIP species of 27/28 kDa (c-FLIPshort) was also detected in most cell lines. All forms of c-FLIP were found to be recruited to the DISC. Expressed at high levels in stable transfectants, c-FLIP completely blocked CD95-mediated apoptosis through inhibition of caspase-8 processing at the DISC. The mechanism of this inhibition involves cleavage of c-FLIP. c-FLIP seems to play only a minor role in apoptosis resistance of activated T cells, since it remained uncleaved in the resistant T cells and could be detected in day 0, day 1, and day 6 T cells at equal amounts.

EXPERIMENTAL PROCEDURES

Cell Lines—The Burkitt lymphomas Raji, BJAB, and SKW6.4; the T cell lymphomas H9, CEM, and Jurkat; the monocytic cell line U937; the cervix carcinoma HeLa; the breast carcinoma MCF-7; the colon carcinoma HT29; the hepatoma cell line HepG2; the myeloblastoma cell line KMY-1; and the small cell lung carcinomas SCLC 16H and NCI N592 were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 50 μg/ml gentamycin, and 5 μg/ml HEPES. The embryonic kidney line 293T was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 μg/ml gentamycin, and 10 μg/ml HEPES. All cells were of human origin.

Cloning of c-FLIP—An expressed sequence tag clone, AA115792, containing the complete c-FLIP open reading frame was identified by searching the public expressed sequence tag data base with a DED search profile. c-FLIP was amplified by polymerase chain reaction using the primers CCGCTCGAGCGGATGTCTGCTGAAGTCATCCCATCATCAAGGTTG and GCTCTAGAGCTAAGTAGGAGAGGATAAGTTTCTT-TCG-3′. The PCR product was cloned into the mammalian expression vector pEFrsFLAG containing a N-terminal FLAG epitope tag sequence. The c-FLIP sequence was determined by automated DNA sequence analysis and found to be identical to the published sequences of FLAMEγ, MRIT, I-FLICE-L, c-FLIPshort, and Casper (26, 30, 32–33).

Fusion Proteins, Antibodies, and Reagents—Using standard polymerase chain reaction and cloning techniques, 6-His-tagged FLIP and GST-caspase-8 fusion proteins (1–194) were generated as fusion proteins and purified as described (9). The affinity-purified rabbit polyclonal antibody RF60 was generated against a peptide spanning amino acids 190–209 as described previously (34). The anti-c-FLIP mAb NF6 (mouse IgG1) was generated against GST-N-c-FLIP as described (35). The anti-CD95 mouse mAb anti-APO-1 (IgG3) recognizes an epitope of the extracellular part of human CD95 (36), and the mouse mAb C15 (IgG2b) recognizes the p18 subunit of caspase-8 (35). Anti-FADD and anti-extracellular signal-regulated kinase-1 mAbs were purchased from Transduction Laboratories, and the anti-extracellular signal-regulated kinase-1/2 polyclonal antiserum was a gift of B. Schraven (University of Heidelberg). The horseradish peroxidase-coupled goat anti-mouse IgG1 and IgG2b antibodies and goat anti-rabbit IgG antibody were purchased from Southern Biotechnologies and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. All chemicals used were of analytical grade and were purchased from Merck or Sigma.

Immunoprecipitation and Western Blot—Immunoprecipitation of the CD95 DISC was carried out as described (37, 38). For immunoprecipitation of c-FLIP, 106 cells either unstimulated or treated with 1 μg/ml anti-APO-1 for 10 min were lysed in lysis buffer (20 mM Tris/HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml of leupeptin, antipain, chymostatin, and pepstatin A) as described (9), and the lysate was incubated with 20 μg of anti-c-FLIP affinity-purified rabbit polyclonal antibody (RF60) coupled to anti-rabbit IgG beads (Sigma) for 1 h at 4 °C.

For Western blot analysis, postnuclear supernatant equivalents of 106 cells or 50 μg of protein as determined by the BCA method (Pierce) were separated by 12% SDS-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech) and blocked with 5% nonfat dry milk in PBS/Tween (0.05% Tween 20 in PBS). After washing with PBS/Tween, the blots were incubated for 16 h with NF6 anti-c-FLIP, C15 anti-caspase-8, anti-FADD, or anti-mitogen-activated protein kinase antibody (1 μg/ml) (960 microfarads, 200 V) using a Gene Pulser™ (Bio-Rad) with control vector (pEFrsFLAG) or c-FLIP expression vector (pEFrsFLAG-c-FLIP). Transfectants were selected in supplemented RPMI 1640 medium containing 1 μg/ml puromycin (Sigma). High expressing clones were identified by Western blot analysis using the anti-c-FLIP mAb NF6.

RESULTS

Identification and Cloning of c-FLIP—Recently, a molecule that was found to be structurally related to caspase-8, c-FLIP, was identified containing two DEDs at its N terminus (Refs. 26–33; see “Experimental Procedures”). The role of c-FLIP in apoptosis signaling is controversial. Some reports have described it as an inducer (27–29, 32), whereas others have found it to be an inhibitor of death receptor-induced apoptosis (26, 27, 30, 31, 33). To clarify this controversy, we generated molecular tools to study the role of c-FLIP in CD95-mediated apoptosis in more detail.

Nine different cDNA variants were reported for c-FLIP. To test which isoforms of c-FLIP are expressed on the protein level, we generated a monoclonal antibody (NF6) against this molecule. Using this antibody, we screened 15 different cell lines for expression vectors for 6-HIS-c-FLIP and 6-HIS-caspase-8. The antibody used recognizes an epitope in the N-terminal half of the molecule. Using this antibody, we screened 15 different cell lines for expression vectors containing a DED-like sequence.

For quantifying the amount of c-FLIP and caspase-8 in cellular lysates, the specific anti-c-FLIP (NF6) and anti-caspase-8 (C15) monoclonal antibodies were used in a Western blot analysis to compare the signal obtained from endogenous expressed c-FLIP and caspase-8 with defined amounts of 6-HIS-c-FLIP and 6-HIS-caspase-8.

For stripping, blots were incubated for 30 min in a buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol at 60 °C. Then the blots were washed six times for 10 min in PBS/Tween and blocked again in 5% nonfat dry milk.

Preparation of Primary T Cells—Human peripheral T cells were prepared as described previously (12). For activation, resting T cells (day 0) were cultured at 2 × 106 cells/ml with 1 μg/ml phytohemagglutinin for 16 h (day 1). Day 1 T cells were then washed three times and cultured for an additional 5 days in the presence of 25 units/ml or 100 units/ml IL-2 (day 6).

Cytotoxicity Assay—For assaying apoptosis, 106 cells were incubated in 24-well-plates with or without 1 μg/ml anti-APO-1 plus 10 ng/ml protein A in medium for 16 h at 37 °C. Cells were centrifuged briefly in a Minifuge (Heraeus) at 4000 rpm for 5 min, washed once with PBS, and resuspended in a buffer containing 0.1% (v/v) sodium citrate, 0.1% (v/v) Triton X-100, and 50 μg/ml propidium iodide (Sigma). After incubation at 4 °C in the dark for at least 16 h, apoptotic nuclei were quantified by FACSscan (Becton Dickinson). Specific apoptosis was calculated as follows: (percentage of spontaneous apoptosis − percentage of spontaneous apoptosis) / 100 (percentage of spontaneous apoptosis) × 100.

Cell Transfections—BJAB cells were transfected by electroporation (Sama-Gene, 200 V) using a Gene Pulse™ (Bio-Rad) with control vector (pEFrsFLAG) or c-FLIP expression vector (pEFrsFLAG-c-FLIP). Transfectants were selected in supplemented RPMI 1640 medium containing 1 μg/ml puromycin (Sigma). High expressing clones were identified by Western blot analysis using the anti-c-FLIP mAb NF6.

To quantify the amount of c-FLIP and caspase-8 in cellular lysates, the specific anti-c-FLIP (NF6) and anti-caspase-8 (C15) monoclonal antibodies were used in a Western blot analysis to compare the signal obtained from endogenous expressed c-FLIP and caspase-8 with defined amounts of 6-HIS-c-FLIP and 6-HIS-caspase-8.
c-FLIP Is Recruited to the CD95 DISC—Since c-FLIP is structurally similar to caspase-8, it was likely that c-FLIP, like caspase-8, was recruited to the CD95 DISC through the adapter molecule FADD. Therefore, we immunoprecipitated either the unstimulated or the stimulated CD95 receptor and tested for associated c-FLIP by Western blot analysis. As shown in Fig. 3A, both c-FLIP and c-FLIPshort, associated with CD95 in a stimulation-dependent fashion. However, the full-length c-FLIP molecule was not detected in the DISC, but only the cleaved form, p43, which was also found in cellular lysates after CD95 triggering (Figs. 2D and 3A). This cleavage product was detected by an antibody against the N terminus of the molecule, and it associated with the CD95 DISC. Therefore, in analogy to caspase-8, p43-c-FLIP very likely represents the cleavage product of c-FLIP after removal of the p10 subunit (Fig. 3B).

We next tested how levels of c-FLIP present in the transfected cells would influence caspase-8 recruitment and process-
forms after their activation at the CD95 DISC (10, 35). Thus, c-FLIP overexpression allows initial cleavage of caspase-8 but blocks further recruitment of procaspase-8 to the DISC, thereby blocking the conversion of cytosolic procaspase-8 to the active enzyme.

**c-FLIP Does Not Interact with FADD and Caspase-8 in the Cytoplasm**—After having established that c-FLIP can act as an inhibitor of CD95 DISC activity, the question remained whether c-FLIP can interact with caspase-8 or FADD in the cytosol, which could be another potential molecular mechanism of c-FLIP-mediated apoptosis resistance. Such a cytosolic interaction has been described by others (26–33) using transient overexpression or in vitro systems. To test if endogenous amounts of c-FLIP were able to interact with FADD or caspase-8, we immunoprecipitated c-FLIP with an affinity-purified rabbit polyclonal anti-c-FLIP antibody from the lysates of either untreated or anti-CD95-treated cells (Fig. 4, anti-c-FLIP (rbAb)). We then analyzed the immunoprecipitates by Western blotting using monoclonal antibodies against c-FLIP, caspase-8, or FADD. Without CD95 triggering, both full-length c-FLIP and c-FLIPshort were immunoprecipitated (Fig. 4A, lane 1). However, no interactions with caspase-8 or FADD could be detected (Fig. 4, B and C, lane 1). After CD95 receptor stimulation, c-FLIP was also immunoprecipitated as the processed p43 form (Fig. 4A, lane 2). In addition, caspase-8 and FADD were now coimmunoprecipitated with c-FLIP (Fig. 4, B and C, lane 2). However, after CD95 triggering, c-FLIP interacted predominantly with the cleavage intermediates of caspase-8 (Fig. 4B, lane 2), which was even more pronounced in cells with high c-FLIP expression levels (Fig. 4B, lane 4). Since these forms of caspase-8 are mostly found at the DISC (compare with Fig. 3B), which is formed after CD95 triggering, we conclude that c-FLIP interacts with caspase-8 and FADD at the receptor level. Therefore, no association between c-FLIP and caspase-8 or FADD could be detected without receptor triggering. The same results were obtained by immunoprecipitating either FADD or caspase-8 and testing for associated molecules by Western blot analysis using antibodies against c-FLIP, caspase-8, and FADD (data not shown). This demonstrates that no stable preformed complex between FADD, caspase-8, and c-FLIP exists in the cytoplasm in vivo.

**Role of c-FLIP in Resistance of Activated T Cells toward CD95-mediated Apoptosis**—We have demonstrated that c-FLIP inhibits caspase-8 processing and thereby apoptosis only when present at elevated levels. We have previously shown that short term activated T cells are resistant to CD95-induced apoptosis caused by a block in caspase-8 activation at the DISC (17). This phenotype would be consistent with c-FLIP being involved in this apoptosis resistance. To investigate this possibility, we isolated peripheral blood T cells (day 0) and activated them with phytohemagglutinin for 16 h (day 1), which resulted in strong up-regulation of CD95. As reported, cross-linking of CD95 in these cells did not result in significant apoptosis induction (Fig. 5A). Continuous culturing for 5 days in the presence of IL-2 (25 units/ml or 100 units/ml) (days 2–6) rendered T cells sensitive to CD95-mediated apoptosis (Fig. 5A) without significant changes in CD95 surface expression (data not shown). During these kinetics, the expression levels of c-FLIP and caspase-8 did not change neither at 25 units/ml IL-2 (Fig. 5A) nor at 100 units/ml IL-2 (data not shown).

Testing for caspase-8 cleavage after CD95 triggering in day 1 and day 6 T cells revealed that the resistant day 1 T cells have a defect in caspase-8 activation (Fig. 5B), consistent with previously published data (17). In addition, at day 1 no c-FLIP cleavage could be detected after CD95 triggering. Since apoptosis inhibition by c-FLIP involves the cleavage of this protein
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FIG. 4. c-FLIP interacts with caspase-8 and FADD only at the DISC. BJAB cells transfected with empty vector (BJAB-control) or FLAG-c-FLIP expression plasmid (BLAB-c-FLIP) were left untreated (−) or stimulated with 1 μg/ml anti-CD95 mAb for 10 min (+) and subsequently lysed. c-FLIP was then immunoprecipitated using the affinity-purified rabbit polyclonal anti-c-FLIP antibody (rabAb) RF60 coupled to anti-rabbit IgG beads. The immunoprecipitates (IP) were analyzed by Western blotting (WB) using anti-c-FLIP mAb NF6 (A), anti-caspase-8 mAb C15 (B), or anti-FADD mAb (C). Migration positions of the detected proteins are indicated.

(Fig. 2D), resistance of day 1 T cells cannot simply be due to expression of c-FLIP. A more general defect in DISC formation in the resistant day 1 T cells was detected using Western blot analysis (Fig. 5D). We did not find significant amounts of FADD, c-FLIP, and caspase-8 coimmunoprecipitated with activated CD95 in day 1 T cells, whereas all three molecules were detectable at day 6. Therefore, the resistance toward CD95-mediated apoptosis in day 1 T cells may be caused by reduced DISC formation rather than by expression of c-FLIP.

DISCUSSION

The early events in the signal transduction pathway of the apoptosis-inducing receptor CD95 have been well characterized. Clustering of the receptor leads to formation of the DISC, which involves binding of the adapter molecule FADD to the death domain of CD95 and subsequent recruitment of caspase-8 (9). Binding of caspase-8 to the DISC results in its proteolytic activation, presumably through an autocatalytic mechanism (10). Although caspase-8 is able to undergo auto-proteolytic maturation when cross-linked (39–41), the active caspase-8 enzyme cannot activate its own proform (10). Therefore, cytosolic procaspase-8 has to be recruited to the CD95 DISC to become activated. This implies that the DISC has to be functional throughout the stimulation to transduce the complete apoptotic signal. Disturbance of the DISC leads to a block in ongoing caspase-8 activation reducing the amount of active caspase-8 formed determining whether the cell will survive or undergo apoptosis.

We have previously shown that overexpression of a viral protein, v-FLIP, containing two DEDs interfered with the function of the DISC by competing with caspase-8 for binding to receptor bound FADD (23). Recently, a human homolog of v-FLIP, c-FLIP, was cloned (26–33). Depending on the transient overexpression or the in vitro pull-down systems used, c-FLIP was shown to either promote or to inhibit apoptosis. High overexpression of DED-containing molecules has been shown to induce apoptosis by aggregation and the formation of nonphysiological death effector filaments, leading to the activation of caspases (42). This may be the reason why several groups found a proapoptotic function of c-FLIP in transient overexpression systems (27–29, 32). No stable expression clone has been described in which c-FLIP had a proapoptotic effect.

In all reports, associations of c-FLIP with FADD or caspase-8 were found using transient overexpression or in vitro systems. Using anti-c-FLIP monoclonal antibodies and stable c-FLIP transfectants, we have now studied the function of c-FLIP in vivo. We show that c-FLIP has an antiapoptotic function, and we have elucidated the mechanism of this inhibition. Furthermore, testing endogenous proteins we could not detect any constitutive association between c-FLIP, FADD, and caspase-8. Only after CD95 triggering are all three molecules recruited to the CD95 receptor complex.

The way c-FLIP inactivates the DISC is by blocking further recruitment of caspase-8 into the complex. This leads to inhibition of caspase-8 activation, since procaspase-8 is no longer able to replace the cleavage products at the DISC in order to become activated (Fig. 6). This mechanism provides an explanation of how the amount of c-FLIP determines the degree of apoptosis-resistance. Small amounts of c-FLIP as endogenously present in most cells are only able to block a few receptor complexes, and caspase-8 is still sufficiently activated at the remaining DISCs, leading to apoptosis. The more c-FLIP is expressed, the more receptor complexes are blocked, making the cell resistant to CD95-induced apoptosis.

c-FLIP has a high affinity to bind to the DISC. Despite the fact that c-FLIP is expressed endogenously at low levels, it was readily detectable at the DISC level. It therefore seems that most of the intracellular pool of endogenous c-FLIP binds to the DISC, providing an explanation for the rapid cleavage of this molecule detectable minutes after stimulation of CD95. High c-FLIP expression in the stable c-FLIP transfectants resulted in increased binding of c-FLIP to the DISC, indicating that the DISC has a high capacity to bind c-FLIP as opposed to caspase-8, of which only a fraction can be found bound to the DISC. These data suggest that the affinity of the DISC to bind c-FLIP is higher than to bind caspase-8, consistent with the function of c-FLIP to prevent further recruitment of caspase-8 to the DISC. This model was confirmed by demonstrating that in CD95-stimulated cells caspase-8 can be coimmunoprecipitated with endogenous c-FLIP, probably because both molecules were complexed with FADD at the activated CD95 receptor (Fig. 4). However, in this complex, mainly the caspase-8 cleavage products were found, suggesting that in a receptor
complex containing c-FLIP the recruitment of full-length caspase-8 is inhibited.

c-FLIP has also been shown to block TNF-RI-, DR3-, and TRAIL-R-induced apoptosis (26, 27, 30, 31), implying a similar mechanism of signal transduction by these receptors. To date, nothing is known about the in vivo receptor complex of DR3 or one of the TRAIL receptors. It has been shown that endogenous amounts of the signaling molecules TRADD, RIP, and TRAF2 are recruited to the activated TNF-RI (43–45). However, no FADD or caspase-8 could be detected in this complex (28), although cells lacking FADD or caspase-8 expression are resistant to TNF-induced apoptosis (46–49). Therefore, either the complex TNF-RI with TRADD, FADD, and caspase-8 is too unstable to be detected by immunoprecipitation, or FADD and caspase-8 are involved in an intracellularly formed complex that could then be a target of c-FLIP.

In summary, since the amount of endogenous c-FLIP present in all cells tested is insufficient to block apoptosis in cells with high CD95 expression, c-FLIP could play a role in vivo in blocking CD95-mediated apoptosis in tissues with low receptor expression. Alternatively, c-FLIP might regulate apoptosis in-

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**FIG. 5.** c-FLIP is not responsible for apoptosis resistance of day 1 T cells. A, sensitivity of activated T cells to CD95-mediated apoptosis. The sensitivity of resting T cells (d0), T cells treated overnight with phytohemagglutinin (d1), and T cells subsequently cultured with 25 units/ml (○–○) or 100 units/ml (●–●) IL-2 (d2–d6) to CD95-mediated apoptosis was determined as described under “Experimental Procedures.” 30 μg of cellular lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting using C15 anti-caspase-8 or NF6 anti-c-FLIP mAbs. To normalize for equal protein loading, a Western blot for mitogen-activated protein kinase using a monoclonal anti-extracellular signal-regulated kinase-1 antibody was included. Shown is the analysis with 25 units/ml IL-2. B and C, purified d1 or d6 primary human T cells were stimulated with anti-CD95 mAb (2 μg/ml) for the indicated periods of time and subsequently lysed. 30 μg of cellular lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting using C15 anti-caspase-8 (B), NF6 anti-c-FLIP (C), or polyclonal rabbit anti-extracellular signal-regulated kinase-1/2 (C) antibodies. Migration positions of the detected proteins are indicated. D, Western blot analysis of the CD95 DISC of day 1 and day 6 T cells. 10^6 T cells were triggered for 1 min with 2 μg/ml anti-CD95 mAb at 37 °C at a density of 2 × 10^6 cells/ml and subsequently lysed. CD95 DISC was immunoprecipitated using protein A-Sepharose beads (+), and subsequently the unstimulated CD95 receptor was immunoprecipitated using anti-CD95 mAb covalently coupled to cyanogen bromide-activated Sepharose beads (−). Immunoprecipitates were analyzed by Western blotting using anti-c-FLIP, anti-caspase-8, and anti-FADD mAbs. Migration positions of the detected proteins are indicated.

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**FIG. 6.** Model for c-FLIP-mediated resistance to CD95-induced apoptosis. A, triggering of CD95 with agonistic anti-CD95 mAb leads to the recruitment of FADD and caspase-8 to the receptor. Binding of caspase-8 results in its activation by autoproteolytic cleavage and the release of the active subunits. The remaining caspase-8 prodomain is replaced by uncleaved procaspase-8, which then starts a new activation cycle. B, in the presence of c-FLIP, both caspase-8 and c-FLIP are recruited to the activated receptor. After initial cleavage of both molecules, the cleavage intermediates remain bound to the receptor and can no longer be replaced by procaspase-8. This prevents caspase-8 activation and renders the cell resistant to CD95-induced apoptosis.

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2 F. C. Kischkel, P. H. Krammer, and M. E. Peter, unpublished data.
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...were shown to have elevated levels of Bcl-xL (17, 51), which expressed Bcl-2 (22). It is interesting to note that day 1 T cells Type I cells were shown to undergo CD95-mediated apoptosis strongly up-regulated, and the mitochondria are therefore involved in this process. 2) In our hands, the expression of c-FLIP both on the mRNA level (data not shown) and the protein level (Fig. 5C) was essentially the same when day 1 and day 6 T cells were compared. This was found to be independent of the concentration of IL-2 used to culture the T cells. c-FLIP in day 1 T cells is not cleaved after triggering the CD95 receptor. Both endogenous and overexpressed c-FLIP were cleaved during CD95-mediated apoptosis in the BJAB cells in c-FLIP-inhibited apoptosis. 3) c-FLIP in day 1 T cells is not cleaved after triggering the CD95 receptor. Both endogenous and overexpressed c-FLIP were cleaved during CD95-mediated apoptosis in the BJAB cells in c-FLIP-inhibited apoptosis. 4) Day 1 T cells seem to have a general defect in formation of the DISC including recruitment of FADD. Hence, none of the DISC components ( caspase-8, FADD, or c-FLIP) was efficiently coimmunoprecipitated with the activated CD95 receptor in day 1 T cells. These data strongly indicate that an as yet unidentified component rather than c-FLIP regulates formation of the DISC in the resistant T cells.

In a recent report, we described a defect in DISC formation in these day 1 T cells (17). Using metabolically labeled T cells and analysis of anti-CD95 immunoprecipitates on two-dimensional gels, we concluded that procaspase-8 was not recruited to the activated CD95 receptor in the day 1 T cells. However, the apparent amount of metabolically labeled FADD recruited by CD95 when comparing day 1 and day 6 T cells appeared to be similar. We therefore predicted a protein carrying a DED that would act as a resistance factor preventing binding of caspase-8 to the DISC-bound FADD. However, due to the labeling method, the amount of DISC components recruited could not be quantified. Monoclonal antibodies available against all known DISC components now revealed that day 1 T cells have a more general defect in DISC formation, since the amount of all components (FADD, caspase-8, and c-FLIP) bound to the activated receptor was severely reduced. Such a general defect in formation of the DISC was recently found in certain cell lines that were designated type II cells (22). In these cells, very little active caspase-8 is formed at the DISC, which is not sufficient to directly activate caspase-3 but enough to activate mitochondrial involvement. 

Mitochondrial involvement is therefore essential for the execution of apoptosis in type II cells. Only in these cells could apoptosis be inhibited by overexpression of either Bcl-2 or Bcl-xL that blocked all apoptogenic activity of mitochondria. Type I cells were shown to undergo CD95-mediated apoptosis independently of mitochondrial functions. Apoptosis in these cells with strong DISC formation could not be blocked by overexpressed Bcl-2 (22). It is interesting to note that day 1 T cells were shown to have elevated levels of Bcl-xL (17, 51), which have been shown to block CD95-mediated apoptosis in type II cells with low DISC formation (22). One could therefore hypothesize the following: Day 1 T cells behave like type I cells, since they do not form a proper DISC and cannot generate enough active caspase-8 to activate caspase-3 directly. They depend on the activity of mitochondria. However, Bcl-xL in these cells is strongly up-regulated, and the mitochondria are therefore blocked in their apoptogenic activity. Hence, the cells are CD95 apoptosis-resistant. After prolonged incubation with IL-2, the T cells turn into type I-like cells, since they now form a DISC and recruit and activate caspase-8 at the DISC (17). These cells are probably independent of mitochondrial involvement in apoptosis and have down-regulated Bcl-xL.

In summary, day 1 T cells are probably resistant to CD95-mediated apoptosis because they seem to be type II-like cells that are protected by overexpression of Bcl-xL or, as it was recently suggested, by Bcl-xL (52). We have shown that day 6 T cells can acquire a sensitive phenotype without IL-2-mediated down-regulation of c-FLIP leading to strong caspase-8 activation at the DISC. In summary, we have not found an indication that c-FLIP regulates the apoptosis sensitivity of peripheral T cells. Future experiments will be aimed to identify tissues that express low levels of CD95 in which endogenous levels of c-FLIP regulate apoptosis sensitivity through death receptors to find a physiological function of this molecule. However, the data already show that overexpression of c-FLIP should be a novel tool to protect certain therapeutically useful cells. One example would be to prolong the lifetime of tumorouspecific killer cells.

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