c-FLIP<sub>R</sub>, a New Regulator of Death Receptor-induced Apoptosis*

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c-FLIP<sub>R</sub> (c-FLICE inhibitory proteins) play an essential role in regulation of death receptor-induced apoptosis. Multiple splice variants of c-FLIP have been described on the mRNA level; so far only two of them, c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub>, had been found to be expressed at the protein level. In this report, we reveal the endogenous expression of a third isoform of c-FLIP. We demonstrate its presence in a number of T and B cell lines as well as in primary human T cells. We identified this isoform as c-FLIP<sub>R</sub>, a death effector domain-only splice variant previously identified on the mRNA level. Importantly, c-FLIP<sub>R</sub> is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex upon CD95 stimulation. Several properties of c-FLIP<sub>R</sub> are similar to c-FLIPS<sub>R</sub>; both isoforms have a short half-life, a similar pattern of expression during activation of primary human T cells, and are strongly induced in T cells upon CD3/CD28 costimulation. Taken together, our data demonstrate endogenous expression of c-FLIP<sub>R</sub> and similar roles of c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> isoforms in death receptor-mediated apoptosis.

Apoptotic cell death is common to multicellular organisms. Apoptosis can be triggered by a number of factors including UV or γ-irradiation, chemotherapeutic drugs, and signaling from death receptors. Eight members of the death receptor family have been characterized so far: tumor necrosis factor-R1 (DR1, CD120a, p55, p60), CD95 (DR2, APO-1, Fas), DR3 (APO-3, LARD, TRAMP, WSL1), TRAIL<sup>‡</sup> (tumor necrosis factor-related apoptosis-inducing ligand)-R1 (DR4, APO-2), TRAIL-R2 (DR5, KILLER, TRICK2), DR6, ectodysplasin A receptor, and nerve growth factor receptor (1–5).

Cross-linking of CD95 with its natural ligand CD95L (CD178) or with agonistic antibodies such as anti-APO-1 (7) induces apoptosis in sensitive cells. A death-inducing signaling complex (DISC) is formed upon CD95 stimulation within seconds (8). The DISC consists of oligomerized, probably trimerized, CD95 molecules, the adaptor molecule FADD (Fas-associated DED), two isoforms of procaspase-8 (procaspases-8A and -8B), procaspase-10, and c-FLIP<sub>L</sub> or c-FLIP<sub>S</sub> (c-FLICE inhibitory protein) (5). The interactions between molecules at the DISC are based on homotypic contacts. The death domain (DD) of the receptor interacts with the DD of FADD, whereas the death effector domain (DED) of FADD interacts with the N-terminal tandem DEDs of procaspases-8 and -10 and c-FLIP<sub>L</sub>S. The binding of procaspase-8 to the DISC results in processing of the zymogen. As a result the active heterotetramer p102-p182 of caspase-8 is released into the cytosol to propagate the apoptotic signal (9).

Death receptor-mediated apoptosis can be blocked at different levels. One of the well described inhibitors is c-FLIP, which is also known as FLAME-1/FLICE/CASPER/CASH/MRTT/CLARP/Usurpin (10). On the mRNA level c-FLIP seems to exist in multiple splice variants, whereas on the protein level only two forms, c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub>, have been detected so far (11, 12). c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> contain two DED domains that are structurally similar to the N-terminal part of procaspase-8. The C terminus of c-FLIP<sub>L</sub> consists of two catalytically inactive caspase-like domains (p20 and p12), whereas the short C terminus of c-FLIP<sub>S</sub> shows no homology to procaspases-8 or -10. Both isoforms of c-FLIP are recruited to the DISC by DED interactions (13–15). c-FLIP<sub>L</sub> blocks caspase-8 processing and activation at the DISC and CD95-induced apoptosis. The role of c-FLIP<sub>S</sub> at the DISC is controversial (16, 17). Some reports characterize c-FLIP<sub>L</sub> as an anti-apoptotic molecule that functions in a way analogous to c-FLIP<sub>S</sub>, whereas others ascribe pro-apoptotic functions to c-FLIP<sub>L</sub> (16), referring to its assistance in the autacatalytic activation of procaspase-8 at the DISC. This pro-apoptotic function would help to explain the phenotype of c-FLIP-deficient mice characterized by heart failure and death at embryonic day 10.5. The same phenotype is described for caspase-8- and FADD-deficient mice (12, 18–20). c-FLIP<sub>S</sub> was shown to play an important role not only in CD95 signaling but also in tumor necrosis factor- and TRAIL-induced apoptosis (21).

Several reports show a correlation between the resistance of freshly activated T cells to CD95-mediated apoptosis or activation-induced cell death (AICD) and increased expression levels of c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub>. In vitro restimulation of preactivated primary human T cells induces expression of c-FLIP<sub>S</sub>, rendering the proportion of cells that survive induction of AICD again resistant to CD95 ligation (22). However, the observed induction of c-FLIP expression does not in itself prevent AICD. Therefore, the exact roles of different c-FLIP isoforms in resistance of short-term activated T cells remain to be determined.

We considered the fact that there are multiple splice variants of c-FLIP on the mRNA level that might contribute to the establishment of sensitivity or resistance toward apoptosis, and we decided to investigate the presence of other c-FLIP isoforms on the protein level. Using a monoclonal antibody directed against the DED-containing N terminus of c-FLIP, we detected a short isoform of c-FLIP in a number of cell lines that was different from c-FLIP<sub>S</sub>. We identified this isoform as c-FLIP<sub>R</sub>. It was initially cloned in 2001 by Djerbi et al. (11). In addition, we observed differential expression of c-FLIP<sub>R</sub> and...
c-FLIP<sub>R</sub> in several cell lines. In some cell lines only one isoform was detected, whereas in others both were present. c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub> demonstrated comparable biochemical characteristics with respect to their recruitment to the CD95 DISC. Furthermore, both isoforms possessed similar anti-apoptotic properties with respect to CD95-mediated apoptosis. We also observed similar characteristics of c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub> with respect to half-life and CD3/CD28-induced up-regulation in primary human T cells. Thus, we demonstrate for the first time the presence of c-FLIP<sub>R</sub> protein in a number of T and B cell lines as well as in primary human T cells and show that its role in apoptosis is comparable with that of c-FLIP<sub>S</sub>.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The T cell lines HUT78, CEM, H9, Jurkat FADD-deficient (clone I.21), Jurkat caspase-8-deficient (clone I.192), Jurkat wild type clone A3, and Jurkat clone J16, the B lymphoblastoid cell lines SKW6.4, Raji, and BJAB, and the pre-B cell line Boe<sup>k</sup> were maintained in RPMI 1640 (Invitrogen), 10 mM HEPES (Invitrogen), 50 μg/ml gentamycin (Invitrogen), 10% fetal calf serum (Invitrogen) in 5% CO<sub>2</sub>. The caspase-8-deficient Jurkat cell line (clone I.192), the FADD-deficient Jurkat cell line (clone I.21), and the wild type Jurkat clone A3 were obtained from J. Blenis (Harvard Medical School).

**Preparation of Primary Human T Cells, Cell Culture, and CD3, CD28, CD3/CD28 Stimulations**—Human peripheral T cells were prepared as described previously (23). For activation, resting primary human T cells (day 0) were cultured at 2 × 10<sup>6</sup> cells/ml with 1 μg/ml phytohemagglutinin for 16 h (day 1). T cells were then washed three times and cultured for 5 additional days in the presence of 25 units/ml interleukin-2 (day 6). To reactivate day 5 primary human T cells via T cell receptor/CD3, 2 × 10<sup>6</sup> cells/ml were incubated with plate-bound anti-CD3 antibody OKT3 (30 μg/ml). Costimulation via CD28 was achieved by the addition of anti-CD28 antibodies (1.5 μg/ml) and goat anti-mouse cross-linking antibodies (2 μg/ml) to the culture. The anti-CD28 isotype-identical antibody I2D11H was added to cells treated with OKT3 only.

**Antibodies and Reagents**—Anti-FADD monoclonal antibody (mouse IgG1) was purchased from BD Transduction Laboratories. Anti-caspase-8 polyclonal antibody C-20 was purchased from Santa Cruz Biotechnology. The anti-caspase-8 mAb C15 (mouse IgG2b) recognizes the p18 subunit of caspase-8 (24). Anti-AP-1 is an agonistic monomeric antibody (mouse IgG3) recognizing an epitope on the extracellular part of CD95 (Fas) (7). The horseradish peroxidase-conjugated goat anti-mouse IgG1, -2a, and -2b were from Southern Biotechnology Associates (Echingen, Germany). All chemicals used were of analytical grade and purchased from Merck or Sigma.

**Reverse Transcription PCR for c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub>**—5 × 10<sup>5</sup> cells were lysed for 10 min in 1 ml of TRIzol (Invitrogen), and RNA was isolated according to the manufacturer's instructions. 50 ng of RNA was reverse transcribed into cDNA using MuLV reverse transcriptase (Applied Biosystems) according to the manufacturer's instructions. The following primers were used to amplify c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub> from cDNA: c-FLIP sense, 5′-ATGTCTGCTGAAGTCATCC-3′; c-FLIP antisense, 5′-CATGGACAACATTTCCAAG-3′; c-FLIP<sub>R</sub> antisense, 5′-TGCTGGATTCTCATAGT-3′. Primers were purchased from MWG.

**Cloning of c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub>**—c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub> were cloned into the pEF4 expression vector (Invitrogen) using the polymerase chain reaction and the following primers: c-FLIP sense, 5′-ggggtacctccATGTCTGCTGAAGTCATCC-3′; c-FLIP<sub>R</sub> antisense, 5′-gggtacctccATGTCTGCTGAAGTCATCC-3′; c-FLIP<sub>R</sub> antisense, 5′-gctgagggctcaCATGGACAACATTTCCAAG-3′; c-FLIP<sub>R</sub> antisense, 5′-gctgagggctcaCATGGACAACATTTCCAAG-3′. Cloning was performed using KpnI and XhoI (Fermentase).

**CD95 DISC Analysis by Immunoprecipitation**—The composition of the CD95 DISC was determined as follows. 5 × 10<sup>5</sup> cells were either treated with 2 μg/ml anti-APO-1 (IgG3) for 5 min at 37 °C or left untreated, washed twice in 1 × PBS, and subsequently lysed in lysis buffer (30 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Roche Applied Science), 1% Triton X-100 (Serva, Germany), and 10% glycerol). CD95 was immunoprecipitated with anti-APO-1 antibody and Protein A-Sepharose beads (Sigma) at 4 °C overnight. Beads were washed five times with 20 volumes of lysis buffer and subjected to autoradiography.

**Stable Transfection of BJAB Cells**—Stable transfection of BJAB cells was performed using expression vectors encoding c-FLIP<sub>S</sub>, c-FLIP<sub>R</sub>, and the empty vector by electroporation (960 microfarads, 200 V). Selection pressure was added 48 h after transfection (100 μg/ml Zeocin) for 2 weeks. Expression was controlled by Western blot using anti-FLIP mAb NF6.

**Cytotoxicity Assay**—For assaying apoptosis, 5 × 10<sup>5</sup> cells were incubated in 48-well plates with or without the indicated amounts of anti-APO-1 for 4 h at 37 °C. Cell death was measured by forward scatter/side scatter via flow cytometry, and specific cell death was calculated as follows: (percentage of experimental cell death − percentage of spontaneous cell death)/100 × 100%. In **Vitro DISC Recruitment Assay**—The CD95 DISC was immunoprecipitated from 5 × 10<sup>5</sup> HUT78 or SKW6.4 cells as described above, and DISC-containing beads were incubated with in vitro translated [35S]labeled c-FLIP<sub>S</sub> or c-FLIP<sub>R</sub> (TNT, T7-coupled reticulocyte lysate system; Promega) in reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, and 10% sucrose) overnight at 4 °C (26). Reactions were separated into supernatants and beads, and the beads were washed five times in lysis buffer. Beads and supernatants were separated on 15% Laemmli gels, blotted, and subjected to autoradiography.

**RESULTS**

**A New Short Variant of c-FLIP Is Detected on the Protein Level**—Two c-FLIP isoforms, c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub>, were found on the protein level (5, 10). To confirm these data we probed different T and B cell lines for c-FLIP expression (Fig. 1A). Cellular lysates were blotted, and c-FLIP proteins were de-
ected with the mAb NF6 directed against the DED region of c-FLIP. Interestingly, we detected a third prominent band different from c-FLIP <sub>R</sub> and c-FLIP <sub>S</sub> in the B cell lines SKW6.4, Boe<sup>5</sup>, and Raji. The molecular mass of this product is lower than that of c-FLIP<sub>S</sub>. Raji and SKW6.4 mainly contained this new variant of c-FLIP, whereas it is coexpressed with c-FLIP<sub>S</sub> in Boe<sup>5</sup> cells. In the T cell lines HUT78 and JA3, coexpression of c-FLIP<sub>S</sub> and the shorter band could also be detected.

To confirm our data we performed immunoprecipitation with anti-FLIP mAb NF6 (Fig. 1B). Consistent with the results of total cell lysates, we detected both short variants, c-FLIP<sub>S</sub> and the new one in Boe<sup>5</sup> cells, whereas in Raji and SKW6.4 only the new c-FLIP<sub>R</sub> variant was observed. Thus, we revealed a new protein product with a molecular mass lower than that of c-FLIP<sub>R</sub> that was recognized by anti-FLIP mAb NF6.

**Identification of the New c-FLIP Variant as c-FLIP<sub>R</sub>**—Sever<sub>r</sub>al alternatively spliced messenger RNAs for c-FLIP have been reported (10, 11). The molecular mass of the newly observed c-FLIP<sub>R</sub> variant is similar to the predicted molecular mass of the splice variant c-FLIP<sub>R</sub> (11). The mRNA encoding c-FLIP<sub>R</sub> had been cloned from Raji cells, but endogenous expression of the corresponding protein had never been shown so far. c-FLIP<sub>R</sub> possesses two tandem DEDs like c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub> (Fig. 2A). Similar to c-FLIP<sub>S</sub>, c-FLIP<sub>R</sub> does not contain caspase-like domains (Fig. 2A).

The human c-FLIP gene, with an estimated size of ~48,000 bp, consists of 14 exons and gives rise to 13 distinct isoforms after splicing. Fig. 2B shows the exon composition of the coding portions of c-FLIP<sub>R</sub>, c-FLIP<sub>S</sub>, and c-FLIP<sub>R</sub> mRNAs: the first three translated exons encode the two DEDs. The caspase-like domain of c-FLIP<sub>R</sub> is encoded by exons 8–10, 12, and 14.

As the molecular mass of the new c-FLIP<sub>R</sub> variant was similar to that predicted for c-FLIP<sub>R</sub>, we examined the possibility that the identified product was indeed c-FLIP<sub>R</sub>. To address this question we performed reverse transcription PCR. As a reverse primer for c-FLIP<sub>R</sub> we selected the sequence at the beginning of exon 6 and as a reverse primer for c-FLIP<sub>R</sub> the sequence at the beginning of exon 7 (Fig. 3A). In Raji and SKW6.4 cells we observed a product of ~642 bp, which corresponds to the size of c-FLIP<sub>R</sub> mRNA (Fig. 3B) (11). In HUT78 and J16 as well as in primary human T cells, we observed that both isoforms, c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub>, were expressed on the mRNA level, although expression of the protein could not be detected in these cell lines (Fig. 1).

To confirm that the new variant of c-FLIP was indeed c-FLIP<sub>R</sub>, we added in vitro translated c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub> to cell lysates of HUT78 cells, followed by immunoprecipitation with anti-FLIP mAb NF6 (Fig. 3C). The products of immunoprecipitation were analyzed by Western blotting. As a control we used lysates of HUT78 cells that, according to Western blot analysis (Fig. 1A), contain both c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub>, with c-FLIP<sub>R</sub> present in a lower amount. We observed that the molecular mass of in vitro translated c-FLIP<sub>R</sub> was identical to the endogenous c-FLIP<sub>R</sub>. These results provide evidence that the new c-FLIP<sub>R</sub> variant found at the protein level is indeed identical to c-FLIP<sub>R</sub>.
We observed FADD, CD95, c-FLIP<sub>L</sub>, and procaspase-8 (p55/p53) as well as the caspase-8 cleavage products p43/p41 and p18 in the DISC immunoprecipitated from all cell lines. DISC formation in Jurkat cells was lower as these are reported to be CD95 “type II” cells, which are characterized by reduced CD95 DISC formation. Therefore, detection of DISC-associated proteins such as procaspase-8 cleavage products or full-length c-FLIP<sub>L</sub> is difficult (28).

To compare the binding of c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub> to the DISC in a semi-quantitative way, we employed an in vitro recruitment assay. In vitro translated <sup>35</sup>S-labeled c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub> were added to immunoprecipitates containing CD95 DISC proteins from SKW6.4 or HUT78 cells. Protein complexes were washed and recovered, and the amounts of c-FLIP<sub>R</sub> or c-FLIP<sub>S</sub> bound to the DISC were assessed by Western blotting (Fig. 4B). This assay revealed a similar recruitment of c-FLIP<sub>R</sub> (Fig. 4B, lane 5) and c-FLIP<sub>S</sub> (lane 3) into the DISC of HUT78 cells. The same result was obtained when CD95 DISC from SKW6.4 cells was used in this assay (lanes 4 and 6). Thus, we concluded that c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub> have similar biochemical properties in vitro with respect to DISC binding.

To assess the apoptotic properties of c-FLIP<sub>R</sub> in <em>vivo</em>, we constructed stable BJAB cell lines that overexpress c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub>. The sensitivity of the corresponding cell lines toward CD95-mediated apoptosis was checked using different concentrations of anti-APO-1 (Fig. 4C). We observed that c-FLIP<sub>R</sub> protects cells from CD95-mediated death with the same efficiency as observed for c-FLIP<sub>S</sub>.

c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub> Have a Similar Half-life—c-FLIP proteins are reported to have a short half-life (27, 29). To monitor the half-life of c-FLIP<sub>R</sub>, we followed the degradation of c-FLIP<sub>R</sub> upon addition of CHX to SKW6.4 cells (Fig. 5A). In parallel, degradation of c-FLIP<sub>R</sub> in HUT78 cells was monitored. The kinetics of degradation of c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub> were similar. Namely, both c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub> were strongly down-regulated after 4 h of CHX treatment, whereas c-FLIP<sub>L</sub> levels were hardly affected. Identical results were obtained using Boe<sup>R</sup> cells, which coexpress c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub> (Fig. 5B).

To obtain more insight into the function of c-FLIP<sub>R</sub>, we decided to check the correlation between the sensitivity of Boe<sup>R</sup> and SKW6.4 cells toward CD95-mediated apoptosis and the expression level of c-FLIP<sub>R</sub>. Boe<sup>R</sup> and SKW6.4 cells were pre-treated with CHX for 4 h. According to our data this time point corresponds to the strong down-regulation of c-FLIP<sub>R</sub> (Fig. 5, A and B). Subsequently, the cells were incubated for another 12 h with different concentrations of anti-APO-1. In previous studies Boe<sup>R</sup> cells were reported to be resistant toward CD95-mediated apoptosis despite high expression of CD95 (28). Interestingly, addition of CHX sensitized the Boe<sup>R</sup> cells toward CD95-mediated apoptosis (Fig. 5C). Addition of CHX to SKW6.4 cells, which contain only c-FLIP<sub>L</sub>, also resulted in an increased sensitivity to CD95 ligation (Fig. 5D). Thus, apoptosis sensitivity correlates with the decrease in the expression level of c-FLIP<sub>R</sub>.

c-FLIP<sub>R</sub> in Primary Human T Cells—c-FLIP<sub>R</sub> is known to contribute to the resistance of short-term-activated T cells toward CD95-mediated apoptosis (22, 27, 30). In addition, c-FLIP<sub>S</sub> was identified as a new effector of CD28-mediated co-stimulation and as a mediator of resistance toward AICD after
T cell receptor restimulation. Because we found the mRNA of c-FLIPR (Fig. 3B) in primary human T cells, we decided to examine the role of c-FLIPR in T cells in more detail.

To examine the expression of c-FLIPR in primary human T cells we performed immunoprecipitations with anti-FLIP mAb NF6 from T cell lysates of several donors (Fig. 6A). Interestingly, we observed variations in the ratio between c-FLIPR and c-FLIPS from donor to donor. The expression of c-FLIPS was always higher than that of c-FLIPR. Moreover, in some donors c-FLIPR could not be detected.

In addition, we analyzed the expression of c-FLIPR during the activation cycle of primary human T cells before and after both primary stimulation and restimulation on day 6 (Fig. 6B). According to previous reports, expression of c-FLIPL did not undergo drastic changes up to day 6. At the same time, we observed that expression of both short isoforms, c-FLIPS and c-FLIPR, was down-regulated from day 1 to day 6. Restimulation with anti-CD3 antibody resulted in up-regulation of c-FLIPS and c-FLIPR.

To study the effects of CD3 restimulation as well as CD3/CD28 costimulation on FLIPR expression, we performed c-FLIP immunoprecipitation from day 6 primary human T cells (Fig. 6C). We analyzed primary human T cells from several donors, observing strong up-regulation of c-FLIPR and c-FLIPS in response to all three stimuli. The up-regulation of c-FLIPS was higher for CD3/CD28 costimulation as was already reported for c-FLIPS. Interestingly, for donors lacking c-FLIPR (Fig. 6C, left panel) costimulation with CD3/CD28 did not result in detection of c-FLIPR. Thus, we observed comparable expression patterns for both isoforms in primary human T cells, suggesting similar functions for both isoforms in this cellular context.

**Fig. 5.** c-FLIPR and c-FLIPS have a similar half-life and are recruited to the CD95 DISC with comparable affinities *in vitro*. A, $5 \times 10^7$ HUT78 or SKW6.4 cells were treated with 10 μg/ml cycloheximide (CHX) for 0, 2, or 4 h. Total cell lysates were analyzed by Western blot using anti-FLIP mAb NF6. B, $5 \times 10^7$ BoeR cells were treated with 10 μg/ml CHX. The time course of c-FLIP expression was analyzed by Western blotting using anti-FLIP mAb NF6. BoeR (C) and SKW6.4 (D) cells were pretreated with 10 μg/ml CHX for 4 h and subsequently washed and stimulated with indicated concentrations of anti-APO-1 for 12 h. Specific cell death was calculated as described under “Experimental Procedures.”

**DISCUSSION**

c-FLIP plays an essential role in CD95-mediated apoptosis as well as in other death receptor-signaling pathways (tumor necrosis factor R1, TRAILR1, TRAILR2) (10, 21). Thirteen splice variants of c-FLIP were discovered on the mRNA level, and only two of them, c-FLIPL and c-FLIPS, were detected on the protein level (11). The role of c-FLIPS in CD95 signaling is well studied. When recruited to the DISC it inhibits caspase-8 activation and blocks CD95-mediated apoptosis. The role of c-FLIPL in CD95 signaling is controversial. According to recent reports it catalyzes caspase-8 activation and blocks CD95-mediated apoptosis. The role of c-FLIPR in CD95 signaling is controversial. According to recent reports it catalyzes caspase-8 processing at the DISC rather than playing an inhibitory role. In this study we demonstrate endogenous expression of a third splice variant of c-FLIP, c-FLIPS, on the protein level, the mRNA of which had been cloned previously.

c-FLIPR has so far not been reported to exist as an endogenously expressed protein. In the study by Djerbi et al. (11), mRNA of c-FLIPR was found in Raji cells, but protein expression is not shown. However, in the literature, we find that authors often describe c-FLIPR in SKW6.4 cells as c-FLIPS, as these isoforms have very similar molecular masses (27). Moreover, in the study by Scaffidi et al. (14), where the expression of c-FLIP was analyzed in a panel of cell lines, it is evident that the molecular mass of the isoform described as c-FLIPS in Raji and SKW6.4 cells is clearly lower than in other cell lines analyzed. In our work we clearly show that c-FLIPR is detected neither on the mRNA nor on the protein levels in these two cell lines, thus demonstrating exclusive expression of c-FLIPR.

c-FLIPR is structurally highly similar to c-FLIPS. Both isoforms contain two DED at their N terminus (Fig. 2A) but differ...
in their C termini (Fig. 2). The C terminus of c-FLIP<sub>R</sub> has a fragment corresponding to translation of the small part of intron 6 that is shorter than the C terminus of c-FLIPS resulting from the translation of the fragment of exon 7. The actual sizes of the different C termini are 11 and 19 amino acids for c-FLIP<sub>R</sub> and c-FLIPS, respectively. Therefore, we expected c-FLIP<sub>R</sub> to have a similar affinity to the CD95 DISC as c-FLIPS. As expected, we observed c-FLIP<sub>R</sub> recruitment to the CD95 DISC in Raji and SKW6.4 cell lines. Moreover, in vitro translated c-FLIPS and c-FLIP<sub>R</sub> bound to the CD95 DISC with comparable affinity. Most likely, c-FLIP<sub>R</sub> blocks activation of procaspase-8 at the CD95 DISC by a mechanism similar to c-FLIPS. In a study by Djerbi et al. (11), retroviral expression of c-FLIP<sub>R</sub> and c-FLIPS inhibited death receptor-dependent apoptosis with comparable efficiency in the murine lymphoma cell line A20. We observed a similar inhibition of CD95-induced apoptosis upon overexpression of c-FLIP<sub>R</sub> and c-FLIPS in the B lymphoblastoid cell line BJAB. This seems plausible in light of the high similarity between those two isoforms and the fact that both short variants of c-FLIP seem to be expressed independently of each other.

c-FLIP isoforms are characterized by a short half-life. It has been proposed previously that mechanisms of differential degradation of c-FLIP isoforms might be important for apoptosis sensitivity (27). Therefore, we were interested in the half-life of c-FLIP<sub>R</sub>. Our data demonstrate that c-FLIPS and c-FLIP<sub>R</sub> have similar proteolytic degradation rates, strengthening our hypothesis of functional redundancy between both short variants of c-FLIP.

At the same time we have demonstrated that the anti-apoptotic properties of c-FLIP<sub>R</sub> are similar to that of c-FLIPS. We observed that down-regulation of both isoforms in CHX-treated Boe<sup>+</sup> cells rendered them sensitive toward CD95-mediated apoptosis. Moreover, down-regulation of only the c-FLIP<sub>R</sub> isoform in SKW6.4 cells resulted in an increase of their sensitivity. Sensitization of those two cell lines with CHX was reported before, but the correlation with down-regulation of c-FLIP<sub>R</sub> was not found. Thus, most probably c-FLIP<sub>R</sub> plays an important role in the resistance toward CD95-mediated apoptosis, similar to that of c-FLIPS. Moreover, one might speculate that the resistance of Boe<sup>+</sup> cells is based on the presence of both short c-FLIP isoforms, which provide a very efficient blockage of the CD95 DISC by unclear mechanisms. This question should be addressed in future studies.

Recently, CD95 was demonstrated to trigger the NF-κB pathway (31–33). Moreover, c-FLIPS and c-FLIP<sub>R</sub> were shown to be important regulators of CD95L-induced NF-κB (31). Both isoforms blocked CD95L-induced NF-κB activation. The structural similarity of c-FLIPS and c-FLIP<sub>R</sub> and the redundancy of their anti-apoptotic functions in CD95 signaling make it most likely that both short isoforms play similar roles in CD95L-induced NF-κB activation. Addressing this issue in detail in future experiments would help to better understand the role of c-FLIP proteins in NF-κB signaling.

Interestingly, we detected the expression of both short isoforms, c-FLIPS and c-FLIP<sub>R</sub>, in primary human T cells. c-FLIPS was reported to rescue cells from AICD in primary human T cells (22). We studied expression of c-FLIP<sub>R</sub> during T cell re-stimulation and found that, similar to c-FLIPS, it also undergoes up-regulation after CD3 or CD28 triggering and CD3/CD28 costimulation. Thus, c-FLIP<sub>R</sub> and c-FLIPS seem to be similar in primary human T cells.

In this study we have shown the presence of c-FLIP<sub>R</sub> protein in a number of cell lines. In two cell lines we observed the exclusive presence of c-FLIP<sub>R</sub>, suggesting functional redundancy between both short variants of c-FLIP. The mechanism by which differential expression of splice variants in different cell lines is regulated requires clarification.

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REFERENCES

8. Kischkel, F. C., Heilbrunn, S., Behrmann, I., Germer, M., Pawlita, M., Kram-