Rapid Turnover of c-FLIPshort Is Determined by Its Unique C-terminal Tail*

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The caspase-8 inhibitor c-FLIP exists as two splice variants, c-FLIPL and c-FLIPS, with distinct roles in death receptor signaling. The mechanisms determining their turnover have not been established. We found that in differentiating K562 erythroleukemia cells both c-FLIP isoforms were inductively degraded by the proteasome, but c-FLIPS was more prone to ubiquitylation and FLIP isoforms were inducibly degraded by the proteasome in differentiating K562 erythroleukemia cells both c-FLIP isoforms. Furthermore the c-FLIPS-specific tail of 19 amino acids, adjacent to the two target lysines, was demonstrated to be the key element determining the isoform-specific instability of c-FLIPS. Molecular modeling in combination with site-directed mutagenesis demonstrated that the C-terminal tail is required for correct positioning and subsequent ubiquitylation of the target lysines. Because the antiapoptotic operation of c-FLIPS was not affected by the tail deletion, the antiapoptotic activity and ubiquitin-mediated degradation of c-FLIPS are functionally and structurally independent processes. The presence of a small destabilizing sequence in c-FLIPS constitutes an important determinant of c-FLIPS/c-FLIPL ratios by allowing differential degradation of c-FLIP isoforms. The conformation-based predisposition of c-FLIPS to ubiquitin-mediated degradation introduces a novel concept to the regulation of the death-inducing signaling complex.

Death receptors are cell surface receptors that belong to the tumor necrosis factor receptor superfamily, the most well known members of which are tumor necrosis factor receptor 1, the CD95/Fas receptor, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors DR4/TRAIL-R1 and DR5/TRAIL-R2 (for a review, see Ref. 1). Upon ligand-mediated oligomerization of CD95/Fas or TRAIL receptors, the Fas-associated death domain protein (FADD) attaches to the death receptor via homophilic death domain interactions. The death effector domain (DED) of FADD is in turn linked to the apoptotic machinery due to its affinity for the initiator caspases, procaspase-8 and procaspase-10. The close proximity of procaspase-8 molecules results in dimerization of the procaspases, the assembly of which forms an enzymatically active site (2, 3). This enzymatic activity induces serial cleavages converting the zymogen into p10 and p18 fragments, forming the proteolytically active caspase-8 heterotrimer. In responsive cells, activated caspase-8 is then able to activate effector caspases thereby initiating apoptosis (for a review, see Ref. 4). In addition to the initiator caspases, the DED in FADD also interacts with the apoptosis modulator cellular FLICE-inhibitory protein (c-FLIP; Ref. 5), which exists as two splice variants, the long splice form of FLIP (FLIPL) and the short splice form of FLIP (FLIPS). Together with the activated death receptor, FADD, and caspase-8 and -10, the FLIP proteins form the core of the death-inducing signaling complex (DISC; Ref. 6), which also contains a number of other proteins, many of which seem to be involved in modulating death receptor signals (for a review, see Ref. 1). The DISC assembly with pro- and antiapoptotic proteins allows for control of death receptor signaling through a number of different routes. Consequently in addition to apoptosis, stimulation of the receptors may also lead to cell survival and proliferation. Modulation of the apoptotic pathway at the level of the activated receptor is required in situations when the extrinsic, but not the intrinsic, apoptosis pathway needs to be inhibited or when localized initiator caspase activity is required for specialized signaling functions. For example, caspase-8 activity is necessary for normal T cell development (7, 8) and is probably acquired through complex regulation of anti- and proapoptotic proteins in the DISC. In addition, some malignant cells are able to convert death receptor stimulation into proliferative signals (9, 10). Therefore, active regulation of the DISC proteins, both transcriptional and post-translational, determines the outcome of death receptor stimulation.

c-FLIPL and c-FLIPS have been characterized as specific inhibitors of death receptor-mediated apoptosis (for a review, see Ref. 11). c-FLIPL is homologous to caspase-8, consisting of two tandemly repeated DEDs and a catalytically inactive caspase-like domain. Although c-FLIPS shares most of its sequences with FLICE, FADD-like interleukin-1β-converting enzyme; FLIP, FLICE-inhibitory protein; c-FLIP, cellular FLIP; FLIPL, long splice form of FLIP; FLIPS, short splice form of FLIP; TRAIL-R, TRAIL receptor; PBS, phosphate-buffered saline; HA, hemagglutinin; PEA-15, phosphoprotein enriched in astrocytes, 15 kDa; E3, ubiquitin-protein isopeptide ligase.

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1 The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DED, death effector domain; DISC, death-inducing signaling complex; DR, death receptor; FADD, Fas-associated death domain protein; FLICE, FADD-like interleukin-1β-converting enzyme; FLIP, FLICE-inhibitory protein; c-FLIP, cellular FLIP; FLIPL, long splice form of FLIP; FLIPS, short splice form of FLIP; TRAIL-R, TRAIL receptor; PBS, phosphate-buffered saline; HA, hemagglutinin; PEA-15, phosphoprotein enriched in astrocytes, 15 kDa; E3, ubiquitin-protein isopeptide ligase.

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ence with c-FLIP\textsubscript{L}, it is considerably shorter, comprising only the two DEDs followed by a short C-terminal sequence and an isoform-specific C-terminal tail of 19 amino acids, the role of which is still unknown (for a review, see Ref. 11). Both c-FLIP isoforms act as inhibitors of caspase-8-mediated apoptosis through binding to DED in FADD (12–14). However, in addition to the well documented antiapoptotic activity of c-FLIPL and c-FLIPS, recent reports have provided evidence for isoform-specific regulatory functions. For example, c-FLIPL is capable of inducing the first cleavage of caspase-8 and itself, thereby leading to membrane-restricted caspase-8 activation, whereas c-FLIPS completely prevents procaspase-8 cleavage (12, 13, 15). Moreover, c-FLIP\textsubscript{L}, but not c-FLIPS, interacts with TRAF2 (tumor necrosis factor receptor-associated factor 2) and induces activation of the NF-\textkappaB signaling pathway (16). Thus, although first considered to be similar inhibitor proteins, the c-FLIP isoforms have now been established as DISC molecules with distinct and even opposite functions.

During development and differentiation it is of great importance for cells to be able to regulate their apoptotic sensitivity. The ability of c-FLIP\textsubscript{L} and c-FLIPS to modulate caspase-8 activity makes them highly adaptable regulators of death receptor signaling. Consequently disturbances in c-FLIP expression have been implicated in certain malignancies. For example, high levels of c-FLIP have been found in some cancer cells, including melanoma and colonic adenocarcinoma (5, 17, 18), and an elevated expression of c-FLIP has been shown to result in the escape of tumors from immune surveillance (19, 20).

c-FLIP expression is carefully regulated at different levels. The transcriptional regulation is linked to a number of growth- and survival-promoting signaling pathways, including NF-\kappB (21, 22), mitogen-activated protein kinase/extracellular signal-regulated kinase (23), and Akt (24, 25). In addition to gene expression, it has been recently reported that the turnover of c-FLIP is actively regulated by ubiquitin-mediated degradation (26, 27). However, very little is known about the mechanisms underlying this regulation or whether there could be differences in isoform stability. In this study, we show that although both c-FLIP isoforms can be degraded via a common inducible pathway, they display clearly distinct half-lives. Our mutational analyses showed that the principal ubiquitin acceptor lysines are different in c-FLIPL and c-FLIPS, and that the unique C terminus of c-FLIPS possesses a destabilizing function. In addition, molecular modeling of the C-terminal segment of c-FLIPS revealed structural features compatible with its preferred ubiquitylation. Although c-FLIPS lacking the C terminus was equally efficient in suppressing TRAIL-induced apoptosis as the wild type protein, the antiapoptotic activity...
and the ubiquitin-mediated degradation of c-FLIP_L is functionally and structurally separated, and ubiquitylation is not associated with the antiapoptotic function of c-FLIP_L but only required for regulating the turnover of the protein. The isoform-specific regulation and turnover of the c-FLIP isoforms add a new dimension to the regulatory mechanisms of the respective c-FLIP isoforms with broad ramifications into understanding the biological functions of c-FLIP_L and c-FLIP_S.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—Human K562 erythroleukemia cells and WM35 melanoma cells were cultured in a humidified 5% CO₂ atmosphere at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum, antibiotics (penicillin and streptomycin), and 2 mm l-glutamine. For WM35 cells, the medium was supplemented with 5 μM epoxomicin. WM35 cells were treated with 7 μg/ml cyclostatin for 12 h followed by treatment with 200 nm epoxomicin for 4 h. c-FLIP protein was detected with Western blot analysis. Samples from 1% Triton X-100 lysates are shown. Hsc70 was used as a loading control.

**Western Blotting**—For Western blot analysis, cells were harvested by centrifugation and washed once with PBS. Cells were lysed either in the Laemmli SDS sample buffer or in lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, Complete mini protease inhibitor mixture (Roche Applied Science)). Resulting Triton X-100 lysis buffer lysates were centrifuged to remove insoluble material, and protein concentrations were determined by Bradford assay. Each lysate containing 30–50 μg of protein was loaded and resolved by SDS-PAGE and transferred to nitrocellulose membrane (Protran nitrocellulose, Schleicher & Schuell) by using a semidry transfer apparatus (Bio-Rad). Western blotting was performed using antibodies against c-FLIP (NF6 FLIP antibody, kindly provided by Peter Krammer, German Cancer Research Center, Heidelberg, Germany; also available from Alexis) and Hsc70 (SPA-815, StressGen). Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Biosciences and Southern Biotechnology. The bands were visualized using the enhanced chemiluminescence method (ECL, Amersham Biosciences).

**Plasmid Constructs**—The FLAG-tagged c-FLIP_L and c-FLIP_S were a kind gift from Dr. Jürg Tschopp (Institute of Biochemistry, University of Lausanne, Lausanne, Switzerland). c-FLIP_L and c-FLIP_S point mutations were made using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. The FLAG-tagged c-FLIP_S deletion mutant was constructed by PCR and cloned into the EcoRI and XhoI sites in-frame with the N-terminal FLAG tag in pB13-Met-Flag. The HA-tagged ubiquitin was a kind gift from Dr. Dirk Bohmann (University of Rochester, Rochester, NY).

**Immunoprecipitation**—For immunoprecipitation of ubiquitylated c-FLIP, the cell pellet from transiently transfected cells was resuspended in 75 μl of boiling 5% SDS in PBS, and the lysate was heated at 100 °C for 5 min. The lysates were suspended 1:10 in 1% Triton X-100 in PBS. DNA was sheared by sonication, and the particulate material was centrifuged for 15 min at 15,000 × g. Samples were taken from the cleared lysates for input control. The lysates were further diluted 1:1 with 1% Triton X-100, 0.5% bovine serum albumin in PBS and incubated with anti-HA (Santa Cruz Biotechnology) antibody and 15 μl of a 50% slurry of protein G-Sepharose under rotation for 2 h. After incubation, the Sepharose beads were washed four times with 1% Triton X-100 in PBS, and the immunoprecipitated proteins were run on an 8 or 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane.
(Protran nitrocellulose, Schleicher & Schuell), and immunoblotted with anti-FLIP antibody.

**Fluorescence-activated Cell Sorter Analysis of Cellular Caspase-3 Activity**—After the treatments, the cells were washed once with ice-cold PBS, and the caspase-3 activity was analyzed with pycoerythrin-conjugated monoclonal active caspase-3 antibody apoptosis kit 1 (BD Pharmingen) according to the manufacturer’s protocol.

**TRAIL-R Immunoprecipitation and DISC Analysis**—To stimulate TRAIL receptors, 4 × 10³ K562 cells/sample were pelleted (500 × g for 7 min) and resuspended in 1 ml of prewarmed RPMI 1640 medium. Thereafter 1 μg of FLAG-tagged TRAIL (Alexis) and 2 μg of anti-FLAG monoclonal M2 antibody (Sigma) were added to the cell suspension. The cells were incubated at 37 °C for 20 min, and the reaction was stopped by adding 10 ml of ice-cold PBS. After washing, the cells were lysed in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 0.2% Nonidet P40, and Complete protease inhibitor mixture (Roche Applied Science)) for 30 min on ice. The cell debris was removed by centrifugation at 15,000 × g for 15 min at 4 °C. The amount of protein was determined by the Bradford assay, and an equal amount of protein from each sample was precleared with 50 μl of Sepharose CL-4B for 2 h at 4 °C. A total of 2.5 μg of monoclonal anti-DR5 and 2.5 μg of monoclonal anti-DR4 (Alexis) were added to samples and immunoprecipitated with 15 μl of protein G beads (Amersham Biosciences) for 2.5 h at 4 °C. The beads were washed six times with 1 ml of lysis buffer, resuspended in 3 × Laemmli sample buffer, and finally boiled for 3 min. The immunoprecipitated samples and corresponding cell lysates were analyzed by 11% SDS-PAGE. Western blot analysis was performed with anti-DR5 (Alexis), anti-FADD (BD Transduction Laboratories), caspase-8 (C15 caspase-8 antibody, a kind gift from P. Krammer, German Cancer Research Center; also available from Alexis), and anti-FLIP antibody.

**Molecular Modeling of c-FLIPS**—All models were built using the Modeller computer program (salilab.org/modeller/modeller.html). The sequence of c-FLIPS contains two conservative DEDs between Ser² (numbering according to Ref. 5) and Val⁷⁹ and between Tyr⁹³ and Gln¹⁷⁶. A model structure for the second DED in c-FLIPS (DED2) was built by using the NMR structural data for phosphoprotein enriched in astrocytes, 15 KDa (PEA-15; Protein Data Bank code 1n3k, Ref. 28) and the NMR structure of the FADD death effector domain (F25Y mutant; Protein Data Bank code 1a1w, Ref. 29). The secondary structure of the c-FLIPS C-terminal segment (amino acids 176–221) was predicted using the following computer programs: PHD: Profile network prediction Heidelberg (30–32), SAM-T99 Secondary Structure Prediction (33), SCRATCH (Saspro) (34), PROF-Secondary Structure Prediction System (35), and ncpredict (36). The loop between the DED2 and the c-FLIPS C-terminal segment has been taken as seen in the structure of PEA-15, the only currently known representative structure covering this region (28). Surface construction and lipophilic potential calculations were done using SYBYL (Tripos Inc., St. Louis, MO). Fig. 8 was produced with MolScript v2.1 (37) and Raster3D v2.4b (38).

**RESULTS**

**Both c-FLIP Isoforms Are Degraded via the Ubiquitin-Proteasome Pathway**—The antiapoptotic functions of the c-FLIP isoforms are tightly associated with their expression levels, and down-regulation of c-FLIP is an important mechanism to sensitize cells to receptor-mediated apoptosis (for a review, see Ref. 11). Previously we have shown that both c-FLIP isoforms are down-regulated in K562 cells undergoing hemin-mediated erythroid differentiation, which sensitizes the cells to TRAIL-mediated apoptosis (12). These results prompted us to examine whether the inducible c-FLIP down-regulation is due to proteasome-mediated degradation. For this purpose, we incubated differentiating K562 cells with the proteasome inhibitor epoxomicin, which efficiently prevented hemin-induced down-regulation of c-FLIPb and c-FLIPa (Fig. 1A). This suggested that the down-regulation of c-FLIP isoforms would be due to degradation through the ubiquitin-proteasome pathway.

Because the 26 S proteasome acts on proteins destined to be degraded by polyubiquitin conjugation, we investigated whether c-FLIP isoforms are inducibly ubiquitylated upon hemin treatment. K562 cell lines 1E5 and 2G11 (12), expressing ectopic FLAG-tagged c-FLIPb and c-FLIPa, respectively, were treated with hemin and epoxomicin. The hemin treatment did not result in down-regulation of the ectopically expressed c-FLIP in the tested cell lines, indicating that the degradation machinery might be saturated. Hemin treatment, however, caused a prominent smear above the FLAG-FLIPS band that was further enhanced by epoxomicin, whereas in FLAG-FLIPb-expressing cells, a prominent slower migrating smear was induced only by treating the cells with both hemin and epoxomicin (Fig. 1B). To verify that the high molecular weight smear indeed represented hemin-induced polyubiquitin conjugation, we transiently coexpressed HA-tagged ubiquitin and FLAG-tagged c-FLIP. The transfected cells were treated with 30 μM hemin for 16 h, and ubiquitin conjugates were immunoprecipitated with HA antibodies. Western blotting with c-FLIP-specific antibodies revealed the ubiquitylation of c-FLIPb and c-FLIPa, which was further enhanced by hemin-mediated differentiation (Fig. 1C). In conclusion, these results show that both c-FLIP isoforms are degraded by the ubiquitin-proteasome pathway, a process that can be activated through a common signal.

**c-FLIPb Has a Markedly Shorter Half-life than c-FLIPa**—Although both c-FLIP isoforms were inducibly ubiquitylated upon hemin treatment, the ubiquitylation of c-FLIPb appeared to be more prominent (Fig. 1B). To study whether the degradation of the c-FLIP isoforms is differentially regulated, we compared the half-lives of c-FLIPa and c-FLIPb by treating K562 cells with the protein synthesis inhibitor cycloheximide for the indicated time periods. Despite the similarity of the isoforms, c-FLIPb appeared to have a markedly shorter half-life.
than c-FLIPL, which does not totally disappear even after 24 h of cycloheximide treatment (Fig. 2A). This result was verified by a quantitation of c-FLIP protein levels, which showed that the half-life of c-FLIPL is ~40 min, whereas the half-life of c-FLIPS is almost 2 h (Fig. 2B). This differential stability of the c-FLIP isoforms is not limited only to K562 cells as a similar difference in the half-lives could be observed in HeLa cells (Fig. 2, C and D). To test whether the different half-lives of the c-FLIP isoforms could contribute to changes in their stoichiometry also in other physiologically relevant cellular backgrounds, we studied WM35 melanoma cells, which have been shown earlier to become sensitized to TRAIL upon cisplatin treatment by specific down-regulation of c-FLIPS (39). In agreement with the earlier study (39), cisplatin treatment down-regulated c-FLIPS more efficiently than c-FLIPL (Fig. 2E). Notably this down-regulation could be completely prevented by the proteasome inhibitor epoxomicin (Fig. 2E), indicating that proteasomal degradation is needed for the specific down-regulation of c-FLIPS. Moreover cycloheximide chase experiments revealed that also in WM35 melanoma cells c-FLIPS is significantly less stable than c-FLIPL (Fig. 2, F and G). Taken together, these findings imply that the stability of c-FLIP isoforms is regulated in an isoform-specific manner and that the differential stability can contribute to an altered stoichiometry between the isoforms.

The Unique C-terminal Tail of c-FLIPS Is Indispensable for Ubiquitylation—Both c-FLIP isoforms have a common N terminus consisting of two tandemly repeated DEDs. In c-FLIPL, the DEDs are followed by a caspase-like domain, whereas c-FLIPS has a unique C-terminal tail sequence composed of 19 amino acids (Fig. 3A). To investigate whether the unique C-terminal tail of c-FLIPS contributes to its preferred ubiquitylation, the Δ203–221 mutant lacking the c-FLIPS-specific tail was transiently transfected together with HA-tagged ubiquitin into K562 cells. The effects were analyzed by Western blotting of HA-immunoprecipitated samples with a c-FLIP antibody. Intriguingly whereas wild type c-FLIPS was prominently ubiquitylated, the Δ203–221 deletion mutant exhibited no detectable ubiquitin conjugation (Fig. 3B), demonstrating that the unique C terminus is indispensable for the ubiquitylation of c-FLIPS. The effect was not cell type-specific because when the Δ203–221 mutant was transfected into HeLa cells we could not observe any ubiquitylation of the mutant protein (data not shown).

Fig. 4. The C terminus is not needed for the antiapoptotic function of c-FLIPS A. Western blot analysis of c-FLIP from parental K562 cells, a mock-transfected cell pool (MOCK), and cell lines stably overexpressing c-FLIPS (ΔE5) or Δ203–221 (2B8, 2D9, 5F4, and S8D). B, an analysis of apoptosis in c-FLIPS Δ203–221 cell lines in response to hemin (30 μM for 16 h) and TRAIL (3 h) treatments by using phycoerythrin-conjugated antibody recognizing active caspase-3. The bars show the mean values (mean ± S.D.) from two independent flow cytometry analyses with two replicas. C, the DISC immunoprecipitation analysis of parental K562 cells and lines stably overexpressing c-FLIPS (ΔE5) or Δ203–221 (5F4). G stands for sample with lysate, isotype-specific antibody, and protein G-beads; C stands for control; and T stands for TRAIL-treated sample. The cells were treated with TRAIL and M2 for 20 min and then lysed. TRAIL-R DISC precipitation was controlled by incubating the sample with isotype-matching antibody. The immunoprecipitates were analyzed by Western blotting using anti-G-Sepharose and DR4- and DR5-specific antibodies. The specificity of immunoprecipitation was controlled by incubating the sample with isotype-matching antibody. The immunoprecipitates were analyzed by Western blotting using anti-FADD, anti-caspase-8, anti-c-FLIP, and anti-DR5 antibodies. The presence of the proteins in cell lysates before immunoprecipitation is shown in the right-hand panels. The migration positions of the proteins are indicated. IP, immunoprecipitate.
221 deletion mutant were strongly protected against TRAIL-induced apoptosis after hemin treatment (Fig. 4B). This result demonstrates that the \c{H}9004^203–221 mutant harbors antiapoptotic activity similar to c-FLIPS and that the unique c-FLIPS C terminus is purely a determinant of ubiquitylation. It should be noted that, in contrast to endogenous c-FLIPS, ectopically expressed wild type c-FLIPS is not significantly down-regulated upon hemin treatment (30 \muM for 16 h) was analyzed by transient transfection and HA immunoprecipitation as described above. Ubiquitylated c-FLIPS was detected by Western blotting as shown in the upper panel. The presence of proteins in the cell lysates before immunoprecipitation is shown in the lower panel. Hsc70 was used as a loading control. C, the ubiquitylation of the cluster mutants with and without hemin treatment (30 \muM for 16 h) was analyzed by transient transfection and HA immunoprecipitation as described above. Ubiquitylated c-FLIPS was detected by Western blotting in the middle panels. The presence of proteins in cell lysates before immunoprecipitation is shown in the lower panels. M stands for mock-transfected cells. Hsc70 was used as a loading control.

DISC immunoprecipitation analyses of the 5F4 clone showed that c-FLIPS \Delta203–221 was avidly recruited to the DISC, and it inhibited procaspase-8 cleavage similarly to wild type c-FLIPS (Fig. 4C). These observations also validated the results from the ubiquitylation experiments as the mutant protein was obviously fully functional, implying that the decreased ubiquitylation of the c-FLIPS \Delta203–221 deletion mutant is not due to abnormal protein conformation, localization, or other problems that could arise from aberrant functions of a mutated protein. Taken together, this analysis shows that the antiapoptotic activity and the regulation of ubiquitylation of c-FLIPS are two distinct functions that reside at physically separated domains. The far C terminus of c-FLIPS is a determinant of ubiquitylation with no direct effects on the antiapoptotic properties of c-FLIPS.

FIG. 5. Lys^{192} and Lys^{195} are ubiquitylated in c-FLIP\(_S\) but not in c-FLIP\(_L\). A, schematic presentation of the lysine residues in c-FLIPS. To find out the target residues for ubiquitylation, in addition to single, double, and triple mutants, four lysine to arginine cluster mutants were used. The mutants were named accordingly. B, ubiquitylation of the K214R mutant was analyzed by transient transfection and HA immunoprecipitation as described for Fig. 1C. Ubiquitylated c-FLIPS was detected by Western blotting as shown in the upper panel. The presence of proteins in the cell lysates before immunoprecipitation is shown in the lower panel. Hsc70 was used as a loading control. C, the ubiquitylation of the cluster mutants with and without hemin treatment (30 \muM for 16 h) was analyzed by transient transfection and HA immunoprecipitation as described above. Ubiquitylated c-FLIPS was detected by Western blotting in the middle panels. The presence of proteins in cell lysates before immunoprecipitation is shown in the lower panels. M stands for mock-transfected cells. Hsc70 was used as a loading control. D, the ubiquitylation of the c-FLIPS K192R,K195R mutant was analyzed and controlled as in Fig. 1C. E, the ubiquitylation of the c-FLIP\(_L\) K192R,K195R mutant was analyzed and controlled as for Fig. 1C. Ubi, ubiquitin; WT, wild type; IP, immunoprecipitate; WB, Western blot; K192,195R, K192R,K195R, K192,K195,214R, K192R,K195R,K214R.

221 deletion mutant were strongly protected against TRAIL-induced apoptosis after hemin treatment (Fig. 4B). This result demonstrates that the \Delta203–221 mutant harbors antiapoptotic activity similar to c-FLIPS and that the unique c-FLIPS C terminus is purely a determinant of ubiquitylation. It should be noted that, in contrast to endogenous c-FLIPS, ectopically expressed wild type c-FLIPS is not significantly down-regulated upon hemin treatment (30 \muM for 16 h) was analyzed by transient transfection and HA immunoprecipitation as described above. Ubiquitylated c-FLIPS was detected by Western blotting as shown in the upper panel. The presence of proteins in the cell lysates before immunoprecipitation is shown in the lower panel. Hsc70 was used as a loading control. C, the ubiquitylation of the cluster mutants with and without hemin treatment (30 \muM for 16 h) was analyzed by transient transfection and HA immunoprecipitation as described above. Ubiquitylated c-FLIPS was detected by Western blotting in the middle panels. The presence of proteins in cell lysates before immunoprecipitation is shown in the lower panels. M stands for mock-transfected cells. Hsc70 was used as a loading control. D, the ubiquitylation of the c-FLIPS K192R,K195R mutant was analyzed and controlled as in Fig. 1C. E, the ubiquitylation of the c-FLIP\(_L\) K192R,K195R mutant was analyzed and controlled as for Fig. 1C. Ubi, ubiquitin; WT, wild type; IP, immunoprecipitate; WB, Western blot; K192,195R, K192R,K195R, K192,K195,214R, K192R,K195R,K214R.
clusters (Fig. 5A). Surprisingly three of the cluster mutants (DED1, DED2A, and DED2B) had little or no effect on ubiquitylation of c-FLIPS (Fig. 5C). In contrast, in the TAIL mutant, carrying only three C-terminal lysine residues (192, 195, and 214) mutated to arginines, the ubiquitin conjugation was efficiently, albeit not fully, inhibited (Fig. 5C). As the K214R mutation on its own did not affect ubiquitylation of c-FLIPS, it seemed likely that Lys192 and Lys195 would be the principal ubiquitin acceptors in c-FLIPS. Indeed the K192R,K195R double mutant displayed a clearly reduced level of ubiquitylation (Fig. 5D). As Lys192 and Lys195 are also present in c-FLIPL, we examined whether the same lysines are targets for ubiquitylation in c-FLIPL. However, the c-FLIPL K192R,K195R double mutant was ubiquitylated at a level similar to the wild type (Fig. 5E), indicating that the major ubiquitin target residues are distinct in c-FLIPS and c-FLIPL. These results provide direct evidence for differential regulation of ubiquitin conjugation in the two c-FLIP isoforms.

C Terminus of c-FLIPS Is Indispensable for Its Degradation—As ubiquitylation has recently been implicated in processes not related to proteasomal degradation (for a review, see Ref. 41), we examined whether the decreased ubiquitylation of the Δ203–221 mutant would be manifested as an increased half-life of the mutant protein as compared with the wild type c-FLIPS. Due to a stabilizing effect of c-FLIPS overexpression, the expression levels of both constructs were titrated close to the endogenous level, and cells were treated with cycloheximide for the indicated time periods (Figs. 6, A and B). As expected, the tail deletion Δ203–221 strongly stabilized c-FLIPS, demonstrating that the unique C terminus is indispensable for the ubiquitin-mediated degradation of the less stable c-FLIP isoform. It should be noted that in all experiments the stability of Δ203–221 mutant was substantially increased when compared with wild type c-FLIPS even if the half-lives of exogenously expressed proteins varied between experiments (most likely due to differences in protein expression levels and transfection efficiency). We also observed that the stability of the Δ203–221 mutant was elevated in the WM35 melanoma cell line as indicated by consistently higher expression levels of the mutant protein (data not shown).

Structural Analysis of the c-FLIPS C Terminus—To better understand the contribution of the unique C-terminal tail to the turnover and the isoform-specific function of the lysine residues Lys192 and Lys195, we analyzed the structural features of the C-terminal segment (amino acids 176–221) of c-FLIPS containing the critical determinants for the specific regulation of c-FLIPS. Amino acids 176–202 of c-FLIPS are identical in sequence with the c-FLIPL isoform, and residues Tyr182–Asp196 are strongly predicted by five independent secondary structure prediction computer programs to have an α-helical secondary structure (Fig. 7). To search for possible sites of interaction between the α-helix and the DED2, we constructed a molecular model of the DED2 based on the known DED structures of PEA-15 (Protein Data Bank code 1n3k) and the FADD death effector domain (Protein Data Bank code 1a1w). The loop region Ala178–Arg183, located between the predicted α-helix and the DED2, is very flexible in the NMR structure for PEA-15 (28). Thus, the conformation of this loop does not define the proper positioning of the predicted α-helix relative to the DED2. According to the model presented in Fig. 8A, both lysines, Lys192 and Lys195, reside along the same, polar side of the predicted amphipathic α-helix; the opposite face of the helix is hydrophobic (Fig. 8A, gray spheres), strongly suggesting that the hydrophobic face of the helix must interact with hydrophobic regions on the DED2 or with some other structure. In the model structure, Lys192 and Lys195 are accessible to solvent and could accept polyubiquitin chains equally well.

To further elucidate the functional motifs in c-FLIPS that could be involved in regulating isoform-specific turnover, we analyzed the hydrophobic/hydrophilic characteristics of the surface of the modeled structure of c-FLIPS. There are two lipophilic clusters along the C-terminal end of FLIPS (Fig. 8B). Lipophilic surface I, including residues Tyr182, Leu190, Ile190, and Leu191, forms the hydrophobic face of the α-helix described above. A second prominent hydrophobic surface, lipophilic surface II, is formed by residues Phe201, Met203, Tyr207, and Cys210; Met203, Tyr207, and Cys210 are unique to c-FLIPS. The surface of the DED2 has two hydrophobic grooves (Fig. 8C). Lipophilic grooves I (Val156, Tyr158, Met159, Leu152, Val155, Val151) and II (Val144, Phe146) and II (Tyr119, Met120, Leu141, Leu143, and Leu154) would be able to accommodate lipophilic surfaces I and II, thus functioning to stabilize the C-terminal region. Because the deletion of the unique C-terminal tail of c-FLIPS (amino acids 203–221) prevented ubiquitylation of c-FLIPS, it is plausible that the unique tail facilitates the ubiquitylation by anchoring the α-helix containing the Lys192 and Lys195 in a proper position. In comparison, the amino acids in c-FLIPL corre-

![Fig. 6. The C-terminal tail destabilizes c-FLIPS](Image 161x67 to 460x130)

![Fig. 7. Prediction of the secondary structure of the c-FLIPS C-terminal segment](Image 99x215 to 266x398)
The molecular modeling together with our experimental data strongly suggests that the structural differences in the region composed of amino acids 176–221 determines the differential ubiquitin-mediated degradation of the c-FLIP isoforms.

**The α-Helical Region of c-FLIPα Is Required for Its Efficient Ubiquitylation and Fast Turnover**—As the molecular model suggested that the α-helix and its proper positioning may de-
We demonstrate that proteasomal degradation can be used to rapidly and specifically regulate the stoichiometry of the c-FLIP isoforms. This newly characterized isoform-specific regulatory feature is well in accordance with the diverging roles of c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub>. The c-FLIP isoforms have been perceived primarily as caspase-8 inhibitors with a direct relationship between c-FLIP protein levels and death receptor sensitivity (for a review, see Ref. 11). However, there are a number of reports demonstrating that c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> have different molecular modes of action (15, 45). Initially both c-FLIP isoforms were believed to simply compete with procaspase-8 binding to the DISC, thereby inhibiting caspase-8 activation. Recently several reports have indicated that rather than preventing procaspase-8 recruitment to the DISC, c-FLIP has isoform-specific effects on the cleavage-dependent activation of procaspase-8 (13, 14). For example, whereas c-FLIP<sub>S</sub> completely prevents the proximity-induced cleavage of procaspase-8, c-FLIP<sub>L</sub> induces the first cleavage of caspase-8 to the p43/41 form but prevents the further cleavage of caspase-8 (15, 45). A differential regulation may also be required for the diverging roles of c-FLIP isoforms in conveying signals from antiapoptotic signaling pathways; for example, in response to CD3 stimulation, NF-κB is preferentially activated by c-FLIP<sub>L</sub> and mitogen-activated protein kinase/extracellular signal-regulated kinase is preferentially activated by c-FLIP<sub>S</sub> (46). The region critical for the activation of NF-κB signaling resides in the caspase-8-cleaved p43 fragment of c-FLIP<sub>L</sub>. This fragment specifically interacts with TRAF2 and induces NF-κB activation (16). Given the striking functional differences between the two c-FLIP isoforms, the regulation of their expression will be an important determinant for cells to adjust their apoptotic threshold and responsiveness to extracellular signals.

Our discovery of the shorter half-life of c-FLIP<sub>S</sub> compared with that of c-FLIP<sub>L</sub> exposes a versatile regulatory feature of this protein pair that cells can use to regulate their death receptor signals. In the cell models that we used, c-FLIP<sub>S</sub> was the more actively regulated isoform. However, given suitable preconditions, this type of regulation could be used to reverse the ratio between the c-FLIP isoforms. Accordingly in SHEP neuroblastoma cells, the half-life of c-FLIP<sub>S</sub> has been shown to be shorter than that of c-FLIP<sub>L</sub> (47), suggesting that the half-life of c-FLIP isoforms can also be regulated in a cell type-specific manner. As c-FLIP<sub>S</sub> was less stable in our cell models, we wanted to unravel the molecular determinants that are responsible for triggering the ubiquitylation-mediated degra-
dation of c-FLIP<sub>S</sub>. In many cases, ubiquitylation is primed by phosphorylation of the substrate protein. We tested this possibility because c-FLIP has been reported to be phosphorylated (48, 49). Although c-FLIP<sub>S</sub> showed serine-directed phosphorylation, it was not associated with the inducible ubiquitylation of c-FLIP<sub>L</sub> (data not shown). Therefore, the triggering mechanisms for ubiquitylation remain to be determined.

The shorter half-life and preferred ubiquitylation of c-FLIP<sub>S</sub> are clearly determined by its unique C-terminal tail (amino acids 203–221), which does not act by providing a target lysine (Lys<sub>214</sub>) but is instead required for correct positioning and stabilization of the α-helix that contains the principal target lysyl residues, 192 and 195, on the surface of the DED2. Thus, the c-FLIP<sub>S</sub>-specific tail is the determinant that favors the ubiquitylation of Lys<sub>192</sub> and Lys<sub>195</sub> in c-FLIP<sub>S</sub> but not in c-FLIP<sub>L</sub>. It is, however, notable that despite the decreased ubiquitylation of c-FLIP<sub>L</sub> in the K192R,K195R mutant lacking the principal ubiquitin acceptor sites, this mutant did not display a significantly longer half-life when compared with wild type c-FLIP<sub>S</sub> (data not shown). Therefore, the triggering mechanism of c-FLIP<sub>S</sub> with its ubiquitin ligase(s).

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REFERENCES

1. Öozo, N., and El-Deiry, W. S. (2003) Semin. Cancer Biol. 13, 135–147
2. Boatright, K. M., Renatus, M., Scott, F. L., Sperandio, S., Shin, H., Pedersen, J. M., Ricci, J. E., Edida, E., Bohmann, D. P., Green, D. R., and Salvesen, G. S. (2003) Mol. Cell. 11, 529–541
3. Chang, D. W., Xing, Z., Capacio, V. L., Peter, M. E., and Yang, X. (2003) EMBO J. 22, 4132–4142
4. Dallal, N. N., and Korsmeyer, S. J. (2004) Cell 116, 205–219
5. Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, E., and Tschopp, J. (1997) Nature 388, 190–195
6. Medema, J. P., Scalfi, C., Kischkel, F. C., Schvechenko, A., Mann, K., Krammer, P. H., and Meier, P. M. (1997) EMBO J. 16, 2794–2804
7. Chun, H. J., Zheng, L., Ahmad, M., Wang, J., Speirs, C. K., Siegel, R. M., Dale, J. K., Puck, J., Davis, J., Hall, C. G., Skolda-Smith, S., Atkinson, T. P., Strauss, S. A., and Lenardo, M. J. (2002) Nature 419, 395–399
8. Salmena, L., Lemmens, B., Hakem, A., Matsuyuki-Zablocki, E., Murakami, K., Au, P. Y., Berry, D. M., Tamblyn, L., Shkeladze, A., Migon, E., Wakeham, A., Bouchard, D., Yeh, W. C., McCabe, J. G., Ohashi, P. S., and Hakem, R. (2005) Genes Dev. 19, 883–895
9. Holmstrom, T. H., Tran, S. E., Holmstrom, T. H., Ahonen, V. L., Ahn, N. G., Chou, S. C., and Eriksson, J. E. (1999) Mol. Cell. Biol. 19, 5991–6002
10. Tran, S. E., Holmstrom, T. H., Ahonen, M., Kahari, V. M., and Eriksson, J. E. (2001) J. Biol. Chem. 276, 16448–16490
11. Krüger, A., Baumann, S., Krammer, P. H., and Kirchhoff, S. (2001) Mol. Biol. Cell. 12, 8247–8254
12. Hietakangas, V., Poukkula, M., Heiskanen, K. M., Karvinen, J. T., Sistonen, L., and Eriksson, J. E. (2003) Mol. Cell. 12, 1278–1291
13. Krüger, A., Schmitz, I., Baumann, S., Krammer, P. H., and Kirchhoff, S. (2001) J. Biol. Chem. 276, 20633–20640
14. Scalfi, C., Schmitz, I., Krammer, P. H., and Peter, M. E. (1999) J. Biol. Chem. 274, 1541–1548
15. Mischeu, O., Thome, M., Schneider, P., Holler, N., Tschopp, J., Nicholson, D. W., Briand, C., and Grutter, M. G. (2002) J. Biol. Chem. 277, 45162–45171
16. Kataoka, T., and Tschopp, J. (2004) Mol. Cell. Biol. 24, 2627–2636
17. Bollani, R. R., Huard, B., Viard-Leveugle, I., Byers, H. R., Irmler, M., Saurat, J. H., Tschopp, J., and French, E. (2001) J. Invest. Dermatol. 117, 390–394
18. Ryu, B. K., Lee, M. G., Chi, S. G., Kim, Y. W., and Park, J. H. (2001) J. Pathol. 194, 19–15
19. Djuris, M., Sereganti, V., Catrina, A. I., Bogen, B., Biferfeld, P., and Grandien, A. (1999) J. Exp. Med. 190, 1033–1038
20. Kreuz, S., Siegmund, D., Schereich, P., and Wajant, H. (2001) Mol. Cell. Biol. 21, 3964–3973
21. Mischeu, O., Lens, S., Saide, G., Alevizopoulos, K., and Tschopp, J. (2001) Mol. Cell. Biol. 21, 5299–5304
22. Yeh, J. H., Hsu, S. C., Han, S. H., and Lai, M. Z. (1998) J. Exp. Med. 188, 1795–1802
23. Panku, D. J., Maon, T., Stahura, T., Walsh, K., and Mier, J. W. (2001) J. Biol. Chem. 276, 6893–6896
24. Skurk, C., Maatz, H., Kim, H. S., Yang, J., Abid, M. R., Aird, W. C., and Walsh, K. (2004) J. Biol. Chem. 279, 1513–1525
25. Kim, Y., Stuh, N., Sporns, M., and Reed, J. C. (2002) J. Biol. Chem. 277, 22320–22329
26. Perez, D., and White, E. (2003) J. Virol. 77, 2651–2662
27. Hill, J. M., Vaidyanathan, H., James, J. W., Ginsberg, M. H., and Werner,
M. H. (2002) *EMBO J.* 21, 6494–6504
29. Eberstadt, M., Huang, B., Chen, Z., Meadows, R. P., Ng, S. C., Zheng, L., Lenardo, M. J., and Fesik, S. W. (1998) *Nature* 392, 941–945
30. Rost, B., and Sander, C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 7558–7562
31. Rost, B., and Sander, C. (1994) *Proteins* 19, 55–72
32. Rost, B., and Sander, C. (1993) *J. Mol. Biol.* 232, 584–599
33. Karplus, K., Sjölander, K., Barrett, C., Cline, M., Haussler, D., Hughey, R., Holm, L., and Sander, C. (1997) *Proteins Suppl.* 1, 134–139
34. Pollastri, G., Przybylski, D., Rost, B., and Baldi, P. (2002) *Proteins* 47, 228–235
35. Ouari, M., and King, R. D. (2000) *Prot. Sci.* 9, 1162–1176
36. Kneller, D. G., Cohen, F. E., and Langridge, R. (1990) *J. Mol. Biol.* 214, 171–182
37. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* 24, 945–949
38. Merritt, E. A., and Bacon, D. J. (1997) *Methods Enzymol.* 277, 505–524
39. Schnell, J. D., and Häcke, L. (2003) *J. Biol. Chem.* 278, 35857–35860
40. Holmström, T. H., and Eriksson, J. E. (2000) *Crit. Rev. Immunol.* 20, 121–152
41. Jesenberger, V., and Jentsch, S. (2002) *Nat. Rev. Mol. Cell. Biol.* 3, 112–121
42. Fukazawa, T., Fujitani, T., Uno, F., Tsuji, T., Kadowaki, Y., Ishihama, T., Takata, Y., Kagawa, S., Roth, J. A., Tsuji, J., and Tanaka, N. (2001) *Oncogene* 20, 5225–5231
43. Chang, D. W., Xing, Z., Pan, Y., Algeciras-Schimnich, A., Barnhart, B. C., Yaish-Ouhl, S., Peter, M. E., and Yang, X. (2002) *EMBO J.* 21, 3704–3714
44. Kataoka, T., Budd, R. C., Holter, N., Thome, M., Martinou, F., Irminger, M., Burns, K., Hahne, M., Kennedy, N., Kovacs, M., and Tschopp, J. (2000) *Curr. Biol.* 10, 640–648
45. Fulda, S., Meyer, E., and Debatin, K. M. (2000) *Cancer Res.* 60, 3947–3956
46. Higuchi, H., Yoon, J. H., Grambihler, A., Wernburg, N., Bronk, S. F., and Gores, G. J. (2003) *J. Biol. Chem.* 278, 454–461
47. Yang, B. F., Xiao, C., Roa, W. H., Krammer, P. H., and Hao, C. (2003) *J. Biol. Chem.* 278, 7043–7050
48. Pickart, C. M. (2001) *Annu. Rev. Biochem.* 70, 503–533
49. Krügerhoff, S., Muller, W. W., Krueger, A., Schmitz, I., and Krammer, P. H. (2000) *J. Immunol.* 165, 6293–6300
50. Schmitz, I., Weyd, H., Krüger, A., Baumann, S., Pas, S. C., Krammer, P. H., and Kricheff, S. (2004) *J. Immunol.* 172, 2184–2200
51. Leverkus, M., Walczak, H., McLellan, A., Fries, H. W., Terbeck, G., Bröcker, E. B., and Kampgen, E. (2000) *Blood* 96, 2628–2631
52. Willems, F., Asmaoui, Z., Vanderheyde, N., Verhasselt, V., Aksoy, E., Scaffidi, C., Peter, M. E., Krammer, P. H., and Goldman, M. (2000) *Blood* 95, 3478–3482
53. MacFarlane, M., Harper, N., Snowden, R. T., Dyer, M. J., Barnett, G. A., Pringle, J. H., and Cohen, G. M. (2002) *Oncogene* 21, 6809–6818
54. Thomas, R. K., Kallenborn, A., Wickenhauser, C., Schultze, J. L., Draube, A., Vockerodt, M., Re, D., Diehl, V., and Wolf, J. (2002) *Am. J. Pathol.* 160, 1521–1528
