

# cFLIP<sub>L</sub> Inhibits Tumor Necrosis Factor-related Apoptosis-inducing Ligand-mediated NF- $\kappa$ B Activation at the Death-inducing Signaling Complex in Human Keratinocytes\*

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Human keratinocytes undergo apoptosis following treatment with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) via surface-expressed TRAIL receptors 1 and 2. In addition, TRAIL triggers nonapoptotic signaling pathways including activation of the transcription factor NF- $\kappa$ B, in particular when TRAIL-induced apoptosis is blocked. The intracellular protein cFLIP<sub>L</sub> interferes with TRAIL-induced apoptosis at the death-inducing signaling complex (DISC) in many cell types. To study the role of cFLIP<sub>L</sub> in TRAIL signaling, we established stable HaCaT keratinocyte cell lines expressing varying levels of cFLIP<sub>L</sub>. Functional analysis revealed that relative cFLIP<sub>L</sub> levels correlated with apoptosis resistance to TRAIL. Surprisingly, cFLIP<sub>L</sub> specifically blocked TRAIL-induced NF- $\kappa$ B activation and TRAIL-dependent induction of the proinflammatory target gene interleukin-8. Biochemical characterization of the signaling pathways involved showed that apoptosis signaling was inhibited at the DISC in cFLIP<sub>L</sub>-overexpressing keratinocytes, although cFLIP<sub>L</sub> did not significantly impair enzymatic activity of the receptor complex. In contrast, recruitment and modification of receptor-interacting protein was blocked in cFLIP<sub>L</sub>-overexpressing cells. Taken together, our data demonstrate that cFLIP<sub>L</sub> is not only a central antiapoptotic modulator of TRAIL-mediated apoptosis but also an inhibitor of TRAIL-induced NF- $\kappa$ B activation and subsequent proinflammatory target gene expression. Hence, cFLIP<sub>L</sub> modulation in keratinocytes may not only influence apoptosis sensitivity but may also lead to altered death receptor-dependent skin inflammation.

Apoptosis is a highly regulated physiological process crucial for tissue homeostasis. It is initiated by a multitude of stimuli such as UV irradiation, growth factor deprivation, or chemotherapeutic drugs (1). Based on the nature of the initiating

stimulus, apoptotic signaling pathways have been distinguished as “intrinsic” or “extrinsic” (2). The extrinsic pathway is initiated by ligation of so-called death receptors, whose ligands, TNF $\alpha$ ,<sup>1</sup> CD95L, and TNF-related apoptosis-inducing ligand (TRAIL), are members of the TNF superfamily. They have been studied intensively over the past decade, and their role in activation-induced cell death, autoimmune disorders, immune privilege, and tumor evasion from the immune system is now well established (reviewed in Refs. 3–5). The TRAIL system, consisting of the ligand and four different membrane-bound cellular receptors, has attracted attention for its ability to preferentially kill tumor cells but not normal cells (6, 7). Binding of TRAIL to its receptors TRAIL receptor 1 (TRAIL-R1) and TRAIL-R2 on the cell surface leads to recruitment of adaptor proteins as well as the initiator caspase-8 and -10 to the death-inducing signaling complex (DISC). This subsequently results in effector caspase activation and ultimately apoptosis (8). By contrast, TRAIL-R3 lacks a cytoplasmic domain and is bound to the cell surface via a glycosylphosphatidylinositol anchor. TRAIL-R4 contains an incomplete death domain (DD) and is also unable to transduce a death signal, although it may have additional yet unknown signaling capabilities (6, 9). Whereas most studies have focused on the role of TRAIL as a death ligand, there is evidence that TRAIL-R1, TRAIL-R2, and TRAIL-R4 can also mediate activation of the transcription factor NF- $\kappa$ B. In most cells, TRAIL-induced NF- $\kappa$ B activation is most prominent when cell death pathways are inhibited by the use of peptidyl caspase inhibitors (for a review, see Ref. 9). In addition to its potent proinflammatory function, NF- $\kappa$ B has been demonstrated to regulate the transcription of numerous antiapoptotic target genes (10). In most cell types, inhibition of NF- $\kappa$ B sensitizes cells to death ligands such as TNF $\alpha$ . In contrast, sensitization to TRAIL-induced apoptosis by NF- $\kappa$ B inhibition appears to be cell type-restricted (for a review, see Refs. 9 and 10).

Death receptor-mediated apoptosis is effectively inhibited by cFLIP (cellular fas-associated death domain-like interleukin-1 $\beta$ -converting enzyme-inhibitory protein), an intracellular homologue of the initiator caspase-8. Similar to caspase-8, the long form of cFLIP (cFLIP<sub>L</sub>) contains two amino-terminal death effector domains and a carboxyl-terminal caspase homol-

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<sup>1</sup> The abbreviations used are: TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DISC, death-inducing signaling complex; DD, death domain; IL, interleukin; Ab, antibody; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; FACS, fluorescence-activated cell sorting; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; RIP, receptor-interacting protein.

ogy domain but lacks enzymatic activity. cFLIP<sub>L</sub> is recruited to the CD95 and the TRAIL DISC and inhibits full cleavage and release of active caspase-8 and caspase-10 from the DISC (11, 12). Overexpression studies have suggested that in the presence of cFLIP<sub>L</sub>, additional signaling molecules such as TRAF-1, TRAF-2, receptor-interacting protein (RIP), and RAF-1 are recruited to the DISC, thus explaining how cFLIP<sub>L</sub> might activate NF- $\kappa$ B and mitogen-activated protein kinase signaling pathways in response to death ligands (13). Therefore, cFLIP<sub>L</sub> not only may play a role in apoptosis regulation but may modify other cellular responses, including inflammatory and proliferative signaling pathways of the cell.

Keratinocytes express all necessary constituents of the apoptosis machinery and can activate this program following various insults (for a review, see Ref. 14). Pathological modulation of apoptosis signaling in the skin may therefore lead to disorders such as psoriasis, alopecia areata, or skin cancer (for a review, see Refs. 15 and 16). Several death receptors are expressed in keratinocytes, and a function has been documented for TNF-R1, CD95, TRAIL-R1, or TRAIL-R2. In addition, autocrine apoptosis induction by keratinocyte-derived CD95L has been suggested for toxic epidermal necrolysis (17). The physiological role of TRAIL in the skin remains to be determined. TRAIL was shown to overcome the relative resistance of senescent keratinocytes to apoptosis, and it was suggested that TRAIL may play an important role in epidermal homeostasis (18). In line with *in vivo* data (19), TRAIL-resistant primary keratinocytes express high levels of cFLIP<sub>L</sub>, whereas cells of the highly TRAIL-sensitive transformed keratinocyte line HaCaT only express marginal levels of cFLIP<sub>L</sub>, suggesting a potential for TRAIL in the treatment of skin neoplasias (20).

Previous reports have demonstrated that inhibition of caspases fully blocks apoptosis induction after TRAIL stimulation while leading to an increased activation of NF- $\kappa$ B and its target gene, IL-8. These data suggested that gene induction is a distinct apoptosis-independent signal elicited by TRAIL receptors in the skin (21, 22). We have thus investigated the impact of cFLIP<sub>L</sub> expression for TRAIL-mediated apoptotic and nonapoptotic signaling pathways in human keratinocytes. cFLIP<sub>L</sub> dose-dependently inhibited TRAIL-mediated apoptosis and NF- $\kappa$ B activation at the level of the DISC in keratinocytes. Furthermore, cFLIP<sub>L</sub> inhibited TRAIL-induced activation of NF- $\kappa$ B as well as induction of target genes such as IL-8 without interfering with the enzymatic activity of the DISC. Rather, cFLIP<sub>L</sub> overexpression reduced the recruitment of RIP to the DISC as well as its subsequent modification in the DISC. Taken together, our data suggest that cFLIP<sub>L</sub> acts as an inhibitor of TRAIL-mediated NF- $\kappa$ B activation by directly interfering with RIP recruitment to the DISC.

#### EXPERIMENTAL PROCEDURES

**Materials**—The following antibodies (Abs) were used: Abs to caspase-8 (C15), cFLIP (NF-6; Alexis, San Diego, California), FADD, I $\kappa$ B $\alpha$  (Transduction Laboratories, San Diego, CA), caspase-10 (4C1; MBL International, Watertown, MA), ERK (C-14) and I $\kappa$ B $\alpha$  (C-21) (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA), CPP32 (kindly provided by D. W. Nicholson, Merck Frost, Quebec, Canada), RIP (BD Biosciences), and Bid Abs (kindly provided by X. Wang, Howard Hughes Medical Center, Houston, TX). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG Abs were from Pharmingen (Hamburg, Germany), and horseradish peroxidase-conjugated goat anti-mouse IgG1 and IgG2b Abs were obtained from Southern Biotechnology (Birmingham, AL). TRAIL-R1 (HS 101), TRAIL-R2 (HS 201), TRAIL-R3 (HS 301), and TRAIL-R4 (HS 402) monoclonal Abs for FACS-Scan analysis of surface receptor expression were used as described (21) and are available from Alexis (San Diego, CA). Recombinant LZ-TRAIL and FLAG-TRAIL were produced as reported (23). Recombinant human TNF $\alpha$  was obtained from Strathmann Biotech (Hannover, Germany). The protease inhibitor Z-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk) was purchased from Bachem (Heidelberg, Germany).

**Cell Culture**—The spontaneously transformed keratinocyte line HaCaT was kindly provided by Dr. N. Fusenig (DKFZ; Heidelberg, Germany) and cultured as described (24).

**Retroviral Infection and Generation of HaCaT Cell Lines Stably Expressing cFLIP<sub>L</sub>**—For overexpression of cFLIP<sub>L</sub> in HaCaT cells, we used the retroviral vector PINCO (kindly provided by Dr. Francisco Grignani) containing cFLIP<sub>L</sub> cDNA (25, 26). Retroviral infection of HaCaT cells was essentially performed as described (21, 22). Briefly, the amphotrophic producer cell line  $\phi$ NX was transfected with 10  $\mu$ g of the retroviral vectors by calcium phosphate precipitation. To select transfected producer cells, 2.5  $\mu$ g/ml puromycin (Sigma) was added to the culture medium for 7–14 days to obtain >95% green fluorescent protein-positive producer cells. Cell culture supernatants containing viral particles were generated by incubation of producer cells with HaCaT medium (Dulbecco's modified Eagle's medium containing 10% fetal calf serum) overnight. Following filtration (45- $\mu$ m filter; Schleicher & Schuell), culture supernatant was added to HaCaT cells seeded in 6-well plates 24 h earlier in the presence of 1  $\mu$ g/ml Polybrene. HaCaT were centrifuged for 3 h at 21  $^{\circ}$ C, and the viral particle containing supernatant was subsequently replaced by fresh medium. After an 10–14-day recovery of bulk infected cultures, single cells were seeded in 96-well plates. Successfully infected individual cell clones were identified by green fluorescent protein expression using inverse fluorescence microscopy (Zeiss, Jena, Germany), and wells containing single cell clones were chosen for further expansion. FACS analysis for green fluorescent protein expression (data not shown) and Western blot analysis (Fig. 1) were performed on expanded cell clones to confirm cFLIP<sub>L</sub> expression.

**FACS Analysis**—For surface staining of TRAIL receptors (TRAIL-R1 to TRAIL-R4), cells were trypsinized, and  $2 \times 10^5$  cells were incubated with monoclonal Abs against TRAIL-R1 to TRAIL-R4, or isotype-matched control IgG for 30 min followed by incubation with biotinylated goat anti-mouse secondary Abs and Cy5-phycoerythrin-labeled streptavidin (Caltag, Burlingame, CA) as described (21). For all experiments,  $10^4$  cells were analyzed by FACS-Scan (BD Biosciences).

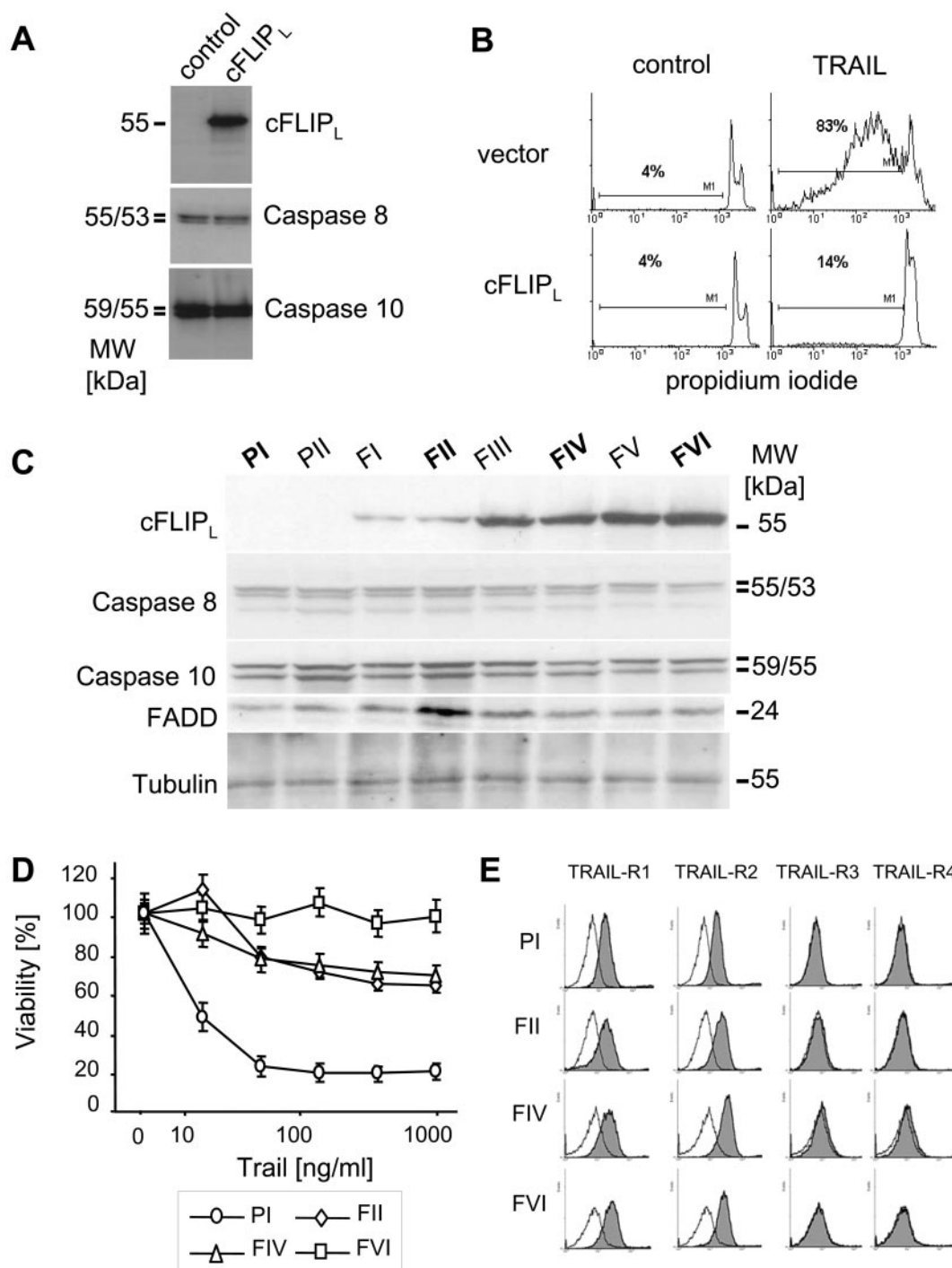
**Western Blot Analysis**—Cell lysates were essentially prepared as described (22). 10–50  $\mu$ g of total protein were loaded on SDS-polyacrylamide gels, separated by electrophoresis, and transferred to nitrocellulose membranes. Blocking of membranes and incubation with the indicated primary and appropriate secondary Abs were performed essentially as described elsewhere (21, 27). Bands were visualized with an ECL detection kit (Amersham Biosciences).

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSAs were performed using nuclear extracts of HaCaT keratinocytes as described previously (22, 28).

**RNAse Protection Assays**—Total RNA was extracted using a Qiagen RNeasy Kit<sup>®</sup> according to the manufacturer's recommendation, and 5  $\mu$ g of total RNA were processed using the Pharmingen (San Diego, CA) RNase protection assay system (hCK-5) according to the manufacturer's instructions. Gels were dried on filter paper and sealed in Saran Wrap, and image data were collected with a phosphor imager (Fuji, Tokyo, Japan). IL-8 mRNA expression levels were normalized against L32 mRNA by densitometric analysis.

**DISC Analysis**—For the precipitation of the TRAIL DISC,  $5 \times 10^6$  HaCaT keratinocytes were used for each condition. Cells were washed once with RPMI medium at 37  $^{\circ}$ C and subsequently incubated for the indicated time periods at 37  $^{\circ}$ C in the presence of 1  $\mu$ g/ml FLAG-TRAIL precomplexed with 2  $\mu$ g/ml anti-FLAG M2 (Sigma) for 15 min or, for the unstimulated control, in the absence of FLAG-TRAIL. DISC formation was stopped by washing the monolayer twice with ice-cold phosphate-buffered saline. Cells were lysed on ice by the addition of 1 ml of lysis buffer (30 mM Tris-HCl, pH 7.5, at 21  $^{\circ}$ C, 120 mM NaCl, 10% Glycerol, 1% Triton X-100, Complete<sup>®</sup> protease inhibitor mixture (Roche Applied Science)). After 15 min of lysis, the lysates were centrifuged at 20,000  $\times$  g for 15 min to remove cellular debris. DISC complexes were precipitated from the lysates by co-incubation with 20  $\mu$ l of protein G beads (Roche Applied Science) for 12 h on an end-over-end shaker at 4  $^{\circ}$ C. For the precipitation of the nonstimulated receptors, 200 ng of FLAG-TRAIL and 400 ng of anti-FLAG M2 were added to the lysates prepared from nonstimulated cells to control for protein association with nonstimulated receptor(s). Ligand affinity precipitates were washed five times with lysis buffer before the protein complexes were eluted from the beads by the addition of 15  $\mu$ l of 2 $\times$  standard reducing sample buffer. Subsequently, proteins were separated by SDS-PAGE on 8–16% Tris-HCl gradient gels (Bio-Rad) before detection of DISC components by Western blot analysis.

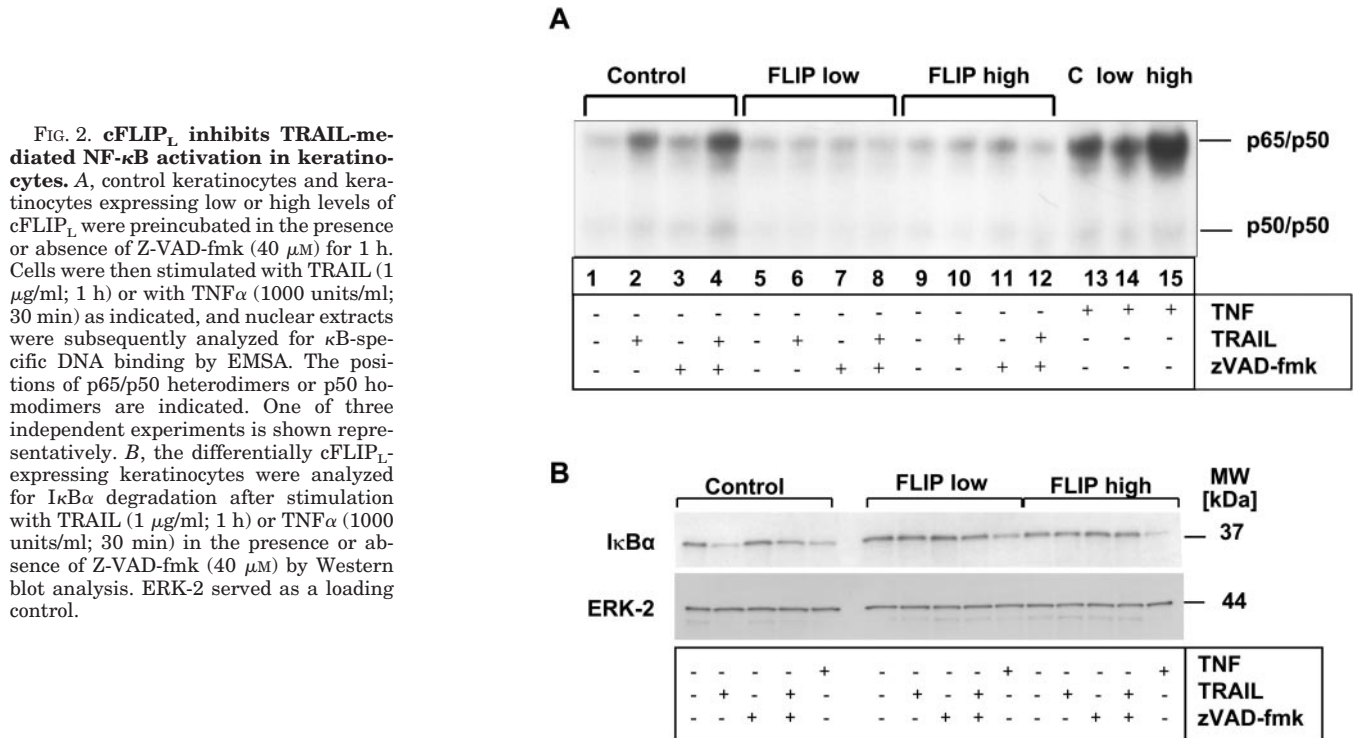
**Preparation of Recombinant Proteins and in Vitro Cleavage Assay**—For the preparation of recombinant Bid, full-length human Bid cDNA



**FIG. 1. Sensitivity to TRAIL-mediated apoptosis correlates with cFLIP<sub>L</sub>/caspase-8 ratio in keratinocytes.** *A*, HaCaT keratinocytes were retrovirally transduced with cFLIP<sub>L</sub> or control vector as described under "Experimental Procedures." 50 μg of protein of total cellular lysates were separated by Western blotting and subsequently analyzed for cFLIP<sub>L</sub>, caspase-8, and caspase-10 expression. *B*, infected keratinocyte lines were either left untreated or stimulated with 1 μg/ml LZ-TRAIL for 3 h, harvested, and examined for hypodiploidy by FACS analysis. *C*, differentially cFLIP<sub>L</sub>-expressing subclones were generated as described under "Experimental Procedures." DISC-associated proteins (namely cFLIP<sub>L</sub>, caspase-8, caspase-10, and FADD) were analyzed in two control clones (PI and PII) and six cFLIP<sub>L</sub>-expressing clones (FI-FVI). PI, FII, FIV, and FVI were chosen for further functional analysis. Tubulin expression was used to confirm equal loading. *D*, cells were seeded in 96-well plates in duplicates and stimulated with 12–1000 ng/ml LZ-TRAIL for 16–24 h. Cellular viability was assessed using the crystal violet assay as described under "Experimental Procedures." The percentage of living cells was normalized to mock-stimulated cells (~100%). One of three independent experiments is represented (mean ± S.D.). *E*, cell surface expression of TRAIL-R1–4 was analyzed by FACS for green fluorescent protein-positive/propidium iodide-negative cells. One of four independent experiments is shown.

was cloned into the pET15a expression vector (Novagen, Darmstadt, Germany) and expressed as an N-terminal His fusion protein in *E. coli* BL21(DE3) pLysS (Novagen, Darmstadt, Germany). Bid was purified from the soluble fraction on Talon-agarose (Clontech) according to the manufacturer's instructions. Active caspase-8 was produced essentially as described elsewhere (29). To detect the enzymatic activity of ligand affinity precipitates, the cleavage of recombinant human Bid was mon-

itored. Ligand affinity precipitates from  $1.5 \times 10^7$  keratinocytes were prepared for each condition as described above. Washed protein complexes were resuspended in 20 μl of CLB buffer (50 mM HEPES-KOH, 2 mM EDTA, 10% sucrose, 0.1% CHAPS, 5 mM dithiothreitol), and recombinant Bid (2 ng) was added to the lysates and incubated overnight at 21 °C. As a positive control, recombinant Bid was mixed with different amounts of recombinant caspase-8, whereas Bid alone served



**FIG. 2. cFLIP<sub>L</sub> inhibits TRAIL-mediated NF- $\kappa$ B activation in keratinocytes.** *A*, control keratinocytes and keratinocytes expressing low or high levels of cFLIP<sub>L</sub> were preincubated in the presence or absence of Z-VAD-fmk (40  $\mu$ M) for 1 h. Cells were then stimulated with TRAIL (1  $\mu$ g/ml; 1 h) or with TNF $\alpha$  (1000 units/ml; 30 min) as indicated, and nuclear extracts were subsequently analyzed for  $\kappa$ B-specific DNA binding by EMSA. The positions of p65/p50 heterodimers or p50 homodimers are indicated. One of three independent experiments is shown representatively. *B*, the differentially cFLIP<sub>L</sub>-expressing keratinocytes were analyzed for I $\kappa$ B $\alpha$  degradation after stimulation with TRAIL (1  $\mu$ g/ml; 1 h) or TNF $\alpha$  (1000 units/ml; 30 min) in the presence or absence of Z-VAD-fmk (40  $\mu$ M) by Western blot analysis. ERK-2 served as a loading control.

as negative control. After 16–24 h, the activity assay was terminated by the addition of loading buffer. Cleavage of Bid to p15/p14 fragments was monitored by Western blot analysis.

**Apoptosis and Cytotoxicity Assays**—Crystal violet staining of attached, living cells was performed 16–24 h after stimulation with different concentrations of LZ-TRAIL (12–1000 ng/ml) in 96-well plates as described (20). Subdiploid DNA content was analyzed as described by Nicoletti *et al.* (30). Briefly, cells from a 35-mm dish were cultured until reaching 70% confluence and were subsequently stimulated with LZ-TRAIL for 3 h. Cells were then detached, washed with cold phosphate-buffered saline, and resuspended in buffer N (0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, 50  $\mu$ g/ml propidium iodide). Cells were kept in the dark at 4  $^{\circ}$ C for 48 h, and then diploidy was measured by FACScan analysis.

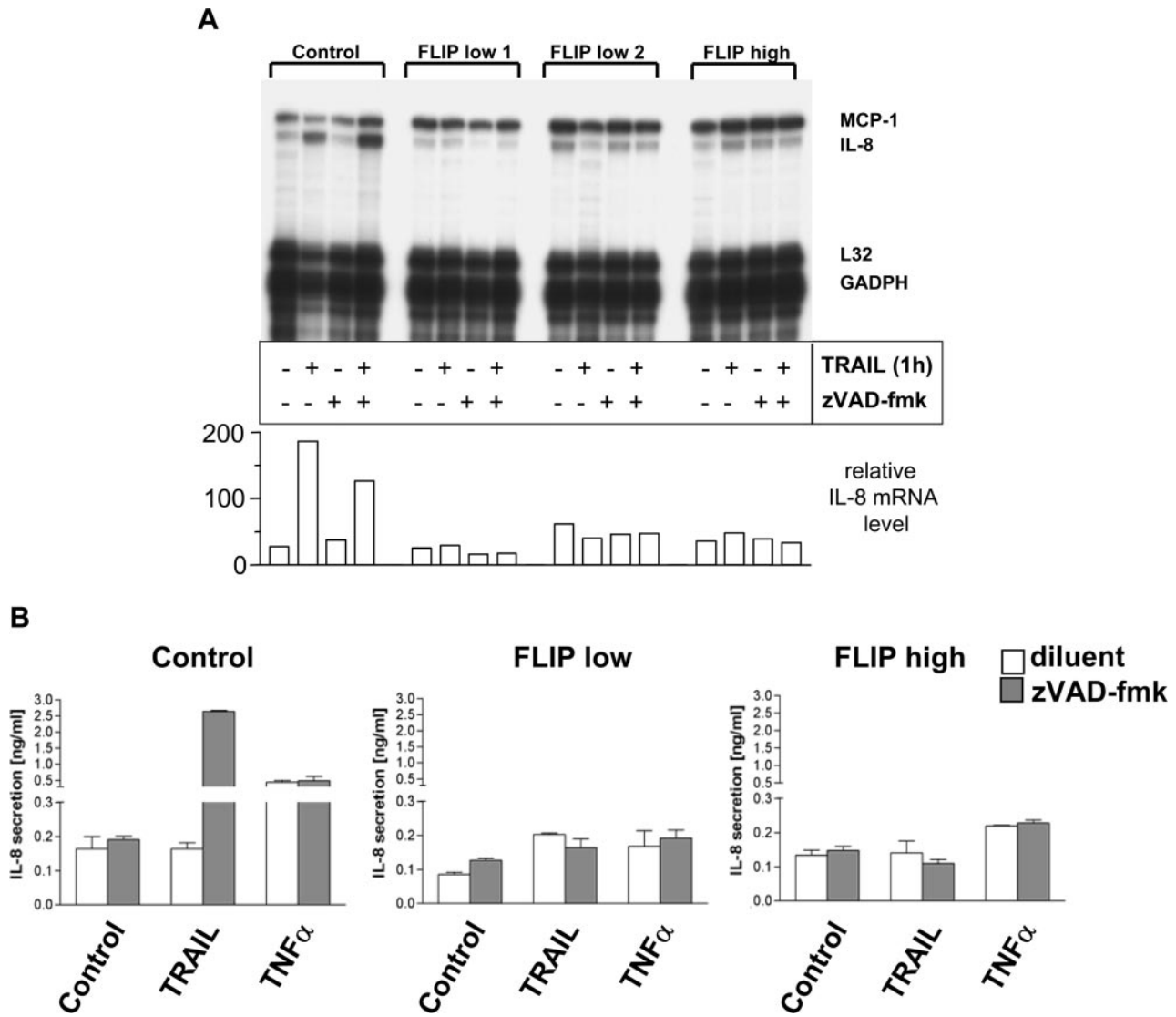
**Determination of IL-8 Secretion**—IL-8 secretion from keratinocyte cultures was analyzed by enzyme-linked immunosorbent assay (ELISA; R&D Biosystems, Minneapolis, MN) as described (22, 31).

## RESULTS

**Sensitivity to TRAIL-mediated Apoptosis Correlates with Caspase-8/cFLIP<sub>L</sub> Ratio in Keratinocytes**—cFLIP<sub>L</sub> is known to inhibit death receptor-mediated apoptosis and is highly expressed in primary human keratinocytes when compared with transformed HaCaT keratinocytes (HaCaT) (20). TRAIL has been demonstrated not only to activate an apoptotic program, but also nonapoptotic signals (e.g. via the activation of the transcription factor NF- $\kappa$ B). Activation of NF- $\kappa$ B is enhanced when caspases are pharmacologically inhibited, which excludes the possibility that this signal is solely an epiphenomenon of apoptosis induction (21, 22, 32, 33). In order to study the impact of caspase inhibition on these different signals in a system that is closer to the physiological situation, we investigated the role of cFLIP<sub>L</sub> in these signaling pathways. We first established cFLIP<sub>L</sub>-expressing HaCaT by retroviral transduction. Polyclonal cFLIP<sub>L</sub>-expressing populations of HaCaT were highly resistant to TRAIL-induced apoptosis when compared with control-infected cells (Fig. 1, *A* and *B*). It was suggested that the ratio of cFLIP<sub>L</sub> to caspase-8 determines the sensitivity to death receptor-mediated apoptosis (11, 12). We therefore established monoclonal cFLIP<sub>L</sub>-expressing HaCaT subclones. Two control lines and six differentially cFLIP<sub>L</sub>-expressing lines were identified by Western blotting (Fig. 1*C*). One control (PI;

control), two intermediate (FII and FIV; FLIP<sub>low</sub>) and a highly cFLIP<sub>L</sub>-expressing cell line (FVI; FLIP<sub>high</sub>) were selected for further analysis. In line with our previous report in primary keratinocytes (20), FLIP<sub>low</sub> cells were relatively resistant to TRAIL. In contrast, FLIP<sub>high</sub> cells were fully resistant to TRAIL-induced apoptosis even at the highest concentration (1  $\mu$ g/ml) tested (Fig. 1*D*). These differences of TRAIL sensitivity were neither due to clonal differences of expression levels of crucial components of the apical death receptor signaling pathways (Fig. 1*C*) nor by differential expression of TRAIL receptors (Fig. 1*E*). Taken together, these experiments demonstrate that the ratio of cFLIP<sub>L</sub> to caspase-8 correlates with resistance to TRAIL-mediated apoptosis in keratinocytes.

**cFLIP<sub>L</sub> Inhibits TRAIL-induced NF- $\kappa$ B Activation**—We have previously reported that inhibition of caspases by pharmacological agents blocks TRAIL-mediated apoptosis but allows for activation of NF- $\kappa$ B (20). Because cFLIP<sub>L</sub> inhibits TRAIL-induced apoptosis as effectively as caspase inhibitors, we hypothesized that TRAIL-induced NF- $\kappa$ B activation should be similarly augmented in cFLIP<sub>L</sub>-expressing cells when compared with TRAIL-stimulated control cells in the presence of Z-VAD-fmk. However, when we measured NF- $\kappa$ B-specific DNA binding activity after stimulation with TRAIL using an electrophoretic mobility shift assay, we surprisingly found that cFLIP<sub>L</sub>-overexpressing keratinocytes failed to activate NF- $\kappa$ B following TRAIL treatment (Fig. 2*A*, lanes 5–12). In line with our previous reports (21, 22), NF- $\kappa$ B was activated following treatment with TRAIL in control cells, and this was enhanced in the presence of the caspase inhibitor Z-VAD-fmk (Fig. 2*A*, lanes 1–4). Interestingly, this effect was specific for TRAIL, since all keratinocyte lines examined potently induced NF- $\kappa$ B following treatment with TNF $\alpha$ , irrespective of their expression levels of cFLIP<sub>L</sub> (Fig. 2*A*, lanes 13–15). NF- $\kappa$ B activation following TNF $\alpha$  or TRAIL treatment is dependent on I $\kappa$ B $\alpha$  degradation (for a review, see Ref. 10). To further characterize at which level TRAIL-mediated NF- $\kappa$ B activation is inhibited by cFLIP<sub>L</sub>, we analyzed cytoplasmic I $\kappa$ B $\alpha$  degradation after stimulation with TRAIL or TNF $\alpha$ . As shown in Fig. 2*B*, TRAIL-induced I $\kappa$ B $\alpha$  degradation was abrogated in cFLIP<sub>L</sub>-expressing



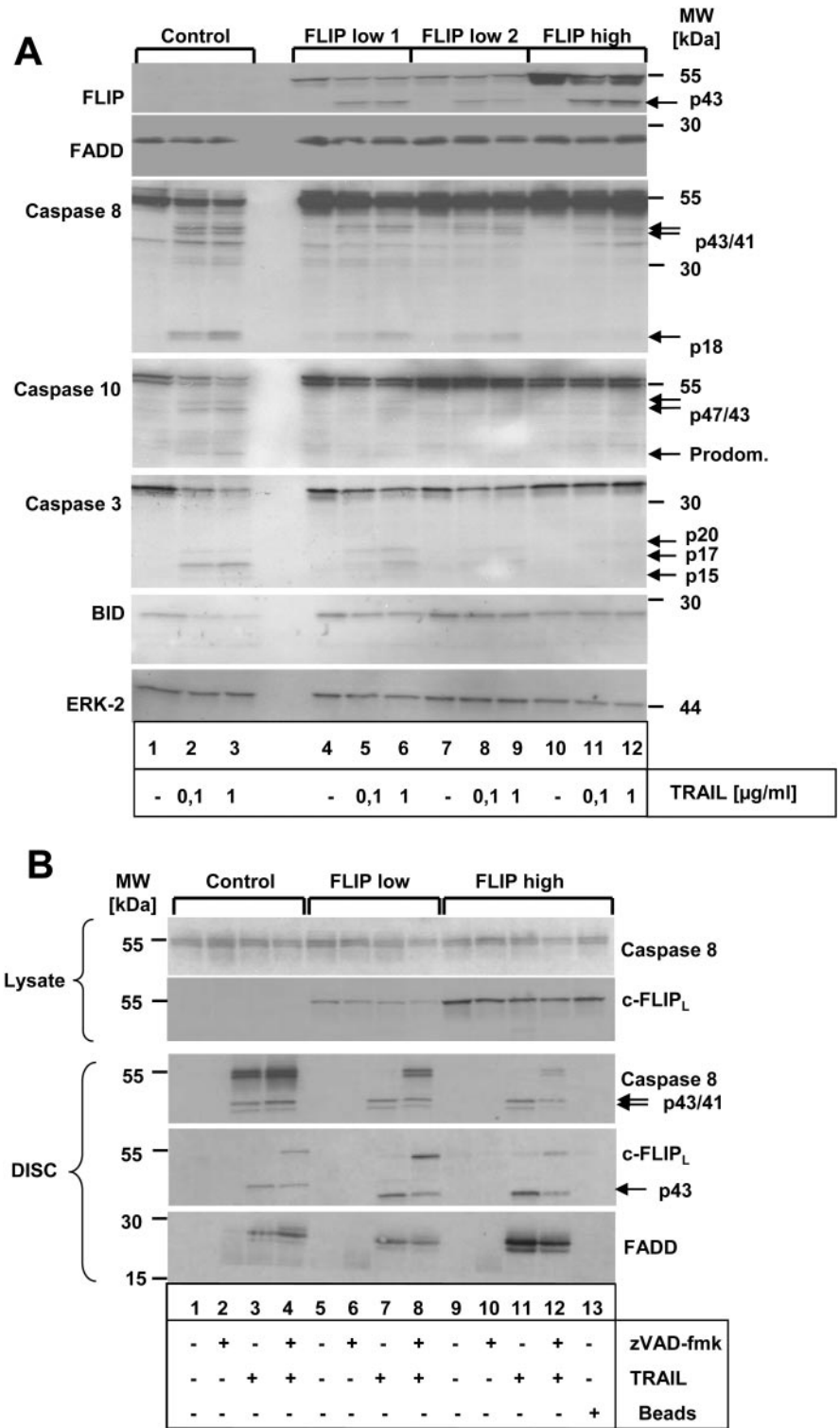
**FIG. 3. cFLIP<sub>L</sub> blocks TRAIL-dependent IL-8 induction.** *A*, total RNA of control or cFLIP<sub>L</sub>-overexpressing keratinocytes stimulated with TRAIL in the presence or absence of Z-VAD-fmk (40  $\mu$ M) were analyzed by RNase protection assays. The protected fragments for MCP-1 and IL-8 mRNAs or control mRNA L32 are indicated. Relative IL-8 mRNA expression levels were normalized against L32 mRNA by densitometry and depicted in relative units. One representative experiment of three independent experiments is shown. *B*, TRAIL-induced IL-8 secretion is inhibited by cFLIP<sub>L</sub>. Following preincubation with either diluent alone (light bars) or 40  $\mu$ M Z-VAD-fmk (dark bars) for 1 h, control or cFLIP<sub>L</sub>-overexpressing keratinocytes were either treated with 1  $\mu$ g/ml TRAIL or 1000 units/ml TNF $\alpha$  for 24 h. Supernatants were assayed for IL-8 protein by ELISA. Shown are mean  $\pm$  S.D. of a representative experiment of a total of four independent experiments.

keratinocytes, whereas cFLIP<sub>L</sub> did not interfere with TNF $\alpha$ -induced I $\kappa$ B $\alpha$  degradation. Taken together, these data indicate that cFLIP<sub>L</sub> specifically blocks TRAIL-mediated I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation, whereas TNF $\alpha$ -mediated NF- $\kappa$ B activation is unaffected by the expression level of cFLIP<sub>L</sub>.

*cFLIP<sub>L</sub> Inhibits TRAIL-mediated Induction of IL-8*—TRAIL induces the proinflammatory cytokine IL-8 in an NF- $\kappa$ B-dependent manner (22). Having shown that cFLIP<sub>L</sub> blocks TRAIL-induced NF- $\kappa$ B activation, we next investigated whether transcriptional target genes induced via TRAIL-R1 and TRAIL-R2 are modulated by cFLIP<sub>L</sub>. We therefore examined IL-8 expression after TRAIL stimulation. In line with the data shown in Fig. 2*A*, induction of IL-8 mRNA after TRAIL stimulation was fully abrogated in FLIP<sub>low</sub> and FLIP<sub>high</sub> keratinocytes, whereas IL-8 mRNA is potently induced upon TRAIL stimulation in control cells (Fig. 3*A*). To further characterize the inhibition of IL-8, we quantified protein levels of IL-8 in cFLIP<sub>L</sub>-overexpressing keratinocytes. Since control cells undergo apoptosis following TRAIL stimulation, we also measured IL-8 induction in the presence of the pancaspase inhibitor

Z-VAD-fmk. In line with our previous findings (22), IL-8 protein is potently induced after stimulation with TRAIL as well as TNF $\alpha$  under these conditions in control cells (Fig. 3*B*, left). In contrast, TRAIL-induced IL-8 secretion is strongly reduced in cFLIP<sub>L</sub>-overexpressing keratinocytes. Interestingly, TNF $\alpha$ -mediated induction of IL-8 protein was also partially inhibited (Fig. 3*B*), suggesting that apart from NF- $\kappa$ B activation, an additional signaling pathway might be required for efficient IL-8 protein secretion after TNF treatment. Taken together, these data demonstrate that cFLIP<sub>L</sub> specifically blocks TRAIL-mediated IL-8 induction by interference with TRAIL-induced NF- $\kappa$ B activation.

*cFLIP<sub>L</sub> Inhibits Full Caspase-8 Processing at the DISC in Keratinocytes, whereas Partial Cleavage Is Unaffected*—cFLIP<sub>L</sub> blocks caspase-8 activation at the DISC by interfering with full cleavage of caspase-8 (34). To investigate the intracellular mechanism responsible for cFLIP<sub>L</sub>-mediated inhibition of NF- $\kappa$ B activation, we next analyzed the activation of initiator caspases following TRAIL treatment in cellular lysates of cFLIP<sub>L</sub>-expressing keratinocytes (Fig. 4*A*). In control cells, full



**FIG. 4. TRAIL-induced caspase-8 activation is inhibited in cFLIP<sub>L</sub>-overexpressing keratinocytes.** A, differentially cFLIP<sub>L</sub>-expressing keratinocytes were mock-treated or stimulated with either 100 or 1000 ng/ml TRAIL for 3 h. Total cellular lysates were analyzed for the activation of cFLIP<sub>L</sub>, FADD, caspase-8, caspase-10, caspase-3, and Bid. Molecular weights of full-length proteins and cleavage products are indicated. B, differential composition of the TRAIL DISC in cFLIP<sub>L</sub>-expressing keratinocytes. DISC analysis was performed from a total of 5 × 10<sup>6</sup> cells. The upper two blots show caspase-8 and cFLIP<sub>L</sub> expression in total cellular lysates used for DISC analysis (lower three blots). Lysates of non-stimulated cells or of cells precipitated in the presence of Protein G beads alone (beads) served as specificity controls for ligand affinity precipitates. Cell lysates were collected from cells preincubated for 1 h with or without Z-VAD-fmk (40 μM). Control cells, one FLIP<sub>low</sub> cell line and FLIP<sub>high</sub> keratinocytes were characterized for cFLIP<sub>L</sub>, caspase-8, and FADD recruitment by Western blotting.

processing of caspase-8 and caspase-10 was readily detectable following TRAIL stimulation (Fig. 4A, lanes 1–3). FLIP<sub>low</sub> keratinocytes showed reduced levels of fully processed caspase-8 (Fig. 4A, lanes 4–9), and full caspase-8 processing to p18 was undetectable in FLIP<sub>high</sub> cells (Fig. 4A, lanes 10–12). Interestingly, partial processing of cFLIP<sub>L</sub> to the p43 fragment as well as partial processing of caspase-8 was observed upon TRAIL stimulation in all cFLIP<sub>L</sub>-expressing lines, indicating that these cleavage events are not blocked by cFLIP<sub>L</sub> overexpression. Known caspase-8 substrates such as Bid and caspase-3 were only cleaved in control cells and, to a lesser extent, in

FLIP<sub>low</sub> keratinocytes, whereas their cleavage was fully abrogated in FLIP<sub>high</sub> cells (Fig. 4A, lanes 10–12). These results confirmed that full caspase-8 processing following TRAIL stimulation is inhibited by cFLIP<sub>L</sub>, comparable with our previous findings using the caspase inhibitor Z-VAD-fmk (20). More importantly, we could not detect any differences in the cytosol with respect to activation of caspases or Bid, which could explain why cFLIP<sub>L</sub>, but not the caspase inhibitor Z-VAD-fmk, blocks TRAIL-induced NF-κB activation. Taken together, our data suggest that cFLIP<sub>L</sub> directly interferes with signals generated at the level of the DISC required for NF-κB activation.

*cFLIP<sub>L</sub> Modifies the Composition of the TRAIL DISC*—Whereas studies using deficient mouse embryonic fibroblasts have provided compelling evidence about molecules necessary for TNF $\alpha$ - or TRAIL-induced NF- $\kappa$ B activation (35, 36), the exact local membrane-associated mechanisms necessary to initiate TRAIL-induced NF- $\kappa$ B activation are not fully understood. To further characterize the level at which NF- $\kappa$ B activation is inhibited by cFLIP<sub>L</sub> in keratinocytes, we next analyzed the composition of the TRAIL DISC in our cellular model (Fig. 4B). FADD recruitment to the DISC did not significantly differ between the keratinocyte lines, although somewhat higher levels of FADD were detected in the DISC of FLIP<sub>high</sub> cells (Fig. 4B, lanes 11 and 12). In line with our previous findings in primary keratinocytes (21), caspase-8 p55/53 was readily detectable in the TRAIL DISC of control cells. In contrast, DISC precipitates of cFLIP<sub>L</sub>-overexpressing keratinocytes mainly contained the processed p43/41 fragments of caspase-8, in line with a previous report for the CD95 DISC (34). The amount of caspase-8 recruited to the DISC correlated with the TRAIL sensitivity of these cell lines. In contrast to the findings in cellular lysates (Fig. 4B, upper two blots), DISC recruitment of the p43 fragment of cFLIP<sub>L</sub> was also detectable in control cells, although FLIP<sub>high</sub> cells showed higher levels of cFLIP<sub>L</sub> p43 fragment in the DISC. These data indicate that even low levels of endogenous cFLIP<sub>L</sub> are recruited to the DISC with high affinity. We had noted that the pancaspase inhibitor Z-VAD-fmk had no effect on TRAIL-induced NF- $\kappa$ B activation in cells expressing cFLIP<sub>L</sub> but increased activation in control cells (Fig. 2A) (22). We therefore compared the difference between TRAIL-treated control cells in the presence of Z-VAD-fmk with TRAIL-treated cFLIP<sub>L</sub>-expressing cells at the DISC level. DISC analysis in the presence of the pancaspase inhibitor Z-VAD-fmk (Fig. 4B, lanes 4, 8, and 12) readily demonstrated the proform of cFLIP<sub>L</sub> as well as the cleavage fragment p43 in the DISC of all keratinocyte lines. Moreover, no significant changes between cell lines expressing different amounts of cFLIP<sub>L</sub> were detected in the presence of Z-VAD-fmk. Therefore, the cleavage pattern of cFLIP<sub>L</sub> in the DISC did not explain the differences for NF- $\kappa$ B activation. However, when we characterized the cleavage pattern of caspase-8 in the DISC, FLIP<sub>high</sub> cells contained largely caspase-8 p43/41, and in the presence of Z-VAD-fmk, the DISC contained larger amounts of the caspase-8 proforms p55/53 in these cells. In contrast, Z-VAD-fmk did not significantly modify the amount of DISC-associated proforms and cleaved fragments of caspase-8 in control cells. These data indicate that Z-VAD-fmk blocks DISC-associated caspase-8 cleavage more effectively in cFLIP<sub>L</sub>-expressing cells.

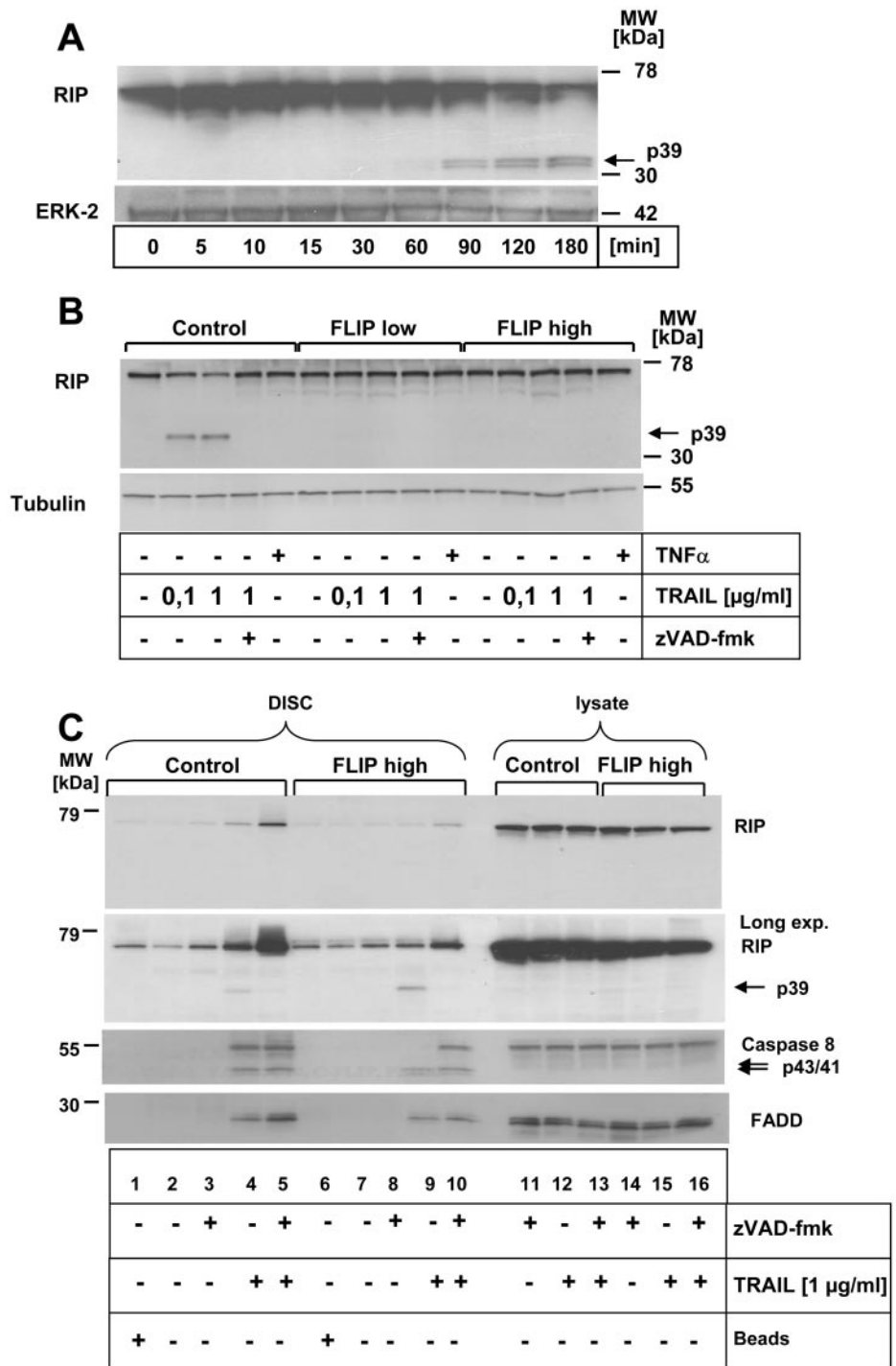
*cFLIP<sub>L</sub> Interferes with DISC Recruitment of RIP in Keratinocytes*—RIP is a known substrate of caspase-8 (37) and is crucial for TNF $\alpha$ - and TRAIL-induced NF- $\kappa$ B activation (35). Caspase-8 cleaves RIP and creates a fragment containing the RIP death domain, which has been associated with a dominant-negative function (37, 38). Moreover, RIP is recruited to the TRAIL DISC in HEK293 and HeLa cells as well as to the membrane-associated TNF-induced signaling complex (32, 39–41). Thus, we next investigated the effect of cFLIP<sub>L</sub> on the cleavage of RIP following TRAIL treatment in keratinocytes. In line with our findings for TRAIL-mediated caspase-8 activation, RIP cleavage was detectable within 60–90 min after stimulation with TRAIL (Fig. 5A), whereas it was undetectable in cFLIP<sub>L</sub>-overexpressing keratinocytes (Fig. 5B). RIP cleavage was dependent on caspase activity, because it was undetectable in cellular lysates of keratinocytes pretreated with Z-VAD-fmk (Fig. 5B). However, these findings did not explain why TRAIL-induced NF- $\kappa$ B activation was absent in cFLIP<sub>L</sub>-expressing

cells, whereas NF- $\kappa$ B activation was rather increased in caspase inhibitor-treated control cells. To further explore the causal relationship between cFLIP<sub>L</sub> and the ability of TRAIL to signal for NF- $\kappa$ B activation, we therefore next examined the recruitment of RIP to the TRAIL DISC. We first compared the extent of caspase-8, FADD, and RIP recruitment relative to the cellular protein levels in lysates (Fig. 5C). The enrichment of RIP in the DISC was moderate, whereas robust enrichment was detected for FADD and caspase-8. In line with the findings of Harper *et al.* (32, 40), RIP was strongly recruited to the TRAIL DISC only in control cells when they were preincubated with Z-VAD-fmk, whereas FADD or caspase-8 recruitment was essentially unchanged (Fig. 5C, lanes 1–5). Moreover, the TRAIL DISC of control cells also contained the cleaved fragment of RIP that was undetectable when the DISC was precipitated from cells pretreated with Z-VAD-fmk. The TRAIL DISC of FLIP<sub>high</sub> keratinocytes contained cleaved RIP at a higher proportion when compared with the full-length form, indicating that RIP is specifically recruited but also rapidly cleaved within the TRAIL DISC of cFLIP<sub>L</sub>-expressing cells (Fig. 5C, lanes 6–10). In marked contrast, the strong increase of 74-kDa RIP seen in the DISC of caspase inhibitor-treated control cells was largely reduced (Fig. 5C, lane 10). Taken together, these findings suggest that DISC recruitment of RIP is reduced in the presence of cFLIP<sub>L</sub>, whereas the DISC-associated cleavage is rather increased. These data provided an explanation for how cFLIP<sub>L</sub> blocks TRAIL-mediated NF- $\kappa$ B activation but also suggested that cFLIP<sub>L</sub> does not block enzymatic activity of the TRAIL DISC.

*cFLIP<sub>L</sub> Expression Does Not Interfere with DISC-associated Caspase Activity*—Recent reports have indicated that the function of cFLIP<sub>L</sub> at the DISC might be more complex than initially thought and that cFLIP<sub>L</sub> may play a role in caspase-8 activation (42, 43). Hence, we addressed the question of whether cFLIP<sub>L</sub> interferes with the enzymatic activity of the DISC. To this end, we used an *in vitro* cleavage assay. Recombinant Bid was added to protein G-Sepharose beads containing TRAIL DISC precipitates and thereby used as a substrate to determine DISC-associated caspase activity. Surprisingly, DISC precipitates of control cells as well as of FLIP<sub>high</sub> keratinocytes were capable of cleaving recombinant Bid to a similar extent (Fig. 6, lanes 2 and 5). In line, we found that the fully cleaved caspase-8 form p18 in ligand affinity precipitates irrespective of the amount of cFLIP<sub>L</sub> present in the DISC. These results indicate that fully cleaved fragments of caspase-8 are already formed at the DISC independent of cFLIP<sub>L</sub> and confirm and extend recently published findings for the CD95 DISC (44). When the precipitation of the DISC was performed in the presence of Z-VAD-fmk, its activity was not altered in control cells but was largely reduced in cFLIP<sub>L</sub>-expressing cells, although caspase-8 was also detectable as fully mature p18 fragment in the DISC irrespective of cFLIP<sub>L</sub> expression (Fig. 6, lanes 3 and 6). These data suggest that the major role of cFLIP<sub>L</sub> is not the direct interference with caspase-8 activity at the DISC.

## DISCUSSION

Dysregulation of apoptosis is an important pathogenic mechanism in many skin diseases and probably involves the activation of death receptors like TNF-R1, CD95 or TRAIL-R1 and TRAIL-R2 (14, 21). Thus, the understanding of regulatory pathways orchestrating the outcome of death receptor triggering is of crucial importance in skin biology. Accumulating evidence suggests that besides its proapoptotic properties, TRAIL also signals for nonapoptotic responses such as NF- $\kappa$ B activation in a cell type-specific manner (6, 22, 32, 45–47). However, the role of distinct molecules recruited to the receptor complex



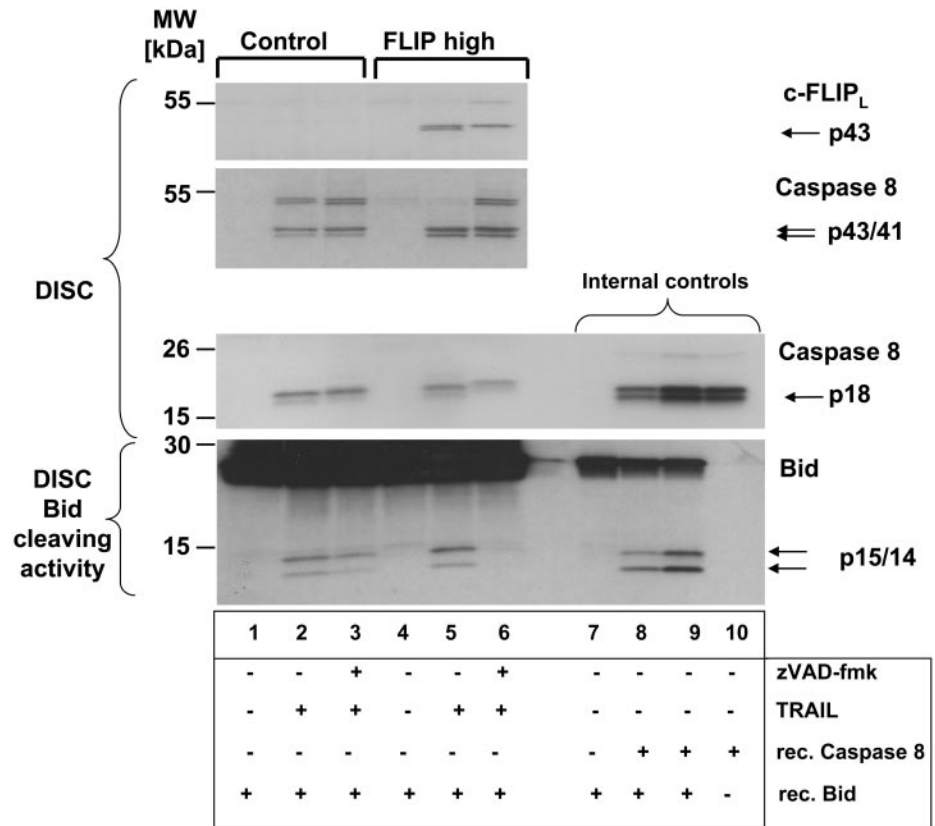
**FIG. 5. cFLIP<sub>L</sub> interferes with RIP recruitment to the TRAIL DISC.** *A*, HaCaT cells were treated for the indicated time periods with LZ-TRAIL (1 μg/ml) and subsequently analyzed for expression and cleavage of RIP. *B*, differentially cFLIP<sub>L</sub>-expressing keratinocytes were either left untreated or were preincubated with Z-VAD-fmk (40 μM; 1 h) and were then treated with TRAIL (0.1 or 1 μg/ml) or TNFα (250 units/ml) for 3 h. Total cellular lysates were analyzed for RIP expression and cleavage by Western blot as described. *C*, inhibition of RIP recruitment to the TRAIL DISC in cFLIP<sub>L</sub>-expressing keratinocytes. DISC analysis was performed from a total of 1 × 10<sup>7</sup> cells. Cell lysates were collected from cells preincubated for 1 h with or without Z-VAD-fmk (40 μM). Lysates of nonstimulated cells in the presence or absence of Z-VAD-fmk (40 μM) or of cell lysates precipitated in the presence of Protein G beads alone (*Beads*) served as specificity controls. Ligand affinity precipitates were characterized for FADD, caspase-8, and RIP recruitment to the TRAIL DISC by Western blotting. For the detection of 39-kDa RIP cleavage fragment, blots were intentionally overexposed (*long exp.*).

following TRAIL stimulation is not fully understood and prompted us to further investigate these signaling pathways in human keratinocytes. In particular, we have studied the consequences of cFLIP<sub>L</sub> expression for death receptor-mediated nonapoptotic signals. Although cFLIP<sub>L</sub> was initially described as a caspase-8 inhibitor (48–50), recent reports have implicated cFLIP<sub>L</sub> as an activator of caspase-8 function (42, 51). It was proposed that the differential stoichiometry between cFLIP<sub>L</sub> and initiator caspase may account for a dual function of cFLIP<sub>L</sub> as either a caspase-activating or caspase-inhibitory protein (42). We show that stable expression of cFLIP<sub>L</sub> renders keratinocytes resistant toward TRAIL-mediated apoptosis and additionally interferes with TRAIL-induced NF-κB activation. In our cellular system, the ectopic expression of cFLIP<sub>L</sub> is likely

to remain at physiologically relevant levels, since we could overcome TRAIL resistance by increasing concentrations of TRAIL, similar to our previous findings in primary keratinocytes (20). In addition, apoptosis resistance correlated with cellular cFLIP<sub>L</sub> expression. Furthermore, although DISC recruitment of caspase-8 is modified, cFLIP<sub>L</sub> does not completely abolish caspase-8 recruitment but rather induces stoichiometric changes of the different cleaved and full-length forms of caspase-8 in the TRAIL DISC. Taken together, these data suggest that the levels of cFLIP<sub>L</sub> and caspase-8 are probably in a physiological range and may allow comparison with primary human keratinocytes. In addition, our data confirm that an important function of cFLIP<sub>L</sub> is the interference with full processing of caspase-8, ultimately leading to



**FIG. 6. *In vitro* enzymatic activity of DISC precipitates is independent of cFLIP<sub>L</sub>.** The enzymatic activity of the DISC of differentially cFLIP<sub>L</sub>-expressing keratinocytes (control and FLIP<sub>high</sub>) was determined by an *in vitro* cleavage assay. The upper two blots show cFLIP<sub>L</sub> and caspase-8 present in DISC precipitates from control cells and FLIP<sub>high</sub> keratinocytes, whereas the third blot shows the presence of caspase-8 p18 in the DISC. Cleavage activity of the DISC was assessed by addition of the known caspase-8 substrate Bid to the ligand affinity precipitates. Comparable Bid cleavage is detectable in control cells (lane 2) as well as in cFLIP<sub>L</sub>-overexpressing keratinocytes (lane 5). Preincubation of cells with Z-VAD-fmk for 1 h (lanes 3 and 6) significantly reduced Bid cleavage only in FLIP<sub>high</sub>. Lane 7 serves as a negative control containing recombinant Bid alone. As a positive control, recombinant Bid was incubated with 0.1 ng (lane 8) or 0.5 ng (lane 9) recombinant caspase-8, whereas lane 10 contains 0.5 ng of recombinant active caspase-8 alone. Cleavage of Bid to p15/p14 fragments was monitored by Western blotting as previously described (22).



decreased levels of active processed caspase-8 homotetramers in the cytoplasm.

Although much effort has been put into the characterization of proapoptotic signals emanating from TRAIL receptors, their ability to activate nonapoptotic signaling pathways remains poorly defined. Previous studies by several groups have shown that apoptosis induction interferes with proinflammatory gene expression elicited by TRAIL, because the addition of caspase inhibitors such as Z-VAD-fmk either leads to increased induction of target genes or is needed for TRAIL-dependent transcription (22, 32, 33, 52). Using the physiological caspase-8 inhibitor cFLIP<sub>L</sub>, we show that cFLIP<sub>L</sub> potently inhibits proinflammatory TRAIL-induced target genes such as IL-8 and thereby demonstrate that the effect exerted by the caspase inhibitor Z-VAD-fmk is clearly different from the potential of cFLIP<sub>L</sub> to interfere with TRAIL receptor signaling. Understanding of this difference is of great importance, since the clinical use of caspase inhibitors may result in potentially deleterious proinflammatory signals exerted by death receptors when apoptosis induction is blocked by Z-VAD-fmk *in vivo* (53). It is currently controversially discussed how cFLIP<sub>L</sub> influences activation of NF- $\kappa$ B (6, 13, 54–56). In this report we have studied how cFLIP<sub>L</sub> modulates TRAIL-mediated gene induction in keratinocytes. cFLIP<sub>L</sub> was described as a constitutive activator of NF- $\kappa$ B exerted by cFLIP<sub>L</sub>-mediated recruitment of TRAF-2 to the caspase-like domain of cFLIP<sub>L</sub> (13). It was proposed that heterodimers of the p43 fragment of cFLIP<sub>L</sub> and the p43/41 fragments of caspase-8 are formed at the CD95 DISC and thereby activate NF- $\kappa$ B (57). However, this study investigated the direct effect of overexpressed p43 cFLIP<sub>L</sub> rather than the impact of death receptor ligation in the presence of different amounts of full-length cFLIP<sub>L</sub>. In contrast, another group suggested that death receptor-mediated NF- $\kappa$ B activation was inhibited by cFLIP<sub>L</sub> (33). We did not detect changes in basal NF- $\kappa$ B DNA binding in cells expressing different ratios of cFLIP<sub>L</sub> to caspase-8 (Fig. 2A). The discrepancy

with the report in 293 cells might be explained by the direct effect of overexpressed cFLIP<sub>L</sub> in this study (57). Our results are in line with the findings by Wajant *et al.* (33) demonstrating an inhibitory role of cFLIP<sub>L</sub> for death receptor-induced NF- $\kappa$ B activation.

We now show that only TRAIL-induced and not TNF $\alpha$ -induced NF- $\kappa$ B activation is affected by cFLIP<sub>L</sub>. This difference is also reflected by differential ligand-mediated I $\kappa$ B $\alpha$  degradation, thereby placing cFLIP<sub>L</sub>-mediated inhibition upstream of or at the signalosome (58). What may be the reason for the difference between TNF $\alpha$  and TRAIL-mediated NF- $\kappa$ B activation? Since we detect NF- $\kappa$ B activation and I $\kappa$ B $\alpha$  degradation within 15–30 min after stimulation with soluble TNF $\alpha$ , the activation of NF- $\kappa$ B via TNF-R2 by endogenous membrane-bound TNF $\alpha$  is unlikely. Therefore, our results imply a direct activation of NF- $\kappa$ B via TNF-R1 rather than induction of endogenous TNF $\alpha$  (59). Thus, nonapoptotic death receptor signaling by TNF-R1 is not abrogated by cFLIP<sub>L</sub> but rather specifically inhibited for TRAIL, whereas cFLIP<sub>L</sub> efficiently blocks proapoptotic signals of both TNF $\alpha$  and TRAIL (60, 61).<sup>2</sup> These data confirm that proapoptotic and gene-inductive signaling pathways utilized by TNF $\alpha$  and TRAIL are not identical, similar to a very recent report for CD95L (56). It will be interesting to determine whether these distinct signaling capabilities are related to the recently described ability of TNF-R1 to form receptor-associated complexes containing RIP as well as cytoplasmic complexes containing FADD and caspase-8 (39, 41).

What are the functional differences in DISC-activated nonapoptotic signaling pathways of cFLIP<sub>L</sub>-expressing keratinocytes when compared with TRAIL-treated control cells in the presence of caspase inhibitor? Since we did not detect differences in the cytoplasmic caspase activation pattern that could

<sup>2</sup> A. Kerstan, T. Wachter, M. Sprick, H. Walczak, and M. Leverkus, unpublished data.

explain our findings for TRAIL-mediated gene induction, we investigated the receptor-proximal events. DISC analysis revealed that predominantly p43/41 fragments of caspase-8 are present in the DISC of apoptosis-resistant cFLIP<sub>L</sub>-expressing keratinocytes. In contrast, the DISC of highly sensitive cells able to induce NF-κB contained large amounts of full-length caspase-8. These results confirm that cFLIP<sub>L</sub> rather facilitates the cleavage of caspase-8 at the TRAIL DISC but that this processing step in the DISC is neither sufficient for apoptosis induction nor predominantly leads to gene induction. The explanation for these data may be that caspase-8 bound in the caspase-8-cFLIP<sub>L</sub> heterodimer does not dissociate from the DISC and therefore only cleaves DISC-associated proteins (34, 42, 51). To obtain more direct evidence for the enzymatic activity of the TRAIL DISC, DISC-associated caspases were assessed for their ability to cleave the prototypical substrate protein Bid. Interestingly, when we analyzed ligand affinity precipitates for the presence of the fully cleaved p18 fragment of caspase-8, we detected comparable amounts of p18 in the DISC independent of the expressed levels of cFLIP<sub>L</sub>. These results indicate that fully cleaved fragments of caspase-8 are already formed at the DISC irrespective of the expression of cFLIP<sub>L</sub>. Our data are in line with similar findings recently reported for the CD95 DISC (44). Our data clearly demonstrate that the presence of cFLIP<sub>L</sub> does not substantially modify the activity of DISC-associated caspases. This indicates that DISC-associated cFLIP<sub>L</sub>, caspase-8, and RIP cleavage occurs also in the DISC of cFLIP<sub>L</sub>-expressing cells. Our findings thereby underline that the local enzymatic activity of the membrane-bound complex is not the decisive factor that determines when TRAIL-induced NF-κB activation can occur. Based on our results, caspase-8-cFLIP<sub>L</sub> heterodimers in the DISC are enzymatically active but inhibit the release of active caspase-8 into the cytoplasm. Our data are in line with a recent *in vitro* study that demonstrates the activation of initiator caspases by cFLIP<sub>L</sub> (43). Because the turnover of DISC-associated proteins is decreased in the presence of cFLIP<sub>L</sub>, this may result in a reduction of the release of DISC-cleaved caspases or other potential DISC-modified substrates (see below). What may be the explanation for the increased gene induction in the presence of Z-VAD-fmk? These findings, made in several cell types, have generally been attributed to the ability of Z-VAD-fmk to efficiently block caspase activation in the cytoplasm, thereby inhibiting the degradation of molecules necessary for NF-κB activation (for a review, see Ref. 10). However, a recent report suggested that the proform of caspase-8 is potentially enzymatically active in the DISC and that Z-VAD-fmk promotes the association of the proforms of cFLIP<sub>L</sub> and caspase-8 at the DISC (51). Our data clearly show that the local activity of caspase-8 at the DISC is not as efficiently blocked by Z-VAD-fmk as the activity of caspases present in the cytoplasm (compare Fig. 4). Thereby, this inhibitor may rather stabilize the full-length homo- or heterodimers of caspase-8 and cFLIP<sub>L</sub> possibly required for the induction of nonapoptotic signals at the DISC. Further studies are required to delineate this point in more detail.

Crucial components for NF-κB activation by TNF-R1 are the adaptor molecules TRAF-2 and RIP (36, 62). Additionally, RIP has been shown to be recruited to the TRAIL DISC (32, 40). Therefore, RIP clearly represents a candidate molecule that may explain why cFLIP<sub>L</sub> interferes with TRAIL-induced NF-κB activation. Although TNF receptor complexes contain large quantities of RIP (40, 41), our analysis revealed that only in the presence of caspase inhibitors are significant amounts of RIP found in the TRAIL DISC, in line with recent reports (32,

40). Here we show that cleaved RIP was detected in the TRAIL DISC, indicating a rapid DISC-associated inactivation of RIP by active caspases (32). In cFLIP<sub>L</sub>-expressing cells, RIP is also efficiently cleaved at the DISC and present in a higher proportion when compared with the full-length form. These data argue against the proposed role of cFLIP<sub>L</sub> as an activator of NF-κB at the DISC (13) and rather support recent data for CD95-induced NF-κB activation and its inhibition by different forms of cFLIP (56). Our results indicate a broader inhibitory role of cFLIP for death receptor-mediated gene induction, whereas TNF-R1-mediated NF-κB activation is not altered by cFLIP. Interestingly, the complete lack of RIP in Jurkat cells inhibits CD95-mediated gene induction (56) but not CD95-mediated κB-specific DNA binding (63). Future experiments using knockdown technology will have to determine the role of RIP for TRAIL- and CD95-mediated gene induction in more detail. Taken together, our data rather suggest that efficient turnover of DISC-associated proteins is blocked by cFLIP<sub>L</sub>, because RIP cleavage in cellular lysates is only detected in control cells. If DISC-associated RIP cleavage does not correlate with gene induction, which DISC-modified signal might be more important in this respect? Recent evidence indicates that RIP undergoes extensive modifications following recruitment to TNF-R1 or TRAIL-R1/TRAIL-R2 (32, 39–41). Although the nature of these modifications is currently unknown, they might include ubiquitination, as indicated by experiments using proteasome inhibitors (40). Interestingly, the presence of ubiquitinated RIP in the TNF receptor complex correlates with TNF-induced NF-κB activation (64). We detect decreased recruitment of full-length and modified RIP to the TRAIL DISC when cFLIP<sub>L</sub> is expressed at high levels. These data imply that the cFLIP<sub>L</sub>-caspase-8 heterodimers may be less efficient for RIP recruitment and/or modification. Thus, our data provide an explanation of why cFLIP<sub>L</sub>-expressing cells fail to activate NF-κB. Modification of RIP and the release of these modified forms may be necessary for effective NF-κB activation, similar to findings for TNFα (40, 41). The reduced turnover of DISC-associated proteins in the presence of cFLIP<sub>L</sub> might thereby provide an alternative explanation for our findings. We conclude that cFLIP<sub>L</sub> blocks not only TRAIL-mediated apoptosis but also the NF-κB signaling pathway at the DISC level.

There is increasing evidence that expression of cFLIP promotes tumor growth and facilitates immune escape of tumors (34, 65–67). Moreover, neutrophilic attraction is critical for CD95L-mediated tumor clearance (68). In addition, a recent report indicates that CD95L-mediated nonapoptotic signaling pathways are critical for tumor motility and invasion when tumor cells are resistant to CD95L-mediated apoptosis (63). Our data demonstrating the inhibition of NF-κB activation as well as chemotactic cytokines by cFLIP<sub>L</sub> may be of crucial importance for the understanding of tumor progression. Specific modulation of cFLIP<sub>L</sub> or the use of caspase inhibitors may thus offer new tools for anti-cancer therapy not only by reactivation of apoptosis signaling pathways but also allowing for death receptor-dependent gene induction attracting neutrophils (69, 70). This might be of particular relevance when downstream portions of the apoptosis pathway, as previously shown for primary keratinocytes (21), are efficiently blocked by modulation of inhibitor-of-apoptosis proteins like XIAP. Inhibition of the intrinsic apoptotic pathway by Bcl-2 increases CD95-mediated gene induction (56). It will be interesting to determine whether DISC-associated caspase activation and/or the release of active caspase-8 to the cytoplasm are necessary for gene induction and how apoptosis protection by downstream effectors like inhibitor-of-apoptosis proteins or Bcl-2

family members will affect gene induction in keratinocytes. Since it is currently not known whether keratinocytes are Type I or Type II cells with respect to CD95-mediated apoptosis, future studies are required to delineate this important point *in vitro* and *in vivo*. Moreover, expression of cFLIP<sub>L</sub> may not allow TRAIL-mediated apoptosis or NF-κB activation but rather initiate alternate signaling pathways, as suggested for death receptor-induced mitogen-activated protein kinases (13, 71). Since TRAIL-induced NF-κB signaling and apoptosis induction are inhibited by cFLIP<sub>L</sub> in keratinocytes, it will be of great interest to analyze these alternative receptor-initiated pathways potentially activated in cFLIP<sub>L</sub>-protected cells in the skin or other organ systems.

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