Metabolic Inhibitors Sensitize for CD95 (APO-1/Fas)-induced Apoptosis by Down-Regulating Fas-associated Death Domain-like Interleukin 1-Convertering Enzyme Inhibitory Protein Expression

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ABSTRACT

Protein or RNA synthesis inhibitors are known to sensitize some resistant cells for death receptor-induced apoptosis. However, the molecular mechanisms involved in sensitization have not yet been defined exactly. Here, we report that metabolic inhibitors such as cycloheximide (CHX) or actinomycin D (ActD) sensitize for CD95-induced apoptosis by strongly down-regulating FLIP and RIP expression. Metabolic labeling studies revealed that CHX or ActD inhibited protein or RNA synthesis at concentrations required for sensitization. In contrast to Fas-associated death domain (FADD) or caspase-8, FADD-like interleukin 1-converting enzyme-inhibitory protein (FLIP) and RIP protein levels rapidly decreased upon treatment with CHX or ActD, indicating that both molecules have a high turnover rate. Selective down-regulation of FLIP expression by FLIP antisense oligonucleotides sensitized for CD95-induced apoptosis. Reduction of FLIP levels resulted in undetectable amounts of FLIP at the CD95 death-inducing signaling complex (DISC) upon CD95 stimulation, thereby enhancing the recruitment of caspase-8 to the DISC and caspase-8 activation. CHX- or ActD-mediated sensitization to CD95-induced apoptosis was predominantly found in type I cells in which FADD and caspase-8 are recruited to CD95 upon stimulation but not in type II cells in which no DISC formation is detected. Pretreatment with CHX or ActD sensitized for subsequent CD95 stimulation compared with cells without pretreatment. CHX or ActD also reduced XIAP expression and similarly sensitized for tumor necrosis factor-related apoptosis-inducing ligand- or tumor necrosis factor-α-induced apoptosis. Because blockade of death receptor triggering by FLIP overexpression has recently been implicated in tumorigenesis and treatment resistance in vivo, strategies to inhibit FLIP expression, e.g., by metabolic inhibitors, may prove to be a useful complementary tool for the treatment of cancer.

INTRODUCTION

Cell death by apoptosis plays an important role in the regulation of various physiological and pathological conditions (1). Death receptors of the TNF-nerve growth factor receptor superfamily, such as CD95 (APO-1/Fas), have been shown to induce apoptosis after triggering with agonistic antibodies or with their natural ligands (1–3). Stimulation of CD95 results in clustering of the receptor, which in turn leads to the recruitment of the adaptor molecule FADD and the receptor proximal caspase-8 to the CD95 receptor to form a DISC (4–7). Caspase-8 becomes activated upon recruitment to the DISC and initiates apoptosis by subsequent cleavage of downstream effector caspases (7, 8).

To avoid inappropriate cell death, death receptor signals must be tightly controlled (1). The CD95 signaling pathway can be inhibited at different checkpoints: at the receptor level [by mutations or down-regulation of the receptor, e.g., by oncogenic Ras (9), decoy receptors, or soluble ligands (10)], during signal transduction (by FLIP and Bcl-2-related proteins), or during the effector phase (by IAPs). c-FLIP, which structurally resembles caspase-8, was identified recently as a cellular homologue of viral FLIPs, except that it lacks proteolytic activity (11–13). FLIP is recruited to the CD95 DISC through the adaptor molecule FADD similar to caspase-8, thereby preventing the recruitment of caspase-8 into the complex and subsequent caspase-8 activation (14). At the mitochondrial level, apoptosis can be inhibited by antiapoptotic members of the Bcl-2 family, such as Bcl-2 or Bcl-XL, which inhibit relocation of cytochrome c or apoptosis-inducing factor from mitochondria into the cytosol or binding of cytochrome c to Apaf-1 (15–22). The effector phase of apoptosis can be blocked by IAPs that interfere with activation of effector caspases, e.g., caspase-3 and caspase-7 (23, 24).

Recently, induction of tumor cell death by triggering of death receptors was found to constitute a more prominent mechanism for tumor clearance in vivo than has been thought previously, and blockade of CD95-induced apoptosis has been implicated in tumorigenesis and treatment resistance (25–28). CD95 is expressed on a variety of different cell types, yet many CD95-expressing cells are resistant to CD95-triggered apoptosis (1). Maintaining a state of resistance to apoptosis often requires de novo protein or RNA synthesis (24, 29–31). Thus, treatment with protein synthesis inhibitors, such as CHX, or RNA synthesis inhibitors, such as ActD, is usually required for TNF-α-mediated apoptosis (29). Likewise, treatment with CHX or ActD may render many resistant cells sensitive to CD95 triggering (24, 29, 30). This sensitizing effect is thought to result from inhibition of the synthesis of short-lived regulatory proteins (24, 29, 30). However, it is not exactly known at which stage(s) of the signaling pathway CHX or ActD interferes, which hampers the potential therapeutic application of metabolic inhibitors as sensitizers for death receptor-induced apoptosis. Therefore, to elucidate the mechanism(s) of CHX or ActD-mediated sensitization, we selected SHEP neuroblastoma cells as a prototype cell line susceptible to CHX- or ActD-mediated sensitization. In addition to the parental cell line, we used derivative cells in which receptor-associated signaling events were blocked by FADD-DN or in which the mitochondrial pathway was inhibited by overexpression of Bcl-2.

MATERIALS AND METHODS

Cell Culture. Vector control or Bcl-2-overexpressing SHEP neuroblastoma cells, BJAB Burkitt lymphoma cells, SKW6.4 B lymphoblastoid cells, Jurkat or CEM T leukemia cells, HepG2 hepatocellular carcinoma cells, or NT68, GG62, or CADO Ewing’s sarcoma cells were maintained in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany) as described previously (32).

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3 The abbreviations used are: TNF, tumor necrosis factor; TNF-RI, TNF receptor type 1; FADD, Fas-associated death domain; FADD-DN, dominant negative mutant of FADD; DISC, death-inducing signaling complex; IAP, inhibitor of apoptosis protein; ActD, actinomycin D; CHX, cycloheximide; FACS, fluorescence-activated cell-sorting; FLIP, FLICE-inhibitory protein; PARP, poly(ADP-ribose)polymerase; zVAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; SODD, silencer of death domains.
determination of apoptosis or in 75-cm² flasks (Falcon, Heidelberg, Germany) for protein isolation or immunoprecipitation.

**Determination of Apoptosis.** Cells were incubated for indicated times with 1 µg/ml mouse anti-APO-1 IgG3 monoclonal antibody (2) and 5 ng/ml protein A (Sigma, Deisenhofen, Germany), 10 ng/ml TRAIL (Pepto Tech, Inc., Rocky Hill, NJ), 30 ng/ml TNF-α (Calbiochem, Bad Soden, Germany), or 1 µM staurosporine (Sigma) in the presence or absence of 1 µg/ml CHX (Sigma) or 0.1 µg/ml ActD (Sigma). The broad spectrum tripeptide inhibitor of caspases zVAD.fmk (Enzyme Systems Products, Dublin, CA) was used at a concentration of 50 µM. Quantification of DNA fragmentation was performed by FACS analysis of propidium iodide-stained nuclei as described previously (33) using CELLQuest software (Becton Dickinson, Heidelberg, Germany).

**Metabolic Labeling Studies.** Protein synthesis of untreated or CHX-treated cells (0.01–3 µg/ml CHX) was determined by pulse-chase labeling of cells with [3H]leucine (34). Cells were washed with leucine-free RPMI 1640 (Life Technologies, Inc.) and incubated for 1 h at 37°C in leucine-free RPMI 1640 supplemented with 5% dialyzed FCS to deplete cellular leucine. Cells were pulsed for 30 min with [3H]leucine (0.2 mCi/ml; Amersham Pharmacia, Freiburg, Germany) and incubated for 2 h at 37°C in RPMI 1640 containing excess leucine (15 µg/ml; Sigma). Protein precipitation was performed using trichloroacetic acid (Roth, Karlsruhe, Germany). RNA synthesis of cells, treated with 0.001–0.3 µg/ml ActD or left untreated, was determined by incorporation of 5.6-[3H]uridine (5 µCi/ml; Amersham Pharmacia) into cells. Radioactivity was quantified by scintillation counting (liquid scintillation analyzer 1500 TR; Packard, Frankfurt, Germany). Results are expressed as percentage of inhibition of protein or RNA synthesis as compared with untreated cells.

**Western Blot Analysis.** Cells were lysed for 30 min at 4°C in PBS with 0.5% Triton X-100 (Serva, Heidelberg, Germany) and 1 mM PMSF (Sigma), followed by high-speed centrifugation. Protein concentration was assayed using biocinchonic acid (Pierce, Rockford, IL). Forty µg of protein/lane was separated by 12% SDS-PAGE and electroblotted onto nitrocellulose (Amersham Pharmacia). Equal protein loading was controlled by Ponceau red staining of membranes. After blocking for 1 h in PBS supplemented with 2% BSA (Sigma) and 0.1% Tween 20 (Sigma), immunodetection of caspase-8, caspase-3, FADD, FLIP, PARP, RIP, XIAP, or β-actin was done using mouse anti-caspase-8 monoclonal antibody (Abcam), mouse anti-caspase-3 monoclonal antibody (1:1000; Transduction Laboratories, Lexington, KY), mouse anti-FADD monoclonal antibody (1:500; Transduction Laboratories), mouse anti-CD95 monoclonal antibody (1:1000; Transduction Laboratories), mouse anti-FLIP monoclonal antibody NF6 (1:10 dilution of hybridoma supernatant; Ref. 14), rabbit anti-PARP polyclonal antibody (1:10000; Enzyme Systems Products), mouse anti-RIP monoclonal antibody (1:10000; Transduction Laboratories), mouse anti-XIAP monoclonal antibody (clone H62120; 1:1000; Transduction Laboratories), or mouse anti-β-actin monoclonal antibody (1:5000; Sigma), followed by goat anti-mouse IgG or goat anti-rabbit IgG (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Enhanced chemiluminescence (ECL; Amersham Pharmacia) was used for detection.

**Immunoprecipitation.** Immunoprecipitation of the CD95 DISC was performed as described previously (35). Briefly, cells were treated with 1 µg/ml anti-APO-1 IgG3 monoclonal antibody (2) for 15 min and lysed for protein extraction. Immunoprecipitation of the CD95 DISC was performed using mouse anti-APO-1 IgG3 monoclonal antibody and protein A-Sepharose (Sigma). Beads were washed three times in lysis buffer and proteins were separated on 12% SDS-PAGE. Western blot for FADD, FLIP, or caspase-8 protein was performed as described above.

**Down-Regulation of FLIP Protein Expression by FLIP Antisense Oligonucleotides.** To inhibit FLIP expression phosphorothiate antisense oligodeoxynucleotides to inhibit the FLIP initiation codon, control sense and nonsense oligodeoxynucleotides with the following published sequences were used (36): FLIP antisense, 5'-gacctagacagcacttc-3'; FLIP sense, 5'-cactata-cagacactccg-3'; and FLIP nonsense, 5'-tggatccgaacatgtcaga-3'. The uptake of FITC-conjugated oligonucleotides was measured by flow cytometry after 24-h incubation with oligonucleotides.

**RESULTS**

Metabolic inhibitors such as CHX or ActD are known to increase sensitivity of many cells for apoptosis in response to death receptor stimulation, e.g., CD95 or TNF-RI (29). In initial experiments we observed a variable degree of sensitization to CD95-induced apoptosis by CHX or ActD in different cell types. To determine the step(s) in apoptosis regulated by CHX or ActD, we selected SHEP neuroblastoma cells as a prototype cell line in which sensitivity to CD95-induced apoptosis was profoundly enhanced in the presence of CHX or ActD. In addition to the parental cell line, we used derivative cells in which the receptor pathway was blocked by FADD-DN or the mitochondrial contribution by overexpression of Bcl-2.

**Sensitization to Anti-APO-1-induced Apoptosis.** Apoptosis triggered by an agonistic anti-APO-1 antibody was strongly increased in the presence of CHX or ActD (Fig. 1). Treatment with CHX or ActD alone at the concentrations required for sensitization (1 µg/ml CHX or 0.1 µg/ml ActD) was devoid of any significant toxicity (data not shown). Overexpression of FADD-DN or the caspase inhibitor zVAD.fmk almost completely blocked anti-APO-1-triggered apoptosis or CHX- or ActD-mediated sensitization (Fig. 1, A and B). Overexpression of Bcl-2 initially (24 h) provided protection against CD95-induced apoptosis in the presence or absence of CHX or ActD (Fig. 1C). However, upon prolonged treatment for 48–72 h, overexpression

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**Fig. 1.** Sensitization to anti-APO-1-induced apoptosis by CHX or ActD. SHEP neuroblastoma cells transfected with vector control (closed symbols), FADD-DN (A, open symbols), vector control cells in the presence of 50 µM zVAD.fmk (B, open symbols) or cells transfected with Bcl-2 (C, open symbols) were treated for the indicated periods with 1 µg/ml anti-APO-1 monoclonal antibody in the absence (■, ○) or presence (●, □) of 1 µg/ml CHX or 0.1 µg/ml ActD (●, □). Apoptosis was determined by FACS analysis of propidium iodide-stained DNA content. The percentage of specific apoptosis was calculated as follows: 100 × [experimental apoptosis (%) - spontaneous apoptosis (%)]/100% - spontaneous apoptosis (%). Spontaneous apoptosis was <1%. Data are the mean of triplicates; similar results were obtained in three separate experiments; bars, SD.
of Bcl-2 only partially inhibited CD95-triggered apoptosis and only poorly blocked CHX or ActD-mediated sensitization (Fig. 1C). In control experiments, Bcl-2 was overexpressed in the mitochondrial fraction and blocked loss of mitochondrial $\Delta \Psi_{m}$ (data not shown), indicating that CHX or ActD may affect a step upstream of mitochondria that eventually leads to cell death, even in the absence of functional mitochondria.

Enhancement of Anti-APO-1-induced Caspase Activation. To test whether CHX or ActD act upstream of mitochondria, we moni-
tored activation of the receptor proximal caspase-8 as one of the earliest detectable events after receptor triggering. Treatment with CHX or ActD enhanced cleavage of caspase-8 already at 2 h after stimulation preceding the onset of detectable apoptosis (Fig. 2A). Caspase-8 cleavage was followed by cleavage of the effector caspase-3 and PARP detectable at 6 h, indicating that caspase-8 was the apical caspase in the cascade upon CD95 stimulation (Fig. 2A). In cells overexpressing Bcl-2, increased cleavage of caspase-8 in the presence of CHX or ActD was found at 2 h after CD95 triggering similar to vector control cells, whereas cleavage of caspase-3 and PARP was markedly delayed to 24 h (Fig. 2B). Treatment with CHX or ActD alone did not induce caspase cleavage at any time point tested (data not shown). Thus, caspase-8 cleavage was increased by CHX or ActD independently of mitochondrial functions, suggesting that CHX or ActD act upstream of caspase-8 activation, possibly at the receptor complex.

**Down-Regulation of FLIP, RIP, and XIAP Expression.** The fact that protein or RNA synthesis inhibitors can strongly enhance CD95-induced apoptosis suggests that the cells are synthesizing an intracellular inhibitor(s). In search for labile repressors regulated by CHX or ActD, we next monitored expression levels of various proteins known to modulate CD95-mediated apoptosis. After treatment with anti-APO-1 in the presence of CHX or ActD, FLIP and RIP protein levels were strongly down-regulated as early as 2 h (Fig. 3A). In addition, expression of XIAP protein decreased after 12 h of treatment with CHX or ActD (Fig. 3A). In contrast, no changes in protein expression levels of Bcl-2, Bcl-XL, Bax, CD95, or CD95L and no alterations in CD95 aggregation were detected (data not shown).

On theoretical grounds, the observed decrease in FLIP, RIP, or XIAP protein levels may be attributable to rapid cleavage upon CD95-induced apoptosis or may result from inhibition of protein synthesis by CHX or ActD. Therefore, to test whether these proteins have a high turnover rate within the cell, we monitored expression levels over time in cells exposed to CHX or ActD. FLIP or RIP protein levels already decreased within 2 h of adding CHX or ActD, and XIAP protein levels started to decline within 12 h (Fig. 3B), despite any signs of cell death (data not shown). In contrast, no change in caspase-8 or FADD expression was seen (Fig. 3B), indicating that the intracellular concentration of FLIP relative to other components of the CD95 DISC, in particular caspase-8, may determine the susceptibility to CD95 triggering.

**Increased DISC Formation by Reduction of DISC-bound FLIP.** FLIP has been reported to interact with FADD or caspase-8 at the DISC level upon CD95 stimulation, and no constitutive association of endogenous FLIP, FADD, or caspase-8 was found in the cytoplasm (14). Upon CD95 triggering, FLIP is recruited to the CD95 DISC through the adaptor molecule FADD, similar to caspase-8 (14). Therefore, to test how FLIP levels could influence the susceptibility to CD95-mediated apoptosis, we immunoprecipitated the CD95 receptor and tested for the presence of CD95-associated FLIP by Western blot analysis. FLIP was recruited to the CD95 DISC after receptor triggering (Fig. 4A). However, in CHX- or ActD-treated cells, in which FLIP protein levels were strongly reduced (Fig. 3), no recruitment of FLIP could be detected in response to CD95 triggering (Fig. 4A). Reciprocally, the recruitment of caspase-8 to CD95 upon receptor triggering was strongly increased in the presence of CHX or ActD (Fig. 4). Thus, treatment with CHX or ActD reduces the amount of DISC-bound FLIP, thereby facilitating the recruitment of caspase-8 to the DISC and subsequent caspase-8 activation. Interestingly, in addition to caspase-8, the recruitment of FADD to the CD95 receptor was also enhanced upon treatment with CHX or ActD (Fig. 4). This indicates that other labile repressors in addition to FLIP, such as RIP, may modulate DISC formation at the interaction of FADD and CD95. However, although RIP protein expression was down-regulated by CHX or ActD, we could not detect any CD95-RIP interaction (data not shown), consistent with a previous report (37), suggesting that RIP may indirectly modulate the recruitment of FADD to CD95. Similarly, the recruitment of FADD-DN to CD95 was increased by treatment with CHX or ActD in cells overexpressing FADD-DN (Fig. 4B). However, overexpression of FADD-DN completely blocked binding of caspase-8 to the DISC (Fig. 4B). In contrast, the presence of zVAD.fmk or overexpression of Bcl-2 did not affect DISC formation (Fig. 4B), consistent with unaltered cleavage of caspase-8 found in Bcl-2-overexpressing cells compared with vector control cells (Fig. 2). Altered recruitment of caspase-8, FADD, or FADD-DN to CD95 occurred without any change in protein expression levels (Fig. 4B). These results indicate that CHX or ActD can modulate DISC formation at least at two different levels, at the interaction between CD95 and FADD and at the interaction between FADD and caspase-8.

**Sensitization of Type I Cells to Anti-APO-1-induced Apoptosis.** To further investigate the potential implications of decreased FLIP levels at the DISC, we analyzed in more detail different cell lines that were found to be sensitized for CD95-induced apoptosis to a variable degree by CHX or ActD in initial experiments. Treatment with CHX or ActD sensitized type I BJAB or SKW6.4 cells (35) and HepG2 or NT68 cells to CD95-induced apoptosis (Fig. 5A). In these cells, FADD and caspase-8 were recruited to CD95 upon receptor stimulation and recruitment of FADD, and caspase-8 was further increased in the presence of CHX or ActD (Fig. 5B). Similar to SHEP neuroblastoma cells, treatment with CHX or ActD strongly reduced FLIP and
RIP expression, decreased XIAP expression, and increased activation of caspases (Fig. 5C). In contrast, no sensitization for CD95-induced cell death upon treatment with CHX or ActD was seen in type II Jurkat or CEM cells (35) or in GG62 and CADO cells, in which no recruitment of FADD and caspase-8 was found upon CD95 stimulation, even in the presence of CHX or ActD (Fig. 5, A and B). However, FLIP expression similarly decreased upon addition of CHX or ActD in type I and type II cells, and no differences in constitutive
expression of FADD, caspase-8, or FLIP proteins were found between both cell types (Fig. 5D; compare Fig. 3B), suggesting that down-regulation of FLIP only results in increased DISC formation and caspase-8 activation in type I cells that form a DISC upon CD95 triggering, whereas down-regulation of FLIP was of no consequence in type II cells that do not form a DISC detectable by the method used in our analysis. In addition, these data demonstrate that our findings are relevant for sensitization mechanisms of tumor cells of different origin.

**Down-Regulation of FLIP Expression by FLIP Antisense Oligonucleotides Sensitizes for CD95-induced Apoptosis.**

To establish a causal connection between the reduction of FLIP levels and sensitization to CD95-induced apoptosis, we used antisense oligonucleotides that included a sequence complementary to the start site of the FLIP open reading frame. A similar uptake of the antisense or the control sense or nonsense oligonucleotides was detected by flow cytometry (Fig. 6A). Western blot analysis of antisense oligonucleotide-treated SHEP cells showed a strong suppression of FLIPlong and FLIPshort protein expression compared with medium (mock) or control sense or nonsense oligonucleotide-treated cells (Fig. 6B). Treatment with FLIP antisense oligonucleotides sensitized SHEP cells for CD95-induced apoptosis to a similar degree compared with treatment with CHX, whereas control sense or nonsense oligonucleotides had no effect on CD95-induced apoptosis (Fig. 6C). Sensitization for CD95-triggered apoptosis by FLIP antisense oligonucleotides was also found in other cell types, such as NT68 Ewing’s sarcoma cells, whereas no effect was seen in Jurkat cells (Fig. 6C). These data show that selective down-regulation of FLIP expression can sensitize for CD95-induced apoptosis and suggest that this mechanism might be especially important in certain cell types, e.g., in cells that do form a CD95 DISC upon CD95 triggering.

**Enhancement of Anti-APO-1-induced Apoptosis by Preincubation with CHX or ActD.**

Because down-regulation of FLIP levels was already seen within 2 h of addition of CHX or ActD, we asked whether these changes would correlate with a gain in susceptibility to CD95-induced death. To address this question, we analyzed the amount of apoptosis in response to CD95 triggering in cells pre-treated with CHX or ActD compared with cells without pretreatment. Pretreatment with CHX or ActD for only 2 h markedly enhanced sensitivity to CD95-triggered apoptosis, which increased upon further preincubation (Fig. 7). After preincubation with CHX or ActD for 12 h, apoptosis levels after treatment with anti-APO-1 antibody alone were close to those found after simultaneous treatment with anti-APO-1 antibody and CHX or ActD for 12 h (Fig. 7). These results provide additional support for the premise that inhibition of synthesis of short-lived proteins sensitizes cells for CD95-mediated apoptosis.

**Inhibition of Protein or RNA Synthesis.**

We then performed metabolic labeling studies to test whether CHX or ActD were inhibiting protein or RNA synthesis at the levels found to reduce FLIP protein levels and to induce apoptosis. CHX concentrations to inhibit protein synthesis or ActD concentrations to inhibit RNA synthesis correlated with the levels for induction of apoptosis (Fig. 8). These
Results indicate that the synergistic effect of CHX or ActD on CD95-mediated apoptosis was the result of inhibition of novel synthesis of labile repressors.

Sensitization to TRAIL- or TNF-α-induced Apoptosis. Because FLIP has also been reported to interfere with TRAIL-R- or TNF-R1-induced apoptosis, implying similar mechanisms of signal transduction by these receptors (13), we tested whether CHX or ActD would also have a synergistic effect on apoptosis in response to TRAIL- or TNF-α in SHEP neuroblastoma cells. In the presence of CHX or ActD, TRAIL- or TNF-α-induced apoptosis was strongly increased (Fig. 9, A and B). In addition, pretreatment with CHX or ActD resulted in enhanced apoptosis compared with cells treated with TRAIL or TNF without CHX or ActD pretreatment (data not shown). In contrast, CHX or ActD had no effect on apoptosis induced by the protein kinase C inhibitor staurosporine (Fig. 9C). Because staurosporine is reported to trigger apoptosis independent of death receptor signaling (38), these results further indicate that sensitization by CHX or ActD involves death receptor-associated signaling events.

DISCUSSION

Recent data indicate that induction of tumor cell death by stimulation of death receptors such as CD95 constitutes a more prominent mechanism in the defense against tumors than has been thought previously (25–28). CD95 is expressed on a variety of different cell types; however, many CD95-expressing cells are resistant to CD95-triggered apoptosis (1). Resistance to apoptosis in response to CD95 stimulation has been implicated recently in tumorigenesis and treatment resistance in vivo (25–28). Maintaining a state of resistance often requires de novo protein or RNA synthesis (29). Therefore, treatment with CHX or ActD has been found to sensitize many constitutively apoptosis-resistant cells (29). This sensitizing effect has been interpreted as the consequence of inhibition of the synthesis of labile repressors (29). However, the potential clinical application of metabolic inhibitors as sensitizers for treatment modalities that act via induction of apoptosis requires a molecular understanding of CHX- or ActD-mediated sensitization.

Here, we report for the first time that metabolic inhibitors such as CHX or ActD regulate the susceptibility to CD95-induced apoptosis through down-regulation of FLIP expression at the CD95 DISC. This conclusion is based on a number of independent pieces of evidence: first, FLIP levels rapidly decreased upon addition of CHX or ActD at concentrations that blocked protein or RNA synthesis, indicating that FLIP has a high turnover rate. Expression of FADD and caspase-8 remained unchanged upon treatment with CHX or ActD, thereby increasing the ratio of caspase-8 to FLIP. Thus, the intracellular concentration of FLIP relative to the other components of the DISC, especially caspase-8, may determine the susceptibility of cells to CD95 triggering; second, selective down-regulation of FLIP expression by FLIP antisense oligonucleotides sensitized for CD95-induced apoptosis. Furthermore, down-regulation of intracellular FLIP levels resulted in undetectable levels of DISC-bound FLIP, thereby enhanc-
Fig. 9. Sensitization to TRAIL-induced or TNF-α-induced, but not to staurosporine-induced, apoptosis by CHX or ActD. SHEP neuroblastoma cells were treated for the indicated periods with 10 ng/ml TRAIL monoclonal antibody (A), 30 ng/ml TNF-α (B), or 1 μM staurosporine (C) in the absence (∗) or presence (●) of 1 μg/ml CHX or 0.1 μg/ml ActD (●). Apoptosis was determined by FACs analysis of propidium iodide-stained DNA content as described above. Data are the means of triplicates; similar results were obtained in three separate experiments; bars, SD.

The recruitment of death receptor signaling by CHX or ActD strongly reduced the expression of RIP. RIP has been identified as a death domain-containing receptor, and SODD association with death domains of death receptors may inhibit the intrinsic self-aggregation properties of the death domain receptor complex, whereas the role of FLIP in apoptosis signaling is not exactly known (13). FLIP expression has been implicated to regulate sensitivity to CD95-mediated cell death in T cells upon TCR stimulation (40, 41) or to modulate TRAIL-induced apoptosis in melanoma cells (42, 43). Moreover, inhibition of death receptor signaling by FLIP has recently been implied as a novel mechanism for immune escape of tumors from T-cell immunity in vivo (25–27). In Burkitt’s lymphoma, the tumorigenic potential of EBV was reported to involve the development of resistance to CD95-induced apoptosis through antagonistic regulation of caspase-8 and FLIP expression (28). In addition, in vivo expression of FLIP has been shown to act as a tumor progression factor by promoting tumor establishment and growth through prevention of death receptor-induced apoptosis mediated by T cells (26, 27). FLIP was also found to be involved in the development of resistance in vivo, because resistant tumor cells were selected for elevated FLIP expression (26). Up-regulation of FLIP expression may be involved in the pathogenesis of tumors such as melanomas, because FLIP was found to be overexpressed in malignant melanoma, whereas no FLIP expression was detected in normal melanocytes (11). Thus, FLIP may play an important role in tumorigenesis and in modulation of sensitivity or resistance toward therapy.

Interestingly, in addition to FLIP, treatment with CHX or ActD strongly reduced the expression of RIP. RIP has been identified as a death domain kinase in TNF-RI signaling mediating activation of nuclear factor-κB, which in turn may exert an antiapoptotic function (44). Recently, RIP has been reported to be cleaved in TNF-induced apoptosis, promoting the interaction between TRADD and FADD and increasing the sensitivity to death receptor triggering (45). Reduction of RIP expression by CHX or ActD may similarly enhance the recruitment of FADD to CD95 to facilitate DISC formation upon CD95 stimulation. In line with this, increased FADD recruitment to the CD95 DISC was found in CHX-treated cells. However, we could not detect any CD95-RIP interaction (data not shown), consistent with a previous report (37) suggesting that RIP may indirectly modulate the recruitment of FADD to CD95. Treatment with CHX or ActD also decreased expression of XIAP, which has been shown to suppress the effector phase of apoptosis by directly interacting with and inhibiting caspase-3, caspase-7, and caspase-9, respectively (23, 46). Moreover, other yet undefined short-lived repressors, e.g., SODD-related proteins, may regulate the interaction between the death domain of CD95 and FADD. SODD has been identified recently as a negative regulatory protein that is associated with the death domain of TNF-RI, inhibiting the intrinsic self-aggregation properties of the death domain (47). SODD association with death domains of death receptors may represent a general mechanism for preventing spontaneous signaling by death domain-containing receptors, and SODD-related proteins may play a similar role in preventing spontaneous signaling by CD95. Thus, CHX or ActD may regulate formation of the DISC at two different levels, at the interaction between CD95 and FADD, e.g., through down-regulation of RIP or SODD-like proteins, and at the

A. TRAIL

B. TNF-α

C. staurosporine

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\[3 \text{ S. Fulda and K-M. Debatin, unpublished results.}\]
interaction between FADD and caspase-8 through down-regulation of FLIP.

The synergistic effect of CHX or ActD in CD95-induced apoptosis has been explained as a consequence of inhibition of protein or mRNA synthesis (24, 29, 30). Consistent with this concept, we found a clear correlation between CHX or ActD concentrations necessary to facilitate apoptosis induction and those required for inhibition of protein or RNA synthesis. In addition, FLIP and RIP levels rapidly decreased upon addition of CHX or ActD, and pretreatment of cells with CHX or ActD enhanced CD95-induced apoptosis, supporting that inhibition of short-lived repressors may account for the synergistic effect of CHX or ActD. Alternatively, CHX or ActD have also been reported to modulate apoptosis by activating intracellular signaling pathways (30). Some cell types such as G0/G2 or CADO-Ewing’s sarcoma cells, however, remained completely resistant to anti-CD95-triggered apoptosis, even in the presence of CHX or ActD. In these cells, only minimal constitutive expression of caspase-8 was detected (data not shown), suggesting that in addition to labile repressors, defects in apoptosis pathways, such as an absence of apoptosis signaling molecules, may account for a resistant phenotype in some cells.

Thus, by demonstrating that metabolic inhibitors such as CHX or ActD can sensitize tumor cells to apoptosis after death receptor ligation through down-regulation of FLIP expression, our findings provide new insights into mechanisms that regulate the susceptibility to death receptor signals. Blockade of the CD95 pathway by overexpression of FLIP was shown to offer significant protection against the in vivo tumor response through immune escape from T-cell immunity (25–28). In addition, overexpression of FLIP also promoted the development of resistance in vivo (26). In light of these recent data, our findings that metabolic inhibitors sensitize tumor cells to death receptor-induced apoptosis through reduction of FLIP expression may have important implications for cancer therapy. Current attempts to improve cancer survival depend on strategies to target tumor cell resistance and on the development of new treatment modalities, e.g., gene therapy using the herpes simplex thymidine kinase/ganciclovir system or immunotherapy. Because the potential success of these approaches largely depends on intact death receptor pathways in target cells (48), strategies to inhibit FLIP expression, e.g., by metabolic inhibitors, may prove to be a complementary tool to the treatment of cancer.

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SENSITIZATION FOR DEATH RECEPTOR-INDUCED APOPTOSIS


Metabolic Inhibitors Sensitize for CD95 (APO-1/Fas)-induced Apoptosis by Down-Regulating Fas-associated Death Domain-like Interleukin 1-Converting Enzyme Inhibitory Protein Expression

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