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Common Regulation of Apoptosis Signaling Induced by CD95 and the DNA-damaging Stimuli Etoposide and γ-Radiation Downstream from Caspase-8 Activation*

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The death receptor CD95 (APO-1/Fas), the anticancer drug etoposide, and γ-radiation induce apoptosis in the human T cell line Jurkat. Variant clones selected for resistance to CD95-induced apoptosis proved cross-resistant to etoposide- and radiation-induced apoptosis, suggesting that the apoptosis pathways induced by these distinct stimuli have critical component(s) in common. The pathways do not converge at the level of CD95 ligation or caspase-8 signaling. Whereas caspase-8 function was required for CD95-mediated cytochrome *c* release, effector caspase activation, and apoptosis, these responses were unaffected in etoposide-treated and irradiated cells when caspase-8 was inhibited by FLIP_L. Both effector caspase processing and cytochrome *c* release were inhibited in the resistant variant cells as well as in Bcl-2 transfectants, suggesting that, in Jurkat cells, the apoptosis signaling pathways activated by CD95, etoposide, and γ-radiation are under common mitochondrial control. All three stimuli induced ceramide production in wild-type cells, but not in resistant variant cells. Exogenous ceramide bypassed apoptosis resistance in the variant cells, but not in Bcl-2-transfected cells, suggesting that apoptosis signaling induced by CD95, etoposide, and γ-radiation is subject to common regulation at a level different from that targeted by Bcl-2.

Cells can undergo apoptosis in response to a broad spectrum of stimuli, including receptor stimulation, treatment with cytotoxic drugs, and γ-radiation. Although a given stimulus may activate unique signaling molecules, the current model states that the molecular events in the execution phase of apoptotic cell death are shared. Members of the caspase family, which are aspartate-specific cysteine proteases, are key initiators of this execution phase (1, 2). Their proteolytic action on specific cellular components, including other caspases, structural proteins, and enzymes, leads to the ordered degradation of the cell into apoptotic bodies.

Certain members of the tumor necrosis factor receptor family, including CD95, directly couple to the caspase cascade via their cytoplasmic death domain. Upon multimerization by its trimeric ligand, CD95 recruits the FADD adaptor via its death domain (3). Via its death effector domain (DED),[1] FADD, in

turn, recruits caspase-8 (FLICE), which contains two related DEDs in its amino terminus (4, 5). Recently, several viral proteins, termed v-FLIP (FLICE inhibitory protein), were identified that contain two regions of homology to the DEDs of FADD and caspase-8 (6–8). Mammalian cells express FLIP homologues: FLIP_S, composed of two DEDs, and FLIP_L, which contains, in addition, a nonfunctional caspase domain (9–11). Both viral and cellular FLIP proteins bind to FADD and/or caspase-8 and inhibit death receptor-induced apoptosis, most likely by displacing DED-containing caspases (caspase-8 and/or -10) from the activated death receptor complex (8–10).

Whereas it is to some extent understood how death receptors link to the caspase family, this is less clear for other apoptotic stimuli. In certain cases, the stimulus indirectly activates death receptors. An example is the T cell antigen receptor, which induces synthesis of CD95 ligand and therewith activates CD95 in, for example, Jurkat T cells (12). A similar mechanism has been proposed for apoptosis induction by anticancer drugs (13, 14). Apart from the death-inducing signaling complex (DISC) at the death receptor cytoplasmic tail (4, 15, 16), the mitochondrial membrane is now thought to be a site for initial caspase activation. In response to various apoptotic stimuli, mitochondria release cytochrome *c* (17–19), which, together with Apaf-1 (20) and caspase-9 (21), can activate caspase-3 *in vitro*. The Apaf-1·caspase complex is thought to be located at the mitochondrial membrane since the homologous *Caenorhabditis elegans* proteins CED-4 and CED-3 form a complex together with CED-9, a homologue of mammalian Bcl-2 (22, 23) that resides at this site (24). Mitochondrial cytochrome *c* release can be blocked by the apoptosis inhibitory proteins Bcl-2 and Bcl-xL by an unknown mechanism (17, 18).

Many apoptosis pathways, including those induced by etoposide and γ-radiation, are controlled by the Bcl-2 family, suggesting that, in these cases, mitochondrial participation is essential for the cell death response (25, 26). Etoposide inhibits topoisomerase II and therewith induces double strand DNA breaks (27), whereas γ-radiation also induces DNA damage. Both stimuli activate caspases (28–30), but it is unknown how the signal is transmitted to these enzymes. In certain cell types, DNA damage induces p53-directed *de novo* synthesis of the Bcl-2 antagonist Bax (31), indicating a mechanism for regulation of the mitochondrial caspase pathway. It has also been shown that anticancer drugs can induce expression of CD95 and/or its ligand and therewith activate the CD95 pathway (13, 14). However, this is not a general mechanism since etoposide-induced apoptosis is CD95-independent in murine

[1] The abbreviations used are: DED, death effector domain; DISC,

death-inducing signaling complex; mAb, monoclonal antibody; FLIP, FLICE inhibitory protein; PIPES, 1,4-piperazinediethanesulfonic acid; IR, γ-radiation; Gy, gray.

thymocytes and Jurkat cells (32).

Several studies suggest that the lipid ceramide is relevant for apoptosis signaling in response to death receptors, γ-radiation, and anticancer drugs. Tumor necrosis factor receptor-1 (33); CD95 (34); γ-radiation (35); and daunorubicin, vincristine, and cytosine arabinoside (36–39) can induce accumulation of ceramide. Moreover, failure to generate ceramide has been associated with apoptosis resistance (34, 37, 40, 41). Exogenous short-chain ceramide can induce apoptosis in certain cell types, but it is not clear whether it is equivalent in its action to naturally generated ceramide. The ceramide response induced by CD95 lies upstream from DEVD-inhibitable caspases and nuclear segmentation (42), allowing for the possibility that ceramide contributes to generation of the apoptotic phenotype.

Jurkat cells selected for resistance to CD95-induced apoptosis were found to be cross-resistant to etoposide and γ-radiation. Therefore, we have examined possible convergence of the apoptosis signaling pathways induced by these three stimuli. We find that the pathways share features downstream from DED-containing caspases and are subject to common mitochondrial control. In addition, comparing responses in Bcl-2 transfectants and the variant clones, we find evidence for a Bcl-2-independent mechanism that commonly regulates apoptosis signaling induced by CD95 and the DNA-damaging regimens.

EXPERIMENTAL PROCEDURES

Reagents—L-[3-^{14}C]Serine (54.0 mCi/mmol) and the enhanced chemiluminescence (ECL) kit were purchased from Amersham Pharmacia Biotech; etoposide was from Sigma; and C$_2$-ceramide was from BIOMOL Research Labs Inc. The anti-CD95 monoclonal antibodies (mAbs) CH-11 and 7C11 were purchased from Immunotech (Marseille, France). Anti-caspase-8 serum was raised in rabbits against a synthetic peptide comprising amino acids 2–20 of human caspase-8. Specificity of the antiserum was confirmed as described previously (42, 43). Mouse anti-human caspase-3 mAb was purchased from Transduction Laboratories (Lexington, KY); mouse anti-human caspase-6 (B93-4) and caspase-7 (B94-1) mAbs and anti-cytochrome *c* mAb 7H8.2C12 were from Pharmingen; and anti-actin mAb C4 was from Roche Molecular Biochemicals. Horseradish peroxidase-conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig were obtained from Dako A/S (Glostrup, Denmark).

Cells—The J16 wild-type clone was derived from the human T-acute lymphoblastic leukemia cell line Jurkat by limiting dilution and selected for CD95 sensitivity (32, 42). CD95-resistant JA variant clones were derived by limiting dilution from the Jurkat line, cultured for 5 weeks in the presence of 1 μg/ml anti-APO-1 mAb (44). Clonogenic assays indicated that ~2 in 10^4 cells of the parental line survive this treatment. JA clones were subcloned in medium without selecting stimulus and have remained resistant throughout prolonged culture periods. The expression level of CD95 is within the wild-type range, and its cytoplasmic tail is wild-type according to nucleotide sequencing.[2] Jurkat cells stably transfected with the *bcl-2* cDNA and empty vector-transfected control cells were previously described (45). The Jurkat cell line JFL2, which is stably transfected with *FLIP*$_L$ cDNA (9), was kindly provided by Dr. J. Tschopp (Institute of Biochemistry, University of Lausanne, Epalinges, Switzerland).

Cell Culture and Stimulation—Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics at 37 °C and 5% CO$_2$. *bcl-2* cDNA-transfected and empty vector-transfected Jurkat cells received additional neomycin sulfate (G418) at 200 μg/ml. Prior to stimulation, cells were incubated overnight in serum-free Yssel's medium (46) and seeded in the same medium at 1 × 10^6/ml (200 μl/well) in round-bottom 96-well plates for apoptosis assays and at 5–10 × 10^6/ml in 24-well culture plates for caspase processing and cytochrome *c* release assays. Cells were irradiated using a ^{137}Cs source (2 × 415 Ci; Von Gahlen Nederland, B. V.) with the indicated doses, stimulated with anti-CD95 mAb or etoposide at the indicated concentrations, and incubated at 37 °C and 5% CO$_2$ for various time periods.

Apoptosis Assay—For apoptosis measurements, cells were lysed in 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide (47). Fluorescence intensity of propidium iodide-stained DNA was de-

termined on a FACScan (Becton Dickinson, San Jose, CA), and data were analyzed using Lysys software. Fragmented apoptotic nuclei are recognized by their subdiploid DNA content.

Immunoblot Analysis for Caspase Processing—Cells were lysed in 1% Nonidet P-40 containing lysis buffer (0.01 M triethanolamine HCl (pH 7.8), 0.15 M NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.02 mg/ml trypsin inhibitor, and 0.02 mg/ml leupeptin). Lysates were centrifuged at 13,000 × *g* for 15 min, and supernatants were mixed with concentrated reducing SDS sample buffer. Equivalents of 10^6 cells/lane were separated on 10% SDS-polyacrylamide minigels. Proteins were transferred to nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany), which were subsequently blocked with 5% nonfat dry milk in Tris-buffered saline and 0.05% Tween 20 (TBST). Blots were incubated with anti-caspase-8 serum or anti-caspase-6 mAb at a 1:500 dilution or with anti-caspase-3 or anti-caspase-7 mAb at a 1:1000 dilution in TBST with 1% nonfat dry milk. After subsequent incubation with a 1:7500 dilution of horseradish peroxidase-conjugated swine anti-rabbit Ig, proteins were visualized by ECL.

Immunoblot Analysis for Cytochrome c—After incubation, cells were washed twice with ice-cold phosphate-buffered saline, resuspended in 100 μl of extraction buffer (50 mM PIPES-KOH (pH 7.4), 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM MgCl$_2$, 1 mM dithiothreitol, and protease inhibitors), and allowed to swell on ice for 30 min (19). Cells were homogenized by passing the suspension through a 25-gauge needle (10 strokes). Homogenates were centrifuged in a Beckman Airfuge at 100,000 × *g* for 15 min at 4 °C, and supernatants were harvested and stored at −70 °C until analysis by gel electrophoresis. Ten μg of cytosolic protein, as determined by the Bio-Rad protein assay, were loaded onto a 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose sheets, which were blocked as described above and probed in TBST with anti-cytochrome *c* mAb (1:2000) and anti-actin mAb (1:3000) to confirm equal loading. After incubation with a 1:7500 dilution of horseradish peroxidase-conjugated rabbit anti-mouse Ig, blots were developed by ECL.

Ceramide Quantification—Ceramide levels were determined as described previously (42, 43). Briefly, cells (1 × 10^6/ml) were labeled with L-[3-^{14}C]serine (0.2 μCi/ml) in Yssel's medium for 20–48 h, washed with Yssel's medium, and resuspended at 5 × 10^6-ml in 24-well plates. Following stimulation, total lipids were extracted with chloroform/methanol (1:2, v/v), and phases were separated using 20 mM HAc. Extracts were spotted on Silica Gel 60 thin-layer chromatography plates (Merck). After chromatography, radioactive lipids were visualized and quantitated using a Fujix BAS 2000 TR PhosphorImager and identified using external lipid standards. Ceramide was expressed relative to total radioactivity in phosphatidylserine and phosphatidylethanolamine, which remained unaltered upon stimulation.

RESULTS

A Common Aspect in Apoptosis Signaling Induced by CD95, Etoposide, and γ-Radiation—The human T-acute lymphoblastic leukemia cell line Jurkat is sensitive to multiple apoptotic stimuli, including CD95 triggering, exposure to certain anticancer drugs, and γ-radiation (IR). We have selected variant clones from the wild-type Jurkat line for resistance to CD95-mediated apoptosis. These clones proved cross-resistant to apoptosis induction by etoposide and IR, as shown in Fig. 1*A*. Whereas the wild-type clone J16 undergoes dose- and time-dependent apoptosis in response to anti-CD95 mAb, etoposide, or IR, apoptosis incidence in the variant clone JA1.2 does not exceed background levels, even at high dose stimulation. Yet, the resistant JA1.2 variant cells sense the DNA damage as induced by etoposide and γ-radiation since they arrest in the G$_2$ phase of the cell cycle upon treatment, as wild-type Jurkat cells do. However, in wild-type cells, the G$_2$ arrest is followed rapidly by apoptotic cell death, whereas this does not occur in JA1.2 cells (Fig. 1*B*).

Apoptosis Signaling Induced by Etoposide and γ-Radiation Does Not Require DED-containing Caspases—The cross-resistance suggested that CD95 and the DNA-damaging stimuli etoposide and γ-radiation require common molecular events to induce apoptosis. To find a possible point of convergence in apoptosis signaling induced by these three inputs, we first tested whether the anticancer treatments induced caspase-8

[2] J. G. R. Boesen-de Cock and U. Oettinger, unpublished data.
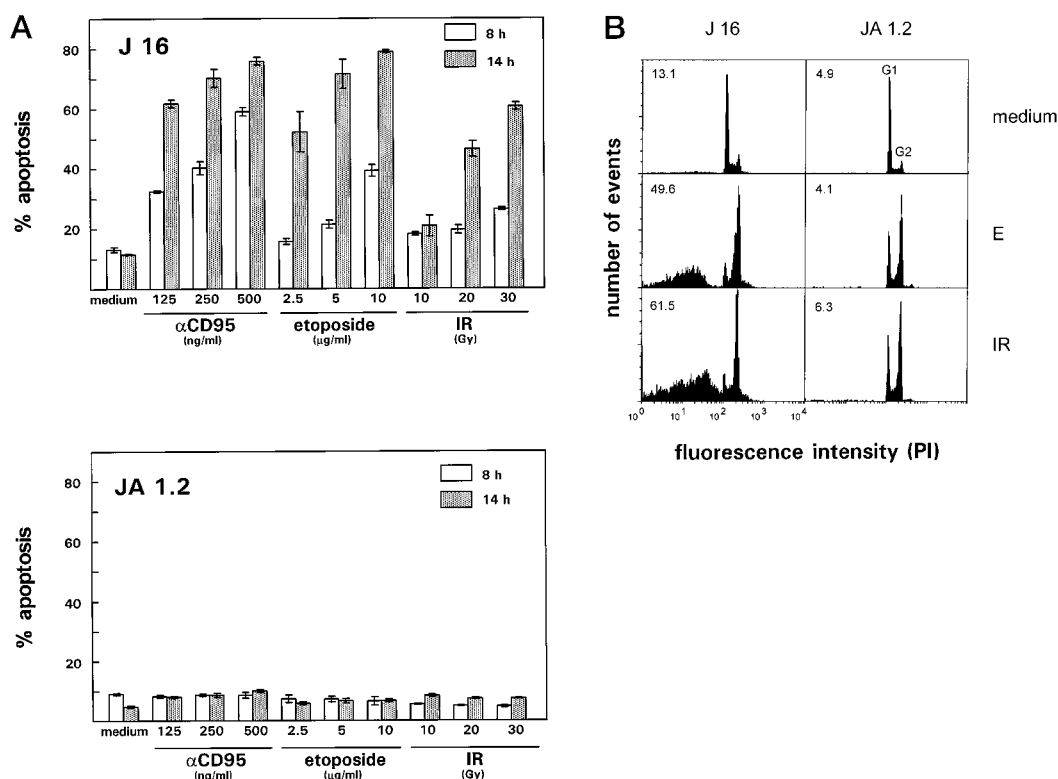
FIG. 1. **CD95-resistant clone JA1.2 senses DNA damage, but fails to undergo apoptosis in response to etoposide or IR.** *A*, apoptosis incidence in the wild-type Jurkat clone J16 and the representative variant clone JA1.2 as read out by nuclear fragmentation (47) at 8 or 14 h after addition of anti-CD95 mAb CH-11 or etoposide or after exposure to IR at the indicated doses. Data are representative of multiple independent experiments. Means ± S.D. from duplicate samples in one experiment are shown. *B*, DNA profiles of nuclei from J16 and JA1.2 cells analyzed 16 h after addition of etoposide (*E*; 2.5 μg/ml) or after IR (10 Gy). Fluorescence intensity of propidium iodide (*PI*)-stained nuclei (47) indicates DNA content and allowed identification of nuclei in the G_1 and G_2 phases of the cell cycle as well as subdiploid fragmented nuclei derived from apoptotic cells. Percentage apoptosis is indicated for each sample.

processing, as does CD95 (15). Immunoblotting of whole cell lysates with an antiserum directed against the amino terminus of caspase-8 allowed detection of two proforms, of ~54 and 50 kDa, prior to stimulation. CD95 stimulation gave rise to a caspase-8 doublet at ~40 and 36 kDa within ~1 h, which increased in intensity in the following hours, whereas the pro-caspase-8 signal decreased concomitantly (Fig. 2). This is consistent with the release of the carboxyl-terminal caspase fragment (15). In addition, a 23-kDa species was detected, which most likely represents the amino-terminal region of caspase-8 after release of the second caspase subunit. Etoposide and IR also induced caspase-8 processing, but to a minor extent. In these cases, only the 40/36-kDa digestion products could be detected, even upon long-term stimulation (Fig. 2). All three stimuli failed to induce caspase-8 processing in the resistant clone JA1.2 (Fig. 2).

To determine whether caspase-8 is instrumental in apoptosis induction by etoposide and IR, we employed Jurkat cells stably transfected with *FLIP*$_L$ cDNA. It was previously demonstrated that apoptosis induction by CD95 and TRAIL receptors is inhibited in these transfectants (9). Fig. 2 shows that in these JFL2 cells, the dramatic caspase-8 processing induced by CD95 was blocked. Also, the minor caspase-8 processing induced by etoposide and IR was no longer observed. Whereas FLIP$_L$ inhibited CD95-induced apoptosis, it did not affect the apoptotic response to etoposide and IR (Fig. 3). Apparently, activation of FLIP$_L$-inhibitable, DED-containing caspases is required for apoptosis induction by CD95, but not for apoptosis induction by etoposide or IR.

Apoptosis Signaling Pathways Induced by CD95, Etoposide, and γ-Radiation Are under Common Mitochondrial Control— Since apoptosis signaling induced by CD95, etoposide, and
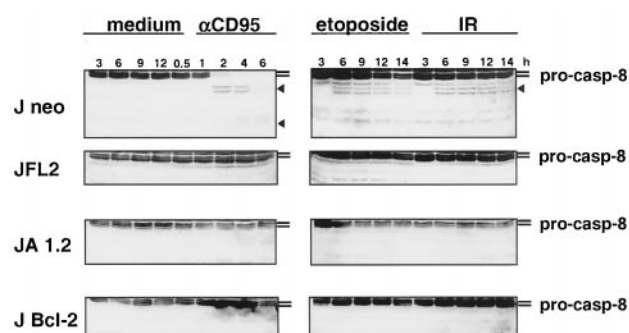
FIG. 2. **Regulation of caspase-8 processing in response to CD95, etoposide, and IR.** Cells were stimulated with anti-CD95 mAb 7C11 at 200 ng/ml, etoposide at 10 μg/ml, or IR at 30 Gy and harvested after the indicated periods of incubation. *J neo* is the control Jurkat clone. JA1.2 is the representative resistant variant clone. JFL2 denotes Jurkat cells transfected with *FLIP*$_L$ cDNA as described (9). *J Bcl-2* indicates Jurkat cells transfected with *Bcl-2* cDNA (45). Total cell lysates were separated by SDS-polyacrylamide gel electrophoresis, and caspase-8 processing was monitored by immunoblotting with an antiserum directed at amino acids 2–20 of human procaspase-8 (*pro-casp-8*) (43). The procaspase-8 forms at 54 and 50 kDa are indicated by *bars*. The caspase-8 digestion products at ~40/36 and 23 kDa are indicated by *arrowheads*.

γ-radiation did not converge at the level of DED-containing caspases, we investigated whether the presumed mitochondrial caspase activation complex might be involved in these three pathways. To directly examine mitochondrial involvement, release of cytochrome *c* into the cytoplasm was determined. Fig. 4 shows that CD95, etoposide, and IR induced release of cytochrome *c* in control Jurkat cells. CD95-induced cytochrome *c* release was inhibited by FLIP$_L$ overexpression, indicating that
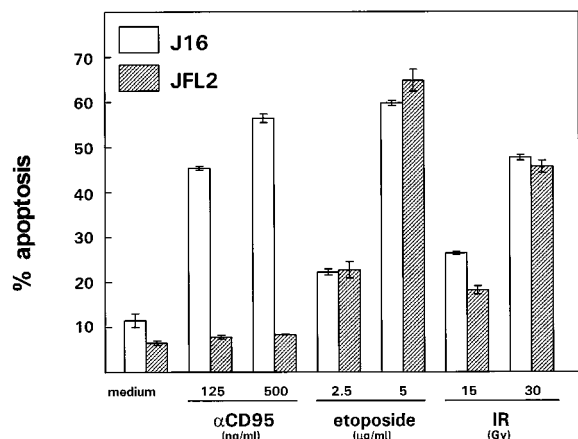
FIG. 3. **FLIP$_L$ inhibits apoptosis induction by CD95, but not by etoposide and IR.** Control Jurkat cells (J16) or Jurkat cells transfected with the human *FLIP$_L$* cDNA (JFL2) were stimulated with anti-CD95 mAb CH-11, treated with etoposide (*E*), or irradiated (*IR*), and apoptosis was read out as nuclear fragmentation (47) 6 h after CD95 stimulation and 14 h after addition of etoposide or after irradiation. The medium controls were measured after 14 h of incubation. Data are representative of three independent experiments and show means ± S.D. from duplicate samples in one experiment.
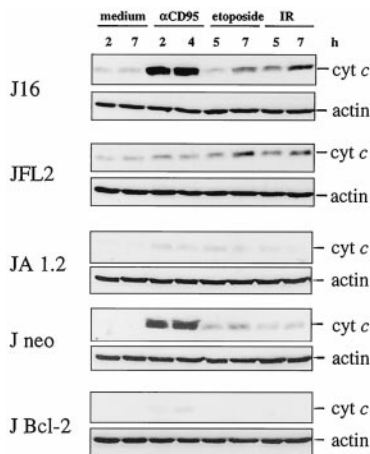

FIG. 4. **CD95, etoposide, and IR induce cytochrome *c* release into the cytoplasm.** Control J16 cells, FLIP$_L$-overexpressing JFL2 cells, Jurkat cells transfected with empty vector (*J neo*) or *bcl-2* cDNA (*J Bcl-2*), and JA1.2 cells were stimulated with anti-CD95 mAb 7C11 at 200 ng/ml, etoposide at 10 μg/ml, or IR at 30 Gy for the indicated time periods. Cells were lysed, and mitochondrion-free cytosol was prepared as described (19). Equal amounts of protein were loaded after quantification, and immunoblotting was performed for both cytochrome *c* (*cyt c*) and actin as an additional loading control. The experiment is representative of three.

CD95 communicates to the mitochondria via caspase-8. FLIP$_L$ did not inhibit cytochrome *c* release in response to etoposide or IR, substantiating the lack of caspase-8 involvement in the DNA damage pathways (Fig. 4).

Cytochrome *c* release in response to all three stimuli was severely reduced in the resistant JA1.2 clone (Fig. 4), suggesting that the apoptosis signaling pathways induced by CD95, etoposide, and IR all depend on a contribution by the mitochondria. Generally, a possible contribution of the mitochondrial pathway to apoptosis induction is assessed by the effects of Bcl-2 family members on the apoptotic response. Whereas apoptosis induction by DNA-damaging regimens such as etoposide treatment and IR is consistently modulated by Bcl-2 family members (25, 26), it has been unclear whether death receptor signaling is similarly regulated. For instance, in transgenic thymocytes, CD95-mediated apoptosis is not affected by Bcl-2
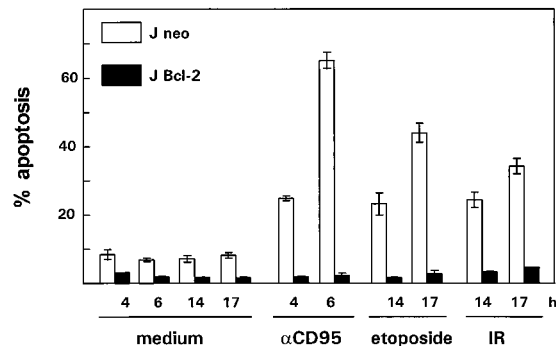

FIG. 5. **In Jurkat cells, Bcl-2 inhibits apoptosis induction by CD95, etoposide, and IR.** Jurkat cells transfected with empty vector (*J neo*) or *Bcl-2* cDNA (*J Bcl-2*) were exposed to anti-CD95 mAb CH-11 (250 ng/ml), etoposide (3 μg/ml), or IR (20 Gy). Apoptosis was read out as nuclear fragmentation (47) at the indicated times after stimulation. Data are representative of two independent experiments and show means ± S.D. from duplicate samples in one experiment.

or Bax overexpression (26, 48), whereas in certain cell lines, Bcl-2 and Bcl-xL were found to inhibit tumor necrosis factor receptor-1- and/or CD95-induced apoptosis (45, 49, 50). Recently, it was suggested that in certain cells (type I), CD95 employs a Bcl-2-independent pathway, whereas in other cells (type II), including Jurkat, Bcl-2 and Bcl-xL inhibit CD95 signaling (51). Using the previously described Bcl-2-overexpressing Jurkat cells (45, 50), we also found that not only etoposide- and IR-induced cytochrome *c* release and apoptosis were inhibited, but also CD95-induced cytochrome *c* release (Fig. 4) and apoptosis (Fig. 5). The combined data suggest a point of convergence in apoptosis signaling induced by CD95, etoposide, and IR at the mitochondria.

Surprisingly, caspase-8 processing induced by CD95 was inhibited by Bcl-2 overexpression (Fig. 2). This has also been observed by Scaffidi *et al.* (50), who suggested that CD95 signaling in Jurkat cells involves rapid, low level caspase-8 activation at the DISC, followed by a slow, more pronounced response, which is under mitochondrial control. Rapid caspase-8 activation within minutes could not be observed in total lysates of Jurkat cells (data not shown) (50). Since FLIP$_L$ inhibits caspase-8 activation at the DISC (9), we cannot assess whether the late, Bcl-2-controlled caspase-8 response contributes to apoptosis induction by CD95. Bcl-2 also inhibited caspase-8 processing as induced by etoposide and IR (Fig. 2).

Regulation of Effector Caspase Processing in Response to CD95, Etoposide, and γ-Radiation—To further delineate the convergence in apoptosis signaling, we set out to identify effector caspases that were under the control of CD95, etoposide, and IR. CD95 effectively induced caspase-3, -6, and -7 processing in control Jurkat cells (Fig. 6*A*). In response to etoposide treatment and IR, caspase-3 and caspase-6 were cleaved to a minor degree, whereas caspase-7 processing was readily detectable (Fig. 6). FLIP$_L$ inhibited CD95-induced caspase-3, -6, and -7 processing (Fig. 6*B*), indicating that a DED-containing caspase (most likely caspase-8) is upstream from these effector caspases in the CD95 pathway. In contrast, effector caspase cleavage in response to etoposide and IR occurred independent of caspase-8 activation (Fig. 6*B*). CD95-, etoposide-, and IR-induced effector caspase processing did not occur in the resistant JA1.2 clone or in Bcl-2-overexpressing Jurkat cells (Fig. 6*B*). We conclude that CD95 and the DNA-damaging regimens can signal to the same effector caspases, but with different efficiencies. CD95 signals to effector caspases via caspase-8, whereas etoposide and IR use a caspase-8-independent route. Downstream from caspase-8, CD95-, etoposide-, and IR-in-
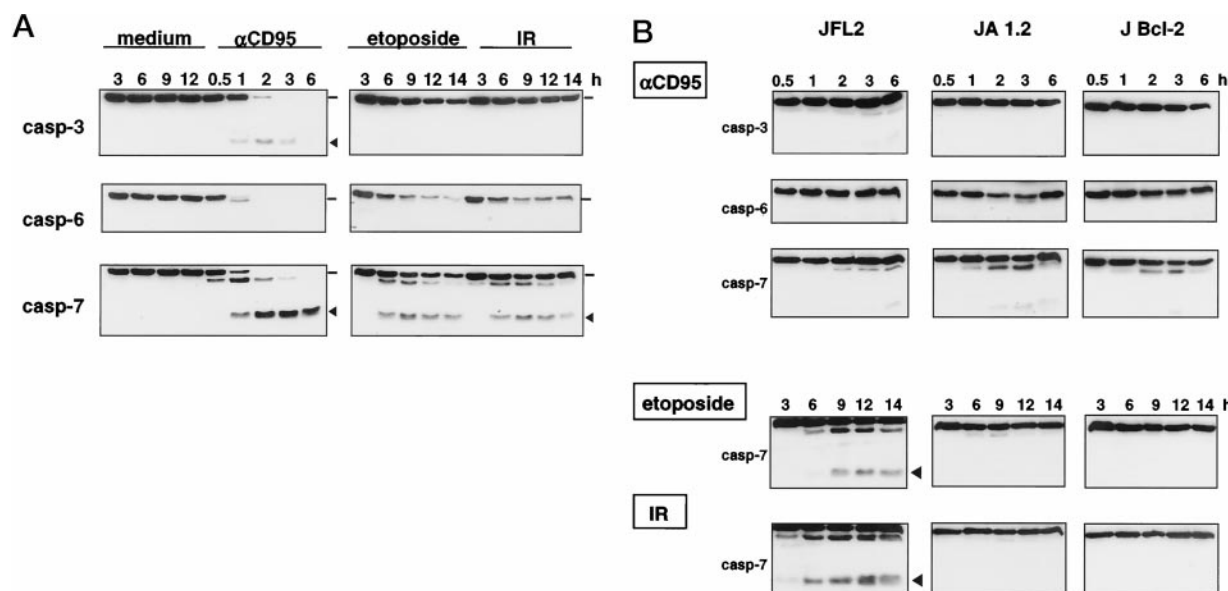
FIG. 6. **Regulation of effector caspase processing in response to CD95, etoposide, and IR in Jurkat cells.** Control Jurkat cells (*A*) and JFL2, JA1.2, and *Bcl-2* cDNA-transfected Jurkat cells (*B*) were stimulated with anti-CD95 mAb 7C11 at 200 ng/ml, etoposide at 10 μg/ml, or IR at 30 Gy and harvested after the indicated periods of incubation. Total cell lysates were separated by SDS-polyacrylamide gel electrophoresis, and caspase (*casp*)-3, -6, and -7 processing was monitored by immunoblotting with specific antibodies. The procaspase forms are indicated by *dashes*, and the degradation products are shown by *arrowheads*.

duced effector caspase activation is subject to common mitochondrial regulation.

Ceramide and Apoptosis Signaling Induced by CD95, Etoposide, and γ-Radiation—Reported common signaling events induced by CD95, certain anticancer drugs, and IR include generation of ceramide, which has been implicated in apoptosis induction. In Jurkat cells, CD95 ligation gives rise to a slow and sustained elevation of ceramide levels upstream from DEVD-inhibitable caspases (42). Fig. 7*A* shows that CD95, as well as etoposide and IR, induced significant ceramide accumulation in wild-type Jurkat cells, but not in the apoptosis-resistant JA1.2 clone.

Exogenous C_2-ceramide induced effector caspase processing (Fig. 7*B*) and apoptosis (Fig. 7*C*) in wild-type Jurkat cells as well as in the JA1.2 clone. More important, whereas C_2-ceramide bypassed apoptosis resistance in JA1.2, the Bcl-2 transfectant was resistant to C_2-ceramide (Fig. 7, *B* and *C*), indicating that apoptosis resistance of JA1.2 is not due to overexpression of Bcl-2 (-related) proteins.

DISCUSSION

Our finding that cells selected for resistance to CD95-induced apoptosis were cross-resistant to apoptosis induction by etoposide and IR suggested that apoptosis signaling induced by these three stimuli has a common aspect. Since CD95 signaling involves FADD-mediated recruitment of caspase-8 and its subsequent proteolytic activation, we examined whether etoposide and IR also induced caspase-8 processing. Caspase-8 was cleaved to a minor extent in etoposide-treated and irradiated cells, but this proved to be dispensable for effector caspase processing and the cell death response. This is an important finding since it was suggested previously that anticancer drugs (13, 14) and γ-radiation (51) can induce apoptosis by activating the CD95 receptor system. Anticancer drugs would do so by inducing synthesis of CD95 ligand (13, 14) and, in certain cases, also the receptor (14). CD95 was implicated in apoptosis induction by γ-radiation based on diminished radiation sensitivity of CD95-deficient splenocytes from *lpr* mice (51).

Such a mechanism would obviously account for the observed cross-resistance. However, involvement of the CD95 receptor-

ligand system in anticancer drug- and γ-radiation-induced apoptosis has been contradicted by several reports (32, 52–54). For instance, in Jurkat cells, we could not detect CD95 ligand synthesis in response to etoposide or doxorubicin, and drug-induced apoptosis could not be inhibited by blocking interaction of CD95 with its ligand (32). In addition, CD95-deficient *lpr* thymocytes are sensitive to etoposide-induced (32) and γ-radiation-induced (52) apoptosis to the same degree as wild-type cells. Based on our experiments using FLIP$_L$-overexpressing cells, we can exclude a role for caspase-8 and therefore for CD95 in apoptosis signaling induced by etoposide and γ-radiation in Jurkat cells, in contrast to an earlier suggestion (55). Moreover, since FLIP$_L$ also inhibits TRAIL receptor-induced apoptosis (9), these death receptors can also be excluded from playing a role in the DNA damage pathways in the cell type examined here.

Caspase-8 cleavage induced by etoposide and γ-radiation was inhibited by Bcl-2 overexpression, suggesting a dependence on mitochondrial components such as cytochrome *c*. Most likely, caspase-8 is processed as a result of the activation of other caspases that are instrumental in etoposide- and radiation-induced apoptosis. Caspase-9 would be a good candidate to initiate the apoptosis signaling pathway induced by etoposide and γ-radiation since it can take part in the Apaf-1 complex (21). However, available antibodies against caspase-9 did not allow detection of this enzyme in Jurkat cells (data not shown).

Our study suggests that, in Jurkat cells, apoptosis signaling induced by etoposide and γ-radiation converges with a CD95-induced pathway downstream from caspase-8, at or upstream from the mitochondrion-dependent caspase complex. It has been debated whether the CD95 pathway can be regulated by the mitochondria as assessed by effects of Bcl-2, Bcl-xL, or Bax overexpression in transgenic or transfected cells (25, 26, 48–50). The discrepant results obtained in these systems were recently reconciled by the suggestion that, in some cell types (*e.g.* SKW6.4 cells), CD95 effectively activates caspase-8 at the DISC and induces activity of effector caspases independent of a mitochondrial contribution. In other cell types (*e.g.* Jurkat cells), caspase-8 activation at the DISC is inefficient, and a
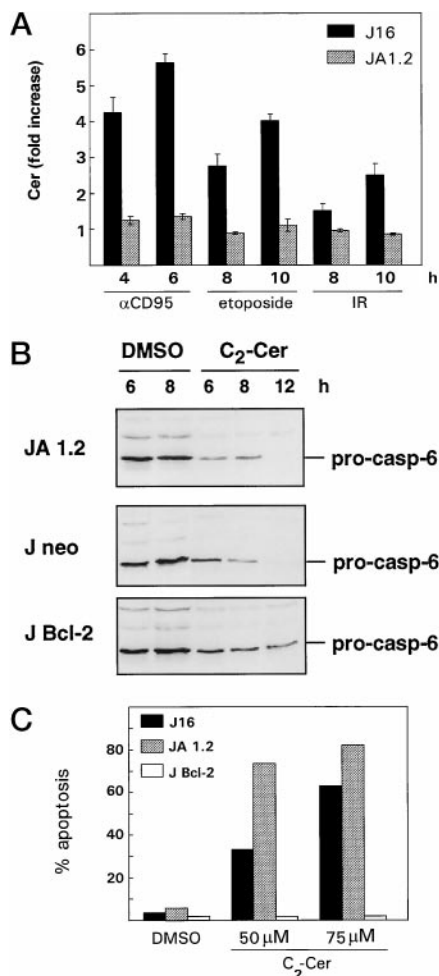
FIG. 7. **Ceramide and apoptosis signaling induced by CD95, etoposide, and IR.** *A*, J16 wild-type cells and the JA1.2 clone were treated with anti-CD95 mAb CH-11 at 500 ng/ml, etoposide at 5 μg/ml, or IR at 15 Gy, and ceramide (*Cer*) levels were determined at the indicated time points and are expressed as -fold increase relative to time-matched controls. Means ± S.D. from duplicate samples in one experiment are shown. *B*, empty vector-transfected (*J neo*) and *bcl-2* cDNA-transfected (*J Bcl-2*) Jurkat cells and JA1.2 were treated with vehicle (dimethyl sulfoxide (*DMSO*)) or 75 μM C_2-ceramide (*C_2-Cer*) for the indicated time periods. Proteolytic processing of caspase-6 was determined by immunoblotting of total cell lysates separated by SDS-polyacrylamide gel electrophoresis. Procaspase-6 (*pro-casp-6*) at 35 kDa is indicated. *C*, cells as indicated were treated with vehicle or C_2-ceramide for 24 h. Apoptosis was read out as nuclear fragmentation (47). Means ± S.D. from duplicate measurements in one experiment are shown.

contribution of the mitochondrial pathway is required for effective induction of apoptosis (50). Our findings in Jurkat cells are consistent with those of Scaffidi *et al.* (50): detectable CD95-induced caspase-8 activation is slow and inhibited by Bcl-2 overexpression. However, we find that CD95-induced cytochrome *c* release is inhibited by FLIP$_L$ overexpression, indicating that caspase-8 signals to the mitochondria. Most likely, undetectable amounts of activated caspase-8 generated in the DISC are sufficient to initiate the apoptosis pathway. Recently, caspase-8 was shown to induce cytochrome *c* release from the mitochondria via its substrate Bid, a Bcl-2-interacting protein (56, 57). Apparently, CD95 signaling can diverge at caspase-8, with one branch of the pathway leading directly to effector caspase activation and the other branch communicating with the mitochondria (50, 56, 57).

Concomitant with its failure to display cytochrome *c* release, effector caspase processing, and apoptosis in response to CD95,

etoposide, and IR, the JA1.2 clone (and other clones from the same resistant culture) fails to mount a ceramide response. Whether ceramide participates in the apoptosis signaling pathway is unknown, but this possibility is suggested by correlations between lack of a ceramide response and a failure to undergo apoptosis (34, 37, 40, 41) as well as by the finding that exogenous ceramide can activate caspases and induce apoptosis. We have recently found that ceramide production in response to CD95, etoposide, and IR occurs in the absence of effector caspase activation, but depends on inducer caspase activity.[3] The failure to mount a ceramide response in the resistant JA clones is therefore not a consequence of defective effector caspase activation, but rather reflects a defect at or downstream from inducer caspase activation.

Apoptosis resistance in the JA clones is most likely not due to overexpression of inhibitory Bcl-2 family members since the variant cells were sensitive to apoptosis induction by exogenous ceramide, whereas Bcl-2-overexpressing cells were resistant (Fig. 7). Moreover, immunoblotting for various Bcl-2 family members did not reveal altered expression in JA cells as compared with wild-type Jurkat cells (data not shown). It is also unlikely that the inhibitor of apoptosis proteins (IAPs) mediate the resistance, since these do not interfere with the release of cytochrome *c* (58). Therefore, it will be of great interest to define the cause of common apoptosis resistance in these variant clones.

Note Added in Proof—Another study recently reported that FLIP does not inhibit apoptosis induced by DNA damaging regimens in Jurkat cells (Kataoka, T., Schroter, M., Hahne, M., Irmler, M., Thome, M., Froehlich, C. J., and Tschopp, J. (1998) *J. Immunol.* **161,** 3936–3942).

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