DNA damage induced by the cancer chemotherapeutic drug etoposide triggers the onset of a series of intracellular events characteristic of apoptosis. Among the early changes observed is the release of cytochrome c from mitochondria, although the mechanism responsible for this effect is unclear. We demonstrate here a role for caspase-2 in etoposide-induced cytochrome c release. In particular, Jurkat T-lymphocytes treated with an irreversible caspase-2 inhibitor, benzyloxy carbonyl-Val-Asp-Val-Ala-Asp-fluoromethyl ketone (z-VDVAD-fmk), or stably transduced with pro-caspase-2 antisense (Casp-2/AS) are refractory to cytochrome c release stimulated by etoposide. Experiments performed using a reconstituted cell-free system indicate that etoposide-induced cytochrome c release by way of caspase-2 occurs independently of cytosolic factors, suggesting that the nuclear pool of pro-caspase-2 is critical to this process. Apart from inhibiting cytochrome c release, undermigrating caspase-2 activity results in an attenuation of downstream events, such as pro-caspase-9 and -3 activation, phosphatidylserine exposure on the plasma membrane, and DNA fragmentation. Taken together, our data indicate that caspase-2 provides an important link between etoposide-induced DNA damage and the engagement of the mitochondrial apoptotic pathway.

Caspases are cysteine-aspartate proteases that play critical roles during the initiation and execution of apoptosis. These enzymes share sequence similarity with the Caenorhabditis elegans cell death protease CED-3 and are synthesized as inactive precursor forms that must be proteolytically cleaved in order to be activated (1). Initiator pro-caspases 2, 8, 9, and 10 are activated with the help of adaptor molecules that bring these proteases into close proximity, permitting autoprocessing (2–4). With the exception of caspase-2, initiator caspases are responsible for cleaving and activating effector pro-caspases 3, 6, and 7. Effector caspases, in turn, cleave various proteins leading to morphological and biochemical features characteristic of apoptosis (5).

In recent years, it has become abundantly clear that caspase-9 activity is required for apoptosis induced by different stimuli (6). Specifically, damage to mitochondria results in the release of cytochrome c, which together with Apaf-1 and dATP lead to the recruitment and activation of pro-caspase-9 (7, 8). Among the cytotoxic stimuli that can initiate the mitochondrial pathway is the chemotherapeutic drug and topoisomerase II poison etoposide (9). Normally, topoisomerase II prevents intertwining of DNA by generating transient double-stranded breaks through which an intact helix can pass (10). Etoposide was designed to exploit selectively the catalytic property of topoisomerase II by increasing the number and duration of DNA cleavage sites, resulting ultimately in permanent double-stranded breaks that are lethal to the cell (11). For several years, it was argued that cytochrome c release induced by etoposide was a caspase-independent event and that caspase-9, in general, was the most apical caspase in chemical-induced apoptosis (9, 12, 13). However, we recently demonstrated that etoposide-induced cytochrome c release involves distinct dose- and caspase-dependent pathways (14). In particular, a low dose (10 μM) of etoposide appears to exert its effect at the nuclear level, resulting in the release of a heat-labile factor(s) that, in turn, interacts with mitochondria to elicit cytochrome c release. Because this effect was inhibited by the general caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), and caspase-2 was the earliest caspase activated in Jurkat T-lymphocytes, we speculated that the release of cytochrome c triggered by a low dose of etoposide may require active caspase-2.

Two mRNA alternative splicing variants encode two caspase-2 proteins, caspase-2L and caspase-2S, with different effects on apoptotic cell death (15, 16). The pro-apoptotic protein caspase-2L (referred to here as caspase-2) is the prevailing isoform expressed in most tissues (15). Subcellular fractionation studies have revealed that pro-caspase-2 is present in several intracellular compartments, including the mitochondrion, Golgi, cytosol, and nucleus (17, 18). It is the only pro-caspase present constitutively in the nucleus. Interestingly, neurons from caspase-2 knock-out mice are actually more sensitive to death than neurons from wild-type mice, whereas caspase-2-deficient oocytes or lymphoblasts from the same animals are resistant to apoptosis induced by chemotherapeutic drugs or granzyme B and perforin, respectively (19).

The aim of the current study was to identify a role for caspase-2 in etoposide-induced apoptosis. The results indicate a
decreased sensitivity to apoptosis induced by 10 μM etoposide in cells with impaired caspase-2 activity. The level of inhibition precedes the engagement of mitochondria, because cytochrome c release was inhibited. Moreover, cells treated with benzoxyloxy carbonyl-Val-Asp-Val-Ala-Asp-fluoromethyl ketone (z-VDVAD-fmk) or stably transected with pro-caspase-2 antisense (Casp-2AS) exhibited significantly lower levels of phosphatidyserine (PS) exposure on the plasma membrane, as well as reduced caspase-9 and caspase-3 activities. In addition, reconstituted cell-free experiments revealed that etoposide-induced cytochrome c release from liver mitochondria was reduced when these organelles were incubated with nuclei isolated from Casp-2AS- versus neonatally transfected cells. Combined, we propose that nuclear caspase-2 is an important upstream promoter of mitochondrial cytochrome c release in response to etoposide.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Wild-type Jurkat T-lymphocytes or cells stably transfected with pro-caspase-2 antisense (Casp-2AS) or control vector (neo) were cultured in RPMI 1640 complete medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2% (v/w) glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified air/CO₂ (19:1) atmosphere at 37 °C. Cells were maintained in a logarthmic growth phase for all experiments. Apoptosis was induced with etoposide (10 μM) (Bristol-Myers Squibb Co.), and ethanol (0.03% final concentration) was used as a vehicle control. In some cases, cells were first treated for 1 h with z-VDVAD-fmk, z-VDVAD-fmk, or z-LEHD-fmk (25 μM) (Enzyme Systems Products, Dublin, CA) to inhibit caspase activity and Me₂SO (0.2% final concentration) was used as a vehicle control.

**Preparation of Cytofl for Cell-free System and Cytochrome c Measurement**—Cells were collected and washed twice in ice-cold phosphate-buffered saline (PBS), resuspended in S-100 buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, and a mixture of protease inhibitors) and incubated on ice for 15 min. Cells were centrifuged at 10,000 g for 15 min at 4 °C. Supernatants were further centrifuged at 100,000 g for 1 h at 4 °C and used for cell-free experiments or Western blot analysis.

**Isolation of Jurkat Nuclei**—Cells were washed once with ice-cold PBS and recovered at 500 × g for 5 min. Pellets were resuspended in buffer A (10 mM HEPES, pH 7.6, 0.1 mM EDTA, 320 mM sucrose, 2 mM magnesium acetate, 1 mM DTT, and a mixture of protease inhibitors) and incubated on ice for 15 min. Cells were centrifuged at 10,000 g for 15 min at 4 °C. Supernatants were further centrifuged at 100,000 g for 1 h at 4 °C and used for cell-free experiments or Western blot analysis.

**Isolation of Rat Liver Mitochondria**—The liver of a male Sprague-Dawley rat was minced on ice, resuspended in 50 ml of MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.5) supplemented with 1 mM EDTA, and homogenized with a glass Dounce homogenizer and Teflon pestle. Homogenates were centrifuged at 600 × g for 8 min at 4 °C. The supernatant was decanted and recentrifuged at 5500 × g for 15 min to form a mitochondrial pellet that was resuspended in MSH buffer without EDTA and centrifuged again at 5500 × g for 15 min. The final mitochondrial pellet was resuspended in MSH buffer at a protein concentration of 80–100 mg/ml. Fresh mitochondria were prepared for each experiment and used within 4 h.

**Apoptosis Measurements**—Phosphatidyserine exposure on the outer leaflet of the plasma membrane was detected using the Annexin V: FITC Apoptosis Detection Kit II (BD PharMingen, San Jose, CA) according to the manufacturer’s instructions. In brief, 5 × 10⁷ cells were pelleted from the treatment and washed in PBS. Next, the cells were resuspended in 100 μl of binding buffer containing annexin V- FITC and propidium iodide. Prior to fluorescence-activated cell sorter analysis, 400 μl of binding buffer were added to the cells. Necrotic cells were excluded by gating before histogram analysis and never accounted for more than 4% of total cells.

Analysis of oligonucleosomal DNA fragmentation was performed as described previously (20). Briefly, 10⁶ cells were collected by centrifugation at 1000 × g, washed once in PBS, and resuspended in 250 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Subsequently, the suspension was mixed with an equal volume of lysis buffer (20 mM EDTA, 0.05% (v/v) Triton X-100, 10 mM Tris-HCl, pH 8.0) and incubated on ice for 30 min. Lysates were centrifuged at 13,000 g for 15 min at 4 °C to separate intact chromatin (pellet) from DNA fragments (supernatant). The supernatant containing fragmented DNA was precipitated with ethanol overnight at −20 °C. Precipitated and lyophylized DNA was resuspended in 20 μl of TE buffer and incubated with 1 μl of 50 mg/ml RNase A for 1 h at 37 °C followed by 1 μl of 25 mg/ml proteinase K. The samples were mixed at 1:5 ratio with loading buffer (40% (v/v) bromophenol blue) and electrophoresed at 60 mA on 18% agarose gels. Separated DNA was stained with ethidium bromide and visualized by UV light.

**Reconstituted Cell-free System**—Standard reactions were carried out in a 35-μl reaction volume with reaction buffer (20 mM HEPES, pH 7.6, 10 mM KCl, 2 mM magnesium acetate, 1 mM EGTA, 1 mM DTT, 250 mM sucrose, 10 mM succinate, 2 mM ATP, 10 mM creatine phosphate, 50 μg/ml creatine kinase, and a mixture of protease inhibitors) in the absence or presence of Jurkat nuclei (10⁶), isolated liver mitochondria (15 μg of protein), and 25 μg of wild-type Jurkat cytosol protein. Nuclei and mitochondria were suspended separately in reaction buffer prior to their addition to the reaction mix. Samples were incubated at 37 °C for up to 2 h. Nuclei and mitochondria were removed by centrifugation at 15,000 g for 10 min. Supernatants and the supernatants were stored at −20 °C until used for Western blot analysis.

**Western Blot Analysis**—Samples were mixed with Laemmli’s loading buffer, boiled for 5 min, and subjected to 15% SDS-PAGE at 130 V followed by electroblotting to nitrocellulose for 2 h at 100 V. Membranes were blocked for 1 h with 5% nonfat milk in phosphate-buffered saline at room temperature and subsequently probed overnight with an anti-cytochrome c (1:2500) (BD PharMingen), anti-caspase-2L (1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Smac/DIABLO (1:500) (Alexis Biochemicals, San Diego, CA), anti-AIF (1:1000), or anti-glyceraldehyde-3-phosphate dehydrogenase (1:3000) (Trevigen, Inc., Gaithersburg, MD) antibody. The membranes were rinsed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h. Following the antibody incubation, membranes were rinsed, and bound antibodies were detected using enhanced chemiluminescence according to the manufacturer’s instructions.

**Measurement of Caspase Activity**—The measurement of DEVD-AMC, VDAD-AMC, or LEHD-AMC (Peptide Institute, Osaka, Japan) cleavage was performed using a modified version of a fluorometric assay reported previously (21). Sample supernatants were resuspended and washed once with ice-cold PBS. For DEVD-AMC cleavage, cells were resuspended in 25 μl of PBS, added to a microtiter plate, and combined with substrate dissolved in a standard reaction buffer (100 mM HEPES, pH 7.25, 10% sucrose, 10 mM dithiothreitol (DTT), 0.1% CHAPS). For VDAD-AMC or LEHD-AMC cleavage, cells were resuspended in 25 μl of PBS, added to a microtiter plate, and combined with substrate dissolved in a standard reaction buffer (100 mM MOPS, pH 6.5, 10% polyethylene glycol, 10 mM DTT, 0.1% CHAPS). Cleavage of the fluorogenic peptide substrates was monitored by AMC liberation in a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden) using 355 nm excitation and 460 nm emission wavelengths. Fluorescence units were converted to pmol of AMC using a standard curve generated with free AMC. Data from duplicate samples were then analyzed by linear regression.

**RESULTS AND DISCUSSION**

The Caspase-2 Inhibitor z-VDVAD-fmk Prevents Apoptosis Induced by 10 μM Etoposide—Uncertainty persists as to how DNA-damaging chemotherapeutic drugs, such as etoposide, initiate apoptosis by invoking the mitochondrial pathway and cytochrome c release. Wrome et al. (14) demonstrated previously that the stimulation of cytochrome c release by etoposide occurs by distinct pathways that hinge upon the concentration of drug being used (14). Specifically, using wild-type Jurkat T-lymphocytes or a reconstituted cell-free system, cytochrome c release stimulated by a low (10 μM) dose of etoposide appeared to require caspase activity and possibly that of caspase-2. To build on our previous study and to evaluate the contribution of caspase-2 to apoptosis induced by 10 μM etoposide, we initially used the irreversibel caspase-2 inhibitor z-VDVAD-fmk. Using
Caspase-2 and Etoposide-induced Apoptosis

Figure 1. Caspase-2 inhibition protects against etoposide-induced apoptosis. A, wild-type Jurkat T-lymphocytes (10^6/ml) either vehicle-treated (solid line) or treated with 10 μM etoposide in the absence (dashed line) or presence (dotted line) of 25 μM z-VDVAD-fmk, were harvested at 6 h and processed for the detection of phosphatidylserine exposure as described under “Experimental Procedures.” B, duplicate aliquots of cells were analyzed for the appearance of oligonucleosomal DNA fragments as described under “Experimental Procedures.” Lane 1, vehicle; lane 2, etoposide alone; lane 3, etoposide + z-VDVAD-fmk. Pretreatment of cells with z-VDVAD-fmk was for 1 h at 37°C.

FITC-labeled annexin V and flow cytometric analysis, we observed that pretreatment of wild-type Jurkat cells with 25 μM z-VDVAD-fmk for 1 h completely blocked etoposide-induced PS exposure on the plasma membrane at 6 h (Fig. 1A). The level of PS exposure in cells treated with etoposide alone was ~37% (Fig. 1A). PS exposure was also evaluated at 3 h, and no difference between control and etoposide-treated cells was observed (data not shown). Consistent with the PS exposure data, 25 μM z-VDVAD-fmk effectively blocked DNA fragmentation as assessed by oligonucleosomal ladder formation (Fig. 1B, lane 3 versus 2). Together, these data provide evidence that caspase-2 is important for etoposide-induced apoptosis.

Caspase-2 is Critical for Etoposide-induced Cytochrome c Release in Intact Cells—A previous study reported the early activation of pro-caspase-2 in response to various stimuli. Activation occurred upstream of caspase-3 activity and thus suggested that pro-caspase-2 may act as an initiator caspase (22). In addition, the authors speculated that caspase-2 may be part of a signaling complex, similar to caspases 8 and 9, involved in transducing signals between death stimuli and downstream apoptotic events, although direct evidence to support this notion was not presented.

To determine whether caspase-2 was important for etoposide-induced cytochrome c release, wild-type Jurkat cells were treated with 10 μM etoposide for 3 h in the absence or presence of 25 μM z-VAD-fmk or z-VDVAD-fmk (Fig. 2). The results indicated that 10 μM etoposide stimulated processing of pro-caspase-2, yielding bands of ~18 and 12 kDa in size (Fig. 2A, lane 2). Pretreatment of cells with either z-VAD-fmk (Fig. 2A, lane 3) or z-VDVAD-fmk (Fig. 2A, lane 4) prevented this event. Processing of pro-caspase-2 was accompanied by the release of cytochrome c into the cytosol (Fig. 2B, lane 2), an effect that was undermined, but not completely prevented, when cells were first incubated with either z-VAD-fmk (lane 3) or z-VDVAD-fmk (lane 4) for 1 h. The absence of total protection by either inhibitor may be explained by recent observations demonstrating a transcription-independent role for p53, where in response to DNA damage this protein localizes to mitochondria to stimulate cytochrome c release and changes in membrane potential (23). Importantly, neither benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (z-DEVD-fmk) nor benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethyl ketone (z-LEHD-fmk), inhibitors of caspase-3-like (referred to here as caspase-3) and caspase-9 activities, respectively, was able to mimic the effect of z-VAD-fmk or z-VDVAD-fmk. Thus, it would appear that caspase-2 acts as an important upstream modulator of cytochrome c release in response to etoposide.

Inhibiting Caspase-2 Interferes With Etoposide-induced Pro-caspase-9 and Pro-caspase-3 Activation—Because caspase-2 inhibition attenuated cytochrome c release in response to etoposide, we conjectured that this would translate into reduced caspase-9 and -3 activities. To test this premise, caspase-2 (VDVADase), caspase-9 (LEHDase), and caspase-3 (DEVDase) activities were measured following treatment of wild-type Jurkat cells with 10 μM etoposide for up to 6 h (Fig. 3). Consistent with our previous results (14), an increase in caspase-2 activity was detected prior to an increase in caspase-9 activity (Fig. 3, A and C). Complementary experiments performed with z-VDVAD-fmk or z-LEHD-fmk revealed that both pro-caspase-9 and -3 activation, stimulated by 10 μM etoposide, depend on active caspase-2 (Fig. 3, B and C). In other words, etoposide-induced caspase-9 and -3 activities were inhibited when cells were pretreated for 1 h with 25 μM z-VDVAD-fmk. It should be noted that 25 μM z-VDVAD-fmk prevented neither cytochrome c release nor pro-caspases 9 or 3 activation in response to 50 μM etoposide, verifying the inability of z-VDVAD-fmk to inhibit these proteases directly. When cells were pretreated for 1 h with 25 μM z-LEHD-fmk, caspase-9 and -3 activities were inhibited (Fig. 3, B and C). Surprisingly, z-LEHD-fmk also partially inhibited caspase-2 activity (Fig. 3A); however, this may reflect a pool of pro-caspase-2 that is normally activated by caspase-3-mediated cleavage (24). Taken together, these data suggest that the ordering of the proteolytic caspase cascade in response to a low dose of etoposide is as follows: caspase-2 → caspase-9 → caspase-3.

Decreased Expression of Pro-caspase-2 Mitigates Etoposide-induced Apoptosis—To assess whether Jurkat cells with a decreased level of pro-caspase-2 were similarly resistant to etoposide-induced apoptosis as wild-type cells treated with 25 μM...
treated or treated with 10 μM etoposide in the absence (●) or presence of 25 μM z-VDVAD-fmk (●) or z-LEHD-fmk (●), were harvested at the indicated time points and processed for caspase activity measurements as described under “Experimental Procedures.” Enzyme activity was monitored by the release of AMC from: A, VDVAD-AMC; B, DEVD-AMC; C, LEHD-AMC. Treated cells are compared with 100% control.

z-VDVAD-fmk, experiments were performed using neo- or Casp-2/AS-transfected cells described previously (Fig. 4A) (25). Although that study demonstrated an apparent role for caspase-2 in Fas-mediated cell death, the authors also reported that down-regulation of pro-caspase-2 had no effect on etoposide-induced apoptotic DNA fragmentation. It was speculated that this might be related to drug-induced transcriptional activation of Casp-2 (26). Although this possibility cannot be excluded, we were interested in whether the lack of protection could be attributed to the concentration (50 μM) of etoposide employed, because we had demonstrated in our previous study that 50 μM etoposide was directly toxic to mitochondria and stimulated a caspase-independent release of cytochrome c as well as earlier and more robust DEVDase activity (14).

Here, neo- and Casp-2/AS-transfected cells were treated with 10 μM etoposide for up to 6 h and evaluated for any difference in apoptosis. Results indicated that both cell lines were sensitive to this concentration of etoposide as measured by PS exposure (Fig. 4B and C). However, ~43% of neo-transfected cells (Fig. 4B) were positive for annexin V binding, whereas only ~28% of Casp-2/AS-transfected cells (Fig. 4C) exposed PS. The fact that Casp-2/AS cells were not entirely resistant to 10 μM etoposide was not surprising because these cells maintain a certain level of pro-caspase-2, albeit considerably less than neo-transfected cells (Fig. 4A). It is worth mentioning that DNA fragmentation was also evaluated, and the results were consistent with those for PS exposure (data not shown). In either case, when neo or Casp-2/AS cells were pretreated with 25 μM z-VDVAD-fmk for 1 h, PS exposure (Fig. 4, B and C) and DNA fragmentation were blocked.

Etoposide-induced Pro-caspase Activation Is Delayed and Less Robust in Casp-2/AS Cells—Because our inhibitor experiments (Fig. 3) revealed that pro-caspase-2 activation apparently occurs upstream of pro-caspases 9 and 3, the next step was to determine whether this ordering of pro-caspase activation was true in cells stably expressing pro-caspase-2 antisense. In other words, does an enforced lowering of pro-caspase-2 translate into reduced or delayed activation of pro-caspases 9 and 3 in response to 10 μM etoposide? To test this possibility, neo- and Casp-2/AS-transfected cells were treated with etoposide for up to 6 h, and fluorometric analysis of different caspase activities was performed (Fig. 5). In agreement with the data presented in Fig. 3, Casp-2/AS cells exhibited lower levels of not only VDVADase but also DEVDase and LEHDase activities when compared with neo cells. In each case, increases in activity were observed earlier and were considerably more pronounced in neo-transfected cells (Fig. 5, A–C). In particular, by 6 h, neo cells exhibited ~500 and ~1600% rise in VDVADase and DEVDase activities, respectively, whereas the rise of these activities in Casp-2/AS cells was more modest at ~250 and ~750%, respectively (Fig. 5, A and B). Moreover, pretreatment of either cell line with 25 μM etoposide was not surprising because these cells maintain a certain level of pro-caspase-2, albeit considerably less than neo-transfected cells (Fig. 4A). It is worth mentioning that DNA fragmentation was also evaluated, and the results were consistent with those for PS exposure (data not shown). In either case, when neo or Casp-2/AS cells were pretreated with 25 μM z-VDVAD-fmk for 1 h, PS exposure (Fig. 4, B and C) and DNA fragmentation were blocked.

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z-VDVAD-fmk for 1 h prior to etoposide completely abolished any increase in VDVADase, DEVDase, or LEHDase activity (Fig. 5, A–C). Taken together, these data offer additional support for the notion that caspase-2 is the most apical caspase in etoposide-induced apoptosis.

**Expression of Casp-2/AS Mitigates Cytochrome c Release in Response to Etoposide**—To extend these results a step further, we were interested in determining whether stable expression of pro-caspase-2 antisense would attenuate etoposide-induced cytochrome c release to an extent similar to z-VDVAD-fmk or z-VDVAD-fmk (Fig. 2B). Two different, albeit complementary, approaches were used to address this question. First, intact neo- or Casp-2/AS-transfected cells were treated with 10 μM etoposide for 3 or 6 h in the absence or presence of 25 μM z-VDVAD-fmk, and cytosolic extracts were prepared for cytochrome c measurement. Second, experiments were performed using a reconstituted cell-free system wherein isolated neo or Casp-2/AS Jurkat nuclei (105), isolated liver mitochondria (15 μg of protein), and/or wild-type Jurkat cytosol (25 μg of protein) were treated with 10 μM etoposide for up to 2 h at 37 °C.

Western blot analysis of cytosols isolated from neo- and Casp-2/AS-transfected cells at 3 h revealed that etoposide-stimulated cytochrome c release in neo but not Casp-2/AS cells (Fig. 6A, lane 2 versus 1 and lane 5 versus 4). Moreover, pretreatment of neo-transfected cells with 25 μM z-VDVAD-fmk attenuated cytochrome c release (Fig. 6A, lane 3 versus 2), an effect that was consistent with results obtained earlier using wild-type Jurkat cells (Fig. 2B, lane 4 versus 2). Whether apoptosis-inducing factor (AIF) or second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein with low pI (Smac/DIABLO) was released at 3 h after etoposide treatment was also investigated. As seen in Fig. 6A, the release profile of Smac/DIABLO was similar to that observed for cytochrome c at 3 h (Fig. 6A), which is consistent with a previous report documenting Smac/DIABLO release as a general feature of apoptosis in Jurkat cells (27). In contrast, AIF was not released during etoposide-induced apoptosis. It should be noted that Western blot analysis of a subcellular
fraction containing mitochondria demonstrated the reactivity of the anti-AIF antibody with human AIF (data not shown). It is also worth mentioning that preliminary data recently generated in our laboratory suggest that AIF is only released during mitochondrial permeability transition pore opening, which may account for the absence of its release in response to 10 μM etoposide.

At 6 h after 10 μM etoposide treatment, neither z-VDVAD-fmk nor pro-caspase-2 antisense alone was able to mitigate cytochrome c release (Fig. 6B, lanes 3 and 5). However, Casp-2/AS cells pretreated with 25 μM z-VDVAD-fmk remained refractory to cytochrome c release (Fig. 6B, lane 6 versus 5). Together, this suggests that pro-caspase-2 antisense significantly delays but does not altogether prevent cytochrome c release induced by 10 μM etoposide. Importantly, these data are in agreement with our previous report demonstrating the inability of 25 μM z-VAD-fmk to prevent etoposide-induced cytochrome c release at 6 h, an effect attributed to the ability of a low dose (10 μM) of etoposide to ultimately target mitochondria directly (14).

Experiments performed using a cell-free system consisting of isolated Jurkat nuclei and liver mitochondria yielded results for cytochrome c release that were consistent with the cytosolic extract data (Fig. 6, panel C versus A). Specifically, 10 μM etoposide stimulated cytochrome c release following a 2-h incubation at 37 °C when the reaction mixture contained nuclei isolated from neo-transfected cells (Fig. 6C, lane 2 versus 1) but not when Casp-2/AS nuclei were present (Fig. 6C, lane 5 versus 4). Interestingly, this effect was not altered by cytosolic factors, because etoposide-induced cytochrome c release occurred to a similar extent when a fraction containing wild-type Jurkat cytosol was present (data not shown). Treating the reaction mixture with 25 μM z-VDVAD-fmk was sufficient to decrease cytochrome c release induced by etoposide (Fig. 6C, lane 3 versus 2). It should be mentioned that 10 μM etoposide did not stimulate cytochrome c release when mitochondria were incubated in the absence of nuclei. Together, these data implicate a role for the nuclear pool of pro-caspase-2 during etoposide-induced cytochrome c release.

Concluding Remarks—That pro-caspase-2 is activated in response to a variety of pro-apoptotic stimuli, including tumor necrosis factor-α, Fas ligand, growth factor withdrawal, and DNA-damaging agents, has been documented previously (22, 25, 28). However, assigning an emergent function to this pro-caspase-2 involves an adaptor molecule known as caspase-2 and receptor-interacting protein (RIP) adaptor with death domain/RIP-associated Ich-1-homologous protein with death domain (CRADD/RAIDD) that is present in the cytosol and the nucleus (33–35), one could envisage a scenario in which activation of pro-caspase-2 occurs in a manner similar to the activation of initiator pro-caspases 8 and 9. Specifically, it is intriguing to think that etoposide-induced activation of pro-caspase-2 might involve the formation of a nuclear signaling complex, wherein upon drug treatment CRADD/RAIDD gathers pro-caspase-2 molecules into close proximity to allow auto-processing. However, additional studies are needed to characterize the mechanism of pro-caspase-2 activation as well as subsequent events leading to the engagement of the mitochondrial apoptotic pathway.

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