The Role of Apaf-1, Caspase-9, and Bid Proteins in Etoposide- or Paclitaxel-induced Mitochondrial Events during Apoptosis

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ABSTRACT

Ectopic overexpression of Apaf-1 (2.5-fold) in human acute myelogenous leukemia HL-60 cells (HL-60/Apaf-1 cells) induced apoptosis and sensitized HL-60/Apaf-1 cells to etoposide- and paclitaxel-induced apoptosis (C. Perkins et al., Cancer Res., 55: 4561–4566, 1995). In this report, we demonstrate that in HL-60/Apaf-1 cells, the activity of caspase-9 and -3 induced by Apaf-1 overexpression was associated with a significant increase (5-fold) in the cytosolic accumulation of cytochrome c (cyt c), loss of mitochondrial membrane potential (ΔΨm), and an increase in the reactive oxygen species. These were also associated with the processing of procaspase-8 and Bid (cytosolic, proapoptotic BH3 domain containing protein). Transient transfection of Apaf-1 into the Apaf-1-containing mouse embryonic fibroblasts (MEFs; Apaf-1+/− MEFs) or Apaf-1−/− MEFs also induced the processing of procaspase-9 and procaspase-8, Bid cleavage, and apoptosis. These events were secondary to the activity of the downstream caspases induced by Apaf-1. This conclusion is supported by the observation that in HL-60/Apaf-1 cells, ectopic expression of dominant negative caspase-9, its inhibitory short isoform caspase-9b, or XIAP or treatment with the caspase inhibitor zVAD (50 μM) inhibited Apaf-1-induced caspase-8 and Bid cleavage, mitochondrial ΔΨm, release of cyt c, and apoptosis. In contrast, a transient transfection of dominant negative caspase-8 or CrmA or exposure to caspase-8 inhibitor zIETD-fmk inhibited the processing of procaspase-8 and Bid but did not inhibit the cytosolic accumulation of cyt c in either the untreated HL-60/Apaf-1 cells or the etoposide-treated HL-60/Apaf-1 and HL-60/neo cells. These results indicate that Apaf-1 overexpression lowers the apoptotic threshold by activating caspase-9 and caspase-3. This triggers the mitochondrial ΔΨm and cyt c release into the cytosol through a predominant mechanism other than cleavage of caspase-8 and/or Bid. This mechanism may involve a cytosolic mitochondrial permeability transition factor, which may be processed and activated by the downstream effector caspases, thereby completing an amplifying feedback loop, which triggers the mitochondrial events during apoptosis.

INTRODUCTION

The family of mammalian caspases (aspartate-specific cysteine proteases) represents the effector arm of the apoptotic program (1, 2). Intracellularly, caspases exist as inactive zymogens (procaspases) that have NH2-terminal prodomains plus large and small catalytically active subunits. Caspases may be subclassified as initiators (e.g., caspase-8, -10, -2, or -9) or effectors, also known as executioners (e.g., caspase-3, -6, or -7), based on whether they have a large or small prodomain (1, 2). Caspases with large prodomains interact with signaling adapter molecules through motifs in the prodomains called CARDs2 (1, 2). For example, interaction between CARDs in the adaptor molecule FADD and the prodomain of procaspase-8 allows the formation of a death-inducing signaling complex during the Fas receptor-induced signaling for apoptosis (3). Recruitment and oligomerization, followed by processing and activation of caspase-8 in the Fas receptor-initiated death-inducing signaling complex, ultimately result in the cleavage and translocation of the cytosolic, proapoptotic BH3 domain-containing Bid protein to the mitochondria (4–7). Here it triggers the preapoptotic mitochondrial events leading to the activation of the executioner, caspase-3 (4, 5). Previous in vitro studies have demonstrated that the enforced expression of adaptor molecules FADD or CRADD triggers the cleavage and activation of executioner caspase-3 and apoptosis by causing oligomerization of the initiator caspases, caspase-8 and caspase-10, or caspase-2, respectively (8, 9). The generation of active caspases requires cleavage at the internal Asp residue present between the catalytic large and small subunit, with further processing at the Asp residue present in the interdomain linkers between the prodomain and the large subunit to remove the prodomain (1, 2). Once activated, caspases can cleave their substrates and other procaspases to generate active subunits (1, 2). The executioner caspases can also proteolytically cleave a number of cellular proteins, e.g., PARP, lamins, DFF, fodrin, gelsolin, protein kinase Ca, Rb, DNA-PK, and so forth, resulting in the morphological features and DNA fragmentation of apoptosis (1, 2, 10).

Recently, Apaf-1 was identified as the human homologue of the Caenorhabditis elegans CED-4 protein and was shown to participate as an adaptor molecule in the sequential activation of caspase-9 and caspase-3 (11–13). The NH2-terminal region of Apaf-1 contains a CARD, which can bind to the corresponding motif in procaspase-9. Apaf-1 also contains a central region homologous to the proapoptotic CED-4 protein, including a conserved P-loop, and a COOH-terminal WD repeat domain that lacks homology with CED-4. In the presence of dATP and cyt c released from the mitochondria by a number of apoptotic stimuli, the monomeric Apaf-1 is transformed into an oligomeric complex made of at least eight subunits (14–16). This Apaf-1-cyt c complex can bind and process procaspase-9, followed by the release of mature caspase-9 (15, 17). This, in turn, can process procaspase-3, triggering the caspase cascade of activities that results in apoptosis (15, 17). Recent studies have demonstrated that an endogenous alternatively spliced isoform of caspase-9 (caspase-9b), a mutation in the active site of caspase-9, or the deletion of caspase-9 from the S-100 fraction of cytosol blocks cyt-c-coupled and Apaf-1-mediated ultimate cleavage and activation of caspase-3 (12, 13, 18, 19). The importance of Apaf-1-induced activity of caspase-9 followed by caspase-3 has been highlighted by reports that demonstrate that Apaf-1 or caspase-9 deficiency results in embryonic lethality due to defective neuronal apoptosis (20–23). Furthermore, inactivation of Apaf-1 or caspase-9 has been shown to substitute for p53 loss in promoting oncogenic transformation of the Myc-expressing cells (24).

Similar to the enforced overexpression of FADD and CRADD, Apaf-1 overexpression was shown to induce apoptosis, through the cleavage and activation of caspase-9 and caspase-3 (25). Higher levels of Apaf-1 were demonstrated to enhance apoptosis induced by chemotherapeutic agents, e.g., etoposide and Taxol, by increasing the sequential cleavage and activities of caspase-9 and caspase-3 (25). However, in these studies, the role of mitochondrial ΔΨm and cyt c.
release in Apaf-1-mediated sensitization of apoptosis was not determined. In the present studies, we investigated whether the lowering of the threshold to trigger apoptosis through Apaf-1 overexpression involves procaspase-8 and Bid cleavage and activity, resulting in the mitochondrial Δψm and the release and cytosolic accumulation of cyt c. Our findings demonstrate that Apaf-1 overexpression mediates mitochondrial Δψm, cytosolic accumulation of cyt c, and apoptosis predominantly through a mechanism that involves the activity of caspase-9 and caspase-3 but not caspase-8 and Bid.

MATERIALS AND METHODS

Reagents. z-VAD-fmk, z-IETD-fmk, and z-LEHD-fmk were purchased from Enzyme Systems Products (Livermore, CA). Anti-Apaf-1 and anti-Bid antibodies (5, 11) as well as cDNA of Apaf-1 and CcrMA were kindly provided by Dr. Xiaodong Wang (University of Texas, Southwestern School of Medicine, Dallas, TX). The recombiant human homotrimeric TRAIL (leucine zipper construct) was a gift from Immunex Corp. (Seattle, WA). Dr. Emad Alnemri (Thomas Jefferson University, Philadelphia, PA) kindly provided us with the cDNA of caspase 9b, DN caspase-9, and Apaf-1, which possesses an additional WD repeat.

Cells and Transfection of the Genes. Human myeloid leukemia HL-60 cells were cultured as described previously (26). Viable HL-60 cells (2–3 \times 10^6) were transiently transfected with 0.5–1.0 μg each of the indicated plasmid DNA using LipofectAMINE PLUS reagent (Life Technologies, Inc., Gaithersburg, MD). Stable HL-60 cell lines overexpressing the human Apaf-1 protein were developed as described previously (25). Lysates from the selected clones were evaluated for Apaf-1 expression by immunoblot analyses (see below). The clones expressing high levels of the specific proteins were further subcloned by limiting dilution. Representative subclones of each of the HL-60 transfectants were passaged twice per week and used for the studies. Data presented are representative of those derived from at least two independent clonal transfectants of HL-60/Apaf-1 cells. Apaf-1-deficient MEFs (a gift from Dr. Tak W. Mak; The Amgen Institute, Toronto, Ontario, Canada) were stably transfected clones of HL-60/Apaf-1 cells. Apaf-1-deficient MEFs (a gift from Dr. Tak W. Mak; The Amgen Institute, Toronto, Ontario, Canada) were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (20). Apaf-1+/− and Apaf−/− MEFs were transfected with 0.5–1.0 μg of the appropriate plasmid DNA using the LipofectAMINE PLUS reagent. Transfection efficiency was 40–60%, based on cotransfection of pCMV-GFP-1.

Western Analyses of Proteins. Western analyses of Apaf-1, Bcl-2, Bcl-xL, caspase-9, caspase-3, caspase-8, Bid, DFF, PARP, and β-actin were performed using specific antisera or monoclonal antibodies according to previously reported protocols (25, 27, 28). Horizontal scanning densitometry was performed on Western blots by using acquisition into Adobe Photo Shop (Apple, Inc., Cupertino, CA) and analysis by the NIH Image Program (NIH, Bethesda, MD). The expression of β-actin was used as a control.

Preparation of S-100 Fraction for the Analysis of Cytosolic Accumulation of Cyt c. Untreated and drug-treated cells were harvested by centrifugation, and the cell homogenates were centrifuged at 100,000 × g for 30 min at 4°C to obtain the S-100 fraction, as described previously. The supernatants were collected, and the protein concentrations of S-100 were determined by using the Bradford method (Bio-Rad, Hercules, CA). Samples were then analyzed for the release of cyt c from the mitochondria into the cytosol by Western blot, as described previously (29).

Apoptosis Assay by Morphology and Annexin V Staining. After drug treatment or the indicated transfections, 5 \times 10^6 to 1 \times 10^7 cells were washed in PBS, and cytosin preparations of the cell suspension were fixed and stained with Wright stain. Morphological evaluation of apoptosis was performed as described previously (26). Cells were also resuspended in 100 μl of staining solution (containing annexin V fluorescein and PI in a HEPES buffer, annexin V-FLUOS staining kit; Boehringer Mannheim, Indianapolis, IN). After incubation at room temperature for 15 min, cells were analyzed by flow cytometry (25). Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of those cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with annexin V) and necrotic cells (stained with both annexin V and PI; Ref. 30).

Measurement of Mitochondrial Potential and ROS. To assess the changes in mitochondrial potential and ROS, 1 × 10^6 cells were incubated for 15 min at 37°C with 40 nM 3,3’-dihexyloxacarbocyanine iodide (DiOC6; Ref. 3) and 5 μM dichlorodihydrofluorescein diacetate, respectively, and analyzed by fluorescence-activated cell sorting as described previously (31, 32).

RESULTS

Ectopic Overexpression of Apaf-1 Causes Mitochondrial Δψm and Cytosolic Accumulation of Cyt c. Stably transfected clones of Apaf-1-overexpressing (HL-60/Apaf-1) or control (HL-60/neo) cells were isolated by limiting dilution (25) and examined for the processing of the caspases and death substrates as well as for the occurrence of preapoptotic mitochondrial events (31, 32). Fig. 1A demonstrates data from a representative clone of each cell type. As compared with HL-60/neo cells, HL-60/Apaf-1 cells showed a significantly greater level of mitochondrial depolarization, as indicated by the decreased MMD (Fig. 1A and 1B, top panels). Similar changes in mitochondrial potential and ROS, 1

Fig. 1. Molecular events of apoptosis mediated by Apaf-1 in HL-60/Apaf-1 versus HL-60/neo cells. Stably transfected Apaf-1-overexpressing cells and control (HL-60/neo) cells were harvested for Western analyses as well as flow cytometric analyses to determine the percentage of cells with low MMP or increase in ROS (see “Materials and Methods”). A, inactive caspase-9 (p35) proform and its active cleaved fragment (p17,000), Zymogen procaspase-3 (p35) and its activated cleaved product (p15,000), DFF45 and its intermediate cleaved product (p17,000), and increased production of ROS (bottom panels) in HL-60/Apaf-1 versus HL-60/neo cells. The arrow points to the positive control after treatment with 10 mmol/liter H2O2.
HL-60.neo cells, HL-60/Apaf-1 cells showed 2.5-fold higher levels of Apaf-1 and demonstrated the processing and activation of procaspase-9 and procaspase-3 (Fig. 1, A and B). This was associated with the cleavage of PARP into its Mr 85,000 fragment and the cleavage of DFF45 (or ICAD) into its Mr 30,000 fragment. DFF45 and PARP are known substrates for caspase-3 (33, 34). As compared with HL-60.neo, Apaf-1 overexpression in HL-60/Apaf-1 cells was associated with a slightly increased expression of Bcl-2 (Fig. 1B) but not of Bcl-xL or Bax (data not shown). Whereas oligomerization of Apaf-1 is known to recruit, bind, and activate caspase-9 and caspase-3, in HL-60/Apaf-1 cells, this was unexpectedly associated with a 5-fold increase in the cytosolic cyt c (Fig. 1C), as well as an increase in the percentage of cells with low MMP and increased ROS (Fig. 1D). These preapoptotic mitochondrial events and caspase activations were also observed in HL-60 cells after transient transfection and overexpression of Apaf-1 or the transfection of its longer, alternatively spliced isomorph (Apaf-1L), which possesses an additional WD repeat (data not shown). Therefore, we used Apaf-1 transfectants in the following experiments and refer to them henceforth as HL-60/Apaf-1 cells.

**Ectopic Overexpression of Apaf-1 Induces Processing of Pro-caspase-8 and Bid and Sensitizes HL-60/Apaf-1 Cells to Etoposide and Paclitaxel.** The activities of caspase-9 and caspase-3 can process and activate procaspase-8 and Bid; the latter, in turn, can cause the mitochondrial release of cyt c (4–6). Therefore we examined whether Apaf-1-mediated activities of the effector caspases would result in the processing of procaspase-8 and Bid, and whether this would sensitize HL-60/Apaf-1 cells to etoposide- or Taxol-induced accumulation of cyt c and apoptosis. As shown in Fig. 2, A and B, Apaf-1 overexpression resulted in the processing of procaspase-8 and Bid, and, as noted previously, Bid processing caused cytotoxic accumulation of cyt c and apoptosis. Fig. 2A also shows that an exposure to Taxol (10 nM for 24 h) or etoposide (1.0 μM for 24 h) induced significantly more apoptosis in HL-60/Apaf-1 cells than in HL-60.neo cells. Cotreatment with 50 μM zVAD, which exerts more potent inhibitory effects against the activities of caspase-9 and caspase-3 than against the activities of apical caspase-8 and caspase-10, also inhibited the processing of caspase-8 and Bid. Consequently, zVAD inhibited the cytosolic accumulation of cyt c and apoptosis induced by either Apaf-1 overexpression or cotreatment with etoposide or Taxol (Fig. 2, A and B). Fig.

![Fig. 2. zVAD blocks downstream events induced by Apaf-1 overexpression in HL-60 cells. Stable HL-60.neo (control) or HL-60/Apaf-1 cells were treated with etoposide, Taxol, and/or zVAD for 24 h, and cells were harvested to determine cell death (apoptosis and necrosis) by annexin V/PI staining and protein levels by Western analyses. A, the percentage of apoptotic (●) or necrotic (□) cells detected by annexin V/PI staining. Error bars, SE for the mean percentage of apoptotic cells under the various treatment conditions. B, Western blot analyses of procaspase-8 (p55), Bid (p22), and its p15 cleaved product as well as the cytosolic levels of cyt c. β-Actin was used as a control for equal protein loading.](cancerres.aacrjournals.org)
3 demonstrates that the mitochondrial release and cytosolic accumulation of cyt c caused by Apaf-1 overexpression in HL-60/Apaf-1 cells or caused by cotreatment with etoposide or Taxol in HL-60/neo and HL-60/Apaf-1 cells is also associated with reduced mitochondrial MMP (ΔΨm) and increased ROS. These mitochondrial perturbations in HL-60/Apaf-1 cells, as in HL-60/neo cells, were also reversed by cotreatment with zVAD, indicating that these were secondary to the activities of the effector caspases.

**Transient Ectopic Expression of Apaf-1 Induces Apoptosis of Apaf-1+/− cells.** We first compared the sensitivity of Apaf-1−/− cells to a number of chemotherapeutic agents, including staurosporine, TNF-α, Fas ligation, and TRAIL. Fig. 4A demonstrates that Apaf-1+/− MEFs, which express Apaf-1 (Fig. 4B), were susceptible to apoptosis induced by etoposide, doxorubicin, and staurosporine, but were minimally susceptible to apoptosis induced by Taxol (1 μM for 24 h; Fig. 4A). Apoptosis of Apaf-1+/− cells was also observed after treatment with TNF-α, Fas ligation, and TRAIL. In contrast, Apaf-1−/− MEFs lacking Apaf-1 (Fig. 4B) were resistant to etoposide, doxorubicin, and staurosporine-induced apoptosis (Fig. 4A). Whereas Fas ligation and TNF-α were equally effective in inducing apoptosis in Apaf-1+/− and Apaf-1−/− cells, TRAIL-induced apoptosis was partially inhibited in Apaf-1−/− cells (Fig. 4A). Etoposide-induced processing of procaspase-9, procaspase-8, and Bid was compared between Apaf-1+/− and Apaf-1−/− cells. Fig. 4B demonstrates that etoposide-induced apoptosis of Apaf-1+/− cells is associated with the processing of procaspase-9, procaspase-8, and Bid, whereas these effects of etoposide are absent in Apaf-1−/− cells. Transient transfection and overexpression of Apaf-1 cDNA increased the percentage of Apaf-1+/− MEFs demonstrating apoptosis. Apaf-1 transfection also sensitized Apaf-1+/− cells to etoposide-induced apoptosis (Fig. 5A). Transient transfection of Apaf-1 also induced apoptosis of Apaf-1−/− cells; Apaf-1 also sensitized these cells to etoposide-induced apoptosis. Fig. 5B shows that the transient transfection of Apaf-1 with or without etoposide resulted in the processing of procaspase-9, procaspase-8, and Bid.

**Mitochondrial Perturbations Induced By Apaf-1 Overexpression Are Caused Primarily by the Activities of Caspase-9 and Caspase-3 and not by Caspase-8 and Bid.** We next determined whether the feedback effects on the mitochondria induced by Apaf-1 overexpression were due primarily to the activities of caspase-9 and caspase-3 or to the activities of processed caspase-8 and Bid. Transient transfection and expression of DN caspase-9 (12), caspase-9b (18), or XIAP, which binds and inhibits caspase-9 and caspase-3 (35, 36), markedly inhibited not only the processing of caspase-9 and caspase-3 but also that of caspase-8 and Bid. Transient transfection of Apaf-1 induced apoptosis of Apaf-1−/− cells; Apaf-1 also sensitized these cells to etoposide-induced apoptosis. Fig. 6A demonstrates that etoposide-induced apoptosis of Apaf-1−/− cells is associated with the processing of procaspase-9, procaspase-8, and Bid, whereas these effects of etoposide are absent in Apaf-1−/− cells. Transient transfection and overexpression of Apaf-1 cDNA increased the percentage of Apaf-1+/− MEFs demonstrating apoptosis. Apaf-1 transfection also sensitized Apaf-1+/− cells to etoposide-induced apoptosis (Fig. 5A). Transient transfection of Apaf-1 also induced apoptosis of Apaf-1−/− cells; Apaf-1 also sensitized these cells to etoposide-induced apoptosis. Fig. 5B shows that the transient transfection of Apaf-1 with or without etoposide resulted in the processing of procaspase-9, procaspase-8, and Bid.
inhibited by transient transfection of the cDNA of DN caspase-8 (37), there was minimal inhibition of the cytosolic accumulation of cyt c and apoptosis (Fig. 7, A and B). In contrast, the processing of procaspase-8 (p55) and Bid was inhibited (Fig. 7B). Procaspase-3 processing was not inhibited by DN caspase-8 in HL-60/neo or HL-60/Apaf-1 cells. Similarly, a transient transfection of the cDNA of CrmA (a cowpox virus encoded serpin-like protease inhibitor), which specifically inhibits caspase-8 activity (5), also did not inhibit cytosolic accumulation of cyt c or apoptosis (data not shown). The dominant role of the activities of caspase-9 and capase-3 over those of caspase-8 followed by Bid in mediating the feedback effects on the mitochondria is further supported by the data presented in Fig. 8, A and B. As shown, the effects of cotreatment with a relatively specific inhibitor of the activity of either caspase-9 (i.e., z-LEHD-fmk) or caspase-8 (i.e., z-IETD-fmk) were compared (38). Again, inhibition of caspase-9 by z-LEHD-fmk inhibited the processing of procaspase-3, procaspase-8, and Bid and markedly inhibited the cytosolic accumulation of cyt c and apoptosis induced by etoposide in HL-60/neo cells or by the ectopic overexpression of Apaf-1 in HL-60/Apaf-1 cells. Inhibition of caspase-8 activity by 50 μM z-IETD-fmk, in contrast, had a minimal effect on the levels cytosolic cyt c and apoptosis in both cell types. The difference in the pattern of the cleavage fragments of procaspase-3 in Fig. 8B versus Fig. 7B may be due to the use of tetrapeptide inhibitors in the studies represented in Fig. 8B but not in those represented in Fig. 7B. z-LEHD-fmk, but not z-IETD-fmk, also reversed the loss of mitochondrial ΔΨm and the increase in ROS induced by the ectopic overexpression of Apaf-1 in HL-60/neo cells (data not shown). The differential effect of treatment with z-LEHD
versus z-IETD does not rule out the possibility that the observed mitochondrial events in HL-60/Apaf-1 cells, including the release of cyt c, might also be due to the activation of other effector caspases, in addition to caspase-3, by caspase-9.

**DISCUSSION**

In the present study, we demonstrate that the stable or transient overexpression of Apaf-1 induces the processing of procaspase-9 and procaspase-3 and causes the cleavage of the death substrates (PARP and DFF45) and apoptosis of the human acute myelogenous leukemia HL-60 and murine Apaf-1−/− cells. The cytosolic Apaf-1 binds and hydrolyzes ATP or dATP (11). Apaf-1 also binds to cyt c (11, 12). The binding to cyt c and the hydrolysis of dATP facilitate oligomerization of Apaf-1 (13–16). The multimeric Apaf-1-cyt c complex binds procaspase-9 with a stoichiometry of 1:1 (14). This results in the activation and processing of caspase-9, which is then released from the Apaf-1-cyt c multimeric complex to process and activate caspase-3 or other caspases including apical caspase-8 (15). There is alternative evidence that suggests that procaspase-9 can also recruit procaspase-3 to the Apaf-1-procaspase-9 complex (16). Using a mutant procaspase-9 disabled for processing, it has also been demonstrated that caspase-9 can be activated without proteolytic processing (38). The spontaneously oligomerizing Apaf-530, which lacks the WD-40 domain, has been shown in vitro to bind and process procaspase-9 but lacks the ability to release the mature caspase-9 or to recruit procaspase-3 (15, 17). In contrast, in the data presented here, we demonstrate that the transient transfection of full-length Apaf-1 in HL-60 cells and in Apaf-1−/− MEFs produces the processing and activities of procaspase-9 and procaspase-3, resulting in apoptosis (25). However, these findings have to be regarded with the caveat that they were observed in an overexpression system, which may have produced an enforced oligomerization of Apaf-1, causing the processing of caspase-9 and caspase-3 and apoptosis. As reported previously, our data demonstrated that Apaf-1−/− cells were resistant to apoptosis induced by chemotherapeutic agents, including etoposide, doxorubicin, and Taxol, but were not resistant to apoptosis induced by TNF-α or Fas ligation by an agonist antibody (20). In contrast, apoptosis induced by TRAIL was only partially inhibited in the Apaf-1−/− cells. This may be because, as compared to Fas ligand or TNF-α, TRAIL-induced processing of procaspase-8 in Apaf-1−/− MEFs may be modest and delayed due to the presence of DcR1 and/or DcR2 (39, 40), allowing Bid processing and the resultant mitochondrial release of cyt c. The presence of Bid in the cytosol clearly would not facilitate caspase-9 and caspase-3 activations in Apaf-1−/− cells (20, 21).

Our data show that Apaf-1 overexpression in HL-60 cells also resulted in the processing of procaspase-8 and Bid. This is most likely due to the activity of caspase-9 and caspase-3, which can result in the processing of procaspase-8 (10, 22). Neither caspase-6 nor caspase-7 has been shown to cleave Bid (41). In addition to the processing of procaspase-8 and Bid, ectopic overexpression of Apaf-1 was also associated with the mitochondrial ∆Ψm and the release of cyt c into the cytosol. As shown in Fig. 9, these preapoptotic mitochondrial events could be due directly to either the activities of caspase-9 followed by caspase-3 or the intervening processing of procaspase-8 and Bid. It should be noted that the inhibition of the activities of caspase-9 and caspase-3 by transient ectopic expression of caspase-9b, DN caspase-9, or XIAP (Fig. 6B) or by cotreatment with the caspase-9-specific inhibitor zLEHD-fmk (Fig. 8B) markedly inhibited not only the processing of procaspase-8 and Bid but also the preapoptotic mitochondrial release of cyt c in HL-60/Apaf-1 cells. In contrast, cotreatment with the caspase-8-specific inhibitor zIETD-fmk (Fig. 8B) or transfection of DHFR (Fig. 7B) or CrmA (data not shown) did not inhibit the mitochondrial release of cyt c. This indicates that the procaspase-8 and Bid processing mediated by ectopic overexpression of Apaf-1 in HL-60 cells does not play a dominant role in causing the feedback effects on the mitochondria. Therefore, the following question arises: how do caspase-9 and/or caspase-3 (or another executioner caspase) activities triggered by ectopic Apaf-1 mediate mitochondrial ∆Ψm and cyt c release, if not through Bid activity? A recent report by Bossy-Wetzel and Green (41)
suggested that the effector caspases such as caspase-3, caspase-6, and caspase-7 that do not act directly on the mitochondria to release cyt c might cleave and activate another cytosolic substrate (other than Bid) that could then promote the mitochondrial ΔΨm and the release of cyt c. Our data also support the existence of such a putative cytosolic MPTF. As shown in Fig. 9, MPTF may be the direct target for processing by caspase-9 and/or caspase-3 or caspase-7. Although Bid cleavage and activity triggered by death receptor signaling has clearly been shown to exert an amplifying role during apoptosis by causing mitochondrial ΔΨm and cyt c release, our findings suggest that this

Fig. 7. DN caspase-8 only partially inhibits Apaf-1- and etoposide-induced apoptosis and cyt c release in HL-60 cells. Stable HL-60/neo and HL-60/Apaf-1 cells were transiently transfected with the cDNA of DN caspase-8 for 24 h. Subsequently, cells were treated with etoposide, and the percentage of apoptotic/necrotic cells was determined by annexin V/PI staining (A); cells were also harvested for Western analysis of procaspase-8, procaspase-3, Bid, and cytosolic cyt c (B).

Fig. 8. As compared to the caspase-8 inhibitor z-IETD-fmk (IETD), the caspase-9-specific inhibitor, z-LEHD-fmk (LEHD), markedly inhibits the processing of procaspase-8, procaspase-3, and Bid and inhibits cytosolic accumulation of cyt c and apoptosis induced by etoposide and the ectopic overexpression of Apaf-1. HL-60/neo and HL-60/Apaf-1 cells were treated with 1.0 μM etoposide and/or 50 μM IETD or with 100 μM LEHD for 24 h, and the cells were harvested for (A) annexin V staining to determine the percentage of apoptotic cells and (B) immunoblot analysis of procaspase-8 (p55), procaspase-3 (p35), Bid, and cytosolic accumulation of cyt c. β-Actin levels were used as a control for protein loading.
may not be the sole mechanism or may be part of the feedback mechanism by which mitochondria are recruited for their role during apoptosis.

Bcl-x\(_L\) (or Bcl-2) has been shown to interact with Apaf-1 and inhibit Apaf-1-mediated activation of caspase-9 and caspase-3 and apoptosis (25, 42, 43). Bcl-x\(_L\) inhibits the binding of Bcl-\(_x\) to Apaf-1. Furthermore, Bak and Bik have been shown to disrupt the binding of Diva to Apaf-1 (44). The inhibitor of apoptosis protein family of proteins, including XIAP, has also been shown to bind and inhibit caspase-9 and caspase-3 (35, 36). Akt-1 kinase-mediated phosphorylation of caspase-9 appears to inhibit its activity and produce a similar effect (46). Taken together, these reports, along with the present data, suggest that a number of molecular determinants may regulate the Apaf-1-mediated caspase activities, which could modulate the downstream feedback activity of the MPTF on the mitochondria.

The importance of the amplifying role of the putative MPTF for mitochondrial input into apoptosis may vary according to the cellular context (47). Its importance is clearly suggested by the role that Apaf-1-mediated activation of caspase-9 and caspase-3 has been demonstrated to play during apoptosis and oncogenic transformation (24). Collectively, these aspects further underscore the greater complexity of the eukaryotic apoptosome as compared with the C. elegans molecular machinery for apoptosis.

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