Caspase-2 is an initiating caspase required for stress-induced apoptosis in various human cancer cells. Recent studies suggest that it can mediate the death function of tumor suppressor p53 and is activated by a multimeric protein complex, PIDDosome. However, it is not clear how caspase-2 exerts its apoptotic function in cells and whether its enzymatic activity is required for the apoptotic function.

In this study, we used both in vitro mitochondrial cytochrome c release assays and cell culture apoptosis analyses to investigate the mechanism by which caspase-2 induces apoptosis. We show that active caspase-2, but neither a catalytically mutated caspase-2 nor active caspase-2 with its inhibitor, can cause cytochrome c release. Caspase-2 failed to induce cytochrome c release from mitochondria with Bid−/− background, and the release could be restored by addition of the wild-type Bid protein, but not by Bid with the caspase-2 cleavage site mutated. Caspase-2 was not able to induce cytochrome c release from Bax−/− or Bak−/− mitochondria either. In cultured cells, gene deletion of Bax/Bak or Bid abrogated apoptosis induced by overexpression of caspase-2. Collectively, these results indicate that proteolytic activation of Bid and the subsequent induction of the mitochondrial apoptotic pathway through Bax/Bak is essential for apoptosis triggered by caspase-2.

Apoptotic cell death, a cellular suicidal process conserved from nematodes to humans, functions to maintain cell homeostasis and eliminate unwanted and damaged cells in organisms (1, 2). It is essential for multiple biological events, and its deregulation in humans can lead to diseases such as cancer, immune disorders, and neurodegenerative diseases (3–6). Biochemically, apoptosis is executed by a family of proteases called caspases (7, 8). Caspases can be categorized into two groups, namely upstream or initiating caspases, which are activated by autocalysis upon sensing death signals, and downstream or executioner caspases, which are activated proteolytically by upstream caspases. In mammalian cells two important caspase activation pathways, the death receptor pathway and the mitochondrial pathway (11, 12), the release of cytochrome c from mitochondria is the central event. When cytochrome c release is induced by various apoptotic stimuli, a protein complex called the apoptosome is formed to recruit and activate the upstream caspase, caspase-9, which subsequently activates caspase-3 and caspase-7 and results in apoptosis. Cytochrome c release from mitochondria is tightly regulated by the Bcl-2 family of proteins (13–17). In this family there are anti-apoptotic members (such as Bcl-2, Bcl-XL, and Mcl-1), Bcl-2 homologous (BH)3 motif-only pro-apoptotic members (e.g. Bid, Bad, Bim, etc.), and multi-BH motif pro-apoptotic members (Bax and Bak). Activation of Bax and/or Bak is essential for cytochrome c release. The anti-apoptotic Bcl-2 members function to inhibit their activation, whereas the BH3-only proteins function to either neutralize the anti-apoptotic Bcl-2 proteins or directly activate Bax/Bak.

Caspase-8/10 and caspase-9 are not the only initiating caspases. Based on protein sequence, caspase-2 is also a putative initiating caspase (7, 8). In fact, caspase-2 was the first cloned human apoptotic caspase (18). However, because targeted deletion of the caspase-2 gene in mice did not cause any apparent phenotype (19), extensive studies of the enzyme had not been pursued previously. Studies of caspase-2 have also been hampered by a lack of specific enzymological and pharmacological tools. Unfortunately, most commercially available and widely used peptide-based caspase-2 inhibitors and substrates (e.g. those based on the VDVAD or LDESD peptide sequence) can also inhibit or be cleaved by caspase-3. Therefore, it is difficult to interpret experiments in which these inhibitors or substrates were used in cultured cells or a crude cell lysate.

More recently, a series of excellent studies have begun to reveal the intriguing mechanism for caspase-2 activation and the prominent role of caspase-2 in apoptosis triggered by cancer chemotherapeutic agents. Evidence supports a critical role of caspase-2 in genotoxic-induced apoptosis in multiple, but not all, tested human cancer cell lines (20). Caspase-2 might act by mediating the function of tumor suppressor p53, which activates transcription of PIDD (21). Up-regulated PIDD, alone with other protein factors such as RAIDD, can form a multicomponent complex named the PIDDosome to recruit and activate caspase-2 (22). This mechanism, similar to those for activation of caspase-9 and caspase-8/10, indicates that induced protein multimerization is a common theme for activation of initiating caspases.

Another important question is how activated caspase-2 causes apoptosis. Biochemical studies have shown that Bid, a BH3 motif-only protein, can be cleaved by caspase-2 (23, 24). Therefore, proteolytic activation of Bid and the subsequent induction of the cytochrome c pathway present one conceivable mechanism for caspase-2 to trigger apoptosis (24). However, this scenario has not been validated in cells. Further, it was proposed recently that caspase-2 can also induce mitochondrial

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2 The abbreviations used are: BH motif, Bcl-2 homologous motif; AMC, 7-amido-4-methylcoumarin; CHO, molecular formula for aldehyde; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast; RNAi, RNA interference.
cytochrome c release in a Bid-independent manner (24–26) and that this action might even be independent of both the enzymatic activity of caspase-2 and the Bcl-2 family of proteins (25, 26). These assumptions were made based on an in vitro cytochrome c release assay using isolated mitochondria with an immunologically non-detectable level of Bid (24–26). Additionally, it is formally possible that caspase-2 might also be able to trigger apoptosis independently of any mitochondrial event. In this current study, we investigated how caspase-2 triggers mitochondrial cytochrome c release in vitro and how it induces apoptosis in cultured cells. We found that proteolytic activation of Bid is essential for cytochrome c release and the subsequent apoptosis induced by caspase-2 and that this whole process is regulated by the Bcl-2 family of proteins.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Anti-cytochrome c (catalog number 556433) and anti-human caspase-2 (catalog number 611022) antibodies were obtained from BD Pharmingen. Anti-tubulin antibody (catalog number CP06) was purchased from Calbiochem. Anti-Bid antibody was a gift from Dr. Xiaodong Wang (27). Caspase-2 peptide substrate Ac-LDESD-AMC (catalog number 218818) and caspase-2 inhibitor Ac-VDVAD-CHO (catalog number 218729) were obtained from Calbiochem.

**Plasmids**—Human caspase-2 cDNA (MGC-2181) was from the American Type Culture Collection. From caspase-2 cDNA, a DNA fragment encoding for truncated caspase-2 (residues 153–435) lacking the pro-domain was amplified by PCR and subcloned into pET28a (Novagen) for bacteria expression, and a DNA fragment encoding for the full-length caspase-2 was amplified by PCR and subcloned into the pCDNA3 vector for mammalian cell expression. pET28b-caspase-8, pET28b-caspase-2, pCDNA3.1-Bid, pCDNA3.1-Bax, and pET15b-Bcl-XL were gifts from Dr. Stanley Korsmeyer (16, 17, 28). HeLa cells and MEFs were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

**Mitochondria Preparation**—HeLa cells or MEFs were trypsinized and harvested by centrifugation at 2,000 × g for 10 min. After washing with phosphate-buffered saline, cell pellets were suspended in 5× volume of buffer B (buffer A supplemented with a mixture of protease inhibitors (Roche Applied Sciences) and 250 mM sucrose). After incubation on ice for 15 min, cells were broken by passage 20 times through 22-gauge needles. The cell lysates were subject to centrifugation at 750 × g for 10 min at 4 °C to remove nuclei and unbroken cells. The supernatants were then centrifuged at 12,000 × g for 10 min at 4 °C. The resulting mitochondrial pellets were re-suspended in Buffer B.

**Caspase-2 Activity Assay**—In the presence or absence of 400 nM caspase-2 inhibitor Ac-VDVAD-CHO the indicated amount of recombinant wild-type or mutant caspase-2 was incubated with 20 μM caspase-2 substrate (LDESD-AMC) in a total volume of 25 μl of buffer B. The generation of fluorescent signal (relative fluorescent units) indicative of caspase-2 activity was measured by an automated spectrophotometer at 30 °C. The initial rate (relative fluorescent units/min) was calculated from three independent experiments.

**Apoptosis Assay**—Mammalian cell culture transfection was performed using a polyethyleneimine reagent (Sigma). MEFs were seeded in 10-cm plates the previous day. When reaching ~70% confluence, the MEFs were transiently transfected with 200 ng of pmaxGFP (Amaxa) and other plasmids as indicated in individual experiments (10 μg of vector plasmid pCDNA3, wild-type, or C303S caspase-2 plasmid; 50 ng of Bax or Bid). The cells were harvested and stained with annexin V-PE and propidium iodide followed by analysis using FlowJo software (Tree Star Inc.). The apoptosis percentage was scored by determining the ratio of annexin-V positive cells within the GFP positive populations. The results were obtained from three independent experiments with S.D.

**Generation of Stable Cell Lines**—HeLa cells were transfected with the pSUPERIOR-Bid plasmid and selected with 0.2 μg/ml puromycin. Most cell lines, derived from puromycin-resistant colonies, stably express a low level of Bid as compared with parental cells. Two of them (Bid RNA interference (RNAi) cell lines iBid-A and iBid-B) were randomly chosen for the in vitro cytochrome c release assay. HeLa cells stably transfected with pSUPERIOR-LacZ were generated in parallel as the controls.

**RESULTS**

**Requirement of the Enzymatic Activity of Caspase-2 for Cytochrome c Release**—It has been proposed that the enzymatic activity of caspase-2 is not required for its function to release mitochondrial cytochrome c in vitro; rather, the autocatalytic processing of caspase-2 is required for this function (25, 26). To examine this possibility, we expressed and purified a series of recombinant caspase-2 proteins (all with the pro-domain deleted) including the wild-type protein, a catalytically inactive C303S mutant (designated Cs in Figs. 1 and 4), an auto-cleavage site mutant D316G mutant (designated DG in Figs. 1 and 4), an auto-cleavage site mutant D316G/Bid-3’ were annealed and subcloned into the pSUPERIOR vector (OligoEngine) by following the manufacturer's standard procedure.

**Recombinant Protein Preparation**—The pET plasmids containing caspase-2, caspase-8, Bid, or Bcl-XL were transformed into Escherichia coli BL21(DE3). Bacteria were incubated at 37 °C until the A_{600} reached 0.6–0.8. Subsequently, the expression of recombinant proteins was induced by incubating with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside for 3–5 h at 30 °C. Cells were pelleted and subject to standard nickel-nitritoltriacetic acid affinity chromatography. All purified recombinant proteins were dialyzed against buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, and 1 mM dithiothreitol) for 2 h at 4 °C. Aliquots were flash frozen with liquid nitrogen and stored at −80 °C.

**Cell Culture**—SV40-transformed wild-type, Bax−/−/Bak−/−, and Bid−/− mouse embryonic fibroblasts (MEFs) were gifts from Dr. Stanley Korsmeyer (16, 17, 28). HeLa cells and MEFs were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO2.

**Mitochondria Preparation**—HeLa cells or MEFs were trypsinized and harvested by centrifugation at 2,000 × g for 10 min. After washing with phosphate-buffered saline, cell pellets were suspended in 5× volume of buffer B (buffer A supplemented with a mixture of protease...
Caspase-2-induced Apoptosis

Catalytic activity of caspase-2 is required for its function to release cytochrome c from isolated mitochondria. A purified, recombinant caspase-2 protein (5 μg each) was resolved by SDS-PAGE and stained with Coomassie Blue R-250. The symbol used are as follows: WT, wild-type; CS, C303S mutant; DG, D316G mutant; DG/D22, D316G/D330G double mutant. B, caspase-2 enzymatic activity was completely inhibited (Fig. 1). Recombinant proteins and 400 nM caspase-2 inhibitor Ac-DAVAD-CHO (WT) were added as indicated. C, mitochondria were isolated from HeLa cells (Mit, HeLa) as described under Experimental Procedures, and the cytochrome c release assay was performed as described under Experimental Procedures. Different amounts of recombinant caspase-2 (Casp2), recombinant proteins and 400 nM caspase-2 inhibitor Ac-DAVAD-CHO (C2 Inh) were added as indicated. Cytochrome c (Cyt.c) and cytochrome oxidase IV (Cyt. Ox. IV) in mitochondrial pellets were blotted as controls.

Caspase-2, LDESD-AMC. As shown in Fig. 1B, wild-type caspase-2 possesses strong catalytic activity that can be inhibited by a low concentration of caspase-2 peptide inhibitor (Ac-VDVAD-CHO; 400 nM); the D316G mutant is also active, though its activity is severalfold weaker than that of the wild-type enzyme. Neither the C303S mutant or the D316G/D330G double mutant is functional. Subsequently, we performed an in vitro mitochondrial cytochrome c release assay by using these recombinant proteins and mitochondria isolated from HeLa cells. Consistent with previous reports, wild-type recombinant caspase-2 resulted in robust cytochrome c release from mitochondria to supernatant, whereas mutation of its catalytic site (C303S mutation) abrogated its ability to release cytochrome c (24, 25) (Fig. 1C). Interestingly, the D316G mutant, which has modest catalytic activity compared with wild-type caspase-2, also induced cleary cytochrome c release when a higher amount of enzyme was added (Fig. 1C). The D316G/D330G mutant, catalytically inactive under this condition, failed to induce cytochrome c release from mitochondria (Fig. 1C). Further, we used caspase-2 peptide inhibitor in combination with the wild-type caspase-2, which is fully processed (Fig. 1A). Under this condition, caspase-2 enzymatic activity was completely inhibited (Fig. 1B), and its cytochrome c releasing function was also blocked (Fig. 1C). Therefore, the enzymatic activity of caspase-2 is required for its function to release cytochrome c from isolated mitochondria, and the autocalytic processing alone is not sufficient for this function. Because the D316G mutant can also cause cytochrome c release (Fig. 1C), whereas the proteolytic processing pattern of this mutant is clearly different from that of wild-type caspase-2 (Fig. 1A), we suggest that autocalytic processing of caspase-2 per se does not play any specific, caspase activity-independent role in inducing mitochondrial cytochrome c release.

Regulation by the Bcl-2 Family Proteins—The Bcl-2 family proteins are the master regulators of mitochondrial apoptotic events (13–17). Therefore, we tested in vitro whether caspase-2–induced cytochrome c release is also regulated by these proteins. Indeed, addition of recombinant Bcl-XL, an anti-apoptotic Bcl-2 family member, prevented cytochrome c release caused by caspase-2 (Fig. 2A). Because activation of pro-apoptotic Bcl-2 members Bax and/or Bak is essential for mitochondrial protein release during apoptosis, we then examined whether they also form the “requisite gateway” for caspase-2–triggered cytochrome c release (17). We used mitochondria isolated from wild-type and Bax−/−Bak−/− MEFs (16, 17) for this purpose. Whereas caspase-2 activity caused cytochrome c release from mitochondria isolated from wild-type MEFs in a Bcl-XL–regulated manner (Fig. 2B), deletion of Bax and Bak genes abrogated caspase-2–induced cytochrome c release, and this loss of function could not be restored by the addition of an excess amount of recombinant Bid, a BH3 motif–only pro-apoptotic Bcl-2 family member (Fig. 2B). These experiments indicate that caspase-2–triggered cytochrome c release is subject to regulation by Bcl-2 family members and is mediated by the activation of Bax and/or Bak.

Requirement of Bid—We next examined how caspase-2 activity leads to activation of Bax/Bak. A common mechanism for activating Bax/Bak is through the action of the BH3–only Bcl-2 family members. Interestingly, it has been reported that caspase-2 can cleave Bax, a BH3–only protein (23, 24); thus, it is possible that Bid can mediate the function of caspase-2 to release cytochrome c (24). However, an alternative mechanism appears to exist, because Bid is mainly a cytosolic protein whereas recombinant, purified caspase-2 can directly release cytochrome c from isolated mitochondria (24, 25) (Figs. 1 and 2). Further, it was reported previously that immunoblotting could not detect Bid in isolated mitochondria, suggesting that Bid is not present there (24). Nevertheless, because Bid could still be present but below the level of detection by immunoblotting in these studies, we conclude that the only definitive way to examine whether Bid is required for caspase-2–triggered cytochrome c release is to utilize mitochondria from cells with the Bid gene specifically deleted. As shown in Fig. 3A, caspase-2 and caspase-8 could not induce cytochrome c release from mitochondria isolated from Bid−/− MEFs (28), whereas the addition of low levels of exogenous Bid protein restored the capability of these caspases to release cytochrome c. As expected, the cytochrome c release induced by caspase-2 and caspase-8 in the presence of Bid could also be inhibited by Bcl-XL (Fig.
Caspase-2-induced Apoptosis

3A). To further test whether cleavage of Bid by caspase-2 is required, we generated a recombinant human Bid protein with the caspase-2 and caspase-8 cleavage site mutated (aspartate 60 to alanine, designated DA in Fig. 3) (23, 24). The addition of the same amount of the mutated, non-cleavable Bid to our assay was not able to mediate caspase-2/8-triggered cytochrome c release from Bid<sup>−/−</sup> mitochondria as the wild-type Bid can do (Fig. 3B). Therefore, the in vitro cytochrome c-releasing function of caspase-2 requires proteolytic activation of Bid by the protease.

Requirement of Bid and Bax/Bak for Caspase-2-induced Apoptosis—Although Bid is essential for caspase-2-induced mitochondrial cytochrome c release in vitro, it is possible that caspase-2 can induce apoptosis in cells in a Bid- or mitochondrial pathway-independent manner. We thus measured apoptosis induced by overexpression of caspase-2 in wild-type, Bid<sup>−/−</sup>, and Bax<sup>−/−</sup>Bak<sup>−/−</sup> MEFs. In these experiments, a plasmid encoding GFP was co-transfected with wild-type or catalytically dead caspase-2, and caspase-2-triggered apoptosis was quantified in the GFP positive population of cells by annexin V staining coupled with flow cytometry. As shown in Fig. 4A, transfected wild-type but not catalytically inactive (C203S) caspase-2 was processed because of autokatalysis. Interestingly, the signal of processed caspase-2 in wild-type MEFs is weaker than that in Bid<sup>−/−</sup> or Bax<sup>−/−</sup>Bak<sup>−/−</sup> MEFs (Fig. 4A); this is because the overexpression of wild-type caspase-2 induced potent apoptosis and subsequent cell breakage and protein leakage only in wild-type MEFs but not in Bid<sup>−/−</sup> or Bax<sup>−/−</sup>Bak<sup>−/−</sup> MEFs (Fig. 4B). Caspase-2-induced apoptosis in Bid<sup>−/−</sup> and Bax<sup>−/−</sup>Bak<sup>−/−</sup> MEFs was restored by co-transfection of low levels of Bid and Bax, respectively (Fig. 4B). Therefore, these experiments demonstrate that in cultured cells caspase-2 induces apoptosis by cleaving Bid and subsequently activating the mitochondrial death pathway through Bax and/or Bak. These Bcl-2 family proteins are essential for the apoptotic function of caspase-2.

Bid-RNAi in HeLa Cells Prevents Caspase-2-induced Cytochrome c Release in Vitro—We showed that Bid mediates caspase-2-induced cytochrome c release and apoptosis in mouse embryonic fibroblasts. However, a legitimate argument is that this could be the case for only certain cell types whereas in other cells, such as HeLa cells (used in previous studies to propose a Bid-independent mechanism) (24), a Bid-independent mechanism might indeed exist. To address this possibility, we created HeLa cell lines stably transfected with a RNAi construct against Bid. As shown in Fig. 5A, the two Bid-RNAi cell lines (iBid-A and iBid-B) had much lower Bid protein levels compared with the control cell lines. Although knocking down Bid in these RNAi cell lines did not completely block apoptosis induced by overexpression of caspase-2 (data not shown), in the in vitro assay using mitochondria isolated from individual stable cell lines as indicated. Recombinant caspase-2 (100 ng) and Bid (10 ng) were added as indicated.

FIGURE 3. Cleavage of Bid by caspase-2 is required for caspase-2-induced cytochrome c release in vitro. A, cytochrome c release assay was performed using mitochondria isolated from Bid<sup>−/−</sup> MEFs (Mit, Bid<sup>−/−</sup>). Recombinant caspase-2 (Casp2; 100 ng), caspase-8 (Casp8; 100 ng), Bcl-X<sub>L</sub> (100 ng), and Bid (10 or 15 ng) were added as indicated. B, cytochrome c release assay was performed as in panel A, and 10 ng of recombinant wild-type (WT) or non-cleavable (DA, D60A) Bid was added as indicated.

FIGURE 4. Bid and Bax/Bak are required for apoptosis induced by caspase-2 in cultured cells. Wild-type (WT), Bax<sup>−/−</sup>Bak<sup>−/−</sup>, and Bid<sup>−/−</sup> MEFs were transfected with, where indicated, vector DNA, wild-type (WT), or catalytically inactive (CS, C203S) caspase-2 (Casp2), Bax, and Bid as described under “Experimental Procedures.” GFP was co-transfected in all samples in order to score transfected population. A, immunoblotting against caspase-2 in transfected cells (using an antibody that recognizes transfected human caspase-2 but not endogenous mouse caspase-2) was performed. Tubulin was immunoblotted as the loading control. B, after transfection, apoptosis was measured as described under “Experimental Procedures.” The GFP and annexin V double positive cells were detected by flow cytometry and scored as transfected apoptotic cells.

FIGURE 5. Caspase-2 fails to release cytochrome c from Bid-RNAi HeLa cell-derived mitochondria. A, HeLa cell lines stably transfected with a LacZ-RNAi (iLacZ) or Bid-RNAi (iBid) were created as described under “Experimental Procedures.” Immunoblotting showed the decreased levels of Bid in two iBid-stable cell lines, iBid-A and iBid-B. B, in vitro cytochrome c (Cyt.c) release assay was performed using mitochondria isolated from individual stable cell lines as indicated. Recombinant caspase-2 (100 ng) and Bid (10 ng) were added as indicated.
DISCUSSION

In this study, we defined the mechanisms by which active caspase-2 triggers mitochondrial cytochrome c release in vitro and by which it induces apoptosis inside cells. By using cells with a definitive genetic background for Bid and Bax/Bak, we showed that Bid is essential for mediating the apoptotic function of caspase-2. Caspase-2 cleaves and activates Bid, which, in turn, activates Bax/Bak to eventually turn on the mitochondrial apoptotic pathway. Previously, in vitro studies have suggested that caspase-2 can release cytochrome c independently of Bid (24, 25). Based on our results, it becomes clear that in those studies Bid was present on isolated mitochondria and that its levels on mitochondria, although non-detectable by immunoblotting (24), must be high enough to mediate caspase-2-triggered cytochrome c release.

Activation of Bid and the mitochondrial apoptotic pathway by caspase-2 also provides an explanation for the puzzling enzymology of caspase-2. Caspase-2, although a putative initiating caspase based on its protein sequence, does not meet the criteria for an initiating caspase because it cannot cleave and activate executioner caspases (24). Alternatively, we can consider that caspase-2 fulfills the mission of an initiating caspase in an indirect way, that is, by causing cytochrome c release caspase-2 activates caspase-9, which, in turn, activates the downstream executioner caspases. This indirect mechanism is also utilized by caspase-8, the initiating caspase for the death receptor-mediated pathway (23, 27), particularly in so-called “type II” cells in which caspase-8 cannot directly cleave executioner caspases and has to go through the mitochondrial pathway by proteolytically activating Bid (29). Strikingly, it was recently reported that caspase-2 is also required for proteolytic activation of Bid in some type II cells during TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced cell death (30). Thus, activation of Bid and the downstream mitochondrial pathway by caspase-2 is a critical component not only for genotoxic-induced apoptosis in selective cancer cells but also for certain specific death receptor-mediated apoptotic events (20, 30).

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