Caspase-2–induced Apoptosis Requires Bid Cleavage: A Physiological Role for Bid in Heat Shock–induced Death^D

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The mechanisms through which Caspase-2 leads to cell death are controversial. Here we show, using a combination of cell-free and cell culture-based approaches, that cleavage of the Bcl-2-family protein Bid is required for the induction of apoptosis by Caspase-2. Caspase-2 promoted cytochrome *c* release from mitochondria in the presence of cytosol from wild-type, but not Bid-deficient, mouse embryonic fibroblasts (MEFs). Recombinant wild-type Bid, but not a noncleavable mutant (D59E), restored cytochrome *c* release. Similarly, Bid-null MEFs were relatively resistant to apoptosis triggered by active Caspase-2, and apoptosis was restored in Bid-null cells by the expression of wild-type, but not D59E, Bid. Finally, Bid-null MEFs were substantially more resistant to apoptosis induced by heat shock, which has been shown to be dependent on apical activation of Caspase-2. The data are consistent with a model in which Caspase-2 induces apoptosis via cleavage of Bid at D59 and the subsequent engagement of the mitochondrial (intrinsic) pathway.

INTRODUCTION

Of all caspases discovered to date, Caspase-2 is the most evolutionarily conserved (Lamkanfi *et al.*, 2002). Despite this, and the fact that it was the second caspase to be identified (Kumar *et al.*, 1994), Caspase-2 remains the least understood in terms of its mechanisms of activation, its target substrates, and its potential roles in physiological cell death.

Like the initiator Caspases-8 and -9, Caspase-2 possesses a large prodomain containing a CARD protein–protein interaction motif (Colussi *et al.*, 1998). RAIDD was identified as a protein that binds to Caspase-2 via a CARD–CARD interaction (Duan and Dixit, 1997). More recently, another protein, PIDD (Lin *et al.*, 2000), was found to interact with RAIDD to form a Caspase-2 activation complex called the PIDDosome (Read *et al.*, 2002; Tinel and Tschopp, 2004), analogous to the apoptosome (a complex containing Apaf-1 and Caspase-9). However, whereas the function of the apoptosome is well established, the role of the PIDDosome, and of Caspase-2 activation itself, in various forms of apoptosis remains unclear.

It is also unclear how Caspase-2, once it is activated, kills cells. Because Caspase-2 neither processes nor activates executioner caspases directly (Guo *et al.*, 2002), it has been suggested that the ability of Caspase-2 to induce apoptosis is dependent upon its engagement of the mitochondrial pathway of apoptosis. Indeed, anti-apoptotic Bcl-2 family members can block Caspase-2–induced death (Guo *et al.*, 2002). It seems plausible that Caspase-2 may exert its effect on mito-

chondria via the BH3-only protein Bid, because Bid has been shown to be a Caspase-2 substrate (Li *et al.*, 1998), and its cleavage at the same site by Caspase-8 is known to engage the mitochondrial apoptotic pathway (Li *et al.*, 1998; Luo *et al.*, 1998) through direct activation of Bax (Kuwana *et al.*, 2002; Kuwana *et al.*, 2005) and possibly Bak. In contrast, some studies have proposed that Caspase-2–induced mitochondrial outer membrane permeabilization (MOMP) occurs independently of Bid and other cytosolic factors (Guo *et al.*, 2002; Enoksson *et al.*, 2004), and even more surprisingly, in one report, independently of Caspase-2 protease activity (Robertson *et al.*, 2004).

To date, few physiological settings have been found in which Caspase-2 plays a demonstrable role in initiating apoptosis. Mice deficient in Caspase-2 display only a mild phenotype that includes apoptotic defects in the ovary (Bergeron *et al.*, 1998). Interestingly, and perhaps related to this, a novel metabolism-related mechanism regulating Caspase-2 activation has recently been discovered in *Xenopus* oocytes (Nutt *et al.*, 2005). In mammalian somatic cells, little evidence for an obligate role of Caspase-2 in initiating apoptosis has been found. However, recently we found that cell death induced by heat shock can involve the early upstream activation of Caspase-2 (Tu *et al.*, 2005). Lymphocytes, in particular, show a marked dependence on Caspase-2 in heat shock–induced death. Furthermore, antiapoptotic Bcl-2 family members inhibit heat shock–induced death, but not Caspase-2 activation. These results argue for a role of Caspase-2 upstream of the mitochondrial apoptotic pathway after heat shock and are consistent with previous observations that mitochondrial cytochrome *c* release and Caspase-3 processing occur in this setting (Mosser *et al.*, 2000).

In the present study, we examine how heat shock and subsequent Caspase-2 activation engage the mitochondrial pathway of apoptosis. First, using a cell-free approach, we demonstrate that Caspase-2 does indeed induce MOMP, but only in the presence of cytosolic proteins, and that the induction of MOMP is completely inhibited by Bcl-x$_L$. Using

cytosol from Bid-deficient cells, we show that the Bid protein is absolutely required for MOMP induction by Caspase-2. Moreover, a cleavage-defective Bid mutant cannot substitute for wild-type Bid in this system. Next, we examine the role of Bid in intact cells and show that Caspase-2–induced cell death is impaired in Bid-null cells. Furthermore, we show that expression of wild-type Bid, but not the cleavage-defective mutant, can restore Caspase-2–mediated apoptosis in these cells. Finally, we demonstrate that Bid-deficient cells are resistant to death after heat shock, underscoring the physiological significance of Bid in cell death mediated by Caspase-2.

MATERIALS AND METHODS

Proteins and Reagents

Recombinant tBid, glutathione S-transferase (GST)-Bid, GST-D59E Bid, and Bcl-xLΔC were produced as described previously (Bossy-Wetzel and Green, 1999; von Ahsen et al., 2000). Antibodies to the following proteins were used: cytochrome c (clone 7H8.2C12; BD Biosciences, San Jose, CA), Caspase-2 (sc-626; Santa Cruz Biotechnology, Santa Cruz, CA), and Bid (Bossy-Wetzel and Green, 1999).

Preparation of Xenopus and Mammalian Subcellular Fractions

X. laevis oocyte cytosol was prepared as described previously (Newmeyer et al., 1994; von Ahsen and Newmeyer, 2000). Cytosols from Jurkat cells, wild-type mouse embryonic fibroblasts (MEFs), and Bid-deficient MEFs were obtained as described in Kluck et al. (2000). Xenopus oocyte mitochondria were prepared as described (Newmeyer et al., 1994; von Ahsen and Newmeyer, 2000). Mouse livers were dissected from young, wild-type C57BL6/J mice and placed in 10 ml of ice-cold mitochondrial isolation buffer (MIB: 68 mM sucrose, 220 mM mannitol, 10 mM KCl, 0.5 mM succinate, 10 mM HEPES/KOH, pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and 2 μM aprotinin), diced, and homogenized using a 15-ml homogenizer with a tight-fitting pestle. The homogenate was centrifuged using a Sorvall centrifuge at $600 \times g$ for 10 min to remove intact cells. Mitochondria were then pelleted from the supernatant at $3500 \times g$ for 15 min. Mitochondrial pellets were resuspended in 15 ml of MIB and centrifuged at $1500 \times g$ for 10 min. The last two steps were repeated twice. The final mitochondrial pellets were resuspended in 400 μl of ice-cold MIB, and protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Activation of Xenopus cytosolic extracts was achieved by supplementation of the extracts with 1 μM horse heart cytochrome c (Sigma-Aldrich, St. Louis, MO), followed by a 5-min incubation at 21°C.

Caspase Activity Assays

Recombinant Caspase-2 and Caspase-8 were purchased from EMD Biosciences (San Diego, CA), and their activities were determined using standard peptide substrate cleavage assays using VDVAD-AFC and IETD-AFC (EMD Biosciences), respectively. Peptide substrate cleavage rates were determined using a SpectraFluor fluorimeter and Select 2.2 software. By titration on their respective preferred peptide substrates (Thornberry et al., 1997; Troy and Shelanski, 2003) and reaction rate analysis, we determined that 160 ng of rCaspase-2 and 0.6 ng of a highly concentrated rCaspase-8 preparation yielded equivalent cleavage activities on their respective preferred peptide substrates. In Figures 1–4 and Supplemental Figures 1 and 3, X denotes this equivalent amount of activity. For effector caspase activity assays, DEVD-AFC cleavage was determined similarly.

In Vitro Reactions

In vitro transcription and translation of pHL30bWT Bid and pHL30bD59E Bid (Li et al., 1998) was performed using T7, TNT coupled reticulocyte lysate system (Promega, Madison, WI), in accordance with the manufacturer's instructions. In vitro-transcribed, -translated, and [^{35}S]methionine-labeled wild-type or D59E Bid reactions were spun through a MicroBio-Spin 30 chromatography column (Bio-Rad). Counts (5000) of radioactivity were then subjected to caspase cleavage in buffer A (250 mM sucrose, 50 mM KCl, 20 mM HEPES/KOH, pH 7.5, 2.5 mM MgCl$_2$, 10 mM dithiothreitol, and 50 μg/ml cycloheximide) at 37°C for 1 h. Samples were subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by phosphorimaging (Storm 860, ImageQuant software; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and autoradiography. Percentage of uncleaved Bid and percentage of maximal cleavage were determined using rCaspase-8 (X)-treated D59E Bid and rCaspase-2 (20X)-treated wild-type (WT) Bid as 100% references, respectively.

Cytochrome c Release Assay

Mitochondria [Xenopus at 4% (vol/vol), mouse liver, 80 μg] in buffer A or mitochondria and cytosol (Xenopus, 20 mg/ml and mammalian, 10 mg/ml) along with an ATP-regenerating system were incubated in the presence or absence of recombinant caspases. (The presence or absence of the ATP-regenerating system had no effect on cytochrome c release; our unpublished data.) Mitochondria were then pelleted by centrifugation at $12,000 \times g$ for 5 min. Supernatants and mitochondrial pellets were resolved by SDS-PAGE and transferred to nitrocellulose.

Cell Culture and Induction of Apoptosis

MEFs were cultured in Iscove's MDM (Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine, 200 μg/ml penicillin, 200 μg/ml streptomycin, 5 mM nonessential amino acids (Invitrogen), 0.1 μM β-mercaptoethanol, and 10% fetal bovine serum (FBS). Jurkat cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 200 μg/ml penicillin, 200 μg/ml streptomycin, and 10% FBS. Cells were maintained at 37°C in a humidified atmosphere with 5% CO$_2$. To induce death, cells were preincubated for 2 h in the presence or absence of caspase inhibitors (50 μM zVAD-fmk or 20 μM Q-VD-OPH [EMD Biosciences]) and treated with UV (UV Stratalinker 2400; Stratagene, La Jolla, CA) at either 2.5 or 3 mJ/cm^2 or by heat shock at 44°C for 1 h.

Transfections

MEFs were transfected using Lipofectamine 2000 following the manufacturer's standard protocol. C2-green fluorescent protein (GFP) and C320G-GFP were kindly provided by Sharad Kumar (Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, Adelaide, Australia) (Colussi et al., 1998). Cell death was assessed 12–24 h posttransfection, as described previously (Colussi et al., 1998). Death percentages were corrected for death induced in untransfected cells. Alternatively, MEFs were loaded with 5 μg of fluorescein isothiocyanate (FITC)-dextrans (approx. 77 kDa) and rCaspase-2 or GST using the Chariot protein transfection reagent (Active Motif, Carlsbad, CA) following the manufacturer's standard protocol. In all cases, the percentage of transfected cells undergoing death is depicted.

Measurement of Cell Death

Cells were treated as indicated, trypsinized, and washed in phosphate-buffered saline (PBS). For propidium iodide (PI) uptake, cells were resuspended in PBS, and 0.5 μg/ml propidium iodide was added just before analysis by flow cytometry. For annexin V binding, cells were resuspended in 200 μl of annexin V binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, and 1.8 mM CaCl$_2$) supplemented with 2 μl of annexin V-APC, -PE, or -FITC (Caltag Laboratories, Burlingame, CA).

RESULTS

rCaspase-2 Induces Mitochondrial Outer Membrane Permeabilization In Vitro

To begin to establish an apoptotic role for Caspase-2 upstream of mitochondria, we tested whether the recombinant enzyme (rCaspase-2) can act as an inducer of cytochrome c release from mitochondria, using a cell-free approach described previously (Newmeyer et al., 1994; Kluck et al., 1997). Mitochondria and cytosolic extracts prepared from X. laevis eggs were mixed together, and rCaspase-2 (Supplemental Figure 1, B and C) was added. After a 2-h incubation, we tested for the induction of MOMP, as reflected in the release of cytochrome c from mitochondria into the soluble fraction. We observed that cytochrome c was completely released within this 2-h period (Figure 1A). Similarly, when added to Xenopus mitochondria incubated in Jurkat cytosolic extracts, rCaspase-2 was able to induce complete cytochrome c release within 5 h (Figure 1B).

To examine whether this phenomenon occurred in a fully mammalian system, we incubated mitochondria isolated from mouse liver with rCaspase-2 in the presence of Jurkat cell cytosol (Figure 1C). rCaspase-2 induced the complete release of mitochondrial cytochrome c in this setting, as did an equivalent activity of rCaspase-8, a known inducer of cytochrome c release from mitochondria (Li et al., 1998). Kinetic analysis revealed that cytochrome c release occurred in as little as 30 min of incubation (our unpublished data).
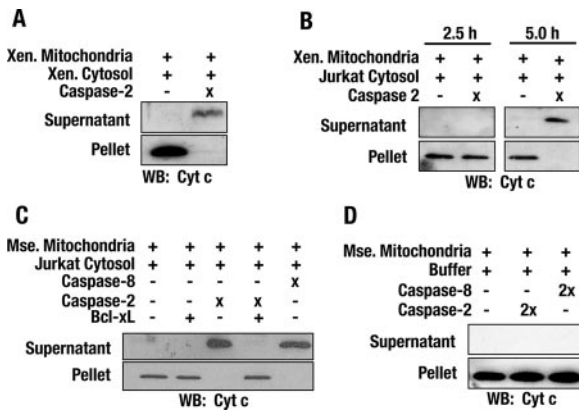
Figure 1. Caspase-2 induces MOMP in isolated mitochondria, in the presence of cytosol, that is blocked by Bcl-x_L. (A) *X. laevis* egg cytosolic extracts (20 mg/ml) and mitochondria (4%) were incubated with or without rCaspase-2 at 21°C for 2 h. (B) Jurkat cytosolic extracts (10 mg/ml) and *Xenopus* egg mitochondria (4%) were incubated with or without rCaspase-2 for 2.5 or 5 h at 21°C. (C) Jurkat cytosolic extracts (10 mg/ml) and mouse liver mitochondria (80 μg) were incubated with the indicated recombinant caspases for 1 h at 37°C. Where indicated, 6 μg of rBcl-xLΔC was used. (D) Mouse liver mitochondria (80 μg) were incubated with the indicated recombinant caspases in buffer for 1 h at 37°C. Supernatants and mitochondrial pellets were resolved by SDS-PAGE, and immunoblots were probed with an anti-cytochrome *c* antibody (BD Biosciences PharMingen, San Diego, CA).

The addition of recombinant Bcl-$x_L\Delta$C (a fragment of Bcl-x_L lacking the C-terminal hydrophobic domain) abolished the ability of rCaspase-2 to induce cytochrome *c* release (Figure 1C), suggesting that rCaspase-2 induces MOMP through a "classical" Bcl-2-inhibitable mechanism. We conclude that rCaspase-2 can induce cytochrome *c* release from both *Xenopus* and mouse liver mitochondria in the presence of *Xenopus* or mammalian cytosolic extracts.

Cytosolic Factors Are Required for Caspase 2–induced MOMP

Others have suggested that Caspase-2 can induce cytochrome *c* release from mitochondria in the absence of cytosol (Guo *et al.*, 2002; Enoksson *et al.*, 2004; Robertson *et al.*, 2004). To test the requirement for cytosolic proteins, we incubated mouse liver mitochondria in buffer along with rCaspase-2 (Figure 1D) and found that cytochrome *c* was completely retained in the mitochondrial pellet fraction. Similarly, rCaspase-8 failed to induce cytochrome *c* release in the absence of cytosol, as reported previously (Bossy-Wetzel and Green, 1999). In addition, treating *Xenopus* mitochondria with rCaspase-2 for 5 h in the absence of cytosol resulted in the complete retention of cytochrome *c* in the mitochondrial pellet fraction (Figure 2A), despite the ability of these mitochondria to respond within minutes to exogenously added recombinant cleaved Bid. Together, these results unambiguously show that the induction of MOMP in vitro by rCaspase-2 depends upon one or more factors present in cytosol.

We then analyzed the time course of MOMP induction, comparing preparations of rCaspase-2 and rCaspase-8 that were calibrated to have equivalent activity, as measured with each enzyme's preferred peptide substrate. As shown in Figure 2B, rCaspase-8 induced cytochrome *c* release from mouse liver mitochondria into the supernatant after 25 min of incubation with Jurkat cytosol. In contrast, rCaspase-2 failed to induce cytochrome *c* release at this early time point
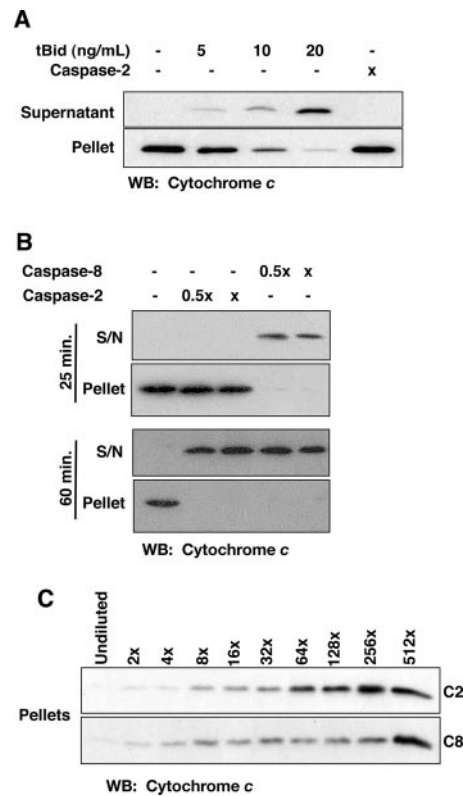

Figure 2. Cytosolic factors are required for Caspase-2–induced MOMP. (A) *X. laevis* egg mitochondria (4%) were incubated in and treated with tBid or rCaspase-2 for 5 h at room temperature. (B) Jurkat cytosolic extracts (10 mg/ml) and mouse liver mitochondria (80 μg) were incubated with the indicated recombinant caspases for 25 and 60 min at 37°C. (C) Jurkat cytosolic extracts (10 mg/ml) were incubated with the indicated recombinant caspases for 60 min at 37°C. Samples were then treated with 100 μM zVAD-fmk and serially diluted with buffer A. Mouse liver mitochondria (80 μg) were added and the samples were incubated for 2 h at 37°C. Mitochondrial pellets are shown. Supernatants and mitochondrial pellets were resolved by SDS-PAGE, and immunoblots were probed with an anti-cytochrome *c* antibody (BD Biosciences PharMingen).

but required 60 min of incubation for complete cytochrome *c* release. These results suggest that rCaspase-8 is more efficient at generating a cytochrome *c*-releasing activity than rCaspase-2. Consistent with this result, we found by serial dilution analysis that rCaspase-8–treated cytosol contained approximately four- to eightfold more cytochrome *c*-releasing activity than cytosol treated with an equivalent amount of rCaspase-2 (Figure 2C).

Bid and Its Cleavage Are Required for rCaspase-2–induced MOMP

Caspase-8 activates the mitochondrial (intrinsic) pathway of apoptosis by cleaving the Bid protein (Li *et al.*, 1998; Bossy-Wetzel and Green, 1999). Because Bid is also known to be a Caspase-2 substrate (Li *et al.*, 1998; Guo *et al.*, 2002), we reasoned that Bid could potentially mediate the induction of MOMP by Caspase-2. To determine whether Bid is required in the cell-free system described above, we incubated cytosolic extracts prepared from wild-type and Bid-deficient MEFs with mouse liver mitochondria and rCaspase-2 (Figure 3A). rCaspase-2 robustly induced cytochrome *c* release from mitochondria in the presence of wild-type cytosol. In

Figure 3. Bid and its cleavage are required for Caspase-2–induced MOMP. (A) Mouse liver mitochondria (80 μg) were incubated in cytosolic extracts (approx. 10 mg/ml) prepared from Bid$^{+/+}$ or Bid$^{-/-}$ MEFs and treated with the indicated recombinant caspases for 1 h at 37°C. (B) *X. laevis* egg mitochondria (4%) were incubated in Bid$^{-/-}$ cytosolic extract (approx. 10 mg/ml) alone or in the presence of 1 or 2 μg/ml either GST, GST-WT Bid, or GST-D59E Bid and subjected to rCaspase-2 treatment for 3 h at 21°C. Mitochondrial pellets and supernatants were resolved by SDS-PAGE and blotted for cytochrome *c* using an anti-cytochrome *c* antibody (BD Biosciences PharMingen).
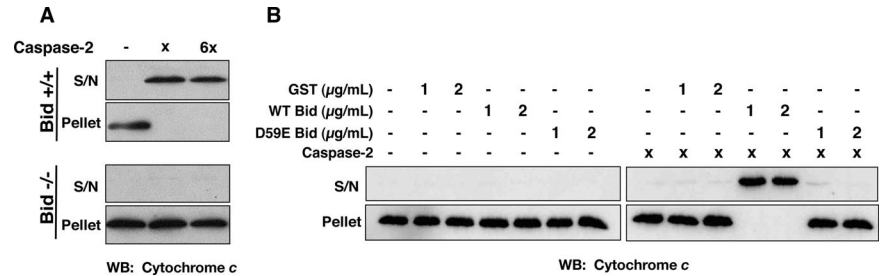
contrast, no cytochrome *c* release was detected in the presence of Bid-deficient cytosol. Similar results were obtained with *Xenopus* egg mitochondria (our unpublished data). Neither increasing the amount of rCaspase-2 used nor increasing the time of incubation to 5 h (*Xenopus* mitochondria; our unpublished data) was sufficient to allow for cytochrome c release by rCaspase-2 in the absence of Bid.

To ensure that the unresponsiveness of Bid-deficient cytosolic extracts was indeed due to the lack of Bid expression in the Bid-null MEFs, rather than some other spurious alteration in these cells, we tested the effect of adding back recombinant Bid protein to the extracts. As shown in Figure 3B, the addition of wild-type GST-Bid in combination with rCaspase-2 was sufficient to induce complete cytochrome *c* release from mitochondria, whereas wild-type GST-Bid added alone had no effect. Thus, the reintroduction of wild-type full-length Bid into Bid-deficient extracts was sufficient to restore rCaspase-2–induced cytochrome *c* release.

We next investigated whether cleavage of Bid is essential for its ability to induce MOMP. Because Caspases-2 and -8 both are known to cleave Bid at D59, we generated recombinant GST-D59E Bid (Supplemental Figure 3). As shown in Figure 3B, the addition of GST-D59E Bid, alone or in combination with rCaspase-2, failed to induce cytochrome *c* release from mitochondria. We conclude that rCaspase-2 not only requires the presence of Bid protein to induce cytochrome *c* release but also requires Bid cleavage.

rCaspase-2 Does Not Cleave Bid Efficiently

Because Caspase-2 is unable to process executioner caspases (Guo *et al.*, 2002), these data suggest that rCaspase-2 itself cleaves Bid at D59 to induce cytochrome *c* release from mitochondria. However, the time course and dilution data in Figure 2, B and C, show that rCaspase-2 cleaves Bid much less efficiently than rCaspase-8. In further experiments, we analyzed Bid cleavage by immunoblot in mitochondria/cytosol mixtures that were incubated with rCaspase-2 or rCaspase-8 (Figure 4A). In samples treated with rCaspase-8 that exhibited complete cytochrome *c* release into the supernatant fraction, a loss of full-length Bid was detected in the supernatant fraction, along with an accumulation of cleaved Bid in the mitochondrial pellet fraction. However, no such cleavage of Bid was detected in samples treated with rCaspase-2, despite the complete release of cytochrome *c* induced by rCaspase-2 under these conditions (Figure 1C). Paradoxically, therefore, Bid cleavage cannot be detected, even though the presence of cleavable Bid is required for rCaspase-2 to induce MOMP. We suspect that in these experiments, rCaspase-2 generated small amounts of cleaved Bid, which, although undetectable by immunoblot analysis, were nevertheless sufficient to induce cytochrome *c* release from mitochondria. Such potency of trace amounts of Bid protein is not unprecedented. Korsmeyer and colleagues

(Zha *et al.*, 2000) showed that minuscule amounts of cleaved Bid can suffice for cytochrome *c* release induction in cells, because the potency of Bid is greatly enhanced by a myristoylation modification that occurs after cleavage (9 fmol of Bid was sufficient to initiate cytochrome *c* release from 1 mg of mitochondria in those studies).

Next, we compared the abilities of rCaspases-2 and -8 to cleave ^{35}S-labeled in vitro-transcribed/translated Bid. Figure 4B shows that rCaspase-2 is considerably less efficient than rCaspase-8 in this assay. These data, along with titrations of rCaspases-2 and -8 against wild-type Bid (our unpublished data) suggest that rCaspase-2 has only approxi-
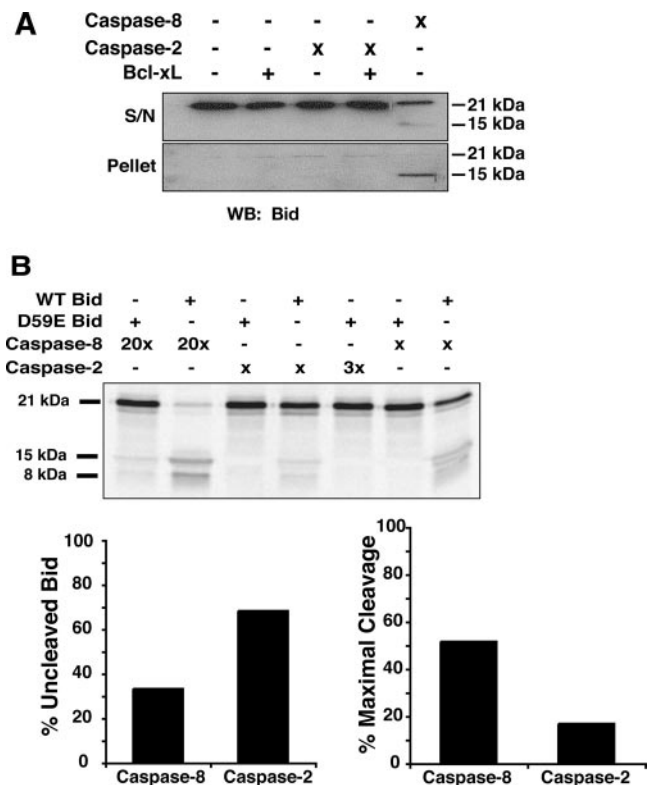

Figure 4. Caspase-2 does not cleave Bid efficiently. (A) Jurkat cytosolic extracts (10 mg/ml) and mouse liver mitochondria (80 μg) were incubated with the indicated recombinant caspases for 1 h at 37°C. Where indicated, 6 μg of rBcl-xLΔC was used. Supernatants and mitochondrial pellets were resolved by SDS-PAGE and blotted for Bid using an anti-Bid antibody (Bossy-Wetzel and Green, 1999). (B) WT or D59E Bid was in vitro transcribed, translated, and labeled with [^{35}S]methionine. Counts (5000) were then subjected to caspase cleavage for 1 h. Samples were analyzed by phosphorimaging (bottom) and autoradiography (top).

mately one-fourth the activity of rCaspase-8 toward Bid as a substrate under these conditions in vitro. For both enzymes, cleavage of Bid was specific, because the D59E mutant failed to be cleaved in either case.

Bid-deficient MEFs Are Resistant to Caspase-2–induced Death

From our data, we can conclude that, at least in vitro, rCaspase-2 requires Bid and its cleavage to induce cytochrome *c* release from mitochondria. Next, we determined whether the genetic ablation of Bid in whole cells is enough to confer resistance to Caspase-2–induced death. Because we found that transfecting cells with plasmids encoding full-length *caspase-2* cDNA induced cell death poorly, we loaded active rCaspase-2 protein into wild-type MEFs, using the Chariot reagent (see *Materials and Methods*). Although there was a fairly high background of death seen in the mock-treated samples, rCaspase-2 produced a substantial increase in apoptosis (38% annexin V-positive cells versus 21% in the mock-treated controls). In contrast, the Bid-deficient MEFs were resistant; when rCaspase-2 was loaded into these cells, we saw no increase in apoptosis over background.

As a control, to show that the Bid-deficient MEFs were not generally compromised in their apoptotic response, we examined the ability of these cells to undergo death in response to a specific stress stimulus, UV-irradiation. Both wild-type and Bid-deficient MEFs were given a dose of UV just above the threshold for apoptosis induction, and a time-course analysis of cell death was performed (Figure 5B). We found that cell death, which was inhibited by the pan-caspase inhibitor Q-VD, occurred with the same kinetics in both wild-type and Bid-deficient MEFs. Thus, these two cell lines exhibited nearly identical apoptotic responses to UV stress. Additionally, these cell lines have been shown to be equally sensitive to various other apoptosis inducers (Zinkel *et al.*, 2005).

As another test of whether Bid-deficiency protects cells from Caspase-2–induced cell death, we transfected wild-type or Bid-deficient MEFs with plasmids encoding C-terminally GFP-tagged full-length Caspase-2 (C2-GFP) or its catalytic mutant (C320G-GFP) (Colussi *et al.*, 1998). It is important to note that these Caspase-2 expression constructs encode full-length Caspase-2, including the prodomain; fusion with GFP apparently increases the spontaneous activation of the expressed Caspase-2. As shown in Figure 5C, few of the wild-type and Bid-deficient MEFs transfected with the catalytic mutant C320G-GFP underwent apoptosis (3 and 2%, respectively, after subtraction of the vector-only background), whereas the wild-type MEFs transfected with C2-GFP exhibited 27% annexin V-positive cells versus 12% for the Bid-deficient cells (Figure 5C). (The residual death seen in Bid-deficient cells transfected with C2-GFP (12%), but not C320G-GFP, presumably reflects the contribution to Caspase-2–induced death of a Caspase-2 substrate other than Bid.) These results underscore the primary importance of Bid for Caspase-2–induced apoptosis. However, they leave open the possibility that other Caspase-2 substrates may engage death pathways in some cells (see *Discussion*).

To determine whether the resistance of the Bid-deficient cells to Caspase-2 was indeed due to the absence of Bid, rather than some undefined adaptation in these cells, we tested the ability of wild-type or D59E noncleavable mutant Bid to rescue cell death induced by Caspase-2 in these cells. As Figure 5D shows, the transfection of wild-type Bid into the Bid-deficient cells was sufficient to rescue death induction by cotransfected C2-GFP (24% annexin V-positive cells compared with 6% in the GFP control transfections). Wild-type Bid cotransfected along with the C320G-GFP catalytic mutant produced only 8% annexin V-positive cells, confirm-
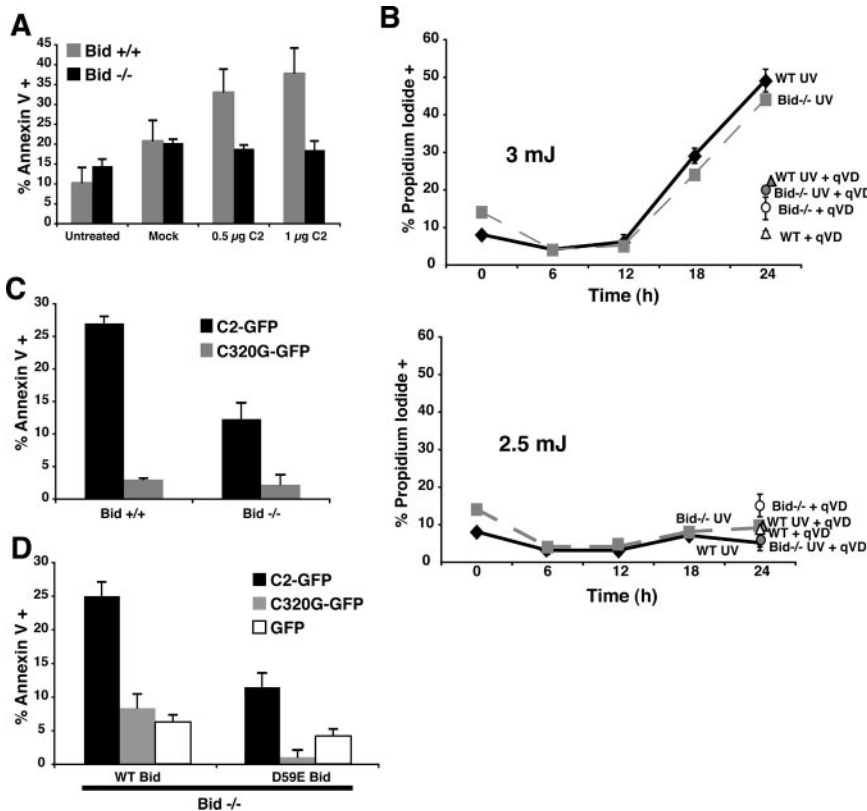

Figure 5. Bid$^{-/-}$ MEFs are resistant to Caspase-2–induced death. (A) Bid$^{+/+}$ and Bid$^{-/-}$ MEFs were transfected with 5 μg of FITC-dextrans and 0.5 or 1 μg of rCaspase-2 using the Chariot reagent. At 5 h posttransfection, cells were analyzed by flow cytometry to determine the percentage of FITC-positive cells that displayed annexin V positivity. The data were obtained from three independent experiments and error bars reflect SE. (B) Wild-type (WT) and Bid$^{-/-}$ MEFs were treated with either 2.5 or 3.0 mJ/cm² of UV. At the indicated times posttreatment, cells were incubated with propidium iodide and subjected to flow cytometric analysis. In parallel, control cells were untreated with UV and/or cultured in the presence of the pan-caspase inhibitor Q-VD (20 μM; 24-h time points only). Error bars reflect SE of triplicate samples. (C) Bid$^{+/+}$ and Bid$^{-/-}$ MEFs were transfected with 2 μg of either C2-GFP or C320G-GFP and subjected to flow cytometric analysis at 12 h posttransfection. The percentage of transfected cells that displayed annexin V positivity is shown. Error bars reflect SE; two independent experiments were performed, each in triplicate. (D) Bid$^{-/-}$ MEFs were cotransfected with either 2 μg of C2-GFP or C320G-GFP and 100 ng of either wild-type or D59E Bid and analyzed by flow cytometry at 12 h posttransfection. The percentage of transfected cells that displayed annexin V positivity is shown. Error bars reflect SE; two independent experiments were performed, each in triplicate.

ing that the catalytic activity of Caspase-2 was required for death (Figure 5D). Furthermore, when D59E Bid was reintroduced into the Bid-deficient cells along with wild-type C2-GFP, apoptosis was considerably less than with wild-type Bid (only 11% of the transfected cells were annexin V-positive compared with 4% in the GFP control). Again, the inability of D59E Bid to sustain Caspase-2–induced death argues that Bid cleavage is required for Caspase-2 to induce cell death. Because Caspase-2 fails to process other caspases directly, these results further suggest that cell death was initiated by the direct cleavage of Bid by Caspase-2.

Bid-deficient MEFs Are Resistant to Heat Shock–induced Death

The experiments described above show that the Bid protein is a key mediator of the mitochondrial death pathway activated by the deliberate introduction of exogenous Caspase-2. However, we also wanted to explore the role of Bid in a natural form of cell death dependent on Caspase-2. Recently, we discovered that Caspase-2 activation is an early initiating step in cell death induced by heat shock (Tu *et al.*, 2005). To examine the role of Bid in heat shock–induced apoptosis, we subjected wild-type and Bid-deficient MEFs to a 1-h heat shock at 44°C in the presence or absence of

Figure 6. Bid$^{-/-}$ MEFs are resistant to heat shock–induced death. (A) Wild-type (WT) and Bid$^{-/-}$ MEFs were heat shocked at 44°C for 1 h and then put at 37°C for the indicated times. At each time point, cells were incubated with propidium iodide and subjected to flow cytometric analysis. In parallel, control cells were pretreated with 20 μM Q-VD and/or not subjected to heat shock (24-h time points only). The percentage of PI-positive cells is shown. Error bars reflect SE of triplicate samples. (B) A model showing the dependence of Caspase-2 on Bid and its cleavage to induce cytochrome *c* release and subsequent cell death. Other Caspase-2 substrates may mediate low levels of Bid-independent cell death or may participate in Bid-dependent death. The pleiotropic effects of heat shock are depicted: Caspase-2 activation, leading to Bid cleavage as well as the sensitization of mitochondria to tBid-induced MOMP (Pagliari *et al.*, 2005).

caspase inhibitor and performed a kinetic assessment of cell death. Figure 6A shows that wild-type MEFs were sensitive to heat shock, because ~45% were dead (PI-positive) within 24 h. This death was greatly reduced by the caspase inhibitor Q-VD, arguing that a large portion of the cells died by caspase-dependent apoptosis. In contrast, Bid-deficient MEFs were comparatively resistant to heat shock–induced death. At 24 h after heat shock, only 17% of the Bid-null cells were PI-positive; moreover, the caspase inhibitor Q-VD was unable to rescue this residual population of cells, suggesting that they underwent caspase-independent, perhaps necrotic, death. (Similar results were obtained after heat shock at 43°C; our unpublished data.) We conclude from this experiment that Bid is a principal mediator of heat-shock-induced apoptosis, a form of cell death largely dependent on Caspase-2.

DISCUSSION

In this study, we demonstrated that the ability of Caspase-2 to induce cytochrome *c* release and subsequent cell death is dependent upon the protein Bid. We showed this first in cell-free experiments, in which we mixed cytosol and isolated mitochondria and added active rCaspase-2 in amounts similar to those of endogenous (unactivated) Caspase-2 present in cytosolic extracts (Supplemental Figure 1A). In these experiments (Figures 1 and 2), we observed that rCaspase-2 induced the release of cytochrome *c* from mitochondria only in the presence of cytosol, regardless of whether the cytosol was prepared from *Xenopus* eggs or mammalian (human or mouse) somatic cells and regardless of whether the mitochondria were isolated from *Xenopus* eggs or mouse liver. We conclude that at least one cytosolic factor is required for the induction of cytochrome *c* release by Caspase-2, in vitro.

Further experiments showed that Bid, a Caspase-2 substrate, is a cytosolic factor essential for cytochrome *c* release induced by rCaspase-2 in vitro. In the presence of cytosol derived from wild-type, but not Bid-deficient cells, mitochondria underwent cytochrome *c* release upon rCaspase-2 treatment. Moreover, the readdition of recombinant wild-type Bid to Bid-deficient cytosol fully restored Caspase-2–induced cytochrome *c* release. In contrast, the noncleavable D59E Bid mutant failed to restore Caspase-2–induced cytochrome *c* release. Thus, our results argue that in this cell-free system, the presence of caspase-cleavable Bid protein is necessary for Caspase-2 to initiate mitochondrial outer membrane permeabilization. Although our results do not directly prove that Caspase-2 is itself responsible for Bid cleavage in extracts, there are two reasons to hypothesize this: first, purified rCaspase-2 can cleave Bid in vitro (Figure 4); and second, the other principal candidate enzyme, Caspase-8, which is known to activate the mitochondrial apoptosis pathway through cleavage of cytoplasmic Bid (Li *et al.*, 1998; Luo *et al.*, 1998; Bossy-Wetzel and Green, 1999), cannot be directly activated by Caspase-2 (Guo *et al.*, 2002).

Previous publications have reported that Caspase-2 can induce cytochrome *c* release from mitochondria in the absence of cytosol (Guo *et al.*, 2002; Enoksson *et al.*, 2004) and also when treated with alkylating agents to produce a 95% reduction in catalytic activity (Robertson *et al.*, 2004), suggesting that Caspase-2 may either cleave a substrate located on the mitochondrial outer membrane, or even act via a surprising noncatalytic mechanism. These results clearly conflict with our data. Moreover, even our own data, at first glance, is paradoxical: on one hand, the presence of caspase-cleavable Bid was required for cytochrome *c* release induced
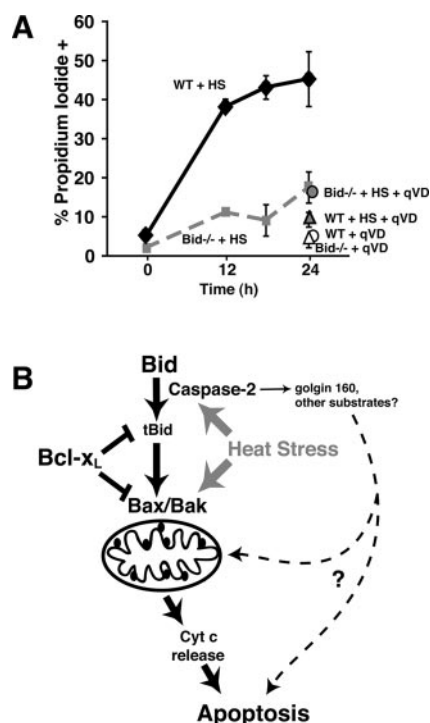
by rCaspase-2 (Figure 3B), and on the other hand, Bid cleavage was undetectable (Figure 4A).

However, a possible resolution for these apparent discrepancies is that, as Korsmeyer *et al.* have shown, the potency of Bid is dramatically enhanced by a myristoylation modification that occurs once Bid is cleaved; as a result, minuscule amounts of cleaved Bid are sufficient to induce robust cytochrome *c* release (Zha *et al.*, 2000). Previous reports have attempted to rule out a requisite role for Bid in Caspase-2–induced cytochrome *c* release on the basis of immunodepletion experiments or have shown only a partial/minimal release of cytochrome *c* in the absence of cytosol using mitochondria isolated from either HeLa cells or rat liver (Guo *et al.*, 2002; Enoksson *et al.*, 2004; Robertson *et al.*, 2004) Tiny amounts of Bid that fail to be detected postimmunodepletion or that contaminate mitochondrial preparations could be sufficient to mediate Caspase-2–induced cytochrome *c* release. Our experiments, using cytosolic extracts from cells genetically deficient in Bid expression, allow us to be confident that not even trace amounts of soluble Bid are present. Furthermore, our mitochondrial preparations from *X. laevis* eggs and mouse liver apparently do not contain appreciable amounts of functional Bid protein accessible to Caspase-2, as rCaspase-2 failed to induce cytochrome *c* release from either type of mitochondria except in the presence of cytosol containing Bid.

Caspase-2 is relatively inefficient at cleaving Bid, as we showed by comparing preparations of rCaspases-2 and -8 that were calibrated according to activity in cleaving their preferred peptide substrates (Figure 4B). Similarly, we saw that rCaspase-2 induced cytochrome *c* release in cytosol/mitochondria mixtures with slower kinetics than equivalent amounts of rCaspase-8 (Figure 2B). Moreover, rCaspase-8–treated cytosol withstood more dilution than rCaspase-2–treated cytosol before losing its ability to induce cytochrome *c* release, suggesting that rCaspase-8 generated higher levels of cytochrome *c*-releasing activity than rCaspase-2 (Figure 2C). Together, these experiments are consistent with the hypothesis that Caspase-2 is less effective than Caspase-8 in initiating the mitochondrial apoptosis pathway, at least in part because Caspase-2 cleaves Bid inefficiently.

Importantly, our results obtained with cell-free systems were corroborated in whole cells. Using two independent systems of protein expression, we showed that Caspase-2–induced death is largely dependent upon Bid. However, overexpression of C2-GFP (but not a catalytically inactive mutant) did induce the death of a small proportion of Bid-deficient cells (Figure 5, C and D). This residual Caspase-2–dependent death was not detected when rCaspase-2 protein was introduced into the Bid-deficient cells (Figure 5A) and may reflect an alternative Caspase-2–induced pathway. For example, Caspase-2 has been shown to cleave Golgin-160 in the Golgi complex, and this cleavage event was required for the disintegration of the Golgi during cell death (Mancini *et al.*, 2000). Perhaps the residual Bid-independent death we observed reflects this pathway. Such alternative apoptotic pathways initiated by Caspase-2, although independent of Bid, may still make use of the Bax/Bak-dependent mitochondrial (intrinsic) pathway, and if so would presumably involve the activation of BH3-only proteins other than Bid. It will be of interest to examine whether cytochrome *c* is released in cells that undergo Bid-independent apoptosis initiated by Caspase-2.

Full-length Caspase-2 possesses a large prodomain that has been shown to interact with other proteins, such as RAIDD in the PIDDosome (Duan and Dixit, 1997; Lin *et al.*, 2000; Tinel and Tschopp, 2004), whereas in rCaspase-2 these

sequences are not present. Possibly, full-length Caspase-2 could induce cell death via a Bid-independent pathway governed by these prodomain interactions. The results in Figure 5, A, C, and D, could reflect this difference. Furthermore, the prodomain of Caspase-2 has been shown to contain nuclear localization sequences (Colussi *et al.*, 1998), and a nuclear role for Caspase-2 in cell death and cytochrome *c* release (Paroni *et al.*, 2002) has been described. Nuclear localization could perhaps influence the ability of Caspase-2 to interact with Bid or other potential substrates. However, it should be noted that the Bid protein is well below the size limit for entry into the nucleus by passive diffusion (Newmeyer *et al.*, 1986) and should therefore gain access to the nucleus, even if it has no nuclear localization signal. Indeed, Bid has recently been shown to function in the nucleus in a nonapoptotic capacity (Kamer *et al.*, 2005; Zinkel *et al.*, 2005). By the same token, Caspase-2– cleaved Bid is predicted to be able to exit the nucleus by passive diffusion.

Heat shock has recently been discovered to be a physiological cell stress that activates Caspase-2 upstream of the mitochondrial apoptotic pathway; lymphocytes deficient in Caspase-2 are resistant to heat shock–induced death (Tu *et al.*, 2005). In support of our contention that Bid is an essential mediator of apoptosis initiated by Caspase-2, we found that Bid-deficient transformed fibroblasts were resistant to heat shock–induced death (Figure 6A). Tu *et al.* (2005) also showed that Bcl-x_L blocks heat shock–induced apoptosis, but not Caspase-2 activation. Together with this result, our data derived from experiments using both cell-free and cell-based approaches argue for a model (Figure 6B) in which Bid plays an essential role in the engagement of the mitochondrial apoptosis pathway downstream of apical Caspase-2 activation, as is the case after heat shock. As a corollary, our data also argue that other Caspase-2 substrates, such as Golgin-160, although important in some forms of apoptosis (Maag *et al.*, 2005), are not sufficient to mediate heat shock–induced death in the absence of Bid, at least under the conditions used in our studies. Alternatively, the observation of a small amount of Bid-independent cell death that occurs upon expression of wild-type, but not catalytically inactive, Caspase-2 (Figure 5, C and D) suggests that, under some conditions, other Caspase-2 substrates can be sufficient to mediate cell death in the absence of Bid, although perhaps inefficiently.

It should be mentioned that heat shock may activate multiple cell death pathways (especially at higher temperatures; our unpublished data), some of which may not be mediated by Caspase-2 and Bid. For example, in Figure 6A, at 24 h postheat shock, the residual death seen in the Bid-deficient cells (17% compared with 45% in the wild-type cells) was not blocked by caspase inhibitor, suggesting that this Bid-independent death was also caspase-independent, i.e., perhaps necrotic.

While this article was in preparation, Gao and colleagues published similar results stressing the importance of Bid in Caspase-2–induced cytochrome *c* release from mitochondria and subsequent cell death; moreover, these authors found, as did we, that a cleavage-defective Bid mutant could not restore cytochrome *c* release in vitro (Gao *et al.*, 2005). Our studies have reached the same conclusion and also extend the model to show that the cleavage, not merely the presence, of Bid in whole cells is essential for Caspase-2 to induce cell death (Figure 5D). Furthermore, our data describe a physiologically significant role for Bid in Caspase-2–dependent, heat stress-induced death (Figure 6A).

Recently, an additional effect of heat shock was observed that may further enhance the ability of trace amounts of

cleaved Bid to effect cytochrome *c* release. Pagliari *et al.* (2005) observed that heat stress can directly activate the multidomain proteins Bax and Bak, an effect that is blocked by the presence of endogenous levels of anti-apoptotic Bcl-2 family members (such as Bcl-x$_L$) in cytosol. Intriguingly, they found that mitochondria subjected to heat in the presence of cytosol, although failing to release cytochrome *c*, became hypersensitive to the effects of cleaved Bid, increasing their sensitivity ~1000-fold compared with untreated mitochondria. In heat-shocked cells, then, it is conceivable that mitochondria become sensitized to small amounts of Bid cleaved by activated Caspase-2. This model accounts for the potential roles for Caspase-2, Bid, and direct effects of heat on Bax and Bak and suggests a unifying scheme to explain how even undetectably small amounts of Bid cleavage by Caspase-2 after heat shock can promote cytochrome *c* release and apoptosis.

ACKNOWLEDGMENTS

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