Rapid Kinetics of tBid-induced Cytochrome c and Smac/DIABLO Release and Mitochondrial Depolarization*

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Mitochondria play a key role in the commitment of cells to apoptosis through the release of cytochrome c, Smac/DIABLO, and other apoptosis-inducing factors from the intermembrane space to the cytosol (cytochrome c, Ref. 1; apoptosis-inducing factor, Ref. 2; caspases, Refs. 3–5; Smac/DIABLO, Refs. 6, 7; endonuclease G, Ref. 8). Released cytochrome c has been identified as binding to Apaf-1 and then activating caspase-9 in the apoptosome (9, 10). Recently, it has also been shown that released Smac/DIABLO promotes caspase activation by binding to IAP, an inhibitor of the amplification of caspase activation (11).

Several mechanisms have been proposed to precipitate the release of cytochrome c and other apoptotic proteins. Among these pathways are Bcl-2 family proteins and/or components of the permeability transition pore (PTP),1 voltage dependent anion channel, adenine nucleotide translocator, and cyclophilin D. In addition to playing a central role in the release of apoptotic proteins, mitochondrial membrane permeabilization also affects the mitochondrial metabolic machinery that provides ATP for many cellular functions including caspase activation. Depletion of cytochrome c impairs H+ extrusion from the mitochondrial matrix, whereas permeabilization of the inner mitochondrial membrane results in dissipation of the H+ gradient that drives the $ATPase. Although analysis of the proteins involved in mitochondrial membrane permeabilization has greatly advanced, formation of the pore is not likely to be the sole determinant for the escape of apoptotic factors from the mitochondria and changes of the metabolic machinery. For example, individual mitochondria are assembled into a complex and dynamic structure that has been visualized as a continuous network in some cell types (12, 13). Furthermore the intermembrane space of each mitochondrion is divided into discrete compartments (14, 15). Thus luminal communication between individual mitochondria and between subdomains of the intermembrane space may also influence the release of the apoptotic factors. To investigate the subcellular spatial pattern of cytochrome c distribution, Heiskanen et al. (16) and Goldstein et al. (17) used cytochrome c fused to green fluorescent protein (GFP) (cyto c-GFP) to show that cytochrome c release occurs in a coordinated manner in single cells and involves either subsets or the entire population of mitochondria in the cell during apoptosis. However, in these studies the time course of cyto c-GFP redistribution from mitochondria to the cytosol included formation of the apoptotic signal delivered to the mitochondria as well as the subsequent permeabilization of the mitochondrial membrane.

1 The abbreviations used are: PTP, permeability transition pore; tBid, truncated Bid; GFP, green fluorescent protein; cyto c-GFP, cytochrome c fused to GFP; TMRE, tetramethylrhodamine ethyl ester; FCCP, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone.
The Bcl-2 family proteins are regulators of cell death in response to a variety of apoptotic stimuli either by acting as anti-apoptotic (e.g. Bcl-2, Bcl-xL) or pro-apoptotic molecules (e.g. Bax, Bak, Bad, and Bid). The N-terminal BH4 domain is restricted to some anti-apoptotic proteins, whereas BH1, BH2, and BH3 domains are commonly found in both the anti- and pro-apoptotic subfamilies. Recent work including structural studies and mutagenesis revealed that the BH1, BH2, and BH3 domains are essential for the dimerization of Bcl-2 family proteins (reviewed in Refs. 18 and 19). The heterodimerization of the anti-apoptotic Bcl-xL with pro-apoptotic Bax requires the BH1, BH2, and BH3 domains of the Bcl-xL and BH3 domain of the Bax (20). Several apoptosis promoters have been identified that have only the BH3 domain. These proteins, named collectively as the "BH3-only" molecules include Bad, Bid, Bik, Bim, Blk, Hrk, and EGL-1 (recently reviewed in Refs. 21 and 22) and can associate with prosurvival Bcl-2/Bcl-xL or with pro-apoptotic Bax/Bak. Translocation of pro-apoptotic Bcl-2 family proteins to the mitochondria results in permeabilization of the outer mitochondrial membrane and induces cytochrome c release (Bax, Refs. 23–27; Bid, Refs. 28, 29; Bak, Ref. 30; Bad, Refs. 31, 32; Bim, Ref. 33).

During Fas and TNFα receptor-mediated cell death, caspase-8 is the initial caspase activated and is important for processing the pro-apoptotic molecule Bid, which in turn evokes cytochrome c release from mitochondria (28, 29, 34–37). Interestingly, granzyme B, a serine protease that also cleaves Bid, induces cytochrome c release and apoptosis (38–40). Specifically, Bid is cleaved into two major fragments, a 15.5-kDa BH3 domain-containing C-terminal fragment (tBid) and a 6.5-kDa N-terminal fragment (28, 29). tBid has been shown to undergo N-myristoylation (41) and translocation to the mitochondria (28, 29). At the mitochondria, tBid interacts with other Bcl-2 family proteins (Bcl-xL, Ref. 42; Bax, Refs. 42–44; Bak, Ref. 45), facilitates cytochrome c release from the intermembrane space to the cytosol (28, 29), and results in respiratory dysfunction (46). Studies with different experimental systems have resulted in numerous models for the mechanism by which tBid induces cytochrome c release such as ion channel activity (47), destabilization of the lipid bilayer (48), interaction of tBid with pro-apoptotic Bcl-2 family proteins (Bax, Refs. 42–44; Bak, Refs. 45), and components of the PTP (49). However, because of the time-consuming process of cell fractionation, the temporal pattern of tBid-induced cytochrome c release and metabolic dysfunction has not been determined.

To better understand the mechanisms of Bid-induced permeabilization of the outer mitochondrial membrane, we established direct measurements of the kinetics of tBid-induced cytochrome c release, Smac/DIABLO release, and ΔΨm loss at a resolution of seconds in permeabilized HepG2 cells. Thus, for the first time, we elucidated the temporal relationships between the tBid-induced release of cytochrome c and Smac/ DIABLO and function of the mitochondrial metabolic machinery.

MATERIALS AND METHODS

Buffers and Reagents—Prior to permeabilization, the cells were washed in a Ca2+-free buffer that was prepared as follows: 120 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 0.2 mM MgCl2, 0.1 mM EGTA, and 20 mM HEPES-NaOH pH 7.4. The permeabilization buffer (intracellular-like medium) consisted of 120 mM KCl, 10 mM NaCl, 1 mM KH2PO4, 20 mM HEPES-Tris pH 7.2, and 1 μg/ml anti-pain, leupeptin, and pepstatin. Digitonin was added to 10 μg/106 cells. In some experiments 5%
Dextran was also included in the medium. The medium was also supplemented with either succinate (2 mM) or malate/glutamate (1 and 5 mM, respectively). The measurements were carried out in the presence or absence of 2.5 μg/ml oligomycin, 2 mM MgATP, and an ATP regenerating system composed of 5 mM phosphocreatine, 5 units/ml creatine kinase. Recombinant proteins, Bcl-xL, full-length Bid, and caspase-8-cleaved Bid (tBid) were produced as described earlier (43, 50).

Monoclonal anti-cytochrome c antibody (clone 7H8.2C12) was from BD PharMingen. The anti-Smac/DIABLO antibody was from a culture supernatant of hybridoma cells (11). The goat anti-mouse horseradish peroxidase-conjugated secondary antibody was obtained from Santa Cruz Biotechnology, Inc.

**Cell Culture and Transfection**—HepG2, human hepatoma cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in humidified atmosphere (CO2, 5%) and were plated for the experiments onto coverslips or 75-cm2 flasks as described previously (51). For transient transfections, cells were transfected using LipofectAMINE 2000 with plasmid DNA (pEGFP-Mito Vector, mito-GFP CLONTECH, or cytochrome c green fluorescence protein) keeping total DNA at 2 μg/ml. After 6 h, the cells were placed in a normal growth medium. Imaging was carried out 48 h after transfection.

**Immunocytochemistry**—Adherent cells transfected with mito-GFP were permeabilized for 5 min and incubated in the presence or absence of 10 nM tBid for 20 min at 35 °C. Fixation of the cells was carried out using 3% paraformaldehyde. Prior to incubation with a monoclonal cytochrome c or Smac/DIABLO antibody (1:200 and 1:500 dilution, respectively) the cells were incubated in the presence of 200 μg/10^6 cells digitonin for 1 h at room temperature. Lengthening incubation or a higher digitonin concentration (up to 1,500 μg/10^6 cells) did not increase labeling of the mitochondrial cytochrome c. To determine the subcellular distribution of the cytochrome c or Smac/DIABLO antibody, the Alexa Fluor 568 anti-mouse signal amplification kit was used as described by the manufacturer (Molecular Probes).

**Confocal Imaging of Cyto c-GFP and Immunofluorescence in Permeabilized HepG2 Cells**—Confocal imaging was carried out using a Bio-Rad MRC1024/2P imaging system equipped with a Kr/Ar-ion laser source (488- and 568-nm excitation) fitted to an Olympus IX70 inverted microscope. Images were captured using a ×60 PlanApo objective. The

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**Fig. 2.** Association of a ΔΨm loss with Bid-mediated cytochrome c release from mitochondria. ΔΨm was monitored in permeabilized HepG2 cells treated with different doses of tBid (A) and full-length Bid (B) for 6 min in the presence of oligomycin. C, dose-response for the ΔΨm loss evoked by tBid and full-length Bid. The data represent mean ± S.E. from three experiments. The permeabilized cells were then centrifuged, and the supernatants (cytosol) were resolved on 15% SDS-PAGE followed by immunoblotting for cytochrome c. D, HepG2-permeabilized cells were treated with different concentrations of tBid, and the released cytochrome c was analyzed as described under "Experimental Procedures." E, similarly, full-length Bid-induced cytochrome c release from mitochondria was analyzed as described above. F, the effect of tBid on cytochrome c distribution was also quantitated by loading three different amounts of cytosol and pellet protein onto the gel. These data are representative of three independent experiments.
pixel size was 0.2 μm, and the axial resolution was ~1 μm. Cyto c-GFP was excited at 488 nm, whereas propidium iodide was excited at 568 nm. In the immunocytochemistry experiments, mito-GFP and Alexa Fluor 568 were excited at 488 nm and 568 nm, respectively. In absence of the primary antibody, the fluorescence signal was minimal (~5%) with the specific settings used to record the images shown in Fig. 1.

Evaluation of ΔΨ<sub>m</sub> and Cytochrome c (Ca<sup>2+</sup>)<sub>i</sub> (Co<sup>2+</sup>)<sub>i</sub> in Permeabilized HepG2 Cells—Fluorimetric measurements of ΔΨ<sub>m</sub> were carried out as described previously (51). Briefly, suspensions of cells (6.5 × 10<sup>6</sup> cells/1.6 ml, equivalent to 2 mg of protein, determined by Bio-Rad protein assay) were incubated in permeabilization medium supplemented with 40 μmol (10 μg/ml) digitonin in the presence of 800 nM JC1 in a fluorometer cuvette. Cell permeabilization was evaluated by tripyn blue exclusion, and after 5 min incubation >95% of the cells were tripyn-positive. Fluorescence was monitored in a multiwavelength-excitation dual wavelength-emission fluorimeter (Delta RAM, PTTI) using 490-nm excitation/535-nm emission for the monomeric form and 570-nm excitation/595-nm emission for the J-aggregate of JC1 (57). ΔΨ<sub>m</sub> is shown as a ratio of the fluorescence of J-aggregate (aqueous phase) and monomer (membrane-bound) forms of JC1.

ΔΨ<sub>m</sub> was also measured using tetramethylrhodamine ethyl ester (TMRE). The cell permeabilization medium was supplemented with 1 μM TMRE. After an 8-min incubation, the cells were centrifuged (125 × g for 5 min) and resuspended in fresh medium devoid of TMRE. Fluorescence was monitored using 540-nm excitation and 580-nm emission. When the time course of the JC1 ratio was compared with the response of the TMRE fluorescence (Fig. 6C, upper panel), the cells incubated with JC1 were also centrifuged after cell permeabilization, but JC1 was added to the medium used to resuspend the pellet of permeabilized cells.

(Ca<sup>2+</sup>)<sub>i</sub> was measured using fura2FF as described previously (51). All of the experiments were carried out with constant stirring at 35 °C (Figs. 1–6, 8) or at 25 °C (Fig. 7).

Detection of Cytochrome c and Smac/DIABLO Release by Western Blotting—At the end of the fluorimetric measurements of ΔΨ<sub>m</sub> in suspensions of permeabilized cells, cytosol was separated from the membranes by centrifugation at 10,000 × g for 10 min. Alternatively, suspensions of the permeabilized cells were rapidly filtered (0.45-μm pore size, cellulose acetate membrane, Whatman) using a syringeless filter device. Supernatant or membrane proteins (25 μg) were resolved on a 15% SDS-polyacrylamide gel, and Western blotting was carried out for cytochrome c. Mouse monoclonal anti-cytochrome c antibody was used with goat anti-mouse peroxidase conjugate for detection. Bound antibody was detected by enhanced chemiluminescence using the SuperSignal reagent (Pierce). Blots were stripped for reprobing by incubation in 0.5 μM Tris/HCl pH 6.8 and 2% SDS (final concentration) containing β-mercaptoethanol for 30 min at 50 °C, followed by brief washing in Tris-buffered saline Tween. Blots were then blocked and reprobed with fresh mouse monoclonal antibody for Smac/DIABLO as described above.

Fluorimetry recordings, Western blots, and confocal images are representative of two to six independent experiments. The data combined from separate experiments are shown as mean ± S.E. Significance of differences from the relevant controls was calculated by the Student’s t test.

RESULTS AND DISCUSSION

Spatial Pattern of tBid-induced Depletion of Mitochondrial Cytochrome c and Smac/DIABLO—Double labeling with a mito-GFP demonstrated that cytochrome c and Smac/DIABLO immunofluorescence was completely coincident with the mitochondria in permeabilized HepG2 cells (Fig. 1, A and B, upper row). Addition of tBid (10 nM) resulted in a loss of cytochrome c and Smac/DIABLO immunofluorescence for all of the intracellular structures that were double-labeled with mito-GFP (Fig. 1, A and B, lower row), showing that tBid evoked an essentially complete release of cytochrome c and Smac/DIABLO from the entire mitochondrial population. In immunostaining studies with intact cells, released cytochrome c increased the global cytosolic signal. However, in our experiments, released cytochrome c diffused to the bulk medium, and so an increase in the cytosolic immunostaining did not complicate evaluation of the extent of mitochondrial cytochrome c depletion. To visualize mitochondrial cytochrome c before and after addition of tBid in the same cells, we used cyto c-GFP-expressing permeabilized cells. As shown in Fig. 1C, the mitochondrial GFP fluorescence disappeared by 15 min after the addition of tBid (10 nM), suggesting that the cyto c-GFP fusion protein was released from mitochondria to the cytosol (Fig. 1C). Taken together, visualization of the mitochondrial cytochrome c and Smac/DIABLO in single cells demonstrated that tBid evoked release of both pro-apoptotic proteins from all regions of the intermembrane space in every mitochondrion. To our knowledge, these experiments provided the first direct evidence for tBid-induced Smac/DIABLO release from mitochondria to the cytosol.

Subnanomolar Doses of tBid Elicit Rapid Release of Cytochrome c and Loss of ΔΨ<sub>m</sub>—Mitochondrial membrane potential is an important measure of the barrier function of the inner mitochondrial membrane and the energy metabolism of the cell. The mechanisms that have been proposed to underlie tBid-induced cytochrome c release include 1) the opening of the permeability transition pore and, in turn, mitochondrial depolarization prior to cytochrome c release (49); 2) selective permeabilization of the outer mitochondrial membrane and loss of ΔΨ<sub>m</sub> secondary to cytochrome c release (46, 52); and 3) release of cytochrome c without depolarization (52, 53). To determine the relationship between the Bid-induced cytochrome c release and ΔΨ<sub>m</sub> loss, we monitored the tBid-induced changes of ΔΨ<sub>m</sub> fluorimetrically in suspensions of permeabilized cells, subsequently separated cytosol from mitochondria, and evaluated cytochrome c release by Western blot analysis. Although extramitochondrial ATP was available in the bathing medium, the presence of an F<sub>0</sub>F<sub>1</sub>-ATPase inhibitor, oligomycin, allowed us to observe depolarization when the oxidative metabolism became impaired.

The loss of ΔΨ<sub>m</sub> after treatment with tBid exhibited both a dose-dependent lag time and rate of depolarization (Fig. 2, A and C). Five minutes of treatment evoked a half-maximal de-
polarization response at 0.5–1 nM tBid (Fig. 2C). In contrast, full-length Bid was unable to facilitate depolarization at low concentrations (Fig. 2B). A delayed and small ΔΨm loss appeared at a 50 nM concentration of Bid. Based on the dose response curves, tBid was ~100-fold more potent at evoking mitochondrial depolarization (Fig. 2C).

After measurement of the ΔΨm, the cells were centrifuged, and cytochrome c was evaluated in the cytosolic fractions. In the control none or a very small amount of cytochrome c was observed in the cytosol (Fig. 2, D and E; 0 nM tBid). Notably, no significant increase in cytosolic c was recorded when a 2-fold higher concentration of digitonin was used for cell permeabilization (n = 2, data not shown). Thus permeabilization of the cells with digitonin did not damage the mitochondrial membrane barrier. As reported for intact cells exposed to tBid-dependent apoptotic agents (17, 28, 29), incubation with tBid resulted in the appearance of cytochrome c in the cytosol. We could visualize essentially maximal cytochrome c release at and above 0.5 nM tBid (Fig. 2D). Similar to the results with ΔΨm, low concentrations of full-length Bid failed to cause cytochrome c release. Bid induced cytochrome c release only at 100-fold higher concentrations as compared with tBid (Fig. 2E). The effect of tBid on cytochrome c distribution was also quantified by loading three different amounts of cytosol and pellet protein to the gel (Fig. 2F). In the naive cells cytochrome c was exclusively in the mitochondria-rich pellet, whereas in tBid-treated cells (2.5 nM for 5 min) cytochrome c was mostly in the cytosol. These results show that tBid-induced cytochrome c release was coupled to mitochondrial depolarization, reflecting an increase in the permeability of the inner mitochondrial membrane or impairment of oxidative energy metabolism. Further, these studies confirmed that C-terminal Bid is considerably more potent than full-length Bid in triggering the mitochondrial phase of apoptosis.

Anti-apoptotic Bcl-xL Prevents Cytochrome c Release and Mitochondrial Depolarization—Anti-apoptotic Bcl-2 family proteins (Bcl-2 and Bcl-xL) interfere with many apoptotic mechanisms at the mitochondrial stage. To assess whether tBid-induced ΔΨm loss and cytochrome c release can be inhibited by Bcl-xL, permeabilized cells were pretreated with 2 μM Bcl-xL for 5 min. Bcl-xL completely prevented the ΔΨm loss induced by 1 nM tBid (Fig. 3A). Bcl-xL pretreatment also resulted in a blockage of cytochrome c release elicited by 1 or 2.5 nM tBid (Fig. 3B). We noted that the ΔΨm loss evoked at a higher concentration of tBid (25 nM) was only partially prevented by 2 μM Bcl-xL (data not shown), and the cytochrome c release was also partially inhibited by Bcl-xL (Fig. 3B). Furthermore, heat-inactivated tBid (95 °C for 30 min) also failed to induce cytochrome c release and ΔΨm loss (not shown), underscoring that the protein structure of tBid is essential for mitochondrial membrane permeabilization. By contrast an inhibitor of PTP, cyclosporin A (1 μM), that has been shown to effectively inhibit Ca2+-induced PTP opening in this experimental model (51) did not abolish the tBid-induced cytochrome c release and loss of ΔΨm (n = 3, data not shown). Collectively, these results suggest that permeabilization of the outer mitochondrial membrane by the tBid protein is controlled by Bcl-2/Bcl-xL, whereas PTP-dependent permeabilization of the inner mitochondrial membrane is not involved in tBid-induced cytochrome c release and mitochondrial depolarization.

The tBid-induced Loss of ΔΨm Is Due to Impairment of Oxidative Metabolism—To gain further insight into the relation between tBid-induced cytochrome c release and ΔΨm loss, we tested the effect of substrate composition on tBid-induced cy-
tochrome c release and $\Delta \Psi_m$ in suspensions of permeabilized cells. As shown in Fig. 4A, permeabilized cells supplied with succinate (complex II substrate) and ATP regenerating system (ATP, creatine phosphate, and creatine phosphokinase) in the absence of oligomycin showed relatively small tBid-induced depolarization. Addition of oligomycin augmented tBid-induced depolarization calculated by subtraction of $\Delta \Psi_m$ recordings obtained in the presence of tBid from the corresponding time control. The data represents mean $\pm$ S.E. of three different experiments. C and D, time course of tBid-induced cytochrome c release as determined by Western blotting of the cytosolic samples. D, three different amounts of protein were loaded to the gel. The data are representative of three independent experiments.

Western blotting of the cytosol and membrane fractions was performed to evaluate whether changes in the substrate composition also affected the cytochrome c release from mitochondria. Cytochrome c increase in the cytosolic fraction and depletion of the mitochondrial compartment was evoked by tBid in all of these conditions without apparent differences (Fig. 4F). These data demonstrate that the $\Delta \Psi_m$ loss was secondary to tBid-induced cytochrome c release and was mediated by impairment of oxidative metabolism.
Until 30 s after addition of 2.5 nM tBid, cytochrome c was not detectable in the cytosol (Fig. 5, C and D). Appearance of cytochrome c in the cytosol started at 30 s, peaked at 50 s, and was sustained for 710 s without any further increment (Fig. 5 C, lanes 4–10, and 5 D). To determine the temporal coupling between cytochrome c release and $\Delta \Psi_m$ loss, we subtracted the recording of the tBid-induced $\Delta \Psi_m$ loss from the time control (Fig. 5 A), and calculated the time course of mitochondrial depolarization (Fig. 5 B). Mitochondrial depolarization started 70 s after addition of 2.5 nM tBid and displayed a monotonous decrease for 10 min (Fig. 5 B). Clearly, the onset of depolarization was observed after a substantial rise in cytosolic cytochrome c appeared (see 30- and 50-s time points in Fig. 5, C and D). Although the lag time of the effects of tBid was somewhat longer in a few experiments (e.g. Fig. 7), the tBid-induced massive cytochrome c release always preceded the onset of mitochondrial depolarization.

One may speculate that a slow response of JC1 to a decrease in $\Delta \Psi_m$ could influence the time course of the tBid-induced $\Delta \Psi_m$ loss. To address this point, first we monitored cytosolic $[Ca^{2+}]_c$ ($[Ca^{2+}]_c$) simultaneously with $\Delta \Psi_m$ (Fig. 6 A). Dissipation of $\Delta \Psi_m$ evoked a release of the mitochondrial $Ca^{2+}$ store, resulting in a $[Ca^{2+}]_c$ rise. After addition of uncoupler, the JC1 ratio displayed a rapid response that preceded the $[Ca^{2+}]_c$ rise, suggesting that JC1 faithfully reported the uncoupler-induced depolarization. Furthermore, we observed that the small depolarization evoked by suboptimal doses of the uncoupler (20 nM FCCP) also appeared as rapid decreases of the JC1 ratio (Fig. 6 B). Finally, we established measurements of $\Delta \Psi_m$ using permeabilized cells preloaded with TMRE. As shown in Fig. 6 C, tBid caused an increase in TMRE fluorescence, reflecting the release of TMRE from the depolarized mitochondria. The time course of TMRE dequenching was very similar to the decrease in JC1 ratio, providing further evidence that the JC1 measure-
tBid-induced Cytochrome c Release

Thus, resolution of the time course of tBid-induced cytochrome c release allowed us to demonstrate that tBid evokes rapid permeabilization of the outer mitochondrial membrane. Importantly, the rapid kinetics did not depend on the presence of digitonin, because we observed a very similar time course when we studied the effect of tBid on isolated HepG2 mitochondria incubated in the absence of digitonin (n = 2, data not shown). Furthermore, because we noticed cytochrome c release before the decay in ∆Ψm, our results show that mitochondrial depolarization occurred downstream to mitochondrial cytochrome c depletion in tBid-treated permeabilized cells.

tBid Induces Temperature-sensitive Release of Cytochrome c and Loss of ∆Ψm—It has been shown that tBid-induced cytochrome c release does not occur at 4 °C (35, 36), whereas Goldstein et al. reported that temperatures ranging from 24–37 °C do not change the duration of cyto c-GFP release in single HeLa cells undergoing tBid-dependent apoptosis (17). However, the delivery of tBid and other permeability-increasing factors to the individual mitochondria may exhibit a complex spatio-temporal control during apoptosis. Thus it is important to determine whether the mitochondrial membrane permeabilization evoked by a global application of tBid is sensitive to the changes of temperature in this range. To address this point we evaluated the kinetics of the tBid-induced cytochrome c release and mitochondrial depolarization at 25 and 35 °C with the rapid filtration technique (Fig. 7). We noticed that 0.5 and 2.5 nM tBid caused cytosolic cytochrome c accumulation both at 25 and 35 °C, but the response was markedly delayed at 25 °C (Fig. 7B). Furthermore, lowering the temperature increased the lag time and decreased the rate of the tBid-induced mitochondrial depolarization (Fig. 7A). Thus, the rapid mitochondrial membrane permeabilization induced by tBid involves steps that are sensitive to relatively small changes in temperature.

Simultaneous Permeation of Cytochrome c and Smac/DIABLO through the Mitochondrial Outer Membrane—The temporal relationship between the release of cytochrome c and Smac/DIABLO is expected to be very important to coordinate caspase activation. However, it remains elusive whether cytochrome c and Smac/DIABLO exhibit differential distribution in the highly compartmentalized intermembrane space and whether the larger Smac/DIABLO permeates the tBid-altered outer mitochondrial membrane as fast as cytochrome c does. To address this question we evaluated cytochrome c as well as Smac/DIABLO in the cytosol fractions generated by the rapid filtration assay. Specifically we carried out the cytochrome c Western blot and stripped and reprobed the membrane for Smac/DIABLO. Cytosolic accumulation was apparent for both cytochrome c and Smac/DIABLO at 30 s after treatment with tBid, and maximal increase was reached at 50 s (Fig. 8). Thus no difference was observed between the release kinetics of these two apoptotic proteins at a resolution of seconds.

The main finding of this study is that we established the rapid time course of tBid-induced permeabilization of the mitochondrial outer membrane, release of apoptotic factors from the intermembrane space to the cytosol, and mitochondrial depolarization. We demonstrated that tBid evokes complete release of cytochrome c from all mitochondria in permeabilized cells. Furthermore, we showed that the release machinery is remarkably sensitive to subnanomolar doses of tBid and permits release of cytochrome c in a few seconds. The released cytochrome c binds to the cytosolic Apaf-1 and forms an oligomeric complex. This complex recruits the procaspase-9, and the activated caspase-9 subsequently activates downstream caspases (9, 54). Recently it has been identified that Smac/DIABLO promotes caspase activation by binding to inhibitors of apoptosis. Once Smac/DIABLO is released from the mitochondria, it prevents MIHA (the human homologue is known as XIAP) from binding to processed caspase-9 and thereby blocks the inhibition of amplification of caspase activation. This study provided direct evidence for tBid-induced Smac/DIABLO release and showed that the release of Smac/DIABLO is synchronized with cytochrome c release. Thus the tBid-induced pore in the outer membrane allows for rapid permeation of the relatively large Smac/DIABLO, and Smac/DIABLO is present for caspase activation simultaneously with cytochrome c.
Studies with different experimental systems have given rise to a wide variety of mechanisms to explain tBid-induced membrane permeabilization, including ion channel activity (47), destabilization of the lipid bilayer by tBid on its own (48), interaction with pro-apoptotic Bcl-2 family proteins (Bax, Refs. 42–44; Bak, Ref. 45), and interaction with components of the PTP (49). Based on our data, formation of the pore in the outer mitochondrial membrane requires only a few seconds and allows for rapid depletion of intermembrane space proteins. Because the intermembrane space is envisioned to be highly compartmentalized and communication between subdomains may be restricted (14, 15), the rapid and complete release of cytochrome c is likely to be mediated by the formation of numerous pores in different regions of the outer membrane. Furthermore, we showed inhibition of tBid-induced permeabilization by Bcl-xL, an observation suggesting that an interaction between tBid and Bcl-2/Bcl-xL controls the increase in outer membrane permeability. However, because Ca2+ failed to prevent the outer membrane permeabilization by tBid, and mitochondrial depolarization appeared only after cytochrome c release, the pore triggered by tBid does not depend on PTP activation (in agreement with Refs. 55 and 56). The manifestation of mitochondrial depolarization only after cytochrome c release and the attenuation of the $\Delta V_m$ loss by extramitochondrial ATP also show that during tBid-mediated apoptosis the $\Delta V_m$ loss reflects the impairment of the oxidative phosphorylation (consistent with Ref. 46). This can be avoided or reversed if an extramitochondrial ATP supply provides the ATP for extrusion of H+ by the F$_{1}$F$_{0}$ ATPase operating in reverse mode. In conclusion, cleaved Bid serves as an effective means to allow cytochrome c and Smac/DIABLO to escape concurrently from mitochondria and in turn, to induce mitochondrial respiratory dysfunction that results in a $\Delta V_m$ loss.

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