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Involvement of Proapoptotic Molecules Bax and Bak in Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL)-induced Mitochondrial Disruption and Apoptosis: Differential Regulation of Cytochrome *c* and Smac/DIABLO Release[1]

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo2L induces apoptosis in a wide variety of cancer and transformed cells. Activation of BID, a "BH3-domain-only" Bcl-2 family member, triggers the oligomerization of proapoptotic family members Bak or Bax, resulting in the release of mitochondrial proteins to cytosol. In this study, we have shown the importance of Bax and Bak in TRAIL-induced apoptosis by studying in murine embryonic fibroblasts (MEFs) from $Bax^{-/-}$ and $Bak^{-/-}$ animals. TRAIL induced cytochrome *c* release and apoptosis in wild-type, $Bid^{-/-}$, $Bax^{-/-}$, or $Bak^{-/-}$ MEFs, but not in $Bax^{-/-}$ $Bak^{-/-}$ double knockout (DKO) MEFs. Bid, which functions upstream of cytochrome *c* release, was cleaved in all of the knockout cells except in $Bid^{-/-}$ MEFs. The release of cytochrome *c* was correlated with caspase-9 activity. TRAIL increased caspase-3 activity in all of the cells except in DKO cells. TRAIL-induced drop in mitochondrial membrane potential was not observed in DKO MEFs. Unlike cytochrome *c* release, TRAIL-induced Smac/DIABLO release was blocked in $Bid^{-/-}$, $Bax^{-/-}$, $Bak^{-/-}$, or DKO MEFs, suggesting the differential regulation of these mitochondrial proteins during apoptosis. The apoptotic events downstream of mitochondria were intact in DKO MEFs, because microinjection of cytochrome *c*, or ectopic expression of mature Smac/DIABLO or pretreatment of Smac N7 peptide completely restored TRAIL sensitivity. In conclusion, the data suggest that Bax and Bak differentially regulate the release of cytochrome *c* and Smac/DIABLO from mitochondria, and Smac/DIABLO can be used to sensitize cells that are deficient in *Bax* and *Bak* genes, or resistant to TRAIL.

INTRODUCTION

Apoptosis, or programmed cell death, is an evolutionarily conserved mechanism to eliminate unwanted cells commonly occurring during development, as well as in many physiological and pathologic processes (1–3). Although apoptosis is a common phenomenon, excessive loss of cells may contribute to acute organ failure and other chronic diseases, and aberrant resistance to apoptosis may lead to the development of neoplasia (3, 4). This endogenous death machinery is triggered via two principal signaling pathways. One involves the death receptors; on ligand binding these receptors associate with an adaptor protein to recruit procaspase-8, forming the DISC[3] (5, 6). The other pathway is stimulated by various anticancer agents, and irradiation to release cytochrome *c* and Smac/DIABLO from the mitochondrial IM space to the cytosol, resulting in activation of effector caspases and induction of apoptosis (7–10). Cross-talk between the death-receptor and mitochondrial pathways is mediated by caspase-8 cleavage of Bid, a Bcl-2 homology 3 containing protein (11–13).

The role of mitochondria in apoptosis was reported in a cell-free system where nuclear fragmentation was induced by cytosolic fractions enriched in mitochondria (14). A vast variety of studies carried out on apoptosis suggest that most of the agents triggering apoptosis affect the mitochondrial PT pore and release of proapoptotic molecules such as cytochrome *c*, Smac/DIABLO, and apoptosis-inducing factor from mitochondria to the cytosol (7–9, 15–17). Released cytochrome *c* binds to Apaf-1 and then activates caspase-9 in the apoptosome (18, 19). Although release of cytochrome *c* and Smac/DIABLO from the mitochondria is an important step in the apoptotic signaling, the proteins directly control the release of cytochrome *c*, and Smac/DIABLO have not been investigated thoroughly. The proteins that may participate in the release of cytochrome *c* and Smac/DIABLO from the mitochondrial IM include proapoptotic genes such as *Bid*, *Bax*, *Bak*, *Bik*, and *Bim*, and antiapoptotic genes such as *Bcl-2* and *Bcl-X_L* (20–25). The cytochrome *c* is involved in apoptosome formation and activation of caspase-9, whereas Smac/DIABLO appears to function by neutralizing the caspase-inhibitory properties of IAP family of proteins (8, 9, 26).

Interestingly, Bid, a substrate of caspase-8, is activated in the Fas, tumor necrosis factor α, and TRAIL receptor-mediated cell death (5, 6, 27–29). Once the Bid is cleaved or activated, it is translocated to mitochondria as tBid and induces cytochrome *c* release (28, 30, 31). Thus, Bid-deficient mice are resistant to anti-Fas-induced hepatic failure and lethality (32). The ability of Bid to induce cytochrome *c* release is mediated by Bax, because Bid can facilitate the insertion of Bax into mitochondrial membrane to form functional oligomers (30, 33). tBid activates proapoptotic members Bax and Bak to release cytochrome *c* from mitochondria (34). DKO MEFs ($Bak^{-/-}$ and $Bax^{-/-}$) are resistant to apoptosis by various agents, and mice deficient in both Bak and Bax survived anti-Fas antibody treatment (34). There is increasing evidence to suggest the involvement of Bak and Bax in the release of cytochrome *c*, and it was reported that mutations in the Bax or Bak gene render cells resistant to apoptosis (35–38).

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[3] The abbreviations used are: DISC, death-inducing signaling complex; IM, intermembrane; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; tBid, truncated Bid; MEF, mouse embryonic fibroblast; DKO, double knockout; PARP, poly(ADP-ribose) polymerase; FADD, Fas-associated death domain; GFP, green fluorescence protein; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt; DAPI, 4′,6-diamidino-2-phenylindole; PMSF, phenylmethylsulfonyl fluoride; $\Delta\Psi_m$, mitochondrial membrane potential; wt, wild-type; MTS, mitochondrial targeting sequence; BIR3, baculovirus IAP repeat; PT, permeability transition; IAP, inhibitor of apoptosis; XIAP, x-linked inhibitor of apoptosis protein; Smac/DIABLO, second mitochondria-derived activator.

Furthermore, various anticancer agents and UV irradiation did not induce apoptosis in cells that were deficient in both Bax and Bak (23, 34).

TRAIL is a member of the tumor necrosis factor family of cytokines that promote cell death in cancer cells (6, 39). In mice, TRAIL effectively reduces mammary adenocarcinoma without apparent side effects (40). We have demonstrated recently that the TRAIL signaling pathway involves mitochondria in both type I and type II cells (6, 31). In the current study, we demonstrate the role of proapoptotic molecules in TRAIL-induced mitochondrial disruption and apoptosis using MEFs that are deficient in Bid, Bak, Bax (single knockout), and both Bak and Bax (DKO) genes.

MATERIALS AND METHODS

Reagents. Anticytochrome *c* antibody and anticaspase-3 antibody were from BD PharMingen (San Diego, CA). Antibodies against Smac/DIABLO and DR5 were from Imgenex (San Diego, CA). Antibodies against PARP, Bid, and caspase-8 were from Santa Cruz Biotechnology Inc., (Santa Cruz, CA). Antihuman FADD antibody was from Biosource International (Camarillo, CA). Antibody against tubulin was from Sigma Chemical Co. (St. Louis, MO). TRAIL was from Biomol (Plymouth Meeting, PA). Antibodies against GFP, and caspase-3, -8, and -9 kits were from Clontech Laboratories, Inc. (Palo Alto, CA). JC-1 dye and anticytochrome *c* oxidase (subunit IV) antibody were from Molecular Probe (Eugene, OR). Enhanced chemiluminescence Western blot detection reagents were from Amersham Life Sciences Inc. (Arlington Heights, IL). Smac-N7 peptide (H-AVPIAQK-P-*RQIKIWFQNRRMKWKK*-OH) and control peptide (H-MKSDFYF-P-*RQIKIWFQNRRMKWKK*-OH) were modified to be cell permeable by linking the lysine COOH terminal to the arginine of *Antennapedia homeodomain* 16-mer peptide via a proline linker. Smac-N7 peptide and control peptide were synthesized at our biopolymer lab. All of the other chemicals were purchased from Sigma Chemical Co.

XTT Assay. Bax and Bak single and DKO MEFs have been described (34). MEFs (1×10^4 in 100 μl of culture medium per well) were seeded in 96-well plates (flat-bottomed), treated with or without TRAIL, and incubated for various time points at 37°C and 5% CO_2. Before the end of the experiment, 50 μl of XTT labeling mixture (final concentration, 125 μM sodium XTT and 25 μM phenazine methosulfate) per well was added, and plates were incubated for an additional 4 h at 37°C and 5% CO_2. The spectrophotometric absorbance of the sample was measured using a microtiter plate (ELISA) reader. The wavelength to measure absorbance of the formazan product was 450 nm, and the reference wavelength was 650 nm.

Transfection. MEFs were plated in 60-mm dishes in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin mixture at a density of 1×10^6 cells/dish. The next day transfection mixtures were prepared. Cells were transfected with expression constructs encoding Smac/DIABLO full-length (pCDNA3-Smac-Flag), Δ55-Smac/DIABLO mature (pCDNA3-Δ55-Smac-Flag), GFP-Smac/DIABLO full-length (pEGFP N1-Smac), GFP-Δ55-Smac/DIABLO mature (pEGFP N1-Δ55-Smac), or the corresponding empty vectors (pCDNA3 or pEGFP N1) in the presence of an expression vector pCMV-LacZ (Invitrogen Life Technologies) expressing β-galactosidase. For each transfection, 2 μg of DNA was diluted into 50 μl of medium without serum. After the addition of 3 μl of LipofectAMINE (Invitrogen Life Technologies) into 50 μl of Opti-MEM medium, the transfection mixture was incubated for 10 min at room temperature. Cells were washed with serum-free medium, the transfection mixture was added, and cultures were incubated for 24 h in the incubator. The next day, culture medium was replaced with fresh RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin mixture, and TRAIL was added. At the end of incubation, cells were washed with ice-cold PBS and harvested for analyses of apoptosis or Western blotting.

Measurement of Apoptosis. MEFs (1×10^4 cells) were cultured in coverslips and treated with TRAIL for 48 h. The treated cells were washed with PBS twice and stained with DAPI (0.5 μg/ml in PBS) for 1 h. DAPI-stained cells (50 cells from three different fields) were counted by fluorescence microscopy (Nikon, Kawasaki, Kanagawa, Japan).

Subcellular Fractionation. Cells were harvested by centrifugation at $1000 \times g$ for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended with 5 volumes of ice-cold buffer [250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/ml PMSF, 8 μg/ml aprotinin, and 2 μg/ml leupeptin (pH 7.4)]. Cells were homogenized with a 22-gauge needle, and the nuclei were pelleted by centrifugation at $750 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $10{,}000 \times g$ for 25 min. The supernatant (cytosolic fractions) were saved, and the pellets solubilized in the same volume of mitochondrial lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 0.2% Triton X-100, 0.3% NP40, 100 μM PMSF, 2 μg/ml leupeptin, and 2 μg/ml aprotinin], followed by pelleting at $100{,}000 \times g$ for 30 min at 4°C. The supernatant from this final centrifugation represents the S100 fraction. The protein concentrations were determined by Bradford method (Bio-Rad, Hercules, CA). The purification of S-100 protein was determined by Western blot analysis using anticytochrome oxidase 2 antibody.

Measurement of $\Delta\Psi_m$. Mitochondrial energization was determined by retention of JC-1 dye (41). Briefly, 5×10^5 cells were loaded with JC-1 dye (1 μg/ml) during the last 30 min of incubation. Cells were washed in PBS twice. Fluorescence was monitored in a fluorometer using 570-nm excitation/595-nm emission for the J-aggregate of JC-1 (42). $\Delta\Psi_m$ was calculated as a ratio of the fluorescence of J-aggregate (aqueous phase) and monomer (membrane-bound) forms of JC-1.

Western Blot Analysis. Lysis of cells was done in a buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, 1% SDS, 1 mM sodium orthovanadate, and a mixture of protease inhibitors (1 mM PMSF, 1 μg/ml pepstatin A, and 2 μg/ml aprotinin). Lysates were sonicated for 10 s, centrifuged for 20 min at $12{,}000 \times g$, and stored at −70°C. Equal amounts of lysate protein were run on 12% SDS-PAGE gels and electrophoretically transferred to nitrocellulose. Nitrocellulose blots were blocked with 5% BSA in Tris Buffer Saline Tween-20 (TBST) buffer [20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 0.01% Tween 20] and incubated with primary antibody in TBST containing 5% BSA overnight at 4°C. Immunoreactivity was detected by sequential incubation with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents. Some of the blots were analyzed on a STORM phosphorimager (Molecular Dynamics) for fluorescence.

Immunofluorescence Analysis of Cytochrome *c*. To colocalize cytochrome *c* in mitochondria, MEFs were cultured on the coverslips and treated with TRAIL for the required period as mentioned in figure legend (Fig. 2*B*). After treatment, the cells were washed with PBS twice, fixed with 2% paraformaldehyde, and permeabilized with 0.1% Triton-X100. The cells were blocked with 5% BSA in PBS for 30 min. The cells were incubated with anticytochrome *c* antibody (1:200 dilution) for 2 h, washed with PBS, and additionally incubated with secondary antibody conjugated with FITC (1:2000 dilution) for 1 h. During secondary antibody incubation, the cells were also incubated with DAPI (0.5 μg/ml) and mitotracker red (50 nM). The cells were then washed and mounted with immunomount solution and visualized by fluorescence microscopy (Nikon).

Caspase Activity. Caspase activity in MEFs was assessed as per the instructions provided by Clontech Laboratories, Inc. Briefly, 2×10^6 cells were lysed with lysis buffer and centrifuged at $12{,}000 \times g$ for 5 min. The supernatants were mixed with reaction buffer containing DTT (10 mM) and caspase-specific substrate, and incubated for 1 h at 37°C. Samples were read at 405 nm using a microplate reader to quantify the caspase activity.

RESULTS

TRAIL Reduces Cell Viability and Induces Apoptosis in wt and Single Knockout MEFs but not in DKO MEFs. Members of the BH3-domain-only proteins of Bcl-2 family members connect proximal death signals to the core apoptotic pathway (4, 24). To evaluate the effect of TRAIL on the viability of wt, $Bid^{-/-}$, $Bak^{-/-}$, $Bax^{-/-}$, and DKO ($Bak^{-/-}$ and $Bax^{-/-}$) MEFs, cells were incubated with various concentrations of TRAIL (1, 10, 50, and 100 ng/ml) for 48 h. wt MEFs were more sensitive to the TRAIL, followed by $Bid^{-/-}$, $Bax^{-/-}$, and $Bak^{-/-}$ MEFs (Fig. 1*A*). TRAIL had no effect on the viability of DKO MEFs up to 48 h of incubation. These data are consistent with the other report where anticancer drugs and UV irradiation did not induce cytotoxicity in DKO MEFs (34).

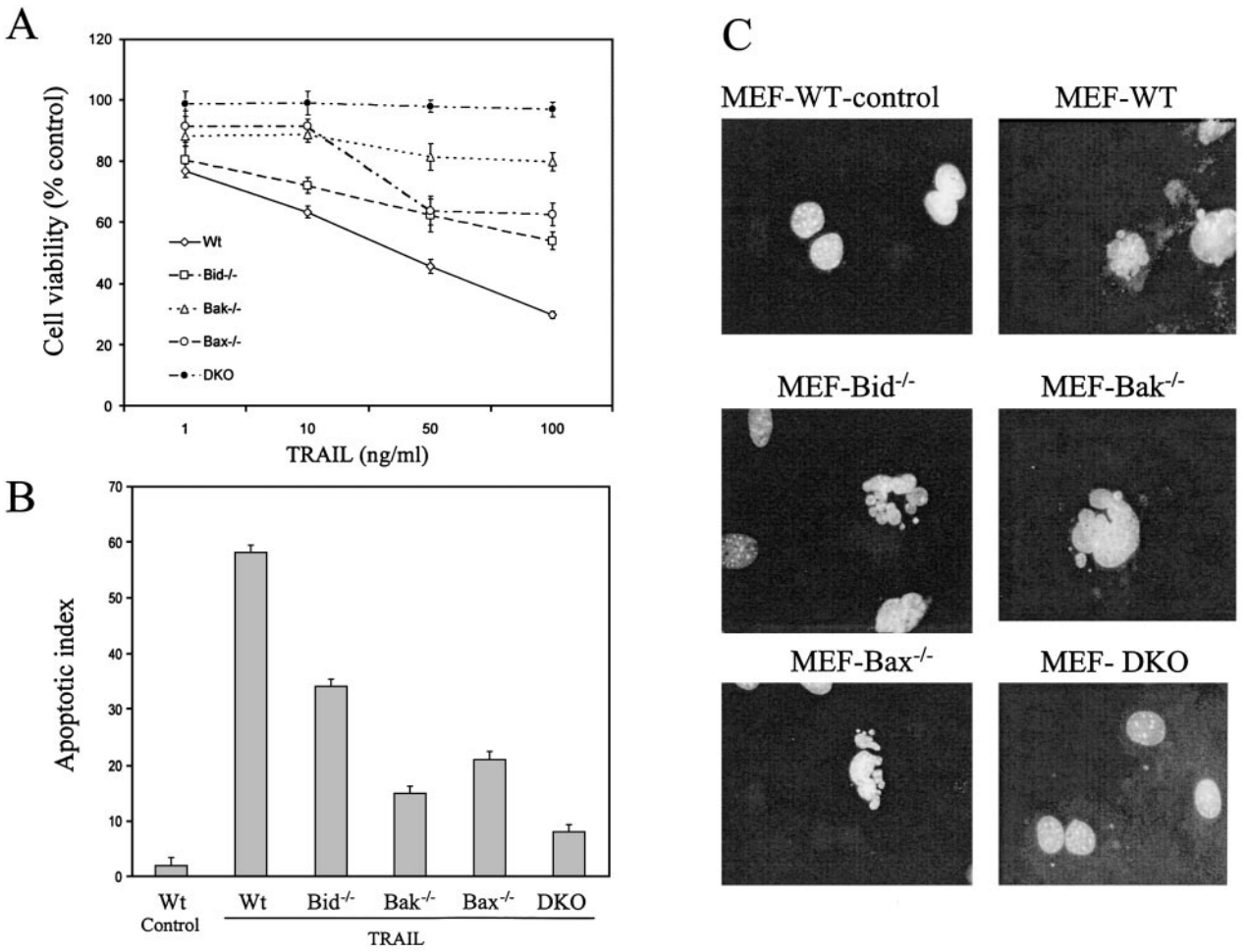

Fig. 1. Sensitivity of MEFs to TRAIL. *A*, TRAIL reduces the cell viability in wt, Bid$^{-/-}$, Bak$^{-/-}$, and Bax$^{-/-}$, but not in Bak$^{-/-}$ Bax$^{-/-}$ (DKO) MEFs. Cells were treated with various doses of TRAIL for 48 h, and survival was measured by XTT assay. Data represent mean; *bars*, ±SE. *B*, TRAIL induces apoptosis in wt, Bid$^{-/-}$, Bak$^{-/-}$, and Bax$^{-/-}$, but not in DKO MEFs. MEFs were incubated with 50 ng/ml of TRAIL for 48 h, and the apoptotic cells were counted by DAPI staining of the nuclei. The data represent mean; *bars*, ±SE. *C*, the fluorescent microscopic pictures of DAPI-stained MEFs after treated with TRAIL (50 ng/ml) for 48 h.

To evaluate the effects of Bid, Bak, and Bax on TRAIL-induced apoptosis, wt, Bid$^{-/-}$, Bak$^{-/-}$, Bax$^{-/-}$, and DKO MEFs were incubated with TRAIL (50 ng/ml) for 48 h. TRAIL induced apoptosis in wt, Bid$^{-/-}$, Bak$^{-/-}$, and Bax$^{-/-}$ cells, but not in DKO cells (Fig. 1, *B* and *C*). DKO MEFs were resistant to apoptosis, whereas wt MEFs showed maximum apoptosis at 48 h of incubation with TRAIL (Fig. 1, *B* and *C*).

TRAIL Induces Cytochrome *c* Release in wt and Single Knockout MEFs but not in DKO MEFs. Induction of cytochrome *c* release is a crucial event of signaling in apoptosis for cells that follow mitochondrial-dependent pathway (6, 31). The release of cytochrome *c* from mitochondria to the cytosol is one of the criteria for determining apoptosis by anticancer drugs (2, 29, 43, 44). To address the mechanism of TRAIL-induced apoptosis in MEFs, we assessed the cytosolic cytochrome *c* levels after 18 h of TRAIL incubation. As in wt cells, all of the single knockout cells showed the presence of cytochrome *c* in the cytosol but DKO MEFs did not (Fig. 2, *A* and *B*). Even after 96 h of TRAIL treatment, DKO MEFs did not release cytochrome *c* to the cytosol (data not shown).

TRAIL Induces Bid Cleavage in MEFs. Because cytochrome *c* release was not found in the cytosol of DKO MEFs, we focused on the signals upstream of cytochrome *c*, such as Bid cleavage. Because tBID facilitates Bax and Bak to release cytochrome *c*, we evaluated whether Bid is cleaved in MEFs treated with TRAIL. Bid cleavage was assessed in wt, single, and DKO MEFs treated with TRAIL (50 ng/ml) for 18 h. wt, Bak$^{-/-}$, Bax$^{-/-}$, and DKO MEFs showed full-length Bid as the Bid antibody could not recognize the tBid in the cytosolic fractions used in the Western blot (Fig. 2*C*). TRAIL induced Bid cleavage in wt, Bak$^{-/-}$, Bax$^{-/-}$, and DKO MEFs, suggesting that the blockage in DKO MEFs did not occur at the level of Bid.

Microinjection of Cytochrome *c* Induces Apoptosis in DKO MEFs. Cytochrome *c* is released from mitochondria to cytosol during apoptosis, and agents that block this event render cells resistant to apoptosis (23, 45, 46). Thus, to evaluate the role of cytochrome *c* in DKO MEFs, we microinjected DKO cells with purified cytochrome *c*, and the apoptotic cells were counted. Microinjection of cytochrome *c* in DKO MEFs facilitates apoptosis equivalent to wt MEFs (Fig. 2*D*). So, the release of cytochrome *c* in Bak$^{-/-}$ and Bax$^{-/-}$ cells (single knockout cells) clearly indicates the role of both genes in apoptosis. DKO MEFs failed to release cytochrome *c* to the cytosol on treatment with TRAIL, suggesting that Bax and Bak are required under physiological conditions, but one can substitute for the other.

Caspase Cascade in MEFs Treated with TRAIL. It has been established that caspases initiate the commitment of cells to apoptosis caused by several stimuli (4, 6). Because the release of cytochrome *c* is the central event in mitochondrial-mediated apoptosis, we intended to study the events upstream and downstream of cytochrome *c* release, the initiator and effector caspases. TRAIL (50 ng/ml) induced caspase-8 activity in all of the MEFs (Fig. 3*A*). Because caspase-8 (initiator caspase) activity is seen on TRAIL treatment, we assessed

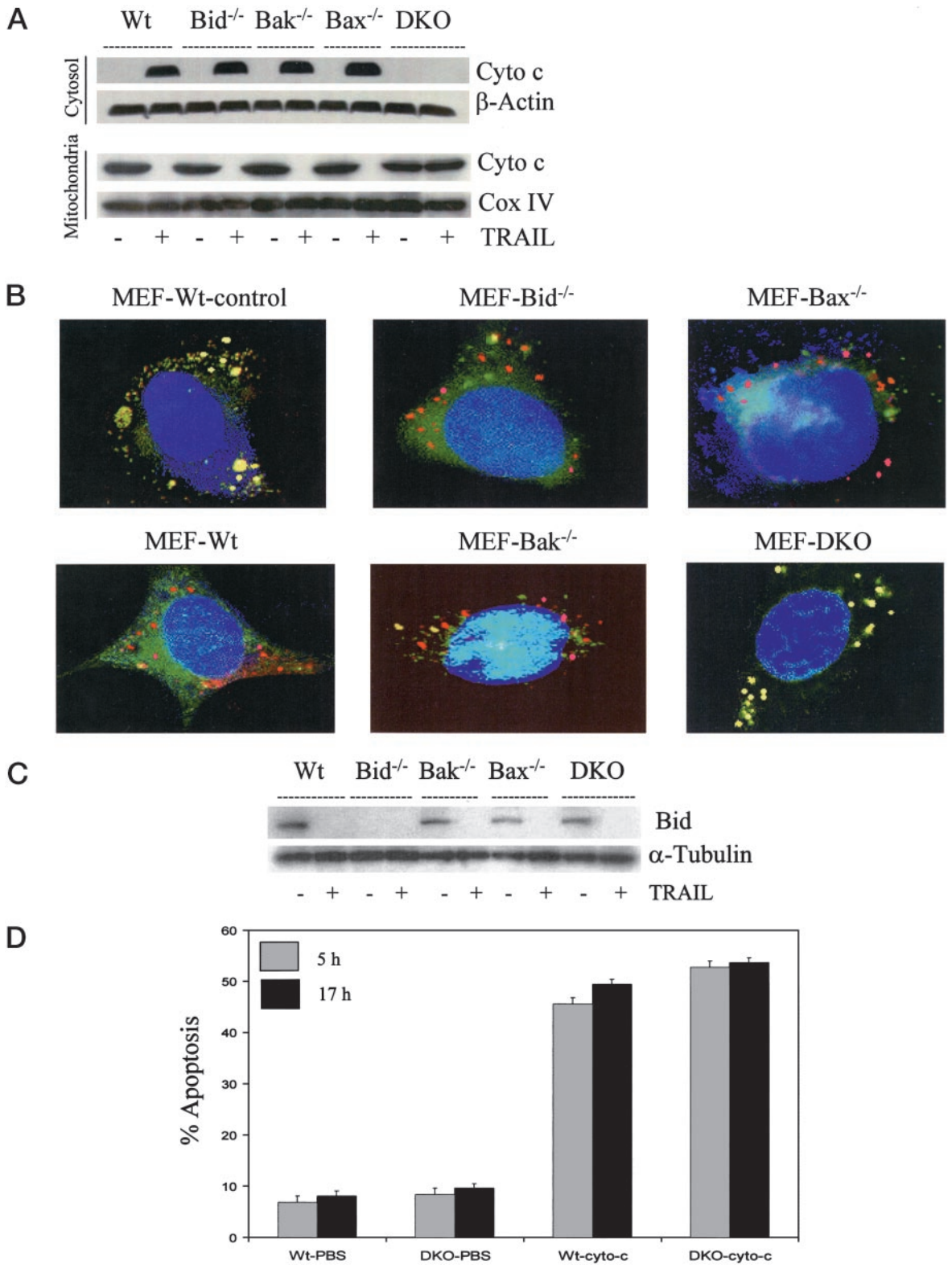

Fig. 2. Mechanism of TRAIL-induced cytochrome *c* release and apoptosis in MEFs. *A*, Western blot analyses showing the presence of cytochrome *c* in the cytosolic fractions of wt, Bid$^{-/-}$, Bak$^{-/-}$, and Bax$^{-/-}$ MEFs treated with TRAIL (50 ng/ml) for 18 h. α-Tubulin antibody was used as a loading control. *B*, immunofluorescence analysis of cytochrome *c* localization in MEFs treated with TRAIL (50 ng/ml) for 18 h. *Green* color indicates cytochrome *c*, *red* color indicates mitochondria, *blue* color indicates nucleus, and *yellow* color indicates the localization of cytochrome *c* into the mitochondria (*green* and *red* = *yellow*). wt control and DKO MEFs show the cytochrome *c* is localized into the mitochondria. Note the presence of some empty mitochondria (*red* color) in wt and single knockout MEFs treated with TRAIL. *C*, immunoblot analysis for the assessment of Bid cleavage in MEFs treated with or without TRAIL (50 ng/ml) for 18 h. Crude proteins were separated by 12% SDS-PAGE, transferred to PVDF membrane, and probed with Bid antibody. The cleavage of Bid was assessed by reduction in whole Bid, as this antibody did not recognize the cleavage product. α-Tubulin was used as loading control. *D*, microinjection of cytochrome *c* into wt and DKO MEFs causes apoptosis. wt and DKO MEFs were cultured in 1% gelatin-coated Petri dishes. Purified cytochrome *c* (1.6 mg/ml) dissolved in PBS or PBS control was mixed with 1:1 with 0.5% FITC and microinjected into the cytoplasm of cells with an Eppendorf 5246 Transjector at a pressure at 150 hPa and an injection time of 0.5 s. MEFs were observed at two time points (5 and 17 h) for apoptotic cells, which detach from the bottom. The cells were also stained with DAPI to confirm the apoptosis. The data represent mean; *bars*, ±SE.

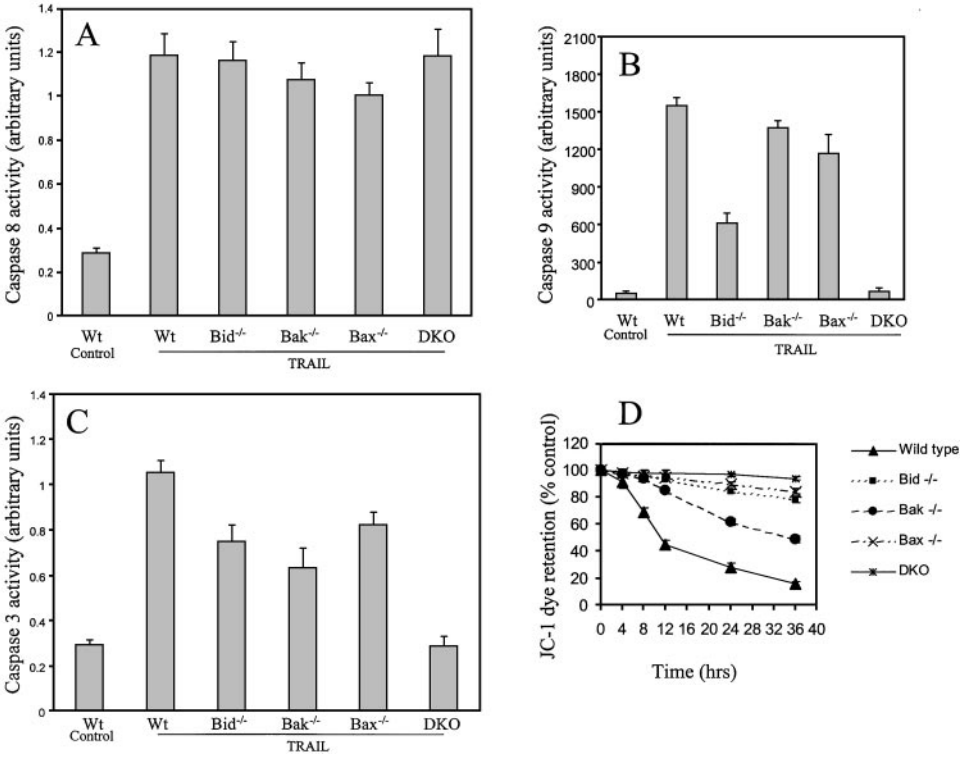

Fig. 3. Function of molecules upstream and downstream of cytochrome *c* on TRAIL-treated MEFs. *A*, caspase-8 activity in MEFs treated with TRAIL (50 ng/ml) for 18 h. *B*, caspase-9 activity in MEFs treated with TRAIL (50 ng/ml) for 18 h. *C*, caspase-3 activity in MEFs treated with TRAIL for 24 h. *D*, measurement of $\Delta\Psi_m$ in MEFs treated with TRAIL for 4, 8, 12, 24, and 36 h. $\Delta\Psi_m$ was measured as described in "Materials and Methods." Caspase-3, -8, and -9 activities were measured as per the manufacturer's directions (Clontech); *bars*, ±SD.

the activity of caspase-9 and caspase-3, which act downstream of caspase-8.

Because caspase-9 is activated in response to mitochondrial disturbance (4, 6), we sought to examine the caspase-9 activity in MEFs. TRAIL (50 ng/ml) induced caspase-9 activity in wt, Bid$^{-/-}$, Bak $^{-/-}$, and Bax$^{-/-}$ MEFs but not in DKO MEFs (Fig. 3*B*).

Caspase-3 can be activated by both mitochondrial-dependent and -independent pathways (31); therefore, we next examined caspase-3 activity in knockout cells. On treatment with TRAIL (50 ng/ml) for 24 h, there was an elevation in caspase-3 activity in wt, Bid$^{-/-}$, Bak$^{-/-}$, or Bax$^{-/-}$ MEFs. Furthermore, caspase-3 activity in DKO MEFs was blocked compared with single knockout MEFs after TRAIL treatment (Fig. 3*C*).

TRAIL Induces Loss of $\Delta\Psi_m$ in wt Bak$^{-/-}$ MEFs but not in Bid$^{-/-}$, Bax$^{-/-}$, and DKO MEFs. $\Delta\Psi_m$ plays an important role in the exchange of ions and various molecules, which may play roles in apoptosis (6, 47, 48). Loss of membrane potential leads to opening of the PT pore leaking the inner components into cytosol, which provide the executing signals for apoptosis (48, 49). Some studies relate the loss of membrane potential and cytochrome *c* release, but others demonstrate that these two events are not related (30). To evaluate the mechanism of TRAIL-induced cytochrome *c* release, we assessed the loss of $\Delta\Psi_m$ in MEFs. Interestingly, TRAIL induced drop in $\Delta\Psi_m$ in wt and Bak$^{-/-}$ MEFs, but not in DKO MEFs (Fig. 3*D*). There was a slight drop in $\Delta\Psi_m$ in Bid$^{-/-}$ and Bax$^{-/-}$ MEFs after 24 h of TRAIL treatment.

TRAIL Induces Smac/DIABLO Release in wt MEFs but not in Bid$^{-/-}$, Bak$^{-/-}$, Bax$^{-/-}$, and DKO MEFs. Recently, a novel protein Smac/DIABLO has been shown to release from the mitochondrial IM space to the cytosol in cells undergoing apoptosis (8, 50). On the basis of *in vitro* studies, Smac/DIABLO appears to function by neutralizing the caspase-inhibitory properties of the IAP family of proteins, particularly XIAP (8, 17, 50–52). Smac/DIABLO, similar to cytochrome *c*, is encoded by a nuclear gene and is subsequently imported into mitochondria (8, 50, 53). To address the mechanism of TRAIL-induced apoptosis in MEFs, we assessed the release of Smac/DIABLO from mitochondria to cytosol. MEFs were treated with TRAIL for 18 h, and cytoplasmic and mitochondrial extracts were prepared. Treatment of wt MEFs with TRAIL resulted in the release of Smac/DIABLO from mitochondria to cytosol (Fig. 4*A*). In contrast, TRAIL did not cause the release of Smac/DIABLO in Bid$^{-/-}$, Bak$^{-/-}$, Bax $^{-/-}$, and DKO MEFs. These results suggest that Bid, Bax, and Bak genes regulate the release of Smac/DIABLO, but the cells lacking these genes were still sensitive to TRAIL-induced apoptosis, indicating the involvement of similar molecule(s) in apoptosis. Interestingly, the release of cytochrome *c* was not blocked in Bid$^{-/-}$, Bak$^{-/-}$, or Bax $^{-/-}$ MEFs as shown above (Fig. 2, *A* and *B*).

Because DKO MEFs were resistant to TRAIL-induced apoptosis, and failed to release cytochrome *c* and Smac DIABLO, we sought to examine the effects of reconstitution of Bax and Bak genes in DKO MEFs (Fig. 4*B*). TRAIL did not cause release of cytochrome *c* and Smac from mitochondria to cytosol in DKO/Neo MEFs. In contrast, reconstitution of Bax and Bak genes in DKO MEFs resulted in cytochrome *c* and Smac release from mitochondria to cytosol.

We examined the effects of ectopic expression of Smac/DIABLO on DISC formation and caspase-8 activation. DKO MEFs were treated with or without TRAIL for 4 h, and analysis of DISC formation was performed as we described elsewhere (31). Ectopic overexpression of Smac/DIABLO did not affect TRAIL-DISC formation (data not shown). Similarly, overexpression of Smac/DIABLO did not affect TRAIL-induced caspase-8 activation in DKO MEFs (Fig. 4*C*).

Mature Smac/DIABLO but not Full-Length Smac/DIABLO Sensitizes DKO MEF to Undergo Apoptosis by TRAIL. The NH_2 terminus of Smac/DIABLO (55 residues containing the MTSs) is removed by proteolysis to generate the mature and functional form (containing 184 amino acids) of the molecule during mitochondrial import (8, 54, 55). We have shown that DKO MEFs were completely resistant to TRAIL; therefore, we examined whether the components of apoptotic pathways downstream of mitochondria are intact in these cells. We examined the ability of full-length (Smac/DIABLO-FL) and

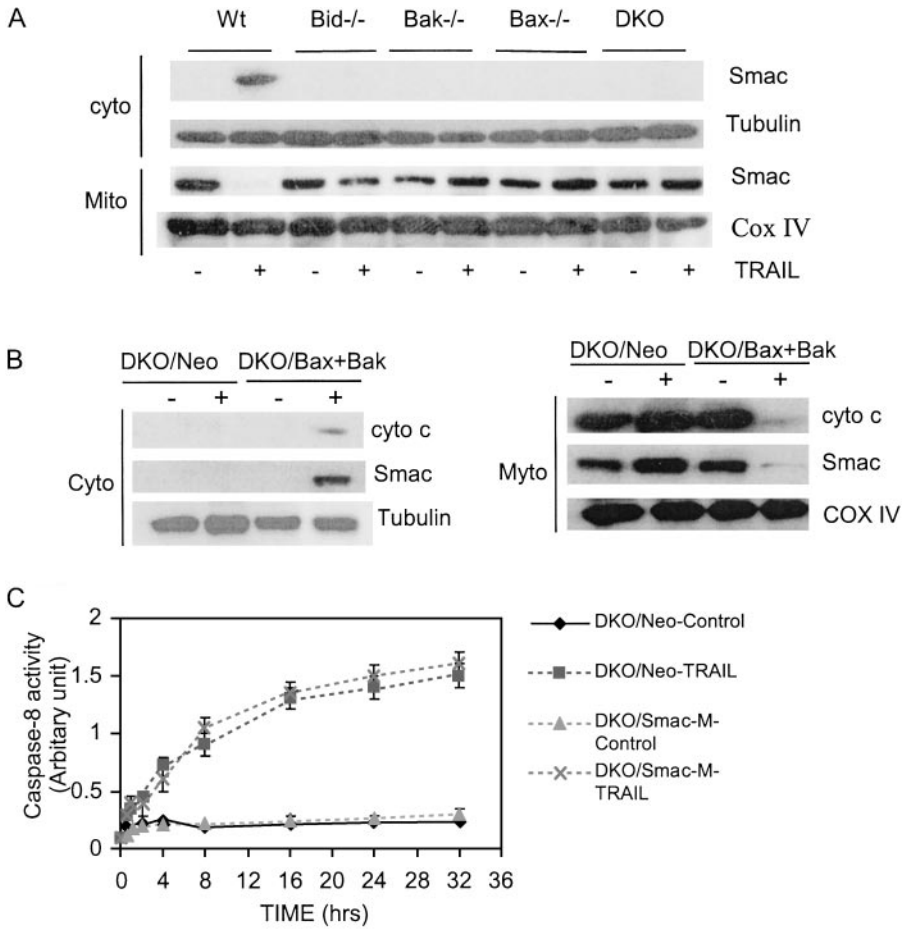

Fig. 4. TRAIL-induced Smac/DIABLO release, and effects of ectopic expression of Smac/DIABLO on DISC formation and caspase-8 activity in MEFs. *A*, wt, Bid$^{-/-}$, Bak$^{-/-}$, Bax$^{-/-}$, and DKO MEFs were treated with TRAIL (50 ng/ml) for 18 h. Cytosolic and mitochondrial fractions were prepared as described in "Materials and Methods." Proteins were subjected to SDS-PAGE, and immunoblotted with Smac/DIABLO, tubulin, or Cox IV antibody. *B*, reconstitution of DKO MEFs with Bax and Bak cDNAs restores TRAIL sensitivity. DKO MEFs were transfected with either empty control vector (pCDNA3/neo), or vectors containing Bax (pCDNA3/Bax) and Bak (pCDNA3/Bak) cDNAs. Cytoplasmic and mitochondrial fractions were prepared as described in "Materials and Methods." *C*, effects of Smac/DIABLO on TRAIL-induced caspase-8 activation. DKO MEFs were transfected as described in *B*. Transfectants were treated with or without TRAIL (50 ng/ml) for various time points. Caspase-8 activity was measured as per the manufacturer's directions; *bars*, ±SD.

mature (Smac/DIABLO-m) Smac/DIABLO to sensitize DKO MEFs to TRAIL. DKO MEFs were transfected with neo, full-length Smac, or mature Smac/DIABLO (Fig. 5*A*). Subcellular fraction analysis showed that full-length Smac/DIABLO was primarily localized to the mitochondria, whereas mature Smac/DIABLO was present in the cytoplasm.

Because the mature form of Smac/DIABLO induces apoptosis by binding to IAPs, we next examined the effects of full-length and mature Smac/DIABLO on the ability of TRAIL to kill cells. Overexpression of neither full-length nor mature Smac/DIABLO had effect on apoptosis (Fig. 5*B*). By comparison, transfection of mature Smac/DIABLO but not full-length Smac/DIABLO restored TRAIL sensitivity in DKO MEFs (Fig. 5*B*). We have shown previously that AVPIAQK sequences (located at the NH_2 terminus after MTS) of Smac/DIABLO are absolutely required for their ability to interact with the BIR3 of XIAP and to promote cytochrome *c*-dependent caspase activation (17). We took the biochemical approach where we treated DKO MEFs with Smac-7 (NH_2 terminus AVPIAQK) or control peptide (MKSDFYF; Fig. 5*C*). Treatment of DKO MEFs with Smac-7 peptide but not with control peptide has a slight effect on apoptosis. Interestingly, cotreatment of DKO MEFs with Smac-7 peptide but not with the control peptide restored TRAIL sensitivity in otherwise TRAIL-resistant DKO MEFs.

The ability of Smac/DIABLO to promote the enzymatic activity of caspases depends on its activation with IAPs (8, 50). It has been shown that the NH_2 terminus deletion mutant of mature Smac/DIABLO is incapable of binding with XIAP, and the NH_2 terminus harbors the caspase-promoting activity of Smac/DIABLO (51, 54). We have generated a functionally active and cytoplasmic Smac/DIABLO expression construct, where a cytoplasmic mature Smac/DIABLO (without MTS) was fused with GFP protein. In this construct (GFP-mature Smac/DIABLO), the IETD site (a caspase-8 cleavage site) was introduced between GFP and mature Smac/DIABLO, which allows the production of authentic Smac/DIABLO in the cytosol after caspase-8 activation in response to TRAIL treatment (17). DKO MEFs were stably transfected with GFP-mature Smac/DIABLO and treated with or without TRAIL to examine the effects of mature Smac/DIABLO on caspase-3 activation, PARP cleavage, apoptosis, and XIAP binding (Fig. 5, *D–F*). Our data revealed that GFP-Smac/DIABLO fusion protein was synthesized in DKO MEFs, and cleaved into GFP and mature Smac/DIABLO proteins by caspase-8 after TRAIL treatment (Fig. 5*D*). Furthermore, TRAIL induced cleavage of caspase-3 and PARP, and apoptosis in DKO MEFs overexpressing mature Smac/DIABLO (Fig. 5, *D* and *E*). Smac/DIABLO was able to interact with XIAP in DKO MEFs treated with TRAIL (Fig. 5*F*), suggesting the ectopically expressed mature Smac/DIABLO is functionally active in inhibiting the activity of XIAP and inducing apoptosis. Because overexpression of Smac/DIBLO sensitizes TRAIL-resistant DKO MEFs, it appears that XIAP-caspase-3 interaction renders these cells resistant to TRAIL, and the release of Smac/DIABLO from mitochondria is necessary to remove the inhibitory effects of XIAP on apoptosis.

DISCUSSION

The results of the present study demonstrate that TRAIL induces apoptosis in MEFs deficient in Bid, Bak, and Bax, but not in DKO MEFs. Several studies have reported the role of these genes on

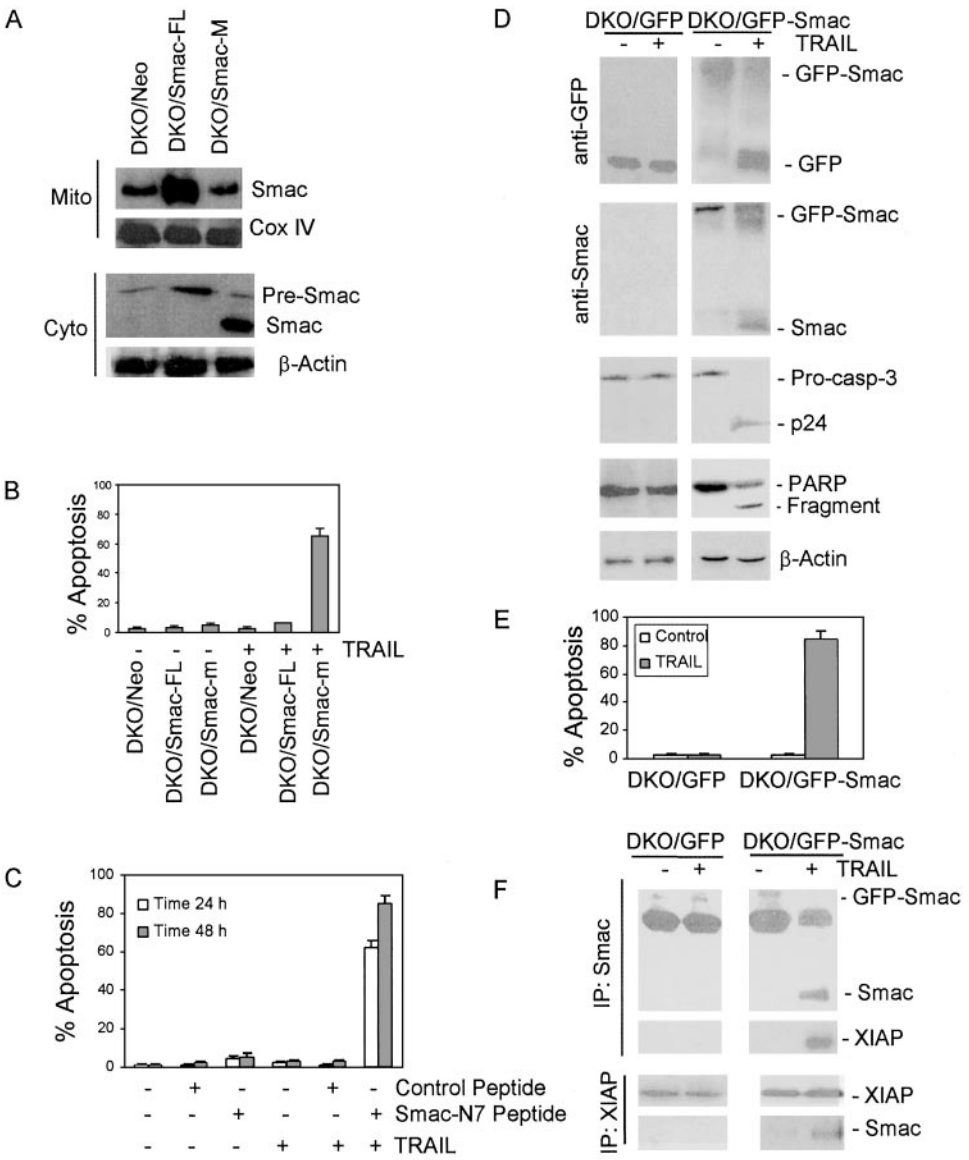

Fig. 5. Mature Smac/DIABLO, but not full-length Smac/DIABLO, sensitizes DKO MEFs to undergo apoptosis by TRAIL. *A*, ectopic expression of full-length Smac (*Smac-FL*) and mature Smac (*Smac-M*). DKO MEFs were transfected with plasmid containing neo, Smac-FL, or Smac-M cDNA. Mitochondrial and cytoplasmic fractions were prepared, subjected to 15% SDS-PAGE, and immunoblotted with Smac, Cox IV, or β-actin antibody. *B*, effects of Smac-FL and Smac-M on apoptosis. DKO MEFs were transfected with plasmid containing neo, Smac-FL, or Smac-M cDNA, and treated with or without TRAIL (50 ng/ml) for 24 h. Apoptosis was measured by DAPI staining. *C*, effects of Smac-N7 peptide on apoptosis. DKO MEFs were pretreated with either control peptide (15 μM) or Smac-N7 (15 μM) peptide (linked with 16-mer of *Antennapedia homeodomain* to enhance permeability) for 3 h, followed by treatment with or without TRAIL (50 ng/ml) for 24 or 48 h. Apoptosis was measured by DAPI staining. The data represent mean; *bars*, ±SE. *D*, expression of mature active form of Smac/DIABLO in the cytosol of DKO MEFs. DKO MEFs, transfected with either GFP or GFP-Smac, were treated with TRAIL (50 ng/ml) for 6 h, and cytosolic extracts were analyzed for expression of GFP, GFP-Smac, and Smac/DIABLO using anti-GFP and Smac/DIABLO antibodies, respectively. Processing and activation of caspase-3 and PARP were analyzed by anticaspase-3 and anti-PARP antibodies. β-Actin was used as a loading control. *E*, effects of TRAIL on DKO/GFP and DKO/GFP-Smac cells. Apoptosis was measured by DAPI staining. The data represent mean; *bars*, ±SE. *F*, presence of Smac/DIABLO-XIAP complex in DKO/GFP and DKO/GFP-Smac cells. Cytosolic extracts, as described in *D*, were immunoprecipitated with either anti-Smac or anti-XIAP antibody, and blotted for Smac/DIABLO or XIAP.

mitochondrial-dependent apoptosis, indicating the involvement of proapoptotic Bcl-2 family members (23, 34, 56). The role of mitochondria in TRAIL-induced apoptosis has been evaluated recently in type I and type II cells (Fig. 6; Refs. 6, 31, 57–60). Furthermore, we have shown for the first time that Bak and Bax genes differentially regulate the release of cytochrome *c* and Smac/DIABLO from mitochondria during TRAIL-induced apoptosis.

Overexpression of Bcl-2 in cancer cells delays or inhibits TRAIL-induced apoptosis in nonlymphoid cells (type II cells) but has no effects in lymphoid cells (type I cells; Refs. 6, 60, 61). Overexpression of BH3-domain-only protein or proapoptotic Bax sensitizes cells to apoptosis (62, 63). We have shown the loss of $\Delta\Psi_m$ in breast and prostate cancer cells on treatment with TRAIL (31, 64). Subsequent to this loss, there is nonselective opening of the mitochondrial PT pore through which proapoptotic molecules are released to the cytosol (6). In contrast, recent studies demonstrate that the release of cytochrome *c* is independent of $\Delta\Psi_m$ and occurs even before the loss of $\Delta\Psi$m (65, 66). The loss of membrane potential and release of cytochrome *c*, mediated by Bax and Bak, are still under debate.

Initially it was demonstrated that tBid was directly involved in the cytochrome *c* release (67), but later it was reported that tBid translocates Bax to mitochondria to form a pore through which cytochrome *c* is released (23, 34). Furthermore, tBid induces cytochrome *c* release independent of Bax (30), and this report is consistent with our findings that TRAIL induces apoptosis in $Bax^{-/-}$ MEFs. In the absence of Bax, tBid induces Bak oligomerization and release of cytochrome *c* from the IM space of mitochondria (12). In the absence of Bax and Bak, TRAIL-treated MEFs (DKO) did not release cytochrome *c* to the cytosol, suggesting that either Bax or Bak is required for the release of cytochrome *c*. Microinjection of cytochrome *c* into DKO cells resulted in apoptosis, indicating that the events downstream of cytochrome *c* release are not affected by the knocking out of Bak and Bax.

On drug treatment, MEFs lacking Bax and Bak genes (DKO) do not release cytochrome *c*, and resist apoptosis when there is a block upstream of cytochrome *c* (34). This prompted us to study the TRAIL-treated Bid knockout MEFs where Bid will act upstream of cytochrome *c*. Surprisingly, we found that TRAIL induces apoptosis in Bid-deficient MEFs. It is not known what triggers the Bax and Bak to release cytochrome *c* from the mitochondria in the absence of Bid on TRAIL treatment. It was suggested that $Bid^{-/-}$ cells are susceptible to apoptosis by various death stimuli indicating that Bid is not the only activator of Bax or Bax, and other upstream activators of Bax and Bak

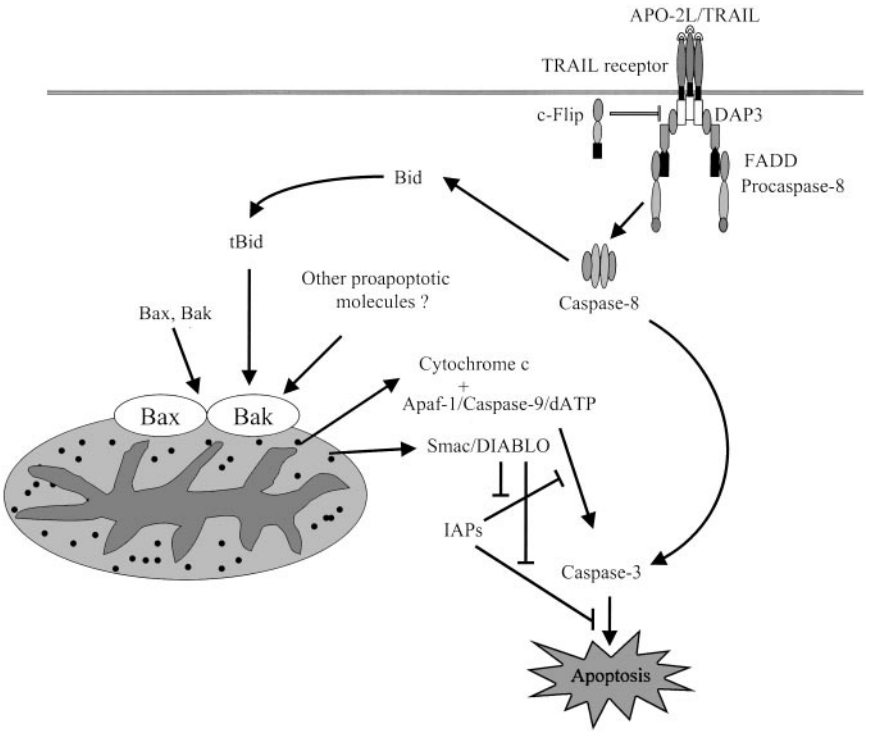

Fig. 6. Involvement of mitochondria in TRAIL-induced apoptosis. Apoptosis pathways activated by TRAIL are depicted. Ligation of death receptors by TRAIL leads to formation of DISC consisting of DAP3 and FADD. c-FLIP inhibits the recruitment of FADD to the complex. Activation of DISC initiates two pathways: (*a*) activation of caspase-8 leading to apoptosis; and (*b*) activation of Bid to tBid regulates mitochondrial dysfunctions. tBID causes oligomerization of BAX and BAK to release of cytochrome *c*. Cytochrome *c* along with Apaf-1 and dATP form apoptosomes, which activate caspase-9. Caspase-9 activates downstream caspases such as caspase-3. These events finally result in apoptosis. Caspase-8 may also cause activation of caspase-3 independent of mitochondria. Smac/DIABLO relieves the inhibitory effects the IAPs on these caspases thereby allowing apoptosis to proceed.

clearly exist (34). Caspase-8, which is upstream of Bid, is almost equally activated in all of the MEFs on treatment with TRAIL, indicating that the DISC is not affected. In our studies, the apoptotic index in TRAIL-treated wt MEFs is higher compared with $Bax^{-/-}$ and $Bak^{-/-}$ knockout MEFs, suggesting an interaction between Bak and Bax in mitochondrial-dependent pathway. The TRAIL induced cell death in $Bid^{-/-}$ MEFs, indicating that Bax and Bak are intact in these cells, and suggesting the possibility of other molecules that may play a regulatory role in triggering Bak and Bax.

It was also shown that loss of membrane potential does not accompany tBid-induced apoptosis (30), but apoptosis mediated by Bax induced a loss of $\Delta\Psi$m (68). Our results demonstrate that wt and $Bak^{-/-}$ MEFs but not DKO MEFs undergo a loss of $\Delta\Psi$m indicating the involvement of specific genes in the regulation of mitochondrial functions in TRAIL-induced apoptosis. It is unclear whether cytochrome *c* release is accompanied by loss of $\Delta\Psi$m or whether these are two independent events in type II cells involving mitochondria. Our results suggest that Bid-mediated Bak oligomerization leads to cytochrome *c* release independent of $\Delta\Psi$m, but Bax translocation may lead to loss of $\Delta\Psi$m. It is likely that the conformational changes that occur on binding of Bid to Bax or Bid to Bak are different for pore formation and for the release of cytochrome *c*. According to the "hit and run model," tBid can induce conformational changes in Bak suggesting the possibility that tBid can interact with both Bak and Bax simultaneously (12). It was also reported that there are possible interactions between Bax and Bak on death stimuli (69), but it is still unclear whether both proapoptotic molecules function independently or cooperatively. Because our data show that wt MEFs are more sensitive to apoptosis than single knockout cells, it is likely that Bak and Bax interact cooperatively for the efficient pore formation and for the release of cytochrome *c*.

During apoptosis, Smac/DIABLO, a mitochondrial protein, is released into the cytosol as a mature protein lacking NH_2 terminus 55-amino acid residues constituting MTSs (8). The mature form of cytosolic Smac/DIABLO inhibits the interaction between BIR3 of XIAP with caspase-9, and linker-BIR2 with caspase-3 or -7, and relieves the inhibitory effects of the XIAP on these caspases thereby allowing apoptosis to proceed (51, 54, 55). The BIR antagonistic action of Smac/DIABLO is because of its binding to BIR3 and linker-BR2 in a mutually exclusive manner with caspase-9, and caspase-3 and -7, respectively. In our studies, TRAIL failed to release Smac/DIABLO from mitochondria to cytosol in MEFs lacking Bid, Bak, Bax, or Bax and Bak both after TRAIL treatment. Furthermore, ectopic overexpression of Smac/DIABLO or pretreatment of cells with Smac N-7 peptide sensitizes TRAIL-resistant DKO MEFs to undergo apoptosis. It appears that the DKO MEFs, lacking mature Smac/DIABLO in the cytosol, are resistant to TRAIL because of strong XIAP-caspase-3 interaction, and mature Smac/DIABLO removes the inhibition of XIAP. The levels of XIAP (possibly cIAP1, cIAP2, or survivin) in cells will determine whether the mitochondrial pathway is essential for death receptor-mediated apoptosis. The mature form of Smac/DIABLO will be required to remove the inhibitory effects of XIAP thereby allowing apoptosis to proceed. MEFs lacking other components of cell death pathway such as Apaf-1 (70, 71), cytochrome *c* (72), and caspase-9 (73) were sensitive to death receptor-mediated apoptosis probably because of functional Smac/DIABLO. Failure of TRAIL to cause Smac/DIABLO release in MEFs lacking Bid, Bax, Bak, or Bax and Bak together, suggest that the release of Smac/DIABLO depends on Bid, which induces Bax and Bak oligomerization, and Bax and Bak are not mutually exclusive in controlling Smac/DIABLO release. Smac/DIABLO-deficient mice were generated recently using homologous recombination in embryonic stem cells (74). These Smac/DABLO knockout mice were viable, grew, and matured normally without showing any histological abnormalities. Furthermore, $Smac/DIABLO^{-/-}$ cells were sensitive to various apoptotic stimuli *in vitro*, and hepatocytes in these knockout mice were underwent to apoptosis by Fas (74). These data are in agreement with our data where $Bid^{-/-}$, $Bax^{-/-}$, or $Bak^{-/-}$ MEFs failed to release Smac/DIABLO into the cytosol, but they were still sensitive to TRAIL-induced apoptosis, suggesting the existence of a similar molecule capable of compensating for a loss of Smac/DIABLO function.

We suggest that the type II pathway is mainly involved in TRAIL-

induced apoptosis in fibroblasts where mitochondria play an important role in amplifying apoptotic signals. In addition, the Bax and Bak differentially regulate the release of cytochrome *c* and Smac/DIABLO from mitochondria. Bax (36, 38) and Bak (35) mutations, and loss of Apaf-1 (75, 76) have been associated with improper mitochondrial activation. Future studies are in progress to assess the clinical significance of Smac/DIABLO in sensitization of drug- or irradiation-resistant human breast and prostate cancer cells. Smac/DIABLO may also enhance the chemotherapeutic potential of antineoplastic drugs and irradiation (77). The optimization of Smac/DIABLO delivery as a small molecule or in gene therapy protocols will be a major achievement to treat various types of cancers. Therefore, the agents that bypass mitochondria such as Smac/DIABLO and XIAP may have therapeutic potentials in treating human diseases.

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