A Role for Mitochondrial Bak in Apoptotic Response to Anticancer Drugs*  

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In the present study a clonal Jurkat cell line deficient in expression of Bak was used to analyze the role of Bak in cytochrome c release from mitochondria. The Bak-deficient T leukemic cells were resistant to apoptosis induced by UV, staurosporin, VP-16, bleomycin, or cisplatin. In contrast to wild type Jurkat cells, these Bak-deficient cells did not respond to UV or treatment with these anticancer drugs by membranous phosphatidylserine exposure, DNA breaks, activation of caspases, or release of mitochondrial cytochrome c. The block in the apoptotic cascade was in the mitochondrial mechanism for cytochrome c release because purified mitochondria from Bak-deficient cells failed to release cytochrome c or apoptosis-inducing factor in response to recombinant Bax or truncated Bid. The resistance of Bak-deficient cells to VP-16 was reversed by transduction of the Bak gene into these cells. Also, the cytochrome c releasing capability of the Bak-deficient mitochondria was restored by insertion of recombinant Bak protein into purified mitochondria. Following mitochondrial localization, low dose recombinant Bak restored the mitochondrial release of cytochrome c in response to Bax; at increased doses it induced cytochrome c release itself. The function of Bak is independent of Bid and Bax because recombinant Bak induced cytochrome c release from mitochondria purified from Bax−/−, Bid−/−, or Bid−/− Bax−/− mice. Together, our findings suggest that Bak plays a key role in the apoptotic machinery of cytochrome c release and thus in the chemoresistance of human T leukemic cells.  

Mitochondrial function is implicated in the two major apoptotic pathways currently accepted as the model(s) for cell death. The death receptor-mediated pathway involves mitochondria mainly as an amplification loop, whereas cellular stress-mediated apoptosis is regulated predominantly at the level of the mitochondria (1). In both pathways the involvement of the mitochondria is manifested by release of cytochrome c, a resident protein in the mitochondrial intermembrane space (2). In the cytoplast, cytochrome c in concert with apoptosis protease-activating factor 1 and dATP activates caspase-9, leading to the subsequent activation of the effector protease, caspase-3 (2, 3). Release of cytochrome c is crucial for the mitochondrial pathway of apoptosis, because cells deficient in cytochrome c are resistant to UV or cytotoxic drug-mediated apoptosis (4). In addition to cytochrome c, other components of apoptosis are sequestered in the mitochondrial intermembrane space, including apoptosis inducing factor (AIF),1 which when released from the mitochondria induces apoptotic nuclear morphology in a caspase-independent manner (5).  

Members of the Bcl-2 family are major regulators of mitochondrial apoptotic events (6). Bcl-2 family proteins can be subdivided into three distinct groups: (i) anti-apoptotic members such as Bcl-2 and Bcl-XL with sequence homology at BH (Bcl-2 homology)1, BH2, BH3, and BH4 domains; (ii) pro-apoptotic molecules, such as Bax and Bak, with sequence homology at BH1, BH2 and BH3; and (iii) pro-apoptotic proteins that share homology only at the BH3 domain, such as Bid, Bik, Noxa, and Bim (7). Pro- and anti-apoptotic Bcl-2 proteins regulate apoptosis in part by controlling cytochrome c release from mitochondria. Expression of Bcl-2 and Bcl-XL prevents the redistribution of cytochrome c in response to multiple death-inducing stimuli (7–9), whereas Bid, Bax, and Bak promote cytochrome c release (10–13). These Bcl-2 family members also regulate apoptotic changes in isolated mitochondria; cytochrome c release is induced by recombinant Bax, Bak, or Bid and prevented by Bcl-2 or Bcl-Xl (10, 14–17). The ratio between pro- and anti-apoptotic proteins determines in part the susceptibility of cells to a death signal (7, 13, 18).  

Bid represents a cytosolic BH3-only protein activated by proteolytic processing. Upon activation of Fas or tumor necrosis factor receptor, Bid is cleaved by caspase-8, and the truncated Bid (tBid) product translocates to the mitochondria and triggers the release of cytochrome c (19–21). Immunodepletion of  

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Bak shows that it is required for caspase-8-induced cytochrome c release (21). A cleavage product of Bid also induces cytochrome c release in response to lysosomal leakage (22) or granulocyte B (23, 24). These findings suggest that Bak may serve as a sensor that allows cells to initiate apoptosis in response to the presence of specific activated proteases.

Bax is a p53-regulated protein (25, 26) that participates in the induction of apoptosis in response to a variety of apoptotic signals (26–28). Mutations in Bax have been identified as a mechanism of protection against apoptosis in cancer cells (29–31). A recent study involving Bax-deficient cells has established an unambiguous role for Bax in the response of human carcinomas to anticancer drugs (18). The absence of Bax in a human colorectal cell line completely abolished the apoptotic response to sulindac sulfone or indomethacin, nonsteroidal anti-inflammatory drugs (NSAID).

Bak, a Bcl-2 homologous antagonist/killer has also been reported to be regulated by p53 (32). Bak gene transduction induced cell death and accelerated apoptosis in response to growth factor deprivation in murine lymphoid, neural, and fibroblastic cells (33–35). It has been reported that gastric and colorectal tumors have reduced Bak protein levels compared with normal mucosa (36, 37). Also, mutations of the Bak gene have been identified in human gastrointestinal cancers (31), suggesting that perturbation of Bak-mediated apoptosis may contribute to the pathogenesis of these tumors.

Although interactions between pro- and anti-apoptotic Bcl-2 family members have been extensively studied, interactions among the pro-apoptotic Bcl-2 family members and their potential interdependence has not yet been fully addressed. Cytotoxicity of Bid was reported to correlate with its ability to interact with Bak (38). The interaction of Bid with Bak has been associated with a change in the conformation of Bak, its translocation to the mitochondria, and the release of mitochondrial cytochrome c (39). However, Bid also induces cytochrome c release from Bak-deficient or Bax knockout cells (38, 40), suggesting the involvement of other proapoptotic targets for Bid in mitochondria. Indeed, insertion of tBid into mitochondria from Bak-deficient cells induced a significant up-regulation in the immunoreactivity of mitochondrial Bak to staining by an N terminus-specific anti-Bak Ab (38). The requirement for Bak in tBid-induced cytochrome c release has recently been demonstrated in hepatocytes from Bak knockout mice (41). In the current study, utilizing Bak-deficient T leukemia cells, we demonstrated the significance of Bak in the apoptotic machinery of cytochrome c release and thus in the chemoresistance of these cells.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Staurosporin, etoposide (VP-16), bleomycin, anti-β-actin mAb (clone AC-15), and the prothrombin carboxyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma; cisplatin was from Bristol Laboratories (Princeton, NJ), and sulindac sulfide and indomethacin were from ICN Pharmaceutical (Costa Mesa, CA). A caspase-8-specific mAb (57F) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY); mAbs anti-caspase-7 (clone 51), anti-caspase-2 (clone 47), and rabbit anti-caspase-3 were from Transduction Laboratories (Lexington, KY). An additional rabbit anti-caspase-3 Ab, anti-cytochrome c Ab, and Ac-DEVD-AMC caspase substrates were from BD-PharMingen. Rabbit anti-Bid Ab was generated as described previously (42); FITC-annexin V and propidium iodide were from CLONTECH (Palo Alto, CA); anti-human Bak Ab were from Oncogene (Ab-1, mouse clone AM03, generated against recombinant BakΔC), Pharmingen (Ab-2, polyclonal Ab generated against recombinant BakΔC), StressGen (Ab-3, polyclonal Ab generated against residues 2–14 of human Bak), and Santa Cruz (Ab-4, polyclonal Ab generated against peptide within an internal region of human Bak). Anti-mouse Bak Ab was from Pharmingen; anti-Bax Ab (N-20) and goat anti-AIF Ab were from Santa Cruz; anti-cytochrome c oxidase (Cox) IV was from Molecular Probes (Eugene, OR).

**Preparation of GST-Bax, GST-Bak, and His-tagged tBid—Mouse BakΔTM (amino acids 1–173) cDNA was ligated into a pGEX-2T (Amersham Pharmacia Biotech) GST expression vector. The resulting construct encoded a GST-BaxΔTM fusion protein with GST at its N terminus. *Escherichia coli* strain DH5α (Life Technologies, Inc.) cells were transformed with the ligated plasmid and cultured at 37 °C until pellets were resuspended and sonicated in a buffer containing 50 mM Tris-HCl, pH 8.0.

**Mouse tBid** (amino acids 60–195) was cloned into a pET23dHis vector modified from the parental pET23d (+) vector (Novagen, WI). The expressed tBid has His<sub>6</sub> tag at its N terminus. *E. coli* strain BL21(DE3) cells were transformed and cultured at 37 °C in Terrific Broth. The induction of expression was started at an *A*<sub>600</sub> of 0.4 by isopropyl β-D-thiogalactoside (1 mM) with continued incubation of the culture at 37 °C for 2–3 h. The bacterial pellets were resuspended and sonicated in a buffer containing 5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9. His-tagged tBid was eluted with elution buffer containing 400 mM imidazole, 500 mM NaCl, and 20 mM Tris-Cl, pH 7.9, and was further purified using a Sephadex G-50 column equilibrated with phosphate-buffered saline.

**Cell Lines and Clones—**The Jurkat T leukemia cell line was obtained from American Type Culture Collection (Manassas, VA). Jurkat cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM l-glutamine, and 100 units/ml each of penicillin and streptomycin (complete medium). A clonal cell line isolated from wild type Jurkat cells was found to be Bak-deficient.

**Transduction of Jurkat Cells by Ad/GT-Bak and Ad/GV16—**Construction of the Ad/GT-LacZ, Ad/GT-Bak, and Ad/GV16 vectors was reported previously (43, 44). A binary adenoviral vector system was used to overcome Bak-mediated apoptosis in the packaging 293 cell line, as described previously (44). In this binary system, the vector Ad/GT-Bak contains a Bak gene under the control of the GAL4/TATA (GT) promoter and the GAL4/GV16 fusion protein. Bak gene expression could then be induced in target cells by coadministration of the Ad/GT-Bak vector with the second adenoviral vector Ad/GV16, which produces the Ad/GV16 fusion protein. The transduction efficiencies of the adenoviral vectors were determined by assessing the titer needed to infect the cells with Ad/GT-LacZ and Ad/GV16.

**Determination of Caspase Activity—**Wild type and clonal Jurkat cells were treated with VP-16 (20 μM), staurosporin (0.5 μM), bleomycin (17 μM), and cisplatin (100 μM) for 4, 12, or 24 h. The cells were Dounce homogenized in extraction buffer (20 mM HEPES, pH 7.2, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% sucrose, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) in ice. Insoluble proteins were removed by centrifugation. The samples were maintained at −80 °C until assayed. Cytosolic proteins (10–30 μg) were diluted in extraction buffer at a final volume of 200 μl containing 12 μM Ac-DEVD-AMC in a microtiter plate. Release of AMC was detected in a PerkinElmer Life Sciences fluorescence spectrophotometer at an excitation value of 380 nm and emission of 440 nm following 10, 30, or 60 min of incubation at 37 °C.

**Western Blot Analysis**—To generate whole cell extracts, the cells were lysed in 0.5% Nonidet P-40, 10 mM HEPES, pH 7.4, 142 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The proteins were resolved by separation through 15% polyacrylamide gel and transferred to polyvinylidene difluoride membranes as described previously (45). Following probing with a specific primary Ab and horseradish peroxidase-conjugated secondary Ab, the protein bands were detected by enhanced chemiluminescence (Pierce).

**Subcellular Fractionation**—To obtain subcellular fractions, including S-100 and HM, Jurkat cell suspension in isotonic mitochondrial buffer (20 mM sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA) was maintained at 4 °C until assayed. Cytosolic proteins (10–30 μg) were diluted in extraction buffer at a final volume of 200 μl containing 12 μM Ac-DEVD-AMC in a microtiter plate. Release of AMC was detected in a PerkinElmer Life Sciences fluorescence spectrophotometer at an excitation value of 380 nm and emission of 440 nm following 10, 30, or 60 min of incubation at 37 °C.
MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin) was Dounce homogenized by 15–20 strokes and centrifuged at 500–1100 g for 5 min at 4 °C to obtain a pellet containing nuclei and remaining intact cells. The postnuclear supernatant was centrifuged at 10,000–11000 g for 30 min at 4 °C to obtain the HM pellet. The supernatant was further centrifuged at 100,000 g for 1 h at 4 °C to pellet the remaining membrane (P-100) containing endoplasmic reticulum and to yield the final soluble cytosolic fraction, S-100. HM and S-100 subcellular fractions were assessed for the presence of cytochrome c by Western blot analysis. The nuclei, P-100, S-100, and HM were assessed for Bak expression.

Mitochondria Purification—To obtain purified mitochondria, the Jurkat cells were suspended in mitochondria buffer (MIB) composed of 0.3 M sucrose, 10 mM MOPS, 1 mM EDTA, and 4 mM KH₂PO₄, pH 7.4, and lysed by Dounce homogenization as described previously (46). Briefly, nuclei and debris were removed by a 10-min centrifugation at 650 × g, and a pellet containing mitochondria was obtained by two successive spins at 10,000 × g for 12 min. The washed mitochondrial pellet was resuspended in MIB and layered on a Percoll gradient consisting of four layers of 10, 18, 30 and 70% Percoll in MIB. After centrifugation for 35 min at 13,500 × g, the purified mitochondria were collected at the 30/70 interface. Mitochondria were diluted in MIB containing 1 mg/ml bovine serum albumin (at least a 10-fold dilution required to remove Percoll). The mitochondrial pellet was obtained by a 30-min spin at 20,000 × g and used immediately.

Alkali or Digitonin Treatment of Purified Mitochondria—Following
incubation of purified mitochondria (100 μg) with recombinant tBid, Bax, or Bak, mitochondria were pelleted and incubated in 100 μl of 0.1 M Na2CO3, pH 11.5, for 20 min on ice (47). Alternatively, mitochondria were incubated with 100 μl of digitonin (1.2 mg/ml) for 20 min on ice (48). Supernatants and mitoplasts were separated by centrifugation and dissolved in 0.5% Nonidet P-40 lysis buffer. The fractions were analyzed by immunoblotting.

Cytochrome c Release Assay—Purified mitochondria (100 μg of protein) were incubated with recombinant His-tBid, GST-BaxATM, or GST-BakAC at various doses as indicated in 20 μl of MIB at 30 °C for 30 min. Mitochondria were pelleted by centrifugation at 4,000 × g for 5 min. The resulting supernatants or mitochondria were mixed with lysis buffer and analyzed by SDS/PAGE and immunoblotting for the presence of cytochrome c or AIF.

RESULTS

Deficient Expression of Bak Protein in Clonal Jurkat Cells—A clonal cell line obtained from the American Type Culture Collection wild type Jurkat cell line was found to be Bak-deficient. The deficiency was determined by immunoblotting of whole cell lysates by three different anti-Bak Abs specific for distinct epitopes (Fig. 1A). To further analyze the expression of Bak, lysates of wild type or clonal Jurkat cells were fractionated to yield nuclei, P-100 (endoplasmic reticulum), S-100 (cytosol), purified mitochondria, purified mitochondria treated with alkali to remove loosely attached proteins, cellular extract without HM, or crude HM containing mitochondria. The various protein fractions were assessed for the expression of Bak by Western blot analyses. Whereas expression of Bak was detected in all mitochondria-containing fractions of wild type Jurkat cells, only minor expression of Bak was observed in a similar quantity of purified mitochondria from Bak-deficient cells (Fig. 1B). To assess the mitochondrial localization of Bak, purified mitochondria from either wild type or Bak-deficient cells were treated with alkali to remove proteins nonspecifically attached to the mitochondria (47). In wild type Jurkat cells, Bak is a mitochondrial integral membrane protein because it was detected in the pellet of alkali-treated mitochondria (Fig. 1C). Because pretreatment of mitochondria with tBid has been reported to enhance Bak localization to the mitochondria (39), we assessed the effects of His-tBid on mitochondrial expression of Bak. However, treatment of mitochondria with His-tBid did not effect Bak localization. In contrast to wild type Jurkat cells, minor or no expression of Bak was detected in mitochondria of Bak-deficient cells, although they express Cox IV or VDAC at levels equal to those of wild type cells (Fig. 1C).

To further analyze the mitochondrial localization of Bak, wild type or Bak-deficient mitochondria were treated with digitonin (48). Mild digitonin treatment partially lyses the outer membrane but not the inner mitochondrial membrane. In wild type Jurkat cells treated with a low concentration of digitonin, Bak was associated with the pellet of digitonin-treated mitochondria, whereas VDAC, an outer membrane protein, was partially removed from the mitochondria into the digitonin-sensitive fraction (Fig. 1D). Partial removal of both Bak and VDAC into the digitonin-sensitive fraction was observed in wild type mitochondria treated with increased digitonin concentrations (data not shown). These findings suggest that in wild type Jurkat cells Bak is a mitochondrial integral membrane protein that is less sensitive than VDAC to removal by mild digitonin treatment. However, the expression of Bak is deficient in the clonal Jurkat cell line.
Abrogation of Mitochondrial Apoptotic Pathways in Bak-deficient Jurkat Cell—Because the Bcl-2 proapoptotic family member Bak resides in the mitochondria, we examined the susceptibility of the Bak-deficient cells to apoptotic agents known to initiate mitochondrial pathways of apoptosis. Wild type and Bak-deficient cells were treated with various doses of cytotoxic drugs, including VP-16 (20–80 μM), staurosporin (0.2–1 μM), bleomycin (50–300 μM), or cisplatin (5–100 μM) for 24 h. Whereas wild type cells demonstrated susceptibility to the lowest dose tested for these cytotoxic drugs, the Bak-deficient cells were found resistant even to the highest dose tested for each of the drugs. Following 24 h of treatment with high dose cytotoxic drugs, the Bak-deficient Jurkat cells remained negative to staining by FITC-annexin V or propidium iodide, whereas high levels of apoptotic cells were detected in wild type Jurkat cells (Fig. 2A). No apoptotic cells were detected among the Bak-deficient cells at 48 or 72 h after exposure to UV or anticancer drugs. No processing of caspase prodomains, as assessed by immunoblot analysis of caspase-8, -3, -2, and -7, was detected in Bak-deficient Jurkat cells treated with staurosporin or VP-16 for 14 h (Fig. 2B). Also, no caspase activity as assessed by cleavage of the fluorescent peptide substrate Ac-DEVD-AMC was detected in Bak-deficient Jurkat cells treated with cytotoxic drugs (Fig. 2C). These results demonstrate that the Bak-deficient cells are absolutely resistant to apoptotic signals delivered by an array of anticancer drugs.

Transduction of Bak-deficient Cells with Ad/GT-Bak and Ad/GV-16 Vectors—Using a binary adenoviral vector system to avoid the toxic effects of Ad/Bak on 293 packaging cells, we successfully produced large amounts of Ad/GT-Bak, whose gene product (Bak) was under the transcriptional control of the GT promoter and GAL4/GV16 fusion protein (43, 44). The binary adenoviral LacZ vector system (Ad/GT-LacZ and Ad/GV16) was used to determine transduction efficiency. As detected by immunoblotting (Fig. 3A), Bak expression was induced when Ad/GT-Bak and Ad/GV16 were administered, but...
not when Ad/GT-LacZ and Ad/GV16 were used. A high level of Bak expression has been reported to induce rapid cell death (33). As assessed by flow cytometry of cells stained with propidium iodide and FITC-conjugated annexin V, we also observed a substantial level of apoptotic cell death (40–60%) in both wild type and Bak-deficient Jurkat cells 24 h after transduction with Ad/GT-Bak + Ad/GV16 vectors but not in mock infected cells (Fig. 3B). However, at 48 h post infection, when the majority of the initial population of apoptotic cells detected at 24 h has already been disintegrated, a population of cells susceptible to VP-16- or cisplatin-mediated apoptosis was detected in Bak-deficient cells administered with Ad/GT-Bak and Ad/GV16 vectors but not with the LacZ mock vectors (results for VP-16 are shown in Fig. 3C). It is plausible that a high level of Bak expression is associated with accelerated cell death, whereas cells with a lower level of Bak expression develop susceptibility to VP-16. Bak-mediated apoptosis was also confirmed by detection of caspase-3 activation and poly(ADP-ribose) polymerase cleavage in Bak-deficient cells infected with Bak but not in mock infected cells (Fig. 3A). These results confirm the role of Bak in the observed resistance to VP-16- and cisplatin-mediated apoptosis.

Abrogation of Cytochrome c Release in Bak-deficient Jurkat Cells—Because the mitochondrial apoptotic cascade requires the release of intermembrane mitochondrial cytochrome c to the cytosol, the Bak-deficient Jurkat cells were assessed for mitochondrial cytochrome c release in response to VP-16 or UV irradiation. In contrast to wild type Jurkat cells, no cytochrome c was detected in the cytosolic S-100 fraction of Bak-deficient cells treated with VP-16 or UV irradiation (Fig. 4A).

Bid, Bax, and Bak are proapoptotic Bcl-2 family members reported to induce release of cytochrome c when applied directly to mitochondria (14, 16, 48). To gain insights into the nature of the block in the cytochrome c release mechanism, purified mitochondria obtained from wild type or Bak-deficient Jurkat cells were treated with recombinant Bax. Purified mitochondria (100 μg) obtained from wild type or Bak-deficient Jurkat cells were treated with GST-BaxΔTM at the indicated concentrations for 30 min at 30 °C. The supernatants (Mit-Sup) were boiled in reducing Laemmli buffer and assessed by immunoblotting for the presence of cytochrome c. Lysed mitochondria from each of the two cell lines tested served as positive controls. C, no release of cytochrome c from Bak-deficient mitochondria in response to recombinant tBid. Purified mitochondria from wild type or Bak-deficient Jurkat cells were treated with the indicated concentration of His-tBid. Mitochondria from wild type Jurkat cells release cytochrome c in response to a dose of His-tBid as low as 5 nM (not shown). D, release of cytochrome c from wild type or Bak-deficient mitochondria treated with the protonophore CCCP. Purified mitochondria from wild type or Bak-deficient cells were treated with CCCP (50 μM) for 30 min at 30 °C. The supernatants and mitochondria were assessed for the presence of cytochrome c.
in the mitochondria (39, 47). Upon induction of apoptosis, Bax translocates to the mitochondria (7, 16, 47, 51). Consistent with these reports, utilizing mitochondria purified from either wild type or Bak-deficient cells, we found that endogenous Bax was attached to nonapoptotic mitochondria of either wild type or Bak-deficient cells. Purified mitochondria (100 μg) obtained from wild type or Bak-deficient Jurkat cells were treated with 0.1 M Na₂CO₃, pH 11.5, for 20 min on ice. The supernatants (S), which contained alkali-sensitive, mitochondria-attached proteins, were separated from the pellets (P) of alkali-treated mitochondria, which contained alkali-resistant proteins inserted into the mitochondria. The proteins were resolved by SDS/PAGE and assessed by immunblotting for the presence of endogenous Bax. The membrane was stripped and successively reprobed with anti-Cox IV Ab and anti-VDAC mAb. C, insertion of recombinant Bax into wild type or Bak-deficient mitochondria. Purified mitochondria from wild type or Bak-deficient cells were treated with GST-BaxΔTM. Following alkali treatment, alkali-sensitive and alkali-resistant mitochondrial fractions were assessed for localization of recombinant Bax. The membrane was stripped and immunoblotted for VDAC as a marker for mitochondrial fraction and as a loading control. The appearance of at least two bands of recombinant Bax is likely due to partial degradation of the fusion protein by bacterial proteases. D, insertion of His-tBid into wild type or Bak-deficient mitochondria. Purified mitochondria from wild type or Bak-deficient cells were treated with His-tBid, followed by exposure to alkali, as described above. Alkali-sensitive and alkali-resistant mitochondrial fractions were assessed for localization of recombinant tBid. tBid was only detected in mitochondria when exogenously applied (data not shown). The membrane was stripped and successively immunoblotted for the presence of Cox IV and VDAC as markers for mitochondrial fractions and as loading controls. WT, wild type.

**Restoration of Cytochrome c Release from Bak-deficient Mitochondria by Recombinant Bak**—As shown in Fig. 3, viral expression of Bak resulted in either a rapid induction of cell death or an acquired susceptibility of Bak-deficient cells to VP-16-mediated apoptosis. We also investigated the possibility of restoring the cytochrome c releasing capability of purified Bak-deficient mitochondria following insertion of recombinant Bak. We first examined the ability of recombinant Bak (GST-BakΔC) to insert into the mitochondria of Bak-deficient cells. When applied to purified mitochondria, recombinant Bak had similar patterns of localization in either wild type or Bak-deficient Jurkat cells. GST-BakΔC was detected both as an attached and as an inserted protein in either wild type or Bak-deficient mitochondria (Fig. 6A). The insertion of GST-BakΔC withstood mild treatment with digitonin, because it was detected not only in the supernatant but also in the pellet.
Bak-dependent Response to Anticancer Drugs

Fig. 6. Insertion of GST-BakΔC into mitochondria of wild type or Bak-deficient cells. Purified mitochondria obtained from the two Jurkat cell lines were incubated with GST-BakΔC (8 μM) for 30 min at 30 °C. In A the mitochondria were pelleted, washed, and treated with 0.1 M Na₂CO₃, pH 11.5. Alkali-sensitive (mitochondria together with GST-Bax/H9004 successively reprobed with anti-Cox IV and anti-VDAC mAbs. In B the mitochondria were stripped and with anti-Bak (Ab-1, Oncogene) for the presence of endogenous Bak. Purified mitochondria obtained from the two or Bak-deficient cells. GST-BakΔC/H9004/Jurkat cell lines were incubated with GST-Bak in Bak activity, we examined the effect of GST-BakΔC on cytochrome c release from liver mitochondria of either Bid⁻/⁻, Bax⁻/⁻, or Bid⁻/⁻ Bax⁻/⁻ mice (28, 52). Bak-induced cytochrome c release from wild type liver mitochondria as well as from mitochondria deficient in Bid, Bax, or both Bid and Bax (Fig. 9A). These results suggest that mitochondrial Bak is capable of releasing cytochrome c independently of Bax or Bid. However, it appears that the release of cytochrome c is enhanced when Bak is applied to purified mitochondria in combination with Bax and tBid (Fig. 9A). The addition of recombinant Bax or tBid also induced cytochrome c release from mitochondria of each of the knockout mice. However, mouse liver mitochondria from wild type, Bid⁻/⁻, Bax⁻/⁻, or Bid⁻/⁻ Bax⁻/⁻ all expressed similar levels of Bak (Fig. 9B).

DISCUSSION

The present study demonstrates a role for Bak in the mechanism of releasing mitochondrial intermembrane proteins by human leukemic cells in response to cytotoxic drugs. The deficiency in Bak endowed Jurkat leukemic cells with a potent mechanism of resistance to UV and drug-mediated apoptosis, which was reversed in viral transduced cells that express Bak. The block in the apoptotic cascade in Bak-deficient cells was localized to the mitochondrial mechanism(s) of releasing intermembrane proteins. No cytochrome c was detected in the cytosol of Bak-deficient cells treated with various doses of VP-16, staurosporin, cisplatin, bleomycin, or UV irradiation. Further, in contrast to wild type mitochondria, those isolated from Bak-deficient cells did not release either cytochrome c or AIF in response to recombinant Bax or tBid in vitro. The deficiency in Bak was directly responsible for the arrest in cytochrome c release, because this capability was restored to the Bak-deficient mitochondria by the insertion of recombinant Bak into purified mitochondria from these cells. Furthermore, synergistic activity was detected in the presence of combinations of suboptimal doses of recombinant Bak and Bax, suggesting that in the presence of a low dose of recombinant Bak the resistance of these mitochondria to Bax-mediated cytochrome c release was reversed. As recently reported for hepatocytes from Bax⁻/⁻ mice (41), Bak-deficient Jurkat cells were also resistant to tBid induction of cytochrome c release, suggesting that in these leukemic cells Bak is involved in mitochondrial cytochrome c release induced by either tBid or Bax.

It has been suggested that tBid acts partly by inducing conformational changes in Bax (38, 39). However, as reported previously (38, 40) and confirmed by this study, recombinant tBid can also induce cytochrome c release independently of application of combined low doses resulted in release of cytochrome c and AIF, which were not released by either Bak or Bax alone at these concentrations (Fig. 7D). Thus, the presence of recombinant Bak restored the capability of Bak-deficient mitochondria to release intermembrane proteins in response to recombinant Bak.

Resistance of Bak-deficient Cells to Nonsteroidal Anti-inflammatory Drugs—It has recently been reported that the apoptotic response of colorectal cells to NSAID, including indomethacin and sulindac sulfide, depends on the presence of functional Bax (18). Utilizing Bak-deficient Jurkat cells we investigated a potential role for Bak in resistance to NSAID. Bak-deficient cells were resistant to indomethacin and sulindac sulfide, whereas wild type Jurkat cells were highly susceptible (Fig. 8). These results suggest that in this variant of T leukemic cells the response to NSAID is regulated by Bak.

Bak-mediated Cytochrome c Release Is Independent of Bid or Bax—The expression of Bax and/or tBid in Bak-deficient mitochondria might have enabled cytochrome c release mediated by recombinant Bak. To investigate the role of Bax and/or tBid in Bak activity, we examined the effect of GST-BakΔC on cytochrome c release from liver mitochondria of either Bid⁻/⁻, Bax⁻/⁻, or Bid⁻/⁻ Bax⁻/⁻ mice (28, 52). Bak-induced cytochrome c release from wild type liver mitochondria as well as from mitochondria deficient in Bid, Bax, or both Bid and Bax (Fig. 9A). These results suggest that mitochondrial Bak is capable of releasing cytochrome c independently of Bax or Bid. However, it appears that the release of cytochrome c is enhanced when Bak is applied to purified mitochondria in combination with Bax and tBid (Fig. 9A). The addition of recombinant Bax or tBid also induced cytochrome c release from mitochondria of each of the knockout mice. However, mouse liver mitochondria from wild type, Bid⁻/⁻, Bax⁻/⁻, or Bid⁻/⁻ Bax⁻/⁻ all expressed similar levels of Bak (Fig. 9B).

of digitonin-treated mitochondria (Fig. 6B). These observations suggest that recombinant Bak was similarly inserted into wild type and Bak-deficient mitochondria. The multi-band appearance of recombinant Bak was most likely due to degradation of GST-BakΔC by bacterial proteases.

Because recombinant Bak has been reported to induce cytochrome c release from liver mitochondria (16), we examined its effect on Bak-deficient mitochondria. Exogenously added recombinant Bak, but not Bax or tBid, induced cytochrome c release from Bak-deficient mitochondria (Fig. 7A). Recombinant Bak had a dose-dependent effect on cytochrome c release in both wild type or Bak-deficient mitochondria (Fig. 7B). It also induced the release of another mitochondrial intermembrane protein, AIF, which was detected in the supernatant of Bak-deficient mitochondria (Fig. 7C). To investigate the ability of Bak to restore mitochondrial response to recombinant Bax, GST-BakΔC at 0.8 μM, a dose found to be too low to induce cytochrome c release, was applied to purified Bak-deficient mitochondria together with GST-BaxΔTM (2 μM). Recombinant Bak was found to synergize with recombinant Bax, because...
FIG. 7. Release of intermembrane proteins, cytochrome c, and AIF from Bak-deficient mitochondria in response to recombinant Bak. A, Bak-mediated release of cytochrome c from Bak-deficient mitochondria. Purified mitochondria obtained from Bak-deficient Jurkat cells (100 μg/20 ml MIB) were incubated with GST-BakΔC (8 μM), GST-BaxΔTM (2 μM), or His-tBid (200 nM) for 30 min at 30 °C. The supernatants were separated from mitochondria, and the proteins of each fraction were resolved by SDS/PAGE and immunoblotted for the presence of cytochrome c. B and C, dose-dependent release of cytochrome c (B) or AIF (C) from wild type or Bak-deficient mitochondria in response to GST-BakΔC. The supernatants of mitochondria treated with various doses of recombinant Bak were assessed for the presence of cytochrome c or AIF. D, synergism between recombinant Bak and Bax in release of cytochrome c or AIF from Bak-deficient mitochondria. Purified mitochondria obtained from Bak-deficient Jurkat cells were treated with recombinant Bak and/or Bax at the indicated doses. The resultant supernatants as well as a sample of mitochondria were assessed by immunoblotting for the presence of cytochrome c. The membrane was stripped and reprobed with anti-AIF Ab. WT, wild type; Bak(-), Bak-deficient.

FIG. 8. Resistance of Bak-deficient cells to sulindac sulfide or indomethacin. Wild type (WT) or Bak-deficient cells (Bak(-)) were treated with sulindac sulfide (200 μM) or indomethacin (500 μM) for 36 h. The cells were then stained with propidium iodide and annexin V and assessed for the presence of positive cells by flow cytometry.
Bax. Indeed, Desagher et al. (38) have noted that conformational changes in mitochondrial Bak were induced by tBid in mitochondria from Bax-deficient cells. The definitive requirement for Bak in Bid-mediated cytochrome c release from mitochondria was recently reported by Wei et al. (41). In that study, by treating Bak-expressing mitochondria with anti-Bak blocking Ab or utilizing liver mitochondria from Bak knockout mice, tBid was found to bind in a transient manner to mitochondrial Bak. tBid-Bak binding resulted in oligomerization of Bak as part of a cytochrome c release mechanism that was independent of permeability transition (41, 53).

It has recently been reported that Bak−/− mice are developmentally normal and reproductively fit (54). Also Bak−/− mice display limited phenotypic abnormalities (28, 54). However, 90% of mice lacking both Bax and Bak genes died perinatally (54). Bax−/− Bak−/− mice displayed multiple developmental defects, suggesting that Bak and Bax have overlapping roles in regulation of apoptosis during mammalian development (54, 55). These overlapping roles, which were observed in murine models for development of normal cells, remain to be elucidated in human tumor cells. Despite the regulation of Bak and Bax by p53, these tumor suppressors also have pro-apoptotic effects in cells with altered p53 expression (25, 32, 56). Therefore, a redundant function for Bax and Bak, as detected in normal murine cells expressing wild type p53, may be differentially regulated in numerous types of cancer, which are either null or mutated for p53, such as human T leukemia Jurkat cells (57, 58). The study by Vogelstein and co-workers (18) also suggests that the redundancy between Bax and Bak does not apply in this type of cancer cells.

Bak-deficient Jurkat cells and three additional loss-of-function mouse models, including Bax−/−, Bid−/−, and Bid−/− Bax−/− cells were used in the current study to investigate whether the Bak activation cascade is dependent on Bax or Bid. Recombinant Bak induced cytochrome c release from mitochondria of Bid−/−, Bax−/−, or Bid−/− Bax−/− mice, suggesting that it can function independently of both Bid and Bax. However, we noticed an enhanced cytochrome c release from the mitochondria of the various knockout mice examined when treated with a combination of recombinant tBid, Bax, and Bak.

Mounting evidence indicates that apoptosis is the predominant form of cell death triggered by anticancer drugs. Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. In particular, altering a component of the apoptotic machinery has been shown to affect the dynamics of tumor progression, providing a rationale for the inactivation of this machinery during tumor development. Mutations in the Bak gene were detected in human gastric and colorectal cancers (31, 36), and degradation of Bak has been observed in human papillomavirus-positive melanoma (59). However, Bak deficiency as a mechanism for chemoresistance has not yet been addressed. The resistance of Bak-deficient cells to certain anticancer drugs in the face of the susceptibility of wild type Jurkat cells suggests that Bak participates in either the regulation or the execution of the mitochondrial apoptotic response in this type of cancer cells.

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