Many studies have demonstrated a critical role of Bax in mediating apoptosis, but the role of Bak in regulating cancer cell apoptotic sensitivities in the presence or absence of Bax remains incompletely understood. Using isogenic cells with defined genetic deficiencies, here we show that in response to intrinsic, extrinsic, and endoplasmic reticulum stress stimuli, HCT116 cells show clear-cut apoptotic sensitivities in the order of Bax+/Bak− > Bax+/Bak− > Bax−/Bak− > Bax+/Bak−. Small interference RNA-mediated knockdown of Bak in Bax-deficient cells renders HCT116 cells completely resistant to apoptosis induction. Surprisingly, however, Bak knockdown in Bax-expressing cells only slightly affects the apoptotic sensitivities. Bak, like Bax, undergoes the N terminus exposure upon apoptotic stimulation in both Bax-expressing and Bax-deficient cells. Gel filtration, chemical cross-linking, and co-immunoprecipitation experiments reveal that different from Bax, which normally exists as monomers in unstimulated cells and is oligomerized by apoptotic stimulation, most Bak in unstimulated HCT116 cells exists in two distinct protein complexes, one of which contains voltage-dependent anion channel (VDAC) 2. During apoptosis, Bak and Bax form both homo- and hetero-oligomeric complexes that still retain some VDAC-2. However, the oligomeric VDAC-2 complexes are diminished, and Bak does not interact with VDAC-2 in Bax-deficient HCT116 cells. These results highlight VDAC-2 as a critical inhibitor of Bak-mediated apoptotic responses.

Bax and Bak are important mediators of cell death as bax/bak double knockout cells are resistant to multiple apoptotic inducers (1, 2). In most cells, Bax is normally localized in the cytosol or loosely associated with the outer mitochondrial membrane, whereas Bak is mostly localized in the outer mitochondrial membrane and remains inactive perhaps because of binding to VDAC-2 (3). BH3-only Bcl-2 proteins such as tBid and Bim, upon activation or up-regulation by apoptotic stimuli, cause translocation of Bak to the outer mitochondrial membrane and subsequent conformational changes and oligomerization of Bax and Bak. The activated Bax and/or Bak facilitate the release of some (e.g. cytochrome c and Smac) (4) but not all (e.g. AIF and Endo G) (5) proapoptotic proteins from the mitochondria by forming pores or channels or otherwise altering mitochondrial membrane permeability and structural architecture of the organelles (6, 7). Recent work suggests that Bak and Bak localized on the ER also play a role in maintaining Ca2+ homeostasis and in ER-mitochondria cross-talk during apoptosis induction (8).

Numerous studies have demonstrated a critical role for Bax in determining tumor cell sensitivity to apoptosis induction and in tumor development. For example, somatic mutation in bax, which occurs in colon (9, 10), prostate (11, 12), hematopoietic (13), and other (14) cancer cells, confers on tumor cells a survival advantage and promotes clonal expansion. Increased Bax degradation through proteosome-dependent mechanisms (15) and reduced Bak expression (12, 16) have also been reported in prostate cancers. In animal models, Bax has been shown to suppress tumorigenesis (17) and to be required for tBid-induced hepatocyte apoptosis (18). Loss of Bax alters tumor spectrum (from lymphoma to carcinoma) (19) and impairs Myc-induced apoptosis during Myc-mediated lymphomagenesis (20). Even loss of a single allele of bax has been shown to accelerate mammary tumor development (21). Somatic knocking out of bax renders HCT116 colon cancer cells resistant to both intrinsic and extrinsic apoptotic inducers as well as to ER stressors (22–27). Bax has also been shown to play an important role in apoptosis induced by death receptors (25, 28–30), p53 (31), ceramide (32), and BH3-only protein Bik (33).

Compared with Bax, fewer studies have been dedicated to Bak. Somatic bax mutations have been reported only in gastric and colon cancer cells (34), and reduced Bak expression is observed in prostate cancer cells (16). Some studies have also implicated Bak as a determinant of drug sensitivity (35). In general, most comparative studies suggest that Bax and Bak have independent, nonredundant proapoptotic activities (29–38). However, the role of Bak in regulating cancer cell apoptotic sensitivities in the presence or absence of Bax re-

The abbreviations used are: VDAC, voltage-dependent anion channel; CHX, cycloheximide; KO, knockout; STS, staurosporine; TG, thapsigargin; VP16, etoposide; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; ER, endoplasmic reticulum; siRNA, small interference RNA; mAb, monoclonal antibody; pAb, polyclonal antibody; DAPI, 4,6-diamino-2-phenylindole; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; IP, immunoprecipitation; Rb, rabbit.

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Received for publication, February 7, 2005, and in revised form, March 7, 2005
Published, JBC Papers in Press, March 9, 2005, DOI 10.1074/jbc.M501391200

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mains incompletely understood. In this study, we address this issue using the siRNA-mediated ablation of Bax and also explore the potential underlying mechanisms of action.

MATERIALS AND METHODS

**Cells and Reagents—**HCT116 and HCT116-Bax-KO (22) were provided by Dr. B. Vogelstein and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Wild-type Du145, Du145-mock, and Du145-Bax (32) cells were cultured in RPMI medium supplemented with 10% fetal bovine serum. The primary antibodies include anti-cytchrome c (mAb; BD Pharmingen), anti-holocytochrome c (mAb; R & D Systems), anti-Bax (Rb pAb; BD Pharmingen), anti-Bak NT terminus or NT (Rb pAb; Upstate Biotechnology, Inc.), anti-Bak (Rb pAb; Santa Cruz), anti-Bak NT (Rb pAb; Upstate Biotechnology, Inc.), anti-VDAC-1 (mAb and Rb pAb; Calbiochem), anti-VDAC-2 (goat pAb; Santa Cruz), anti-Smac (Rb pAb; Promega), anti-anapase-9 and -9 (Rb pAb; Bi- omol), anti-Fas cross-linking antibody (clone CH11; Upstate Biotechnol- ogy, Inc.), and anti-actin (mAb; ICN). Secondary antibodies and ECL reagents were acquired from Amersham Biosciences. AlexaFluor 594- or 488-conjugated goat anti-rabbit IgG (H+L) and mitochondrial dye (i.e. MitoTracker Orange CMTMRos) were purchased from Molecular Probes. Fluorogenic caspase substrates DEVD-7-amino-4-trifluoromethyl-couma- rin, IETD-7-amino-4-methyl-coumarin, and general caspase in- hibitor Z-VAD-fmk were bought from Biomol. All other chemicals were purchased from Sigma unless specified otherwise.

**Subcellular Fractionation and Western Blotting—**Whole cell lysate, mitochondrial membrane fractions, and cytosol were prepared, and Western blotting was performed as previously detailed (16, 39–41).

**Quantification of Apoptosis and Caspase Activity Measurement—**Apoptotic nuclei were determined based on apoptotic nuclei upon transfection using cells live with DAPI (12, 16). DEVDase and LEHDase activities were measured as previously described (39–41).

**Establishment of HCT116 Cells Stably Expressing Bak siRNA Using Retroviral Vectors—**Three double-stranded 21-nt siRNAs against human bak were initially made using the Silencer™ kit (Ambion, Austin, TX). The efficiencies were 5'-CTCTGAGTCATAGCGTCG-3', bak2(5'-GCTGAATGACTACTAGGCA-3'), and bak3(5'-ACAGGCTGTTGGAATC-3'). Preliminary experiments revealed the strongest effect with bak3, and this siRNA was then used in most subsequent experiments. A scrambled sequence (5’-CAACAAGCTGCT-GAAGCTCA-3’) for bak3 was used as control. To generate stable expression of siRNA, we first cloned the siRNA sequences corresponding to bak3 and the scrambled control into the pSuper.retro viral vector (42). These vectors were transfected into the Amphi Phoenix packaging cells. 48 h after transfection, the culture medium containing the infectious virions was harvested, filtered (0.2 μm), and directly used to infect HCT116 cells. 48 h later, the cells were selected with puromycin (2 μg/ml), and stable clones were isolated 2–3 weeks later using a cloning ring. Stables clones were analyzed by Western blotting to confirm knock-down of expression.

**siRNA Down-regulation of Bak—**SIGNENOME SMARTpool for Bak (Bak siRNApool; sequence 1, 5'-CAGAGAAGUCCUAGUAGUAAU-3', sequence 2, 5'-UAUGAGAUCAUUCCAAAGAUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAU
suggesting that it might be caspase-independent. In support, DEVDase activity was not detected in either cell type upon irradiation (Fig. 1E). Furthermore, x-ray-induced death in these cells morphologically resembled necrosis (not shown).

Finally, when HCT116 cells were stimulated with TG, an irreversible inhibitor of the ER outer membrane pump SERCA whose function is to maintain the concentration of ER Ca\(^{2+}\) several orders of magnitude above that of the cytosol, >40% of the cells died within 12 h, and ~85% underwent apoptosis by 36 h (Table I). Again, loss of Bax delayed the onset and also decreased the peak level of apoptosis (Table I). As expected, Z-VAD significantly inhibited TG-induced cell death (Table I), and Bax deficiency led to lower DEVDase activity (Fig. 1E).

To determine whether the Bax deficiency conferred resistance is at least partially mediated via decreased release of mitochondrial cytochrome c, we treated HCT116 or HCT116-Bax-KO cells with one extrinsic (i.e. Fas ligation) and one intrinsic (i.e. STS) inducer. As shown in Fig. 1B, the loss of Bax reduced the apoptotic stimuli-induced release of the mitochondrial holocytochrome c, which was detected by an antibody that specifically recognizes holo- but not apo-cytochrome c (39). Altogether, the above results have revealed the following points. First, the loss of Bax delays the apoptosis initiation in that the inhibitory effect of Bax deficiency appears more dramatic at earlier time points (Table I). These observations are consistent with Bax functioning as a critical gateway in the mitochondrial pathway of apoptosis (1, 2). Second, at later time points, the Bax deficiency-rendered apoptosis resistance is somewhat diminished (Table I), likely because of compensation from Bak (see below). Third, Bax might also play a role in caspase-independent cell death (43–45) because HCT116-Bax-KO cells are resistant to x-ray irradiation, which does not activate DEVDase and induces Z-VAD-insensitive cell death (Table I and Fig. 1E).

**Fig. 1.** Bax expression increases holocytochrome c release and caspase activation. A and C, Western blotting of Bax and Bak in HCT116 and HCT116-Bax-KO (A) or Du145 and Du145-Bax (C) with actin as loading control. B and D, HCT116 and HCT116-Bax-KO (B) or Du145 and Du145-Bax (D) cells were treated with STS (0.5 \(\mu\)M) or Fas ligation (FAS). Equal amounts of cytosol (60 \(\mu\)g/lane) were used in Western blotting for holocytochrome c (Holocyto-c). X-protein is an unknown protein detected by the same antibody and used as loading control. E and F, DEVDase activities in HCT116 and HCT116-Bax-KO (E) or Du145-mock and Du145-Bax (F) either untreated (Cont) or treated with STS (24 h), TG (24 h), x-ray irradiation (72 h), VP16 (48 h), or Fas antibody plus CHX (8 h). The mean DEVDase activities were derived from triplicate samples. WT, wild type.

Restoration of Bax Expression Enhances Apoptosis in Bax-deficient, Bak-expressing Du145 Prostate Cancer Cells—Bax-deficient Du145 cells, compared with other prostate cancer cells, are relatively more resistant to apoptosis by some inducers such as starvation (12) and ceramide (32). Restoration of Bax expression using stable retroviral transduction confers sensitivity in Du145 cells to ceramide-induced cell death (32). As shown in Fig. 1C, Du145-mock cells (i.e. Du145 cells subjected to mock infection) (32) were devoid of Bax expression, whereas Du145-Bax cells expressed Bax but both expressed Bak. Bak re-expression enhanced the apoptotic sensitivities of Du145 cells to all five apoptotic stimuli, with different kinetics and to different degrees (Table I). For example, Du145-Bax cells were more sensitive to STS, VP16, and x-ray throughout the treatment period (Table I). By contrast, Du145-Bax cells showed increased sensitivity to Fas ligation only early upon
more, Bax re-expression did not result in increased DEVDase activities to TG (Table I), suggesting that the Bax-regulated responses to TG until 36 h after stimulation (Table I). Consequently, Bax appeared to play a more prominent role in Du145-Bax cells did not show enhanced apoptotic responses to TG until 36 h after stimulation (Table I), probably because caspases other than caspase-3/7. Indeed, TG has been shown to induce apoptosis in the control siRNAs, whereas levels of Bax were not affected siRNAs down-regulated Bak protein expression in HCT116 and HCT116-Bax-KO cells (not shown). Unfortunately, although these siRNAs dramatically down-regulated Bak expression by 24 h, their inhibitory effects essentially disappeared by 72 h (supplemental Fig. S1A). To circumvent this problem, we constructed retroviral vectors encoding either bak3 shRNA or a scrambled control shRNA, which were used to infect HCT116 cells. Of seven stable clones, clones 3 and 4 showed the lowest Bak protein expression (supplemental Fig. S1B). We thus produced scrambled clone 3 (as control) and Bak clone 4 HCT116 cells with STS, Fas/CHX, and TG. Much to our surprise, both clones showed overall similar levels of release of cytochrome c and Smac as well as caspase-3 activation, i.e. generation of the p20/p17 fragments of active caspase-3 (40) (Fig. 2A). Clonogenic survival assays also did not reveal significant differences in cell death and clonogenicity (not shown).

To further establish the role of Bak in Bax-expressing cells, we obtained a new generation of siRNAs from Dharmacon, which have longer lasting and more powerful silencing effects as a pool of target siRNAs is used. A pool of bak siRNAs (containing four siRNA oligonucleotides) or control siRNAs were transfected into HCT116 or HCT116-Bax-KO cells, and Western blot analysis revealed a time-dependent down-regulation of Bak in both cell types by the treatment of Bak-expressing cells might affect cancer cell apoptotic sensitivity. To that end, we designed and synthesized three siRNAs targeting human bak. When tested in Du145 cells, all three bak siRNAs down-regulated Bak protein levels in the order of bak3 > bak2 > bak1 (supplemental Fig. S1A). These bak siRNAs also down-regulated Bak protein expression in HCT116 and HCT116-Bax-KO cells (not shown). stimulation, i.e. at 4 h (Table I). By 8 h after Fas ligation, similar levels of apoptosis were observed in both Du145-mock and Du145-Bax cells (Table I), probably because caspases became activated by Bax-independent mechanisms. On the other hand, Du145-Bax cells did not show enhanced apoptotic responses to TG until 36 h after stimulation (Table I).

Compared with the isogenic pair of HCT116 cells, isogenic Du145 cells showed some interesting differences. For example, Du145 cells were more sensitive to Fas ligation than the HCT116 pair (Table I) in that the former cells, with or without Bax, were nearly completely killed by 8 h, whereas within the same time frame only ~50% of the HCT116 cells were dead. On the other hand, Bax appeared to play a more prominent role in HCT116 cells than in Du145 cells in determining their sensitivities to TG (Table I), suggesting that the Bax-regulated response to ER stress might be cell type-dependent. Furthermore, Bax re-expression did not result in increased DEVDase activities in Du145-Bax cells treated with TG (Fig. 1P), suggesting that Bax restoration may lead to TG-induced activation of caspases other than caspase-3/7. Indeed, TG has been shown to activate the death-receptor pathway leading to caspase-8 activation (25, 27).

To determine whether the Bax restoration-conferred apoptosis sensitivity is at least partially mediated via increased release of mitochondrial cytochrome c, we treated isogenic Du145 cells with Fas ligation or STS. As shown in Fig. 1D, Bax re-expression increased the release of the mitochondrial cytochrome c.

siRNA-mediated Down-regulation of Bak in Bax-expressing Cells Only Slightly Inhibits Mitochondrial Protein Release, Caspase Activation, and Apoptosis—The preceding experiments support a critical role of Bax in determining cancer cell apoptotic sensitivities, as previously reported by others (e.g. 22–33). Nevertheless, both HCT116-Bax-KO and Du145 cells still undergo apoptosis in response to these stimuli except with slower kinetics, suggesting that other molecules, in particular, Bak, are also functional apoptosis regulators. To test this, we carried out reciprocal experiments to examine how ablation of Bak in Bax-expressing cells might affect cancer cell apoptotic sensitivity. To that end, we designed and synthesized three siRNA oligonucleotides targeting human bak. When tested in Du145 cells, all three bak siRNAs down-regulated Bak protein levels in the order of bak3 > bak2 > bak1 (supplemental Fig. S1A). These bak siRNAs also down-regulated Bak protein expression in HCT116 and HCT116-Bax-KO cells (not shown).

Unfortunately, although these siRNAs dramatically down-regulated Bak expression by 24 h, their inhibitory effects essentially disappeared by 72 h (supplemental Fig. S1A). To circumvent this problem, we constructed retroviral vectors encoding either bak3 shRNA or a scrambled control shRNA, which were used to infect HCT116 cells. Of seven stable clones, clones 3 and 4 showed the lowest Bak protein expression (supplemental Fig. S1B). We thus produced scrambled clone 3 (as control) and Bak clone 4 HCT116 cells with STS, Fas/CHX, and TG. Much to our surprise, both clones showed overall similar levels of release of cytochrome c and Smac as well as caspase-3 activation, i.e. generation of the p20/p17 fragments of active caspase-3 (40) (Fig. 2A). Clonogenic survival assays also did not reveal significant differences in cell death and clonogenicity (not shown).

To further establish the role of Bak in Bax-expressing cells, we obtained a new generation of siRNAs from Dharmacon, which have longer lasting and more powerful silencing effects as a pool of target siRNAs is used. A pool of bak siRNAs (containing four siRNA oligonucleotides) or control siRNAs were transfected into HCT116 or HCT116-Bax-KO cells, and Western blot analysis revealed a time-dependent down-regulation of Bak in both cell types by the bak siRNAs compared with the control siRNAs, whereas levels of Bax were not affected.
Bak Regulation of Cancer Cell Apoptosis

FIG. 2. Down-regulation of Bak in Bax-expressing cells only slightly affects the mitochondrial protein release and caspase activation. A, Bak-stable (clone 4) and scrambled control (clone 3) HCT116 cells were treated with STS (0.5 μM), Fas ligation (FAS), or TG (5 μM). Equal amounts of cytosol or mitochondrial (60 μg/ml) were used in Western blotting. X-protein, loading control; Cont, control. B, HCT116 and HCT116-Bax-KO cells were transfected with either control siRNA oligonucleotides (100 nM) or bak siRNA pools (100 nM) for 24–72 h. Whole cell lysates (60 μg/ml) were used in Western blotting. NS, non-specific band. C, HCT116 cells were transfected with either control (Cont and ctl) or bak siRNAs at 100 nM. Forty-eight h after transfection, the cells were treated, harvested, and analyzed as described for A. Cyt. c, cytochrome c.

(unknown 1024x1536 to 563x737)

(unknown 263x485 to 563x737)

(unknown 263x485 to 563x737)

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(unknown 263x485 to 563x737)

(unknown 263x485 to 563x737)
mitochondrial potential collapse (Fig. 4 and supplemental Fig. S2). We observed similar results in stimulated HCT116 cells with respect to Bax N terminus exposure (not shown). Likewise, in both Bax-expressing as well as Bax-deficient Du145 cells, we observed similar levels of Bak staining indicative of N terminus exposure in response to STS (Fig. 5), Fas (Fig. 5), or VP16 (not shown). In both cell types stimulated with STS, most cells with Bak staining were DAPI-positive and also retained MitoTracker labeling (Fig. 5), just as in Bax-activated cells (Fig. 4). Similarly, in Fas (Fig. 5) or VP16 (not shown) stimulated Du145-mock or Du145-Bax cells, many Bak-activated cells had not shown apoptotic nuclei, suggesting that, like Bax (Fig. 4), Bak activation precedes nuclear apoptosis. Similar results on Bak N terminus exposure were obtained in HCT116 and HCT116-Bax-KO cells (data not shown). Collectively, the observations in Figs. 4 and 5 suggest that Bak (and Bax) underwent apoptotic stimuli-induced N terminus exposure in both Bax-expressing and Bax-deficient cells.

Different Bak and Bax Oligomerization Status in Unstimulated Cells and during Apoptotic Stimulation—Next, we examined the second event critical in Bak (and Bax) activation, i.e. oligomerization. Bak is generally thought to exist as monomers in the cytosol (or loosely attached to the outer mitochondrial membrane), and apoptotic stimulation induces its oligomerization in the mitochondria. Indeed, when we utilized gel filtration analysis to characterize Bak, we found that Bak in unstimulated HCT116 cells was mainly expressed as monomers eluted at fractions 14–16 (Fig. 6A). Fas ligation in HCT116 cells resulted in a shift of the protein toward high molecular mass fractions (fractions 2–11) (Fig. 6A) peaking at ~158 kDa (fractions 9 and 10), indicating the formation of Bak oligomers. To provide additional support for Bak oligomerization, we analyzed the formation of a higher order Bak complex by chemical cross-linking. To that end, freshly isolated mitochondria were incubated with bismaleimidohexane, a noncleavable, membrane-permeable homobifunctional maleimide that covalently and irreversibly cross-links sulfhydryl groups (1, 3). As shown in Fig. 7 (A and B), Fas ligation induced formation of homooligomeric Bak proteins in the mitochondria as early as 4 h (7A), and Bak oligomers further increased by 6 h (Fig. 7B). As expected, Bak oligomerization was not observed in HCT116-Bax-KO cells (Fig. 7, A and B) or the cytosol of Fas-treated HCT116 cells (not shown). STS treatment similarly induced Bak oligomerization in HCT116 but not in HCT116-Bax-KO cells (see Fig. 7G, lanes 3 and 9). Likewise, Bak oligomerization was observed in Du145-Bax but not Du145-mock cells treated with either Fas or STS (Fig. 7C and supplemental Fig. S3). Interestingly, apoptotic stimulation slightly increased the Bax protein levels in some experiments (Fig. 7, A–C, and supplemental Fig. S3), which resulted, most likely, from increased translocation to the mitochondria. These data altogether suggest that Bak is activated in our apoptotic systems by both exposing the N terminus and undergoing homo-oligomerization as identified by bismaleimidohexane.

Much to our surprise, the elution profile of Bak in size exclusion chromatography showed a very different pattern from that of Bak in unstimulated HCT116 cells (Fig. 6A). In contrast to Bax, Bak was eluted in fractions 1–14 with 3 relative peaks, i.e. peak 1 (complex I, ~700–2,000 kDa) at fractions 1–3, peak 2 (complex II, ~230 kDa) at fractions 7–9, and peak 3 (monomers, ~67 kDa) at fractions 11–13 (Fig. 6A). These results suggest that most Bak molecules in unstimulated HCT116 cells pre-exist in at least two protein complexes, i.e. complex I and II. Fas ligation in HCT116 cells resulted in the reduction in complex I and monomers and a corresponding increase in complex II, which partially overlapped with the ~158-kDa Bak-containing protein complex (Fig. 6A). These results suggest that: 1) Fas ligation induced increased Bak oligomerization in complex II, 2) the ~230 kDa Bak-containing complex II may represent an apoptosis-related protein complex, and 3) the Bak complex II might contain both Bak and Bak homo-oligomers as well as Bak-Bak hetero-oligomers (see below).

The Bak elution profiles in unstimulated and Fas-activated HCT116-Bax-KO cells were overall similar to those observed in the corresponding wild-type HCT116 cells (Fig. 6A, lower panel). However, we observed relatively less dramatic increase in the Bak-containing complex II formation accompanied by less obvious decrease in complex I in Fas-stimulated HCT116-Bax-KO cells compared with in the HCT116 cells (Fig. 6A).
These observations were sort of opposite to what we had expected and suggest that Bax might actually be involved in facilitating Bak oligomerization, as recently suggested by others (26). Indeed, when we analyzed the oligomerization status of Bak using bismaleimidohexane cross-linking, we observed Bak oligomerization in HCT116 (Fig. 7, E and F, lanes 1–3) or Du145-Bax (Fig. 7F, lanes 4–6) cells treated with STS or Fas ligation. By contrast, little (Fig. 7, E and G) or significantly reduced (Fig. 7F) Bak oligomerization was observed in HCT116-Bax-KO or Du145-mock cells. It is unclear at the moment why chemical cross-linking could not efficiently detect the slightly increased Bak oligomers observed by gel filtration analysis in stimulated Bax-deficient cells (Fig. 7, E–G). Perhaps the cross-linked high molecular mass complexes could not be resolved well on regular polyacrylamide gel. Interestingly, Bak oligomerization in Fas-stimulated cells was observed at 6 h but not 4 h (Fig. 7, D and E), when Bak oligomerization was already apparent (Fig. 7A), suggesting that Bak oligomerization might also precede Bak oligomerization. By contrast, when Bax expression was down-regulated by siRNA, similar levels of Bak oligomerization were still observed in HCT116 cells treated with STS or Fas ligation (Fig. 7G, lanes 1–6). As expected, no significant Bak oligomerization was observed in bak siRNA-transfected HCT116 cells treated with STS or Fas ligation (Fig. 7G, lanes 4–6). It was interesting to note that apoptotic stimulation sometimes also up-regulated Bak protein expression (Fig. 7, E and F).

Different Bak and Bax Oligomerization Statuses: Relationship to the Changes in VDAC-1 and VDAC-2—Both Bak and Bax have been shown to interact with some resident mitochondrial proteins, and these interactions have been proposed to regulate the apoptotic responses of the cell. For example, Bax has been proposed to interact with some components of the permeability transition pore, such as VDAC-1, to mediate mitochondrial protein release during apoptosis (reviewed in Refs. 46 and 47), although some recent studies have challenged this view (48, 49). By contrast, Bak has been shown to bind to VDAC-2 in unstimulated mouse embryonic fibroblasts and apoptotic signals disrupt this “sequestration” and thus release Bak to carry out proapoptotic functions (3). On these considerations, we reasoned that perhaps Bak interactions with these proteins might explain why Bak seems to play a more prominent role in regulating apoptosis in Bax-deficient cells than in Bax-expressing cells.

We first examined the oligomerization status of VDAC-1 and VDAC-2 in our gel filtration experiments. As shown in Fig. 6A,
VDAC-1 in both HCT116 and HCT116-Bax-KO cells pre-existed in gigantic protein complexes peaking at ~2000 kDa (i.e. fraction 1). Apoptotic stimulation induced a clear-cut increase in the low molecular mass VDAC-1-containing complexes and monomers in both cell types without a corresponding decrease in the high molecular mass protein complex (Fig. 6A), suggesting that apoptotic stimulation may up-regulate the VDAC-1 protein levels. Indeed, Western blotting using whole cell lysates revealed increased VDAC-1 in HCT116 cells treated with both Fas ligation and STS (not shown). Different from VDAC-1, VDAC-2 in unstimulated cells existed in two populations, i.e. a major population representing monomers eluted at fractions 10–13 (~67 kDa) and a minor population representing oligomers eluted at fractions 7–9 (~230 kDa) (Fig. 6A). Fas ligat-

|        | Bak | MitoTracker | DAPI | Composite |
|--------|-----|-------------|------|-----------|
| Control|     |             |      |           |
| STS    |     |             |      |           |
| Fas    |     |             |      |           |

**Fig. 5.** Bak N terminus exposure during apoptosis. Du145-Bax and Du145-mock cells were treated with STS (0.5 μM, 12 h) or Fas ligation (4 h). The cells were then immunostained with rabbit anti-Bak (NT) antibody. Shown are representative images of Bak (green), MitoTracker labeling of mitochondria (red), DAPI labeling for apoptotic nuclei (blue) and composites. Original magnifications, ×200.
Fig. 6. Oligomerization of Bax and Bak. A, HCT116 and HCT116-Bax-KO cells were treated with Fas ligation for 6 and 12 h, respectively. Fractions (0.6 ml each) were collected, and a portion (20 μl) of fractions 1–25 was analyzed by Western blotting. B, co-IP experiments. HCT116 cells were treated with Fas ligation (12 h) or STS (0.5 μM, 12 h). Cleared lysates were used in IP using the antibodies indicated at the top. The IP products were then analyzed in Western blot analysis for the molecules indicated on the right. C, co-IP experiments in HCT116-Bax-KO cells as described for B.

...tion caused formation of larger VDAC-2-containing complexes eluted at fractions 7–9, which coincided with the Bak complex II (Fig. 6A), suggesting that the two proteins might co-exist in this complex. Of interest, the Fas ligation-induced formation of larger VDAC-2 complex in fractions 7–9 in HCT116-Bax-KO cells was much less obvious than in HCT116 cells (Fig. 6C).

To help elucidate the complex composition, we carried out co-immunoprecipitation (co-IP) experiments in both HCT116 (Fig. 6B) and HCT116-Bax-KO (Fig. 6C) cells either untreated or stimulated with Fas ligation or STS. We focused on the potential interactions between Bak and VDAC-2 because conflicting data have been presented about the role of VDAC-1 in regulating apoptosis (46–49). In all of our co-IP experiments, we first cleared cell lysates with Rb or goat IgG conjugated to the agarose beads (see “Materials and Methods”), thereby stringently eliminating any nonspecific interactions. In unstimulated HCT116 cells, IP using the anti-Bak antibody that preferentially recognizes the N terminus-exposed Bak (but also some native Bak protein) pulled down prominent VDAC-2 but not Bak (Fig. 6B, lane 1). Reciprocal co-IP using anti-VDAC-2 antibody similarly pulled down Bak but not Bax in unstimulated HCT116 cells (Fig. 6B, lane 4). These results are consistent with gel filtration data showing co-elution of Bak with VDAC-2 but not Bak in fractions 7–9 (Fig. 6B, lane 1). Reciprocal co-IP experiments during Fas activation did not result in any specific products (Fig. 6B, lanes 7–9).
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It is also unclear why the absence of Bax renders Bak critical in mediating the apoptotic responses. One possibility is that in the absence of Bax, more activated Bak might be available for apoptosis induction because of reduced inhibitory effect of VDAC-2. This possibility is supported by the gel filtration data showing significantly reduced formation of the high molecular mass VDAC-2 complex that co-elutes with the proapoptotic Bak complex II in HCT116-Bax-KO cells (Fig. 6A). More importantly, in Bax-expressing cells, not all VDAC-2 dissociates from Bak, and some VDAC-2 remains bound to Bak during apoptosis induction (Fig. 6B). However, in Bax-deficient cells, there is no significant interaction between Bak and VDAC-2 either with or without apoptotic stimulation (Fig. 6C). Because VDAC-2 has recently been shown to be a major inhibitor of the proapoptotic functions of Bak (3), our observations potentially explain why the siRNA-mediated down-regulation of Bak manifests such a dramatic inhibitory effect on apoptosis. Absence of Bak-VDAC-2 interactions in Bax-deficient cells is unlikely caused by the Bak deficiency per se because VDAC-2 does not directly interact with Bak (Fig. 6B). Nevertheless, the absence of Bak-VDAC-2 interactions and thus the lack of inhibitory effects of VDAC-2 may underlie the critical role of Bak in dictating the apoptotic sensitivity of Bax-deficient cells.

The mechanistic studies here also shed some interesting light on how Bax and Bak may be activated and differentially involved in apoptosis. Although both Bak and Bax activation involves N terminus exposure, the two proteins differ significantly with regards to oligomerization. Bax exists mainly as monomers in unstimulated cells, and apoptotic stimulation induces apparent oligomerization on the mitochondria. In contrast, most Bak pre-exists in several protein complexes of different sizes in unstimulated cells, and apoptotic stimulation results in increased formation of the ~230-kDa complex II with corresponding decreases in the monomers and the ~700-kDa complex I. That Bak preexists in several protein complexes of different sizes raises the possibility that the constitutively expressed, mitochondrially localized, and VDAC-2-bound Bak (Ref. 3 and this study) might also play a nonapoptotic function(s) in unstimulated cells. Recent studies showing a neuroprotective function for Bak (50) supports this possibility. This possibility also resonates well with recent demonstrations that several proapoptotic molecules including Omi (51), Fasl (52), and Bad (53, 54) also play important physiological or even pro-survival functions. In this sense, the increase in VDAC-2 complex formation in stimulated HCT116 cells (Fig. 6, A and B) might be considered as a prosurvival mechanism activated by apoptotic stimuli, as we have recently shown in several other systems (55). In HCT116-Bax-KO cells, this putative prosurvival mechanism (i.e. VDAC-2 oligomerization) is diminished upon apoptotic stimulation (Fig. 6, A and C), thus allowing Bak to play a full-throttle role in mediating apoptosis.

Acknowledgments—We thank B. Vogelstein for providing HCT116 and HCT116-Bax-KO cells and members of the Tang lab for support and helpful discussion.

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FIG. 7. Oligomerization of Bax and Bak by cross-linking analysis. A–F, HCT116 and HCT116-Bax-KO (A, B, D, and E) or Du145-mock and Du145-Bax (C and F) cells were treated with STS (0.5 μM) or Fas ligation for the time intervals indicated (STS and FAS treatment in C and F was 12 and 6 h, respectively). The arrows indicate Bax or Bak oligomers, and the asterisks indicate nonspecific bands. G, HCT116 and HCT116-Bax-KO cells were first transfected with either control (ctl) or bak siRNA pool (100 nM each). Forty-eight h post-transfection, the cells were treated with STS (0.5 μM for 12 h), Fas ligation (6 h). The arrows indicate Bax or Bak oligomers, and the asterisks indicate nonspecific bands.
