PUMA Dissociates Bax and Bcl-X<sub>L</sub> to Induce Apoptosis in Colon Cancer Cells*

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PUMA is a BH3-only Bcl-2 family protein that plays an essential role in DNA damage-induced apoptosis. PUMA interacts with anti-apoptotic Bcl-2 and Bcl-X<sub>L</sub>, and is dependent on Bax to initiate apoptosis. In this study, we investigated how the interactions of PUMA with the antiapoptotic proteins coordinate with Bax to initiate apoptosis in HCT116 colon cancer cells. We found that Bcl-X<sub>L</sub> was most effective among several antiapoptotic proteins in suppressing PUMA-induced apoptosis and PUMA-dependent apoptosis induced by the DNA-damaging agent adriamycin. Mutant Bcl-X<sub>L</sub> that cannot interact with Bax was unable to protect cells from PUMA-mediated apoptosis. Knockdown of Bcl-X<sub>L</sub> by RNA interference significantly enhanced PUMA-mediated apoptosis in HCT116 cells but not in PUMA-knockout cells. Furthermore, Bax was found to be dissociated preferentially from Bcl-X<sub>L</sub> in HCT116 cells but not in the PUMA-knockout cells, in response to PUMA induction and adriamycin treatment. PUMA inhibited the association of Bax and Bcl-X<sub>L</sub> in vitro by directly binding to Bcl-X<sub>L</sub> through its BH3 domain. Finally, we found that wild-type Bax, but not mutant Bax deficient in either multimerization or mitochondrial localization, was able to restore PUMA-induced apoptosis in the BAX-knockout cells. Together, these results indicate that PUMA initiates apoptosis in part by dissociating Bax and Bcl-X<sub>L</sub>, thereby promoting Bax multimerization and mitochondrial translocation.

PUMA is a downstream target of the p53 tumor suppressor gene and a member of the BH3-only group of Bcl-2 family proteins (1, 2). PUMA is normally expressed at a low level but is markedly induced after cells are exposed to DNA-damaging agents, such as chemotherapy drugs and ionizing radiation (1, 2). The activation of PUMA by DNA damage is dependent on p53 and is mediated by the direct binding of p53 to the PUMA promoter region (1). PUMA can also be induced via p53-independent mechanisms by glucocorticoids and serum deprivation (3).

PUMA plays an essential role in p53-dependent and -independent apoptosis induced by a variety of stimuli (4). Deletion of PUMA in HCT116 colon cancer cells abrogated apoptosis induced by p53, the DNA-damaging agent adriamycin, and hypoxia (5). PUMA-knockout mice recapitulate major apoptotic deficiencies observed in p53-knockout mice (6, 7). For example, apoptosis induced by γ-irradiation, DNA-damaging drugs, and the oncogenes c-Myc and E1A is abrogated to a similar extent in PUMA-knockout mice as in the p53-knockout mice (6, 7). PUMA is also involved in apoptosis induced by endoplasmic reticulum stress and human immunodeficiency virus proteins (8, 9) and is necessary for p53-independent apoptosis induced by cytokine withdrawal, glucocorticoids, kinase inhibitors, or phorbol esters (6). Furthermore, PUMA seems to play a role in tumor suppression, because down-regulation of PUMA promotes oncogenic transformation and accelerates lymphomagenesis induced by the Myc oncogene (10).

In mammalian cells, Bcl-2 family proteins play a critical role in regulating apoptosis initiation through the mitochondria (11–16). Bcl-2 family members can be grouped into "multidomain" and "BH3-only" subgroups. The multidomain proteins, such as antiapoptotic Bcl-2 and Bcl-X<sub>L</sub> and proapoptotic Bax and Bak, contain three or four Bcl-2 homology (BH)2 domains (11). The BH3-only proteins, including PUMA and at least eight additional proapoptotic proteins, display sequence homology with other members only within an α-helical BH3 domain, which is essential for apoptosis induction (17). BH3-only proteins function through multidomain Bcl-2 family members to induce apoptosis, either by antagonizing antiapoptotic proteins or by directly activating proapoptotic proteins (18, 19). Proapoptotic Bcl-2 family members trigger a cascade of downstream events to initiate apoptosis, including collapse of mitochondrial membrane potential, release of cytochrome c, SMAC/Diablo, and AIF from the mitochondria into the cytosol, and activation of caspase cascade (12, 14).

Our previous studies in colon cancer cells demonstrated that PUMA is a mitochondrial protein, and its mitochondrial localization is indispensable for apoptosis induction (1, 5). PUMA binds to Bcl-2 and Bcl-X<sub>L</sub> and induces mitochondrial membrane potential change and caspase activation (1, 5). PUMA-induced apoptosis is completely abrogated in BAX-knockout (BAX-KO) HCT116 cells (5). Expression of PUMA leads to translocation of Bax from the cytosol to the mitochondria and formation of Bax multimers in the mitochondria (5). It has also been shown that PUMA can couple the nuclear and cytoplasmic pro-apoptotic functions of p53 by displacing p53 from Bcl-X<sub>L</sub>, thereby allowing p53 to induce mitochondrial permeabilization (20). However, it remains to be determined how these events and their coordination initiate PUMA-induced apoptosis.

To further understand the mechanisms of PUMA-induced apoptosis, we investigated how PUMA functions through Bax and other Bcl-2 family members to initiate apoptosis. We found Bcl-X<sub>L</sub> to be a critical antiapoptotic protein mediating PUMA-induced apoptosis in HCT116

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<sup>2</sup> The abbreviations used are: BH, Bcl-2 homology; siRNA, small interference RNA; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; GFP, green fluorescent protein; HA, hemagglutinin; IP, immunoprecipitation; TM, transmembrane; SMAC, second mitochondria-derived activator of caspase; Diablo, direct inhibitor of apoptosis protein-binding protein with low pl; AIF, apoptosis-inducing factor.
cells. PUMA dissociates Bax from Bcl-X<sub>L</sub>, thereby promoting Bax mitochondrial translocation and multimerization.

**MATERIALS AND METHODS**

**Cell Culture and Transfection**—All of the cell lines were maintained at 37 °C and with 5% CO<sub>2</sub>. HCT116 and derivative cell lines were cultured in McCoy’s 5A medium (Invitrogen). 911 cells were cultured in Dulbecco’s modified Eagle’s medium (Mediatech). All of the cell culture media were supplemented with 10% defined fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

HCT116 and derivative cell lines were transfected with various constructs and small interference RNA (siRNA) by Lipofectamine™ 2000 (Invitrogen) following the instructions of the manufacturer. The siRNA duplex (Dharmacon) used for targeting Bcl-X<sub>L</sub> (Bcl-X<sub>L</sub>471) 5'-GGAGAU-GCAGGUAUUGGUG-3' was as described (21). The Bcl-2 expression plasmid constructed in pCEP4 (Invitrogen), the Bcl-X<sub>L</sub> expression plasmid constructed in pcDNA3.1 (Invitrogen), and the Mcl-1 expression plasmid constructed using p3XFLAG-CMV-10 (Sigma) were previously described (1, 22). The Bcl-X<sub>L</sub> mutant (Bcl-X<sub>L</sub> mt8) that is deficient in binding to other Bcl-2 family proteins was also previously described (19).

**Analysis of PUMA-induced Apoptosis**—The cells were plated in 12-well plates at ~20% density and allowed to attach for 12 h. Adenoviruses expressing PUMA (Ad-PUMA) and a PUMA mutant with a deletion of the BH3 domain (Ad-ΔBH3) were used to infect cells (5). Adriamycin (0.2 μg/ml) was used to treat p21-KO and p21-KO/PUMA-KO cells. Floating and adhering cells were collected at various time points after treatment. For analysis of apoptosis by nuclear staining, the cells were resuspended and fixed in phosphate-buffered saline solution containing 3.7% formaldehyde, 0.5% Nonidet P-40, and 10 μg/ml Hoechst 33258 (Molecular Probes). Apoptosis was assessed through microscopic visualization of condensed chromatin and micronucleation as previously described (23). For analysis of apoptosis by annexin V staining, the cells were stained by annexin Alexa-594 (Molecular Probes) according to the instructions of the manufacturer, counterstained by 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) for cell nuclei, and then analyzed by flow cytometry as described (24).

**Antibodies and Western Blotting**—The antibodies used for Western blotting include antibodies against caspase 8 and caspase 9 (Cell Signaling), anti-caspase 3 antibody (Stressgen), antibodies against Bax (N-20), GFP, HA, and AIF (Santa Cruz), antibodies against cytochrome <i>c</i>, α-tubulin, Bcl-X<sub>L</sub> (BD Biosciences), anti-V5 antibody (Invitrogen), and anti-cytochrome oxidase subunit IV (Cox IV) antibody (Molecular Probes). The anti-SMAC/Diablo antibody was a generous gift from Dr. Eileen White at Rutgers University (Piscataway, NJ). Western blotting analysis was performed as previously described (24).

**Immunoprecipitation**—Twenty-four hours after transfection, the cells were harvested and resuspended in 1 ml of EBC buffer (50 mM...
Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% Nonidet P-40) supplemented with protease inhibitor mixture (Roche Applied Sciences). The cells were disrupted by sonication and then spun at 10,000 \( \times g \) for 10 min to collect the cell lysate. For immunoprecipitation (IP), 1–2 \( \mu g \) of IP antibodies were added to 50 \( \mu l \) cell lysates. The mixtures were mixed on a rocker at 4 °C overnight. The immunocomplexes were captured by the addition of protein G/A-agarose (Roche Applied Sciences) mixed at 1:5 ratio, followed by incubation at 4 °C for 1 h. The beads were washed four times by EBC buffer and then collected by centrifugation at 6,000 \( \times g \). After the final wash, the beads were mixed with 50 \( \mu l \) of 2 \( \times \) Laemmli sample buffer, heated at 95 °C for 15 min, and analyzed by Western blotting.

In some experiments, a peptide (EQWAREIGAQLRRMADDLNA) containing the BH3 domain of PUMA and a control peptide (EQWAREIGAQLRRMADDLNA) with a point mutation (Leu to Glu) in the BH3 domain were added to cell lysates before the addition of the antibodies. The peptides were synthesized and high pressure liquid chromatography-purified by the Peptide Synthesis Core Facility at University of Pittsburgh.

**Generation of BAX Mutants and Stable Cell Lines Expressing BAX Mutants**—Bax expression construct pCEP4-HABAX was used to generate Bax mutant expression constructs (5). To generate Bax deletion constructs BAX-D1 and BAX-D2, the corresponding BAX sequences
were amplified using the pCEP4-HABAX as template. The PCR products were then cloned into the vector pCEP4-HAHA (pHAHA) containing double HA tags (5). To generate point mutation constructs BAX-M1 and BAX-M2, site-directed mutagenesis was performed on the pCEP4-HABAX plasmid with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) following the instructions of the manufacturer. To generate constructs expressing N-terminal GFP fused to wild-type and mutant Bax proteins, the corresponding sequences were amplified using the pCEP4 constructs as template. The PCR products were then cloned into pEGFPN1 vector (Clontech). Details of plasmid construction are available upon request. The inserts of all the constructs were verified by restriction enzyme digestion and DNA sequencing analysis.

To establish stable cell lines expressing wild-type and mutant Bax, BAX-KO cells were transfected with the Bax expression constructs. After transfection, cells were plated out by limiting dilution and selected in the presence of 0.4 mg/ml of G418 (Invitrogen) for 3 weeks. Individual clones were expanded and cell lysates were harvested and examined for Bax expression by Western blotting. Clones exhibiting stable expression of wild-type and mutant Bax were identified and used in the described study.

Localization of Wild-type and Mutant Bax Proteins—911 cells were seeded on glass chamber slides (Nalge Nunc) and transfected with various Bax expression constructs. Twenty-four h following transfection, the cells were incubated with 100 nM of MitoTracker Red (CmxRos; Molecular Probes) at 37 °C for 20 min to label the mitochondria. Cells were then permeabilized with cold acetone, and counterstained by DAPI (2 μg/ml). The mounted slides were subjected to microscopic analysis under a Nikon fluorescence microscope (TS800) equipped with a SPOT camera and imaging software.

Analysis of Cytochrome c, SMAc/DiaBio, and AIF Release—Mitochondrial and cytosolic fractions were isolated by the differential centrifugation method previously described (5). Briefly, cells were washed and resuspended in homogenization buffer (0.25 M sucrose, 10 mM HEPES, pH 7.4 and 1 mM EGTA) and subjected to 40 strokes of homogenization in a Dounce homogenizer. The homogenate was subjected to centrifugation at 1,000 × g for 15 min at 4 °C to pellet nuclei and unbroken cells. The supernatant was subsequently centrifuged at 10,000 × g for 15 min at 4 °C to obtain cytosolic fraction (supernatant) and mitochondrial fraction (pellet). The mitochondrial fraction was resuspended in homogenization buffer following one wash. Both fractions were mixed with equal volumes of 2 × Laemmli sample buffer for Western blotting analysis.

RESULTS

Bcl-X<sub>1</sub> Is Most Effective in Inhibiting PUMA-mediated Apoptosis—It has been suggested that the interactions between proapoptotic and antiapoptotic Bcl-2 family members play an important role in apoptosis initiation (11–15). Our previous studies demonstrated that PUMA interacts with antiapoptotic Bcl-2 and Bcl-X<sub>1</sub> proteins through its BH3 domain (1). To study the role of the antiapoptotic proteins in PUMA-induced apoptosis, we transfected HCT116 cells with Bcl-2, Bcl-X<sub>1</sub>, and Mcl-1, all of which are known to be abundantly expressed in colon cancer cells (25). Western blotting analysis indicated that Bcl-2, Bcl-X<sub>1</sub>, and Mcl-1 were expressed at a high level in the transfected cells (Fig. 1A). After transfection, the cells were infected with an adenovirus expressing PUMA (Ad-PUMA) and a control adenovirus expressing PUMA lacking the BH3 domain (Ad-ΔBH3). Viral titer and infection efficiency were controlled by GFP expressed from the adenoviruses. Apoptosis was analyzed 48 h after infection by counting fragmented nuclei. Transfection of Bcl-X<sub>1</sub> led to over 60% inhibition of apoptosis induced by Ad-PUMA (Fig. 1A). However, transfection of Bcl-2 had virtually no effect on PUMA-induced apoptosis, whereas transfection of Mcl-1 modestly inhibited apoptosis (Fig. 1A). No significant apoptosis was detected in the control cells infected by Ad-ΔBH3, with or without transfection of different plasmids.

To determine whether Bcl-2, Bcl-X<sub>1</sub>, and Mcl-1 have similar effects on apoptosis mediated by endogenous PUMA, we analyzed adriamycin-induced apoptosis in p21-knockout (p21-KO) HCT116 cells. Adriamycin, a DNA-damaging agent that causes single- and double-stranded DNA breaks, can induce PUMA-dependent apoptosis in the p21-KO cells (5). Similar to apoptosis induced by Ad-PUMA in HCT116 cells, transfection with Bcl-X<sub>1</sub> suppressed adriamycin-induced apoptosis by over 60% in the p21-KO cells, whereas transfection of Bcl-2 and Mcl-1 had much less influence on adriamycin-induced apoptosis (Fig. 1B). These results suggest that Bcl-X<sub>1</sub> is effective in inhibiting PUMA-mediated apoptosis and perhaps a major mediator of the proapoptotic function of PUMA.

Knockdown of Bcl-X<sub>1</sub> Enhanced PUMA-mediated Apoptosis—To determine whether Bcl-X<sub>1</sub> plays a role in regulating the proapoptotic function of PUMA, we examined whether down-regulation of Bcl-X<sub>1</sub> enhances PUMA-mediated apoptosis. RNA interference was used to knock down Bcl-X<sub>1</sub> expression in HCT116 and the p21-KO cells.
Transfection of the control GFP plasmid indicated that at least 80% of HCT116 cells can be transfected (Fig. 2A). It was found that Bcl-XL protein expression was significantly suppressed (60–90%) upon transfecting the cells with siRNA specific for Bcl-XL (Fig. 2, B and C). Knockdown of Bcl-XL markedly enhanced PUMA-induced apoptosis in HCT116 cells, as well as adriamycin-induced apoptosis in the p21-KO cells (Fig. 2, B and C). In contrast, transfecting the cells with a control scrambled siRNA did not affect Bcl-XL expression and apoptosis induced by Ad-PUMA and adriamycin (Fig. 2, B and C). These results were confirmed by analysis of apoptosis using annexin V staining (data not shown). To test whether the effect of Bcl-XL on adriamycin-induced apoptosis is mediated by endogenous PUMA, we also knocked down Bcl-XL in HCT116 cells deficient in both p21 and PUMA (p21-KO/PUMA-KO), which were shown previously to be insensitive to adriamycin-induced apoptosis (5). We found that Bcl-XL knockdown did not increase the sensitivity to adriamycin-induced apoptosis in these cells (Fig. 2D). These results suggest that Bcl-XL plays an important role in regulating apoptosis mediated by PUMA.

Bax Is Dissociated from Bcl-XL during PUMA-mediated Apoptosis—Previous studies have shown that there are two groups of BH3-only proteins. The BH3 domains of Bid and Bim can directly activate Bax and Bak, whereas those of Bad and Bik activate Bax and Bak by displacing them from antiapoptotic activities (26). Because Bax is required for PUMA-induced apoptosis in HCT116 cells (5), and Bax is known to interact with Bcl-XL, we hypothesized that PUMA induces apoptosis by modulating the interaction between Bax and Bcl-XL. To test this hypothesis, we analyzed the effect of a Bcl-XL mutant (Bcl-XL mt8) deficient in binding to Bax on PUMA-mediated apoptosis (Fig. 1C) (19). The mutant Bcl-XL did not suppress apoptosis induced by Ad-PUMA and adriamycin (Fig. 1, A and B), suggesting that the inhibition of Bcl-XL on PUMA-mediated apoptosis requires its interaction with Bax or other Bcl-2 family proteins.

We then analyzed whether PUMA induction affects the interactions between Bax and Bcl-XL, between Bax and Bcl-2, and between Bax and Mcl-1. Infection of HCT116 cells by Ad-PUMA caused a significant decrease in the amount of Bcl-XL associated with Bax (Fig. 3A). Similarly, the level of Bcl-XL associated with Bax was much lower in the p21-KO cells after adriamycin treatment (Fig. 3B). Nevertheless, the level of Bcl-XL bound to Bax was not affected by adriamycin treatment in the p21-KO/PUMA-KO cells, indicating that the dissociation of Bax and Bcl-XL was mediated by PUMA (Fig. 3C). No significant association between endogenous Bax and Bcl-2 or between Bax and Mcl-1 was detected in these cells before and after the treatment (Fig. 3). These results indicate that dynamic regulation of Bax and Bcl-XL interaction plays an important role in initiating PUMA-mediated apoptosis.

PUMA Dissociates Bax and Bcl-XL through Competitive Binding to Bcl-XL—To understand how PUMA causes the dissociation of Bax and Bcl-XL, we studied the direct interactions among PUMA, Bax, and Bcl-XL. HA-tagged PUMA and GFP-tagged Bax expression constructs were transfected into the BAX-KO cells to examine whether PUMA binds to Bax. Reciprocal IP with either HA (for PUMA) or GFP (for Bax) did not identify any interaction between PUMA and Bax (Fig. 4A). In contrast, both PUMA and Bax were found to be strongly associated with Bcl-XL under identical conditions (Fig. 4A).

Because both Bax and PUMA interact with Bcl-XL through their BH3 domains, and Bcl-XL interacts with Bax and BH3-only proteins through the same amino acid residues (19, 27), we speculated that PUMA can directly cause the dissociation of Bax and Bcl-XL through competitive binding to Bcl-XL. To test this possibility, protein extracts isolated from the BAX-KO cells cotransfected with Bax and Bcl-XL were incubated with those from cells cotransfected with HA-Bax and V5-Bcl-XL for indicated time. IP was performed with anti-HA antibody to pull down Bcl-XL. Western blotting for HA was used to analyze PUMA and Bax bound to Bcl-XL. Dissociation of Bax and Bcl-XL by the PUMA BH3 domain (Mutant BH3) at indicated concentrations. IP was performed with anti-HA antibody, and Western blotting was used to analyze Bax and Bcl-XL.
unable to dissociate Bax and Bcl-X\textsubscript{L} (Fig. 4B). To directly examine whether the BH3 domain of PUMA has this activity, we analyzed the effects of a peptide containing the BH3 domain of PUMA, and a control peptide containing a point mutation (Leu to Glu) in the lysine residue critical for proapoptotic activity (17), on the interaction between the transfected Bax proteins. GFP-tagged wild-type and mutant Bax constructs were transfected into 911 cells. GFP fluorescence (green) indicated the localization of transfected Bax. Mitochondria were stained by MitoTracker Red (red) with DAPI for counterstaining (blue). D, expression of wild-type and mutant Bax proteins in stable cell lines. Stable cell lines expressing wild-type (Bax) and mutant Bax proteins were isolated as described under “Materials and Methods.” Bax expression was analyzed by Western blotting with anti-GFP antibody. α-Tubulin was used as a loading control. IB, immunoblot.

FIGURE 5. Bax mutants deficient in multimerization and mitochondrial localization. A, schematic diagram of the Bax mutants. Bax-D1 lacks the N-terminal 71 amino acids including the BH3 domain. Bax-M1 contains a point mutation (L63E) in the first lysine residue of the BH3 domain. Bax-D2 lacks C-terminal TM domain. Bax-M2 contains a point mutation (S184K) in the TM domain. It was predicted that Bax-D1 and -M1 are unable to form protein-protein complexes, whereas Bax-D2 and -M2 disrupt its mitochondrial localization. B, homodimerization of Bax proteins. BAX-KO cells were cotransfected with HA-tagged and GFP-tagged Bax proteins. IP was performed to analyze the interactions between the transfected Bax proteins. C, subcellular localization of Bax proteins. GFP-tagged wild-type and mutant Bax constructs were transfected into 911 cells. GFP fluorescence (green) indicated the localization of transfected Bax. Mitochondria were stained by MitoTracker Red (red) with DAPI for counterstaining (blue). D, expression of wild-type and mutant Bax proteins in stable cell lines. Stable cell lines expressing wild-type (Bax) and mutant Bax proteins were isolated as described under “Materials and Methods.” Bax expression was analyzed by Western blotting with anti-GFP antibody. α-Tubulin was used as a loading control. IB, immunoblot.

Disrupting Bax Multimerization or Mitochondrial Localization Blocked PUMA-induced Apoptosis—Our previous studies demonstrated that PUMA promotes the formation of Bax multimers and translocation of Bax from the cytosol into the mitochondria (5). However, it is unclear whether these events, which probably result from the dissociation of Bax and Bcl-X\textsubscript{L}, are important in initiating PUMA-mediated apoptosis. We therefore determined whether disrupting the multimerization or mitochondrial localization of Bax would affect PUMA-induced apoptosis. The formation of Bax multimers is mediated by the BH3 domain near the N terminus of Bax (27). To disrupt Bax multimerization, a Bax mutant lacking the N-terminal 71 amino acids, including the BH3 domain (Bax-D1), and a Bax mutant containing a point mutation (L63E) in the first lysine residue of the BH3 domain (Bax-M1) were constructed (Fig. 5A). On the other hand, the mitochondrial localization of Bax is regulated by the C-terminal transmembrane (TM) domain (28). To disrupt such localization, a deletion mutant (Bax-D2) and a point mutation (S184K, Bax-M2) in the TM domain were also constructed (Fig. 5A). To examine whether Bax mutants can multimerize, IP was performed after the BAX-KO cells were transfected with the Bax mutants tagged with HA or GFP. Bax-D1 and -M1, but not other Bax mutants, were found to completely lose their ability to form Bax homodimers (Fig. 5B). To analyze subcellular localization of the Bax mutants, GFP-fused Bax mutants were transfected into 911 cells. Bax-D2 and Bax-M2 were found to be diffusely distributed in the nuclei and cytoplasm of the cells, with little colocalization with the mitochondria labeled by MitoTracker Red, whereas wild-type Bax and other Bax mutants were localized both in the cytoplasm and at the mitochondria (Fig. 5C). These results were confirmed by probing mitochondrial and cytosolic Bax by Western blotting (data not shown).

To determine whether mutant Bax can restore PUMA-induced apoptosis in the BAX-KO cells, the wild-type and mutant Bax constructs were transfected into the BAX-KO cells, and stable cell lines expressing Bax proteins at a level comparable with the endogenous Bax in HCT116 cells were established (Fig. 5D). Parental HCT116, BAX-KO, and the BAX-KO cells reconstituted with the wild-type and mutant Bax were infected with Ad-PUMA and Ad-ΔBH3. Cells expressing wild-type Bax were found to be completely proficient in apoptosis, whereas those expressing mutant Bax proteins, including Bax-D1, -M1, -D2, and -M2,
FIGURE 6. PUMA-induced apoptosis in stable cell lines expressing Bax mutants. A, apoptosis analyzed by nuclear staining. BAX-KO and stable cell lines expressing wt and mutant Bax proteins were infected with Ad-PUMA and Ad-ΔBH3. The fractions of apoptotic cells were determined 48 h after the treatment by nuclear staining (left panel). The results are the averages of three independent experiments. Representative cells after nuclear staining with Hoechst 33258 (Hoechst) were also shown (right panel). The arrow indicates an example apoptotic cell. GFP fluorescence (GFP) indicated that cells were infected by Ad-PUMA at a similar level. B, apoptosis analyzed by annexin V staining. The cells were infected with Ad-PUMA for 36 h, stained with annexin V, counterstained by DAPI, and then analyzed by flow cytometry. Four subpopulations and their fractions are indicated: early apoptotic cells (R4, lower right), late apoptotic cells (R2, upper right), necrotic cells (R6, upper left), and nonapoptotic cells (R3, lower left). C, caspase activation. Representative cell lines expressing wt and mutant Bax were infected with Ad-PUMA. Activation of caspase 3, caspase 8, and caspase 9 was analyzed by Western blotting at indicated time points. Active caspases are
were found to be deficient in PUMA-induced apoptosis (Fig. 6A). Analysis of apoptosis using annexin V staining confirmed these results (Fig. 6B). We then investigated whether PUMA-induced caspase activation and cytosolic release of the mitochondrial apoptogenic proteins are deficient in the cells expressing mutant Bax. PUMA-induced activation of caspases 3, 8, and 9 was completely blocked in the cells expressing defective in the cells expressing mutant Bax. PUMA-induced activation and cytosolic release of the mitochondrial apoptogenic proteins are deficient in the cells expressing mutant Bax. PUMA-induced activation of caspases 3, 8, and 9 was completely blocked in the cells expressing Bax-M1 (Fig. 6C). Activation of these caspases was partially inhibited in the cells expressing Bax mutants, but not in the cells expressing the wild-type Bax (Fig. 6D). These results suggest that Bax multimerization and mitochondrial localization mediate PUMA-induced release of mitochondrial apoptogenic proteins and activation of caspases. Together, our results demonstrate that PUMA induces apoptosis in part by dissociating Bax and Bcl-X\textsubscript{L}, thereby promoting Bax mitochondrial translocation and multimerization.

**DISCUSSION**

BH3-only Bcl-2 family proteins are evolutionarily conserved and essential mediators of apoptosis initiation. PUMA and another BH3-only protein Noxa were initially identified as downstream targets of p53 and subsequently shown to play an important role in apoptosis (4, 29). PUMA is induced at the transcriptional level in response to DNA damage and other stimuli. We and other groups have shown that PUMA plays an essential role in apoptosis induced by a variety of stimuli in several tissues and cell types (4). Deletion of PUMA in human cancer cells and mice abrogated p53-dependent apoptosis induced by DNA damage, hypoxia, oncogenes, human immunodeficiency virus proteins, or endoplasmic reticulum stress, as well as p53-independent apoptosis induced by cytokine withdrawal, glucocorticoids, kinase inhibitors, or phorbol esters (5–7).

Although the role of PUMA in apoptosis has been well established, the mechanisms of PUMA-mediated apoptosis remain unclear. Our studies demonstrated that similar to other BH3-only proteins, PUMA functions through multidomain Bcl-2 family members, including Bax and Bcl-X\textsubscript{L}. We used two different models, i.e. Ad-PUMA and adriamycin-induced apoptosis, to demonstrate that Bcl-X\textsubscript{L} plays an important role in regulating PUMA-mediated apoptosis in colon cancer cells (Figs. 1 and 2). These observations may be related to the fact that Bcl-X\textsubscript{L} is overexpressed in a large fraction of colon tumors, which confers colon cancer cell resistance to apoptotic stimuli (30, 31). The involvement of Bcl-X\textsubscript{L} in PUMA-mediated apoptosis has also been demonstrated in a recent study in which PUMA was found to interact with Bcl-X\textsubscript{L} and displace p53 from Bcl-X\textsubscript{L} to activate its mitochondrial apoptotic activity (20). In our study, we found that PUMA displaced Bax rather than p53 from Bcl-X\textsubscript{L}. We also found that p53-knockout HCT116 cells are as sensitive as parental HCT116 cells to Ad-PUMA-induced apoptosis, suggesting that p53 is dispensable for PUMA-induced apoptosis (data not shown). The differences between these studies are likely due to the stimuli used for apoptosis induction. Ad-PUMA and adriamycin were used in our study, whereas ultraviolet irradiation was used in the other study, to induce PUMA-dependent apoptosis. These observations suggest that Bcl-X\textsubscript{L} binds to multiple proapoptotic proteins, including Bax and p53, to suppress their proapoptotic functions. In response to apoptotic stimuli, the induction PUMA and binding of PUMA to Bcl-X\textsubscript{L} will relieve the inhibition of Bcl-X\textsubscript{L} on these proteins.

Although Bcl-X\textsubscript{L} seems to be an important mediator of PUMA-induced apoptosis, our data suggest that there is a mechanism of action for PUMA independent of the effects on Bcl-X\textsubscript{L}. Knockdown of Bcl-X\textsubscript{L} did not trigger apoptosis on its own (Fig. 2, B–D). Transient transfection of another antiapoptotic Bcl-2 family protein, Mcl-1, inhibited PUMA-induced apoptosis (Fig. 1, A and B), suggesting that Mcl-1 also mediates PUMA-induced apoptosis. However, the mechanism by which Mcl-1 regulates PUMA-induced apoptosis appears to be different from that of Bcl-X\textsubscript{L}, because no strong interactions between Bax and Mcl-1 could be detected (Fig. 3A). On the other hand, Mcl-1 protein level was found to be significantly decreased after adriamycin treatment for 48 h (Fig. 3B), suggesting that it is regulated by protein degradation. This is consistent with the reports that Mcl-1 is degraded by the ubiquitin-proteasome machinery during the initiation of DNA damage-induced apoptosis (22, 32). Nevertheless, additional experiments are necessary to further delineate how Mcl-1 mediates PUMA-induced apoptosis.

Several observations on PUMA can be explained by its interactions with Bcl-X\textsubscript{L} and other antiapoptotic Bcl-2 family proteins. PUMA is a potent inducer of cell death. Induction of PUMA can trigger apoptosis in human cancer cells within several hours (1). Such potency of PUMA in apoptosis induction may be related to the strength of its interactions with antiapoptotic proteins. It was shown that as much as 50% of transfected Bcl-X\textsubscript{L} and Bcl-2 could be coprecipitated with PUMA (1). In this study, PUMA expressed at a similar level as Bax displaced a great majority of Bax bound to Bcl-X\textsubscript{L} (Fig. 4B). In competitive binding assays, the BH3 peptide of PUMA exhibited lower IC\textsubscript{50} (concentrations required for 50% of binding) for binding to antiapoptotic proteins than those from most other BH3-only proteins, suggesting that PUMA binds to antiapoptotic proteins with high affinity (33). It has also been shown that the BH3 domain of PUMA can promiscuously interact with multiple antiapoptotic Bcl-2 family members, including Bcl-2, Bcl-X\textsubscript{L}, Bcl-w, Mcl-1, and A1, whereas other BH3-only proteins, such as Bad and Bid, selectively interact with a subset of these antiapoptotic proteins (33). The ability of PUMA to interact with different antiapoptotic proteins may explain why it mediates apoptosis in diverse tissues and cell types, because the functions of antiapoptotic proteins are tissue- and cell type-dependent (34).

Our study demonstrated that Bax, which is required for PUMA-mediated apoptosis in colon cancer cells, is dissociated from Bcl-X\textsubscript{L} in response to PUMA induction and adriamycin treatment (Fig. 3). The dissociation of Bax and Bcl-X\textsubscript{L} is dependent on PUMA, because it was not observed in the p21-KO/PUMA-KO cells treated with adriamycin (Fig. 3C). The dissociation also seems to be mediated by competitive binding of the BH3 domain to Bcl-X\textsubscript{L}, rather than through direct interaction of PUMA with Bax (Fig. 4). Thus, PUMA appears to be similar to Bad and Bik, which cannot directly activate Bax and Bak but instead bind to antiapoptotic proteins via their BH3 domains, thereby displacing Bax and Bak and indirectly activating these proapoptotic proteins (26). These BH3-only proteins are different from Bid and Bim, whose BH3 domains are capable of directly activating Bax and Bak by inducing oligomerization of Bax and Bak. Our results are consistent with a recent study showing that the BH3 domain of PUMA alone cannot induce Bax-mediated membrane permeabilization and is incompetent in directly inducing cytochrome c release in cell-free assays (35). However,
our results are in disagreement with another study showing that Bax binds to PUMA through its first α-helical domain in vitro (36). Further experiments will be necessary to resolve whether PUMA directly interacts with Bax and whether such an interaction plays a role in PUMA-mediated apoptosis in vivo.

The dissociation of Bax and Bcl-XL probably releases Bax and activates its proapoptotic function by promoting its mitochondrial translocation and multimerization. These changes in Bax are only observed in cells undergoing PUMA-mediated apoptosis, suggesting that they result from the displacement of Bax from Bcl-XL (5). Although Bax was frequently found to undergo a conformational change, translocate to the mitochondria, and multimerize during apoptosis, it is unclear whether these events are always required for apoptosis. Several studies showed that mutant Bax protein that cannot interact with itself or other Bcl-2 family members can still induce apoptosis (37, 38). In our study, stable cell lines expressing Bax mutants that are unable to form multimers did not undergo PUMA-induced apoptosis (Fig. 6), suggesting that the ability of Bax to form protein-protein complexes is essential for its function in PUMA-mediated apoptosis. Our data also showed that stable cell lines expressing Bax mutants in the TM domain (Bax-D1 and Bax-M2), which disrupt its mitochondrial localization, were resistant to PUMA-induced apoptosis (Fig. 6), suggesting that the mitochondrial localization of Bax is critical for its proapoptotic function. PUMA-induced release of mitochondrial apoptogenic proteins and caspase activation also seem to be dependent on these Bax-related changes (Fig. 6, C and D). Our data support the model in which Bax multimers function as pores in the mitochondria to facilitate the release of apoptogenic proteins (39). In summary, we have demonstrated that PUMA induces apoptosis in part by displacing Bax from Bcl-XL, thereby promoting Bax multimerization and mitochondrial translocation.

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