Pseudomonas Exotoxin A-Mediated Apoptosis Is Bak Dependent and Preceded by the Degradation of Mcl-1[▽]

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***Pseudomonas* exotoxin A (PE) is a bacterial toxin that arrests protein synthesis and induces apoptosis. Here, we utilized mouse embryo fibroblasts (MEFs) deficient in Bak and Bax to determine the roles of these proteins in cell death induced by PE. PE induced a rapid and dose-dependent induction of apoptosis in wild-type (WT) and Bax knockout (Bax^{-/-}) MEFs but failed in Bak knockout (Bak^{-/-}) and Bax/Bak double-knockout (DKO) MEFs. Also a loss of mitochondrial membrane potential was observed in WT and Bax^{-/-} MEFs, but not in Bak^{-/-} or in DKO MEFs, indicating an effect of PE on mitochondrial permeability. PE-mediated inhibition of protein synthesis was identical in all 4 cell lines, indicating that differences in killing were due to steps after the ADP-ribosylation of EF2. Mcl-1, but not Bcl-x_L, was rapidly degraded after PE treatment, consistent with a role for Mcl-1 in the PE death pathway. Bak was associated with Mcl-1 and Bcl-x_L in MEFs and uncoupled from suppressed complexes after PE treatment. Overexpression of Mcl-1 and Bcl-x_L inhibited PE-induced MEF death. Our data suggest that Bak is the preferential mediator of PE-mediated apoptosis and that the rapid degradation of Mcl-1 unleashes Bak to activate apoptosis.**

Apoptosis is a mode of cell death utilized by multicellular organisms to remove unwanted cells. Also, many different cancer treatments, including chemotherapy and radiotherapy, induce apoptosis and result in the destruction of tumor cells. In some cases, apoptosis resistance can contribute to the failure of chemotherapy (14, 20, 24). Immunotoxins are a class of antitumor agents in which a powerful protein toxin is brought to the cancer cell by an antibody or an antibody fragment (for reviews, see references 28, 29, and 32). Several immunotoxins are currently in clinical trials, and one of these, BL22, targeting CD22, has shown excellent activity in drug-resistant hairy-cell leukemia (18, 19). Also, a fusion protein in which a fragment of diphtheria toxin is fused to the cytokine interleukin 2 (IL-2) (Ontak) is approved for the treatment of cutaneous T-cell lymphoma (26). Several studies carried out to determine how protein toxins and immunotoxins containing these toxins kill target cells have reported caspase activation (13, 16, 17, 30, 33). However, the steps leading up to caspase activation by these toxins that inhibit protein synthesis have not been elucidated.

Bcl-2 family members are essential regulators of the mitochondrial (intrinsic) apoptosis pathway (1, 21). Proteins of this family have been divided into pro- and antiapoptotic proteins. Antiapoptotic proteins include the multi-Bcl-2 homology (BH) domain proteins Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, Bcl-b, and Bcl2a1. Proapoptotic members can be further classified into two subfamilies, the multi-BH domain Bax homologues, including Bax, Bak, and Bok, and the BH3-only proteins, including Nbk/Bik, Noxa, Hrk, Bad, Bim, Puma, and Bmf. Bax and Bak are the most extensively studied central mediators in the mitochon-

drial apoptosis pathway (4, 6). Various stimuli, including pathogens, toxic drugs, irradiation, and starvation, induce a conformational change and activation of Bak/Bax, usually via BH3-only proapoptosis proteins. This results in the disruption of mitochondrial membranes and the release of apoptotic factors, such as cytochrome c, SMAC, and apoptosis-inducing factor, which lead to the activation of effector caspases (5, 37, 40, 42, 43).

The roles of Bax and Bak can be redundant or nonredundant, depending on the apoptotic stimuli. Bak and Bax can compensate for each other in apoptosis induced by staurosporine, etoposide, UV irradiation, serum deprivation, tBid, Bim, Bad, or Noxa (37, 43). Bak plays an essential role for apoptosis induced by Semliki Forest virus, gliotoxin, Bcl-x_S, and vinblastine (22, 27, 34, 35), while Bax is favored for apoptosis induced by Nbk/Nik, a combination of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and ionizing irradiation, or TRAIL and 5-fluorouracil (5-FU) (9, 10, 36, 38). Silencing of either Bak or Bax resulted in resistance to apoptosis induced by *Neisseria gonorrhoeae* and cisplatin (15). Sometimes the same stimulus may result in different outcomes in different cell types. NBK/Bik mediated Bax-dependent cell death in one study (9), while in another study, NBK/Bik activated BAK-mediated apoptosis (31).

In the current study, we utilized mutant mouse embryo fibroblasts (MEFs) deficient in Bak, Bax, or both proteins and provided evidence for an essential role of Bak in apoptosis induced by *Pseudomonas* exotoxin A (PE) and other protein synthesis inhibitors. We found that Bak^{-/-} cells are resistant to killing by PE and that Mcl-1, which binds to Bak, controls apoptosis induced by PE.

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MATERIALS AND METHODS

Cells. Simian virus 40 (SV40)-immortalized wild-type (WT), Bak^{-/-}, Bax^{-/-}, and Bak/Bax double-knockout (DKO) MEFs were generously provided by Stan-

ley Korsmeyer, Dana Farber Cancer Center, Boston, MA, and maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS).

Apoptosis and mitochondrial membrane potential assay. MEFs at 2×10^5 /well were seeded in 6-well plates overnight and then incubated with PE (purified in our laboratory), staurosporine, or cycloheximide (Sigma, St. Louis, MO) at the indicated concentrations for the indicated times. At the end of each experiment, floating and adherent cells were collected, and then apoptosis was measured using the Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA). Briefly, cells were washed twice with cold Dulbecco's phosphate-buffered saline (DPBS) and resuspended in 100 μ l of $1 \times$ binding buffer. Annexin V-PE and 7-aminoactinomycin D (7-AAD) (5 μ l) were added and incubated in the dark for 15 min at room temperature. Then, 400 μ l of $1 \times$ binding buffer was added and analyzed on a FACSCalibur (BD Biosciences, San Jose, CA). In some experiments, cells were treated with 10 μ M MG132 (EMD/Calbiochem, Gibbstown, NJ) for 1 h before PE was added. Apoptotic cells were defined as the total of annexin V-positive cells (annexin⁺/7-AAD⁻ and annexin⁺/7-AAD⁺).

The mitochondrial membrane potential was measured with JC-1 (Invitrogen, Carlsbad, CA). Cells were incubated with 4 μ g/ml of JC-1 at 37°C for 1 h and then washed twice with DPBS. The cells were resuspended in DPBS and analyzed on a FACSCalibur for the decrease in red fluorescence and increase in green fluorescence.

Protein synthesis inhibition assay. MEFs at 3×10^3 /well were seeded in 96-well plates overnight and then incubated with different concentrations of PE, ricin, or cycloheximide for 8 h. Cells were harvested after further incubation with 2 μ Ci/well of [³H]leucine (GE Healthcare, Piscataway, NJ) for 4 h. The cpm of triplicate samples were averaged, and the inhibition of protein synthesis was determined by calculating the percentage of ³H incorporation compared with untreated cells.

Immunoblotting and immunoprecipitation (IP). After treatment, both floating cells and adherent cells were collected, washed with cold DPBS twice, and solubilized in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1.0% Triton X-100) with protease inhibitors (Roche Applied Science, Indianapolis, IN). Protein concentrations were determined using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL). Equal amounts of protein were loaded onto 4 to 20% Tris-glycine SDS-PAGE (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. The following primary antibodies were used: rabbit anti-mouse Mcl-1 polyclonal antibody (Rockland Immunochemicals, Gilbertsville, PA), rabbit anti-Bak polyclonal antibody (Millipore/Upstate, Lake Placid, NY; anti-Bak NT), rabbit anti-Bax polyclonal antibody (Millipore/Upstate; anti-Bax NT), mouse anti-Bcl-x_L monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; clone 2H12), and mouse anti- β -actin monoclonal antibody (Abcam, Cambridge, MA; clone 8226). Horseradish peroxidase (HRP)-conjugated secondary antibodies—goat anti-rabbit (Santa Cruz Biotechnology) or sheep anti-mouse (GE Healthcare antibody)—were used and then visualized with an ECL substrate (GE Healthcare).

For immunoprecipitation, cell lysates were prepared in CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} lysis buffer (1% CHAPS, 150 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM EDTA with protease inhibitors) or Triton buffer and incubated with rabbit anti-mouse Mcl-1 polyclonal antibody (Rockland Immunochemicals) or rabbit monoclonal anti-Bcl-x_L antibody (Cell Signaling Technology, Danvers, MA; no. 2764) (12). Rabbit IgG was used as a control. The immunocomplexes were pulled down with protein A-agarose (Santa Cruz Biotechnology). For Western blot analysis of precipitation, the same anti-Mcl-1 antibody, rabbit anti-Bak antibody (Millipore/Upstate; anti-Bak NT) and mouse anti-Bcl-x_L (Santa Cruz Biotechnology; 2H12) were used. Band intensity was analyzed with NIH Image J.

To quantify how much Bak was in complexes, MEF lysates were prepared in 1% CHAPS buffer (4 mg/ml) with protease inhibitor. Cell lysates (100 μ l) were diluted with 300 μ l 1% CHAPS buffer; mixed with rabbit IgG, rabbit anti Bcl-2 (Cell Signaling; no. 2870), rabbit anti Bcl-x_L, and rabbit anti Mcl-1 antibodies; and incubated in a cold room overnight with gentle agitation. Protein A-agarose (20 μ l) was added and incubated in a cold room for 3 h with gentle agitation. The IP pellets were washed three times and dissolved in 50 μ l SDS loading buffer (5 μ l IP products–10 μ l whole-cell lysate [WCL; defined as $1 \times$ WCL]). Fifteen microliters ($3 \times$ WCL) or 5 μ l ($1 \times$ WCL) IP products and 10 μ l ($1 \times$ WCL) and serially diluted WCL were loaded in 4 to 20% SDS-PAGE and transferred to a 0.2- μ m polyvinylidene difluoride (PVDF) membrane.

Overexpression of Mcl-1 and Bcl-x_L. Mouse Mcl-1 and Bcl-x_L were cloned from cDNA clones (Origene, Rockville, MD). After sequencing verification, both were fused into the EGFP-C1 vector (Clontech, Mountain View, CA). All plasmids were prepared from an EndoFree Maxi Kit (Qiagen, Valencia, CA). DNAs (4 μ g) were transfected with Lipofectamine 2000 (Invitrogen) into WT MEFs in 6-well plates. The next day, 50 ng/ml of PE was added and incubated for

FIG. 1. Apoptosis of MEFs induced by PE. (A) WT MEFs were incubated with 1 to 50 ng/ml of PE for 24 h and subjected to apoptosis analysis by phosphatidylserine exposure (annexin V-PE) and 7-AAD uptake. (B) WT MEFs were incubated with 50 ng/ml of PE for 0 to 24 h and then subjected to apoptosis analysis as for panel A.

another 20 h. Apoptotic cells were stained with annexin V-allophycocyanin (APC) (BD Pharmingen), and green fluorescent protein (GFP)-positive populations were gated out as transfected cells with overexpression.

RESULTS

PE induces apoptosis in MEFs. PE and PE-derived immunotoxins are able to induce apoptosis in a wide variety of cells (13, 16, 17). In the current study, we utilized WT MEFs, Bax^{-/-} MEFs, Bak^{-/-} MEFs, and Bax/Bak DKO MEFs and established optimal conditions to monitor PE-mediated apoptosis by monitoring both an early apoptotic event (annexin binding to plasma membrane-exposed phosphatidylserine) and a late event (AAD entry and binding to DNA). As shown in Fig. 1A, 5 to 50 ng/ml of PE induced apoptosis after 24 h of treatment. Both early-stage (annexin⁺/7-AAD⁻) and late-stage (annexin⁺/7-AAD⁺) apoptotic cells increased with the toxin concentration. A time course study showed that no obvious apoptosis was evident 6 h after 50-ng/ml PE treatment

FIG. 2. Bak is required for the apoptosis of MEFs induced by PE. (A) WT, Bax^{-/-}, Bak^{-/-}, and DKO MEFs were incubated with medium only or with 50 ng/ml of PE for 24 h, and the apoptosis was analyzed with annexin V-PE and 7-AAD. (B) Means and standard deviations (SD) of apoptotic cells (annexin V positive) from four independent assays. NT, no treatment (medium only). (C) MEFs were treated with different concentrations of PE for 8 h. The cells were then assayed for protein synthesis using a [³H]leucine incorporation assay. ●, WT; ■, Bak^{-/-}; ▲, Bax^{-/-}; ◆, DKO.

(Fig. 1B), while 42% apoptotic cells were detected after 12 h and 63% after 24 h.

PE-mediated apoptosis requires Bak but not Bax. Bak and Bax are critical proapoptotic proteins that trigger the mitochondrial (intrinsic) apoptosis pathway (4, 6). To investigate the roles of these proteins in PE-induced apoptosis, we utilized Bak and Bax knockout (Bak^{-/-} and Bax^{-/-}) and DKO MEF lines (37, 43). Both WT and Bax^{-/-} MEFs were highly sensitive to PE, whereas Bak^{-/-} and DKO MEFs were resistant (Fig. 2A). At 50 ng/ml, PE killed 45 to 55% of WT and Bax^{-/-} cells, whereas Bak^{-/-} and DKO cells exhibited no more than 5% cell death. Further, when PE-treated and untreated cells were compared, the numbers of apoptotic cells in the Bak^{-/-} and DKO cultures were the same (Fig. 2B). This 5% level of apoptosis represents the normal background of cell death under these culture and experimental conditions. These results suggest that Bak is essential for toxin-mediated apoptosis.

Bax^{-/-} and Bak^{-/-} cells are equally sensitive to PE-mediated inhibition of protein synthesis, but with differential mitochondrial-potential loss. The earliest detectable cytotoxic effect of PE on cells is inhibition of protein synthesis due to ADP-ribosylation and inactivation of elongation factor 2. As shown in Fig. 2C, protein synthesis, measured by the incorpo-

ration of leucine 8 h after PE treatment, was inhibited to similar extents in all four cell lines, indicating that the resistance to apoptosis was not due to an alteration in the PE pathway that prevented access to cytosolic EF2 or NAD.

A step in cell death due to activation of the intrinsic pathway is loss of mitochondrial membrane potential ($\Delta\Psi_m$) (8). Utilizing the potential-sensitive dye JC-1 for flow analysis, only WT MEF and Bax^{-/-} cells showed loss of $\Delta\Psi_m$ after treatment with PE. This was reflected by a decrease in red fluorescence (Fig. 3) and an increase in green fluorescence (data not shown). No obvious change of mitochondrial potential was found for Bak^{-/-} or DKO MEFs. This result confirmed that the effect of PE is at a step before mitochondrial membrane potential disruption.

PE causes a rapid and preferential loss of Mcl-1 and releases Bak. Mcl-1 is a prosurvival protein that turns over rapidly and is an important suppressor of Bak. The rapid degradation of Mcl-1 has been shown to trigger apoptosis by UV irradiation (25). Rapid loss of Mcl-1 has also been reported when cells were treated with cycloheximide (2) or with an immunotoxin directed to the epidermal growth factor (EGF) receptor (3). Because PE is a potent inhibitor of protein synthesis, it seemed likely that the levels of Mcl-1 would fall

FIG. 3. Bak is required to disrupt mitochondrial membrane potential. WT, Bax^{-/-}, Bak^{-/-}, and DKO MEFs were incubated with medium only or 50 ng/ml of PE for 24 h. The MEFs were stained with JC-1. Loss of $\Delta\Psi_m$ is indicated by the decrease in red fluorescence.

rapidly in MEFs. Accordingly we measured the levels of Mcl-1 after PE treatment of WT MEFs and found that Mcl-1 was rapidly degraded (Fig. 4A). To investigate if degradation of Mcl-1 was linked to the activation of Bak in PE-induced apoptosis, we measured the expression of Mcl-1, along with Bak, Bax, and Bcl-x_L, another Bak suppressor protein (25, 39, 40), in WT and mutant MEFs (Fig. 4B). WT and Bax^{-/-} MEFs had similar Bak expression. WT and Bak^{-/-} MEFs had similar Bax expression. PE did not cause detectable changes in the amount of Bak, Bax, or Bcl-x_L in WT or mutant MEFs. Only Mcl-1 was affected, and it was rapidly degraded in all four MEF lines (Fig. 4B), which is consistent with the data showing that PE causes inhibition of protein synthesis in all four lines (Fig. 2C). However, its degradation did not initiate apoptosis in the Bak^{-/-} or DKO MEFs (Fig. 2A), which indicates that loss of Mcl-1 is not a consequence of cell death. Pretreatment of WT MEFs with the proteasome inhibitor MG132 prevented the degradation of Mcl-1 (Fig. 4C) and caused a decrease in apoptotic cells from 35% to background levels (Fig. 4D), suggesting that loss of Mcl-1 is an upstream event in apoptosis in MEFs. After PE treatment, degradation of Mcl-1 occurs in both WT and mutant MEFs, but only WT and Bax^{-/-} cells undergo apoptosis, suggesting that Bak is activated and induces apoptosis when Mcl-1 is no longer bound to it. Because Mcl-1 and Bcl-x_L are the two major BH-3 suppressor proteins that keep Bak in check (39, 40), it seemed likely that the loss of Mcl-1 would result in Bak being unleashed and trigger the downstream apoptosis cascade. To examine the binding of Mcl-1 associated with Bak, we

FIG. 4. Mcl-1 degradation is associated with apoptosis induced by PE. (A) WT MEFs were treated with 50 ng/ml of PE for up to 24 h, and the Mcl-1 expression level was detected by Western blotting. (B) WT, Bak^{-/-}, Bax^{-/-}, and DKO MEFs were treated with 50 ng/ml of PE for 20 h. Expression levels of Mcl-1, Bak, Bax, Bik, and Bcl-x_L were detected by Western blotting. (C) WT MEFs were pretreated with 10 μ M MG132 and then exposed to 50 ng/ml of PE for 20 h. The Mcl-1 expression level was detected by Western blotting. (D) The apoptosis of cells in panel C was analyzed with annexin V-PE and 7-AAD. Means and SD from triplicate wells are shown, representing data from three independent experiments.

FIG. 5. Bak released from suppressor complexes after degradation of Mcl-1. (A) WT MEFs were treated with 50 ng/ml of PE for 20 h. Cell lysates were prepared with 1% CHAPS buffer and immunoprecipitated with anti-Mcl-1 antibody (Ab) (M) or anti-Bcl-x_L antibody (B) combined anti-Mcl-1 and anti-Bcl-x_L antibodies (M+B), or rabbit IgG (R). Bak, Mcl-1, and Bcl-x_L in precipitated pellets were detected by Western blotting. At the bottom are shown endogenous Bak, Mcl-1, and Bcl-x_L in whole-cell lysates (before immunoprecipitation). *, rabbit IgG light chain. (B) Cell lysates were prepared with 1% Triton X-100 buffer, and immunoprecipitation was done as for panel A.

coimmunoprecipitated Bak with anti-Mcl-1 antibody and/or anti-Bcl-x_L antibody in CHAPS buffer (Fig. 5A). In untreated MEFs, 54% of Bak was coprecipitated with Mcl-1 antibody and 35% with Bcl-x_L antibody (lanes 1 and 2). After PE treatment, no detectable Bak was coprecipitated with anti-Mcl-1 antibody, presumably because of degradation of Mcl-1. Bcl-x_L-bound Bak increased to 72%. The total bound Bak was decreased to 70% after PE treatment (lanes 3 and 7), indicating that 30% of Bak was released from suppressed complexes when Mcl-1 was degraded. Control rabbit IgG did not precipitate Bak. When Triton X-100 was used instead of CHAPS, we had similar findings, i.e., that about 50% of Bak was released from total suppressed complexes (Fig. 5B, lanes 3 and 7). These experiments confirmed that Mcl-1 has a key role in regulating Bak activity.

Mcl-1 and Bcl-x_L are the major proteins binding to Bak in MEFs, and overexpression protects MEFs from PE-induced apoptosis. To quantify how much total cellular Bak was in suppressor complexes, the amounts of Mcl-1 and Bcl-x_L, as well as Bak, in the immunoprecipitation pellets were compared with serial dilutions of WCL of WT MEFs on the same membrane blots (Fig. 6A). Mcl-1 and Bcl-x_L immunoprecipitation pellets equaling three times that of the WCL contained about 36% of total cellular Bak, suggesting that about 12% (36%/3) of the Bak forms complexes with Mcl-1 and about 12% complexes with Bcl-x_L. The efficiency of Mcl-1 and Bcl-x_L immu-

noprecipitation was about 80% (Fig. 6A), indicating that about 15% (12%/80%) of the total Bak forms complexes with Mcl-1 and another 15% forms complexes with Bcl-x_L in MEFs. Thus, under these conditions, we found that about 30% of the Bak is in complexes with Mcl-1 and Bcl-x_L. To rule out other possible Bak suppressor proteins, we checked Bcl-2 and VDAC2. Though Bcl-2 was immunoprecipitated efficiently, no Bak was found associated with Bcl-2. Also, we did not detect evident VDAC2 expression in WT MEFs (data not shown).

These experiments confirmed that Mcl-1 and Bcl-x_L are the two major interacting Bcl-2 family proteins for Bak in MEFs. To assess whether ectopic expression of Mcl-1 and Bcl-x_L can protect MEFs from PE-induced apoptosis, we transiently transfected WT MEFs with enhanced green fluorescent protein (EGFP)-mouse Mcl-1 and -Bcl-x_L. MEFs with either Mcl-1 or Bcl-x_L overexpression (GFP-positive populations) showed minimal apoptosis (Fig. 6B) after PE treatment, even though endogenous Mcl-1 was still degraded (Fig. 6C).

Mcl-1 degradation after PE treatment may also release other Bcl-2 proteins bound to Mcl-1. We found that Puma expression levels did not change after PE treatment of WT MEFs, and less than 5% formed a complex with Mcl-1. Such complexes disappeared after PE treatment (Fig. 7). Bim expression levels decreased greatly after PE treatment (Fig. 7). Bim formed complexes with Mcl-1, and these complexes diminished after PE treatment, possibly due to reductions in both Mcl-1 and Bim. Also, we did not detect a Bax-Mcl-1 complex (Fig. 7).

Cellular response to high concentrations of PE. To study whether higher toxin concentrations would induce apoptosis in Bak^{-/-} and DKO MEFs, we added PE at 250 and 1,000 ng/ml. Regardless of the increase in the toxin concentration, we could not detect obvious apoptotic cells at 24 h (Fig. 8A). To investigate the duration of the protective effect of the Bak^{-/-} phenotype, we incubated Bak^{-/-} and DKO MEFs with PE for up to 96 h (Fig. 8B). Though no apoptotic cells were observed after 24 h, 36% of Bak^{-/-} MEFs showed apoptosis after 48 h, and the apoptotic-cell number increased to around 90% after 72 h. For DKO MEFs, there were no detectable apoptotic cells after 24 or 48 h, but after 96 h, about 27% of cells were undergoing apoptosis (Fig. 8B). Since protein synthesis was arrested in these cells for the whole 72-hour period, it is likely that the cells were dying from a pathway independent of Bak/Bax.

The preferential role of Bak and Mcl-1 degradation in the apoptosis induced by other protein synthesis inhibitors. Because several other toxins besides PE kill cells by arresting protein synthesis, although the mechanism of arrest differs from that of PE, we examined the ability of ricin, which arrests protein synthesis by ribosomal attack, to induce apoptosis of Bak^{-/-} cells. As shown in Fig. 9A, in cells treated for 24 h, ricin acted like PE and did not induce apoptosis in Bak^{-/-} cells. We also examined cycloheximide, a well-characterized inhibitor of protein synthesis. The ability of cycloheximide to induce cell death was less than that of PE, but the resistance of Bak^{-/-} cells was still evident. In contrast, cell death induced by the protein kinase inhibitor staurosporine was not prevented in Bak^{-/-} or Bax^{-/-} cells (Fig. 9A), and there was no loss of Mcl-1 (Fig. 9B), indicating that a different pathway is involved in staurosporine-mediated cell death. Moreover, the DKO

FIG. 6. Mcl-1 and Bcl-x_L bound to Bak in MEFs, and overexpression protected MEFs from PE-induced cell death. (A) WT MEF lysates were prepared in CHAPS buffer. Rabbit IgG and antibodies against Bcl-2, Mcl-1, and Bcl-x_L were used for immunoprecipitation. Immunoprecipitates equal to 3× WCL were loaded for Bak Western blots, and immunoprecipitates equal to 1× WCL were loaded for Mcl-1, Bcl-x_L, and Bcl-2 Western blots. Serially diluted WCL was loaded as a quantity determination control. (B) EGFP-C1, EGFP-Ms-Mcl-1, and EGFP-Ms-Bcl-x_L were transiently transfected into WT MEFs; 24 h later, 50 ng/ml PE was added and incubated for 20 h. The means and SD of apoptotic cells in GFP-positive populations from three independent assays are shown. (C) The cell lysates in panel B were prepared in 1% CHAPS buffer. Western blots were done with antibodies against Mcl-1, Bcl-x_L, and GFP.

MEFs, but not either single-knockout MEF, were resistant to staurosporine, suggesting that Bak and Bax are redundant for the apoptosis induced by this compound.

DISCUSSION

Pseudomonas exotoxin A and other protein synthesis inhibitors have been shown to promote cell death via apoptosis (2, 3, 13, 16, 17, 26, 30, 33). In the present study, we demonstrated that Bak is required for MEF apoptosis following the inhibition of cellular protein synthesis, and this requirement was not agent specific, since three distinct “toxins,” PE, ricin, and cycloheximide, acting on different components of the protein synthesis machinery, gave similar outcomes. Our results indicate that there is an absolute requirement for Bak and almost no requirement for Bax in PE-mediated apoptosis.

Bak^{-/-} cells were resistant to toxin-mediated cell death, but the absence of Bak did not prevent the delivery of PE to the cytosol or its ability to inhibit protein synthesis. Thus, Bak acts at a step following the ADP-ribosylation of EF2 and the inhibition of protein synthesis. Further, our results confirmed that Bak has only two major Bcl-2 family-interacting proteins,

FIG. 7. PE treatment did not release a significant amount of other Mcl-1-bound proapoptotic proteins. WT MEFs were treated with 50 ng/ml PE for 20 h. The cell lysates were prepared in 1% CHAPS buffer. Mcl-1 was immunoprecipitated from WCL with or without PE treatment, and Puma, Bax, Bim, and Mcl-1 were detected via Western blotting.

FIG. 8. Bak knockout cannot protect MEFs from prolonged exposure to PE. (A) Bak^{-/-} and DKO MEFs were treated with 50 to 1,000 ng/ml of PE for 24 h and analyzed by fluorescence-activated cell sorter (FACS) for apoptosis. (B) Bak^{-/-} and DKO MEFs were treated with 50 ng/ml PE for up to 96 h and then subjected to apoptosis analysis. At the bottom are shown the means and SD of apoptotic cells from three independent assays.

FIG. 9. The preferential role of Bak and Mcl-1 downregulation in apoptosis induced other protein synthesis inhibitors. (A) WT, Bak^{-/-}, Bax^{-/-}, and DKO MEFs were incubated with 50 ng/ml of ricin, 10 μg/ml of cycloheximide (CHX), or 50 nM staurosporine (STS) for 24 h and then analyzed by FACS for apoptosis. The means and SD of apoptotic cells from four independent assays are shown. NT, no treatment (medium only). (B) MEFs were treated with 50 ng/ml of PE, 50 ng/ml of ricin, 10 μg/ml of cycloheximide (CHX), or 50 nM staurosporine (STS) for 20 h. The expression level of Mcl-1 was detected by Western blotting.

Mcl-1 and Bcl-x_L, which may sequester Bak from activation (39, 40). A total of about 30% of the Bak was found in Mcl-1 and Bcl-x_L complexes, and no Bak was found to be associated with Bcl-2 (Fig. 5C). Although this partial-percentage binding of Bak by antiapoptotic Bcl-2 family members suggests that further interactions may occur subsequent to initial conformational changes in Bak, it is clear that Mcl-1 and Bcl-x_L are likely the primary inhibitors of Bak. The importance of Mcl-1 and Bcl-x_L for Bak regulation was further verified, as overexpression of either Mcl-1 or Bcl-x_L diminished PE-induced MEF death.

Mcl-1 is a short-lived protein and was lost quickly when protein synthesis was inhibited by PE, while no changes were detected for either Bcl-x_L, Bak, or Bax (Fig. 4). After PE treatment, Bcl-x_L-bound Bak increased from 40% to 60% (Fig. 5A) or 29% to 39% (Fig. 5B), possibly because part of the Bak was recaptured by Bcl-x_L after Mcl-1 degradation. However, even with recapture by Bcl-x_L, 30% to 50% of Bak was still released from total inhibitory complexes after PE treatment (Fig. 5A and B, lane 3 versus lane 7). Together, these data indicate that the inhibition of protein synthesis leads to rapid degradation of Mcl-1, leaving Bak unleashed to initiate the apoptosis cascade. The degradation of Mcl-1 is thought to be mediated via the proteasome system. Confirmation of this is provided by evidence that the addition of MG132 led to the stabilization of Mcl-1 and the prevention of toxin-mediated cell

death, indicating that Mcl-1 degradation is an upstream event in apoptosis in MEFs.

Our results indicate that in MEFs, Bak is preferentially involved in apoptosis mediated by inhibitors of protein synthesis. This role of Bak is not due to a greater abundance of Bak, because in these MEFs, the levels of Bak and Bax are comparable (39). Our finding is different from that of the study of Adams and Cooper, who found that both Bak and Bax contributed to cycloheximide-mediated apoptosis in U937 and HeLa cells (2). The contrasting results may reflect cell type differences or the distinction between using knockdown and knockout strategies. We also found that some human cancer cell lines are resistant to PE-based immunotoxin treatment, and their Bak expression levels are lower than those of sensitive cell lines, while other Bcl-2 family proteins, such as Mcl-1 and Bax, are comparable (X. Du and I. Pastan, unpublished data). These findings in human cells suggest that Bak may also play an important role for apoptosis induced by PE and PE-based immunotoxins in cells other than MEFs.

The link between inhibition of protein synthesis and the initiation of apoptosis is centered on Mcl-1. In contrast to our study and the paper from Adams and Cooper (2), Mcl-1 levels remained unchanged during apoptosis induced by protein synthesis inhibition through degradation of cellular mRNA by MazF (31). The importance of Mcl-1 in apoptosis has been emphasized in many studies (2, 7, 25, 29), though discrepancies exist. Mcl-1 degradation induced by Noxa overexpression (39) or downregulation by RNA interference (RNAi) (7, 25) did not trigger significant apoptosis by itself, while Mcl-1 knockdown alone induced apoptosis in other studies (2, 11, 23, 41). Our study indicates that Mcl-1 degradation may elicit apoptosis after PE treatment via Bak released from suppressed complexes, whereas Bcl-x_L is unaffected. Also, Mcl-1 is not always degraded upon stimulus. Noxa overexpression caused Mcl-1 degradation in MEFs, but not in 293T cells and Bcl-x_L^{-/-} MEFs (39). NBK/Bik overexpression stabilized Mcl-1 in DU145 cells (9), but not in 293T cells with MazF (31).

While the loss of Mcl-1 contributed to Bak-mediated apoptosis, we noted that there was not a strict correlation between the loss of Mcl-1 (with a variety of agents) and the percentage of cells undergoing apoptosis (Fig. 9A and B). For example, ricin and cycloheximide each reduced Mcl-1 levels, but ricin, like PE, caused more apoptosis than cycloheximide, suggesting that toxins may have additional effects to selectively activate Bak in addition to a reduction of Mcl-1. It is noteworthy that cell death caused by PE was time dependent and eventually Bak/Bax independent, because there was cell death at 72 h for Bak^{-/-} or DKO MEFs.

Mcl-1 degradation after PE treatment may also release other Bcl-2 proteins bound to Mcl-1. Among the BH3 proteins we tested, PE treatment did not alter Puma expression levels in WT MEFs. Less than 5% formed a complex with Mcl-1, and such complexes disappeared after PE treatment. Bim expression levels decreased greatly after PE treatment. Bim formed complexes with Mcl-1, but these complexes diminished after PE treatment, possibly due to a fall in both Mcl-1 and Bim. We did not detect a Bax-Mcl-1 complex, as reported by others (39). Thus, we did not find a significant amount of other Mcl-1-bound proapoptotic BH3 proteins released after PE treatment.

In summary, our results show that Bak is important for the death of MEFs induced by PE, ricin, and cycloheximide. The rapid degradation of Mcl-1 due to protein synthesis inhibition releases Bak from suppressed complexes and contributes to the Bak-dependent apoptosis.

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