Functional Linkage between NOXA and Bim in Mitochondrial Apoptotic Events*

Jie Han1, Leslie A. Goldstein1,2, Wen Hou3, and Hannah Rabinowich4

From the 1Department of Pathology, University of Pittsburgh School of Medicine, and the 2University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213

NOXA is a BH3-only protein whose expression is induced by certain p53-dependent or independent apoptotic stimuli. Both NOXA and Bim are avid binders of Mcl-1, but a functional linkage between these BH3-only proteins has not yet been reported. In this study, we demonstrate that Mcl-1 binding of endogenously induced NOXA interferes with the ability of Mcl-1 to efficiently sequester endogenous Bim, as Bim is displaced from its complex with Mcl-1. Induced NOXA significantly enhances the UV sensitivity of cells, and the ensuing mitochondrial depolarization is entirely abrogated by Bim knockdown. These results demonstrate a Mcl-1-mediated cross-talk between endogenous NOXA and Bim that occurs upstream of the Bak/Bax-dependent execution of UV-induced mitochondrial depolarization. The current findings demonstrate that the mitochondrial response to an induced expression of NOXA is executed by endogenous Bim and suggest a plausible mechanism for the observed NOXA-Bim linkage.

Mitochondrial involvement in programmed cell death is mediated by mitochondrial alterations that lead to the release of caspase activators and caspase-independent death effectors, which are cumulatively responsible for eventual cell death. The permeability of the mitochondrial outer membrane is regulated by pro- and anti-apoptotic Bcl-2 family members (1–4). Bcl-2 proteins can be divided into three sub-classes on the basis of their function and the number of Bcl-2 homology (BH)3 domains included in their structure. The anti-apoptotic members, including Bcl-2, Bcl-XL, Bcl-W, A1, and Mcl-1 contain three or four BH domains; the pro-apoptotic members, including Bax and Bak, possess three BH domains; and the "BH3-only" pro-apoptotic members, such as Bid, Bim, NOXA, and Puma, share homology only within the BH3 domain (5). Structural studies have suggested that the hydrophobic face of the α-helix formed by the BH3 domain of BH3-only proteins inserts into the hydrophobic groove formed by the BH1, BH2, and BH3 domains of the anti-apoptotic Bcl-2 members (6). In response, Bax and Bak form homo-oligomers that are capable of permeabilizing the mitochondrial outer membrane for an egress of mitochondrial apoptotic proteins. Bid and Bim appear to function upstream of Bax and Bak; ectopic expression of these proteins in cells lacking both Bax and Bak could not induce the mitochondrial release of cytochrome c (7, 8). The function of Bcl-2 proteins is also closely tied (in a yet unresolved manner) to the loss in mitochondrial membrane potential that represents an early phase in the apoptotic process (9, 10). The electrochemical gradient across the inner mitochondrial membrane is critical for normal mitochondrial function and therefore its loss is ultimately associated with cell death.

The BH3-only proteins NOXA and Puma have been identified as transcription targets of p53 that are involved in DNA damage-induced apoptosis (11). NOXA was originally identified as an adult T-cell leukemia-derived phorbol 12-myristate 13-acetate-responsive gene (12) and more recently as a p53-induced gene in x-ray-irradiated murine embryo fibroblasts (13). Expression of NOXA is induced by phorbol ester, p53, UV radiation, and other DNA-damaging agents, such as etoposide and doxorubicin (13–16). Recently, it was reported that proteasome inhibitors induce NOXA in a p53-independent manner (17–19). The human NOXA gene is located on chromosome 18q21 and encodes a 54-amino acid protein with a single BH3 motif, which is in contrast to the murine NOXA protein that possesses two BH3 domains (13). Both the human and the mouse proteins contain a mitochondrial targeting domain. Following its translocation to the mitochondria, NOXA binds preferentially to Mcl-1, and not to Bcl-2 or Bcl-XL (20).

Our previous studies have documented the sequestration of Bim by Mcl-1 under non-apoptotic conditions (21–23). As Mcl-1 is also a preferred binding target of NOXA, we investigated the relationships between NOXA and Bim in Mcl-1 binding and in mediating mitochondrial apoptotic susceptibility. The current study demonstrates a functional linkage between NOXA and Bim, which is suggested by the apoptotic involvement of Bim downstream of NOXA induction.

**EXPERIMENTAL PROCEDURES**

Reagents—Anti-human Mcl-1 Abs were from Oncogene (Boston, MA; mouse clone RC13 generated against recombinant Mcl-1), and from Santa Cruz Biotechnology (Santa Cruz, CA; a polyclonal Ab generated against a 20-amino acid residue

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1 Both authors contributed equally to this work.
2 To whom correspondence should be addressed: University of Pittsburgh Cancer Institute, The Hillman Cancer Center, Research Pavilion, Rm. G17c, 5117 Centre Ave., Pittsburgh, PA 15213. Tel.: 412-623-3212; Fax: 412-623-1119; E-mail: rabinow@pitt.edu.
3 The abbreviations used are: BH, Bcl-2 homology domain; Ab, antibody; mAb, monoclonal antibody; GSI, γ-secretase inhibitor; MOPS, 4-morpholinepropanesulfonic acid; nt, nucleotide(s); RNAi, RNA interference; siRNA, small interference RNA; JC-1, 1',1',3,3′-tetrathyylbenzamidazolocarbocyanin iodide; Tet, tetracycline.
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synthetic peptide of human Mc1-1). Anti-β-actin mAb (clone AC-15) was purchased from Sigma; anti-Cox IV mAb was from Molecular Probes (Eugene, OR); rabbit anti-Bim Ab was from ProSci (Poway, CA); Bim-specific rat mAb was from ApoTech (San Diego, CA; Clone 14A8); anti-NOXA Ab was from Immunex (San Diego, CA). Abs to AIF, Bcl-2, Bcl-XL, p53 (clone DO-1), and β-tubulin were from Santa Cruz Biotechnology; anti-Xpress mouse mAb for lacZ was from Invitrogen; [35S]methionine, Protein A-Sepharose beads, and Protein G-Sepharose beads were from Amersham Biosciences. The γ-secretase inhibitor (GSI) consisting of a tripeptide aldehyde, N-benzyl-oxycarbonyl-Leu-Leu-norleucine-CHO was from Calbiochem.

Cell Lines, Cell Lysates, and Cell Extracts—Jurkat T leukemic cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 20 mM HEPES, 2 mM L-glutamine, and 100 units/ml each of penicillin and streptomycin. HeLa, breast carcinoma CAMA-1, colon cancer Hct116, and T-Rex-293 cells were grown in Dulbecco’s modified Eagle’s medium containing 15% fetal calf serum, 10 mM MOPS, 1 mM EDTA, and 4 mM KH2PO4 (pH 7.4) and lysed by Dounce homogenization as previously described (21). Briefly, nuclei and debris were removed by a 10-min centrifugation at 650 × g and a pellet containing mitochondria was obtained by two successive spins at 10,000 × g for 10 min at 4 °C. The purity was assessed at 95%, with ~5% or less contamination from the microsomal fraction.

Molecular Cloning of Tet-inducible NOXA Expression Plasmid—Total RNA was isolated from Jurkat T-cells using RNA STAT-60 Reagent (Tel-Test B, Inc.). Reverse transcription was carried out with 5 μg of total RNA using an oligodT12–18 primer and SuperScript II RNase H– reverse transcriptase (Invitrogen). PCR was performed using the Expand Long Template PCR System kit (Roche Applied Science). A NOXA amplon containing its open reading frame was generated with the following primer pair (forward and reverse): 5’-CGCGGATCC-GCGGAGATGCTGGGAAAG-3’, extends 6 nucleotides (nt) into 5’-untranslated region and 5’-ACCGTCGACCATGAGT-GCACCTTACATTCC-3’, complementary to nt 437–457 in 3’-untranslated region (extends 119 nt into the 3’-untranslated region). The putative NOXA ampiclon was size-selected using a 1.4% agarose gel, and DNA was purified with the QIAquick gel extraction kit (Qiagen). The purified ampiclon was digested with the restriction enzymes BamHI and SalI and ligated into the Tet-inducible vector, pcDNA4/TO (Invitrogen) that had been previously digested with BamHI and Xhol. Following transformation (Escherichia coli TOP 10F’, Invitrogen), plasmids from randomly picked colonies underwent automated DNA sequence analysis (University of Pittsburgh DNA Sequencing Core Facility) to confirm sequence integrity.

Stable Transfection of T-Rex-293 Cells with Tet-inducible Plasmids pcDNA4/TO, pcDNA4/TO/lacZ, and pcDNA4/TO/NOXA—The cell line T-Rex-293 (Invitrogen) comprises human embryonic kidney cells that stably express the Tet repressor. To generate stable clones that harbor the pcDNA4/TO vector control, pcDNA4/TO/lacZ expression control (Invitrogen) and pcDNA4/TO/NOXA, all plasmids were linearized using the restriction enzymes FspI (pcDNA4/TO and pcDNA4/TO/NOXA) and ScaI (pcDNA4/TO/lacZ). Transfection was carried out using 30 μl of GenePorter Transfection Reagent (Gene Therapy Systems Inc., San Diego, CA) following the manufacturer’s directions and 5 μg of linearized plasmid in 6-well plates with 0.8 × 10^6 cells per well (~60% confluency) that were maintained in the presence of 5 μg/ml blasticidin. At 48 h post-transfection Zeocin was added to a final concentration of 100–200 μg/ml. After ~2 weeks individual colonies were selected, expanded, and harvested. Induction of NOXA and lacZ expression by individual clones was confirmed by immunoblotting after the addition of 1 μg/ml tetracycline for 16 h. Confirmation of stable clones containing integrated pcDNA4/TO vector was obtained by reverse transcription-PCR of their total RNA. We utilized a primer pair that delimits the Zeocin resistance gene open reading frame in pcDNA4/TO (forward and reverse): 5’-ACCATGCGCAAGTTGACCGT-3’, corresponds to nt 2247–2267 and 5’-GAAATTCTCGTAG-CACGTGTCA-3’, complementary to nt 2622–2642. Multiple such clones were utilized in the studies to confirm the described findings.
RNAi Using NOXA, Mcl-1, Bim, Bak, and Bax siRNAs and a Non-targeting siRNA—NOXA, Mcl-1, and Bim siRNAs were obtained as duplexes in purified and desalted form (Option C) from Dharmacon. The three siRNAs had the following sense strand sequences: NOXA, 5′-GUCGAGUGUGCUACUGCAAAUCUtTdTdT-3′; Mcl-1, 5′-GAAACGCGGUAAUCCGAUTdTdT-3′; and Bim, 5′-GACCGAAGAGUAGACAAUUdUdTdT-3′. Bak and Bax siRNAs were also obtained from Dharmacon but as siGENOME SMARTpool reagents M-003305-01 and M-003308-00, respectively. The non-targeting siRNA control used in our RNAi experiments is the siCONTROL Non-Targeting siRNA #1 (D-001210-01) from Dharmacon. Hct116 or T-REx-293 cells were irradiated with UV light from a germicidal lamp, which emitted radiation primarily in the UVC region, with a peak wavelength of 254 nm. The total dose of irradiation was 60 J/m² as measured by a Black-Ray UV meter, model J225 (UVP Inc., Upland, CA).

In Vitro Transcription-Translation—Mcl-1, NOXA, Bim, and Bak cDNAs were expressed in the Tnt T7 transcription-translation reticulocyte lysate system (Promega, Madison, WI). Each coupled transcription-translation reaction contained 1 μg of plasmid DNA in a final volume of 50 μl in a methionine-free reticulocyte lysate reaction mixture supplemented with 35S-labeled methionine according to the manufacturer’s instructions. After incubation at 30 °C for 90 min, the reaction products were immediately used or stored at −78 °C.

Flow Cytometry—Cytofluorometric analyses of apoptosis were performed by co-staining with propidium iodide and fluorescein isothiocyanate-Annexin V conjugates (BD Biosciences). Mitochondrial membrane potential depolarization was measured by using a fluorescent cationic dye, 1,1′3,3′-tetraethylbenzamidezolocarboxyliodide (JC-1, Molecular Probes). The staining was performed according to the manufacturer’s procedures, and loss of mitochondrial membrane potential was quantified by flow cytometric analysis of the decrease in the 590 nm (red)/527 nm (green) fluorescence intensity emission ratio utilizing a Beckman Coulter Epics XL-MCL and analyzed with the EXPO32 software.

RESULTS

Involvement of Mcl-1, NOXA, and Bim in GSI-mediated Changes in Mitochondrial Membrane Potential—To investigate the role of NOXA in mitochondrial apoptotic events, we treated several tumor cell lines with GSI, which was recently reported to mediate apoptosis in association with induced expression of NOXA (18). This drug is a tripeptide aldehyde that was initially identified as an inhibitor of Notch signaling (25, 26) and was recently shown to induce apoptosis in Kapoisi's
sarcoma and melanoma cell lines (18, 25). In our studies, this aldehyde inhibitor induced a significant level of apoptosis in most of the treated tumor cell lines (Fig. 1, A and B). GSI-induced apoptosis was associated with NOXA induction in four of five treated cell lines, whereas only a low level of apoptosis was detected in CAMA-1 cells, where NOXA was not induced.

Induction of p53 accompanied that of NOXA in WT Hct116, but NOXA up-regulation can also be independent of p53, as was determined in a p53 knock-out-Hct116 clonal cell line (p53−/−) and in Jurkat cells (p53-null). Moreover, despite p53 expression, there was no induction of NOXA by GSI in CAMA-1 cells. GSI enhanced the Mcl-1 expression levels in both Hct116 clonal cell lines (WT and p53−/−), in CAMA-1 and in HeLa cells, whereas in Jurkat cells it mediated Mcl-1 degradation. Bim was up-regulated following treatment with GSI in Hct116 clonal cell lines, unchanged in CAMA-1 cells, and down-regulated in Jurkat and HeLa cells. These observations illustrate the occurrence of multiple changes in the expression levels of mitochondrial apoptosis regulatory proteins in response to a single drug and suggest that these differential alterations are cell-type specific.

Because GSI induced a significant apoptotic level in Hct116 cells in association with up-regulated expression of the mitochondrial Bcl-2 members, Mcl-1, NOXA, and Bim, we investigated the roles of these proteins in the regulation of mitochondrial membrane potential in these cells. To this end, RNAi was carried out on WT Hct116 cells with siRNA specific for Mcl-1, NOXA, and Bim or with a non-targeting siRNA control. During

FIGURE 2. Apoptotic implications of mitochondrial translocation of GSI- or Tet-induced NOXA. A, mitochondrial translocation of GSI-induced NOXA is associated with cytochrome c release. GSI-treated Jurkat cells (5 μM, 16 h) were disrupted by Dounce homogenization followed by a purification process for mitochondria and S-100 fractions. The extract, mitochondrial (Mlt) and S-100 fractions of either control or GSI-treated cells were assessed for the expression of the indicated proteins. Expression of β-actin (cytosolic protein) and Cox IV (mitochondrial protein) serve as markers for equal loading and purity of the cellular fractions. B, mitochondrial translocation of Tet-induced NOXA is not associated with release of apoptogenic proteins or changes in expression of Bim or p53. T-REx-293 cells stably transfected with either Tet-inducible NOXA or its vector control were treated with tetracycline (1 μg/ml for 16 h). The cells were then disrupted by Dounce homogenization and subcellular fractionated as described in A. The same membrane was stripped and reprobed successively for the indicated proteins.

FIGURE 3. Co-immunoprecipitation of NOXA with Mcl-1. A, GSI-induced NOXA co-immunoprecipitates with endogenous Mcl-1. Hct116 cells were treated with GSI (5 μM, 16 h). The lysates of control and GSI-treated cells were subjected to immunoprecipitation with Mcl-1-specific mouse mAb. The original lysates, depleted supernatants, and immunoprecipitated pellets were assessed by immunoblotting for the expression of Mcl-1 (using rabbit anti-Mcl-1 Ab, top). The membrane was stripped and reprobed with NOXA-specific mouse mAb (bottom).

B, in vitro translated NOXA co-immunoprecipitates with in vitro translated Mcl-1. The 35S-labeled in vitro translated Mcl-1 was mixed with unlabeled in vitro translated NOXA and subjected to immunoprecipitation with rabbit anti-Mcl-1 Ab. The original mixture, depleted supernatant, and immunoprecipitated pellet were assessed by immunoblotting for the presence of Mcl-1 (top) and NOXA (middle), and by autoradiography for the radioactive signal generated by 35S-labeled Mcl-1 (bottom).

C, Tet-induced NOXA co-immunoprecipitates with endogenous Mcl-1. T-REx-293 cells stably transfected with Tet-inducible control or with Tet-inducible NOXA were treated with tetracycline (1 μg/ml, 16 h). The cells were then lysed, and subjected to immunoprecipitation by rabbit anti-Mcl-1 Ab. The original lysates, depleted supernatants, and immunoprecipitated pellets were assessed by immunoblotting for expression of Mcl-1 and NOXA. β-Actin serves as an equal loading control. All immunoprecipitation procedures were performed after pre-clearance with protein A/G-Sepharose beads and nonspecific rabbit or mouse Ig. The ratio of cell lysate (input) gel loading to that of the immunoprecipitation (IP) pellet was 1:4.
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The last 16 h of the siRNA transfection, the cells were treated with GSI and assessed by immunoblotting for the expression levels of the targeted proteins and by JC-1 flow cytometry for changes in the mitochondrial membrane potential. GSI-1 flow cytometry is a lipophilic fluorochrome that, upon mitochondrial binding, emits a green fluorescent signal, which is further processed to an additional red fluorescent signal only in mitochondria with preserved membrane potential. Thus, mitochondria with intact membrane potential emit high green and high red fluorescence, whereas loss of mitochondrial membrane potential results in reduced emission of red fluorescence while maintaining the high green fluorescence. Mcl-1 siRNA increased the loss in red fluorescence in response to GSI, whereas knockdown of either NOXA or Bim significantly inhibited the GSI-mediated loss in mitochondrial membrane potential. These observations suggest that the changes in mitochondrial membrane potential in response to GSI are jointly regulated by Mcl-1, NOXA, and Bim. However, because of the multiple concurrent changes in the expression of these mitochondrial apoptosis regulators, the drug-induced cell is not an optimal system for analyzing the functional relationship between NOXA and Bim.

Elucidation of NOXA Apoptotic Implications in Tet-induced NOXA-transfected Cells—To elucidate a potential role for NOXA in mitochondrial apoptotic events, we utilized a tetracycline-regulated expression (T-Rex) system consisting of the T-Rex-293 (human embryonic kidney epithelial cells stably transfected with the Tet repressor) clonal cell line stably transfected with NOXA or the empty inducible vector whose expression is switched on by the presence of tetracycline. Similar to GSI-induced NOXA, Tet-induced NOXA accumulated exclusively in the mitochondria (Fig. 2, A and B). In contrast to GSI-induced NOXA, Tet-induced NOXA was not associated with increased expression of Bim, p53, or Mcl-1 (Figs. 2B and 3C). Furthermore, induction of NOXA alone did not result in cell death or a release of mitochondrial apoptogenic proteins.

Recently, NOXA was reported to

bind exclusively to Mcl-1, and not to Bcl-2 or Bcl-XL (20, 27). To investigate the ability of drug-induced NOXA and Tet-induced NOXA to bind Mcl-1, we immunoprecipitated Mcl-1 in three experimental systems, including GSI-treated Hct116 cells (Fig. 3A), a mixture of in vitro translated Mcl-1 and NOXA proteins (Fig. 3B), and T-Rex-293 cells expressing Tet-induced NOXA or Tet-vector control (Fig. 3C). In each of these experimental systems, Mcl-1 was efficiently immunoprecipitated, because it was completely depleted from the remaining supernatants (Fig. 3, A (lane 7 top), B (lane 4 top), and C (lanes 4–6).
UV Sensitivity Induced by NOXA Is Executed by Bim

A
Non-Targeting siRNA

B
Tetracyclin: - + - + Xpress (lacZ)

C
siRNA: Cont Bim Cont Bim

D
Tet-Induction: lacZ NOXA lacZ NOXA lacZ NOXA lacZ NOXA

E
siRNA: Cont Bax Cont Bax

F
% Cells Exhibiting Loss in Av

G
% Inhibition of UV:NOXA-mediated
GSI-induced NOXA was entirely co-immunoprecipitated with Mcl-1 (Fig. 3A, lanes 6–8 bottom), whereas Tet-induced NOXA was partly, although significantly, co-immunoprecipitated with Mcl-1 (Fig. 3C, lanes 3, 6, and 9). It is possible that the expression level of Tet-induced NOXA is excessive relative to that of endogenous Mcl-1, and thus a significant proportion of the induced NOXA remains Mcl-1-free.

Previous studies performed by others (28–31) and by us (21–23) documented the sequestration of Bim by Mcl-1 under non-apoptotic conditions. Because both NOXA and Bim are binding partners for Mcl-1, we investigated the possibility that induced NOXA displaces Bim from its Mcl-1 sequestration. To this end, we utilized two experimental systems that allowed for the presence of an increased dose of NOXA, with no associated changes in the expression levels of either Bim or Mcl-1. Thus, constant doses of in vitro translated Mcl-1 and Bim (5 μl each of in vitro translation extract) were mixed with increasing NOXA doses (0–15 μl) forming ratios of in vitro translation system extracts of Mcl-1:Bim:NOXA (1:1:0, 1:1:1, 1:1:2, and 1:1:3, respectively) and subsequently subjected to Mcl-1 immunoprecipitation (Fig. 4, A and B). (Fig. 4A shows all components of the reaction, including input, depleted supernatant, and immunoprecipitated pellet; Fig. 4B shows a side-by-side run of the immunoprecipitated pellets from the procedure performed with increasing NOXA doses; Fig. 4D shows the densitometric quantitation of the Mcl-1 pellet proteins shown in B.) The amount of Bim co-immunoprecipitated with Mcl-1 was inversely related to the amount of co-immunoprecipitated NOXA. Thus, in our in vitro translation system, the increased presence of NOXA competed off Bim for Mcl-1 binding. The Tet-induced NOXA system was also utilized to test the relationship between endogenous NOXA and Bim (Fig. 4, C and E). The stably transfected T-Rex-293 clonal cell lines (Tet-Vector control and Tet-NOXA) were treated with tetracycline for either 8 or 16 h, and then subjected to Mcl-1 immunoprecipitation. Kinetics-dependent induction of NOXA was observed in the cells stably transfected with NOXA, but not in control cells treated with tetracycline (Fig. 4C, lanes 1–3 top). No change in the Mcl-1 expression level was detected in either of the tetracycline-treated cell lines (Fig. 4C, lanes 1–3), and immunoprecipitation of Mcl-1 completely depleted the supernatants (lanes 4–6) of Mcl-1, which in turn exhibited equal levels in the pellets of tetracycline-treated cells (Tet-vector and Tet-NOXA, lanes 7–9). Co-immunoprecipitation of Bim was again inversely related to the level of induced NOXA, with the highest level in the pellet of control cells (lane 7) and reduced levels in Tet-NOXA cells treated with tetracycline (lanes 8 and 9). The depleted supernatant exhibited a converse image to that of the pellet, with the highest level of Bim being present in the post-immunoprecipitation supernatant obtained from cells with the highest level of NOXA induction (lane 6). These results suggest that induced NOXA is capable of displacing Bim from Mcl-1 sequestration.

Functional Linkage between NOXA and Bim in UV-mediated Apoptosis—UV has been demonstrated to induce NOXA, potentially implicating this BH3-only protein in the mitochondrial apoptotic response to UV (13, 15, 16, 32). Furthermore, mitochondrial apoptotic response to UV, including involvement of Bax and Bak has been demonstrated in numerous cell lines (including HEK293). Hence, we utilized T-Rex-293 cells to investigate the functional linkage between Tet-induced NOXA and endogenous Bim in response to UV. Flow cytometric assessment of JC-1 staining was used as the readout for mitochondrial response. Tet-induced expression of NOXA, but not expression of the lacZ control, significantly increased the UV-mediated loss in the mitochondrial membrane potential (Fig. 5A and F). To investigate the contribution of Bim, Bak, or Bax to NOXA-mediated UV susceptibility, we treated T-Rex-293 clonal cell lines that were stably transfected with either a Tet-inducible lacZ control or Tet-inducible NOXA with siRNA specific for Bim, Bak, or Bax, or a non-targeting siRNA control. The efficient siRNA down-regulation of the indicated proteins is demonstrated by immunoblotting of untreated or UV-treated cells (Fig. 5, C–E). Following siRNA transfection (40 h), Tet-inducible lacZ or Tet-inducible NOXA cells were treated with tetracycline (12 h) and subsequently exposed to UV irradiation. Following an additional 16-h incubation, the loss in mitochondrial membrane potential in response to UV was assessed by flow cytometry of JC-1 staining (Fig. 5A and G). The NOXA-mediated susceptibility to UV (Fig. 5A, panel d1) was completely abrogated by Bim siRNA (panel d2) and partly abrogated by either Bax or Bak siRNA (panels d3 and d4). Notably, not only the Tet-NOXA-mediated sensitivity was abrogated by Bim knockdown, but also the basal mitochondrial sensitivity to UV was significantly blocked (Fig. 5A, panels c1–c4).

Because NOXA is a UV up-regulated gene (13, 15, 16, 32), the basal UV response of T-Rex-293 may as well be executed by NOXA and Bim. Indeed, UV alone induced some expression of NOXA (Fig. 5D) as well as that of Bax and p53 (Fig. 5E). Under the experimental conditions utilized, the expression of Mcl-1 was not altered in response to UV irradiation (Fig. 5D). The involvement of Bax and Bak in the NOXA/Bim-mediated loss in the mitochondrial membrane potential is not surprising, because the requirement for either Bax or Bax downstream of Bim has been documented (8). These observations suggest a previously unknown role for Bim as an intermediate execu-

FIGURE 5. NOXA-mediated loss in mitochondrial membrane potential in response to UV is abrogated by Bim RNAi. A, flow cytometry analysis of JC-1 in response to UV in lacZ- or NOXA-expressing cells. Tet-inducible lacZ control and Tet-inducible NOXA T-Rex-293 clonal cell lines were treated with the indicated siRNA for 40 h. The cells were then treated with tetracycline (1 μg/ml) for 12 h. Subsequently, the cells were exposed to UV (60 J/m²) and incubated for additional 16 h prior to their JC-1 flow cytometry assessment. B, immunoblot analysis for Tet-induced expression of lacZ. T-Rex-293 clonal cell lines stably transfected with lacZ or empty vector control were treated with tetracycline and assessed for lacZ expression using Xpress mAb (Invitrogen). C–E, immunoblot analyses for the expression of Bim (C), Bak (D), and Bax (E) in Tet-induced lacZ or NOXA cells following treatment with the specific siRNAs, tetracycline and UV. Each of the specific siRNAs is compared with the concurrent treatment with non-targeting siRNA (control). Expression of β-tubulin serves as loading control. The membrane shown in D was further probed for NOXA and Mcl-1; the membrane shown in E was further probed for p53, F and G, quantitative representation of the results obtained in A, F, the presence of NOXA, but not of lacZ, enhances significantly the loss in mitochondrial membrane potential in response to UV. Representative results of five independent experiments are shown. G, the mitochondrial membrane potential of Tet-induced NOXA cells treated with UV is differentially altered by Bim, Bak, Bax, or non-targeting siRNA. The values are mean ± S.E. of three to five independent experiments.
tioner between NOXA and Bak/Bax in mediating mitochondrial depolarization in response to UV.

**DISCUSSION**

A potential role for Bim in p53-mediated apoptosis has been suggested by studies performed with Bim knock-out lymphocytes, which were somewhat refractory to γ-irradiation (11,33). Until recently, these observations remained unresolved, because in contrast to NOXA and Puma, Bim is not a p53 target (11). A recent study performed with Puma−/−Bim−/− double knock-out mice has established the cooperation between Puma and Bim in p53-dependent apoptosis (34). Thus, apoptosis induced by γ-irradiation or DNA-damaging drugs was more efficiently blocked in Puma/Bim double-deficient cells when compared with cells lacking the individual genes (34). Our current studies elucidated a role for Bim as a liaison between NOXA and the mitochondrial executioners, Bak and Bax. Elucidation of such a functional linkage between NOXA and Bim was enabled by the significant UV susceptibility gained by T-REx-293 cells through the solitary induction of NOXA in a tetracycline-regulated expression system. The complete abrogation of the gained UV sensitivity by Bim knockdown places Bim downstream of NOXA in implementing the observed loss in mitochondrial membrane potential. Thus, the current studies demonstrate in vivo a functional linkage between these two BH3-only proteins in the execution of UV-mediated mitochondrial depolarization.

The avid binding of both Bim and NOXA to Mcl-1 offers a plausible mechanistic scenario, whereby up-regulated expression of NOXA leads to Bim activation. Under non-apoptotic conditions Bim is sequestered by Mcl-1, which potentially neutralizes its apoptotic activity (21–23). In a recent study, A. Letai’s group (35) has demonstrated the ability of a 20-residue peptide similar in sequence to NOXA BH3 to displace Bim BH3 peptide from sequestration by GST-Mcl-1. Our current studies demonstrate such a displacement of Bim in vivo through interactions between endogenous Mcl-1, Bim, and NOXA. The ability of in vitro translated or Tet-induced NOXA to displace Bim from its complex with Mcl-1 suggests that the binding of these two proteins to Mcl-1 is mutually exclusive: either one or the other can bind to the same Mcl-1 molecule. Thus, induction of endogenous NOXA may result in an increased level of Mcl-1-free Bim capable of mediating Bax/Bak-dependent mitochondrial apoptotic events.

Bim-dependent apoptosis in response to UV may also be facilitated by caspase- or proteasome-mediated degradation of Mcl-1 (21–23, 36, 37). Such a scenario satisfactorily explains the involvement of Bim but does not account for its direct link to NOXA in executing the NOXA-mediated UV susceptibility. In our studies, the solitary induction of NOXA by tetracycline was not associated with any change in the level of Mcl-1 expression. As reported previously (21–23, 36), several mechanisms are involved in the elimination of Mcl-1 expression in response to UV irradiation. However, in T-REx-293 cells, at the time of detection of changes in mitochondrial membrane potential, no changes in the level of Mcl-1 expression had occurred yet.

These observations suggest that the UV apoptotic process in cells with increased expression of NOXA preferentially utilizes Bim, freed from Mcl-1 sequestration, in mediating changes in the mitochondrial membrane permeability. Subsequent involvement of caspase or proteasomal degradation of Mcl-1, as mechanisms for the further release of Bim from Mcl-1 sequestration, is not excluded and seems to occur during a later phase of the apoptotic process.

In summary, the current study identified a novel cross-talk mechanism between two BH3-only proteins, NOXA and Bim, that culminates in enhanced mitochondrial depolarization in response to apoptotic stimuli. Our results suggest that apoptotic sensitivity gained by induced expression of NOXA is executed by Bim, placing NOXA upstream of Bim in mediating Bak/Bax-dependent mitochondrial permeability. The NOXA-mediated displacement of Bim from Mcl-1 sequestration represents a plausible molecular mechanism that underlies the cooperation between Bim and NOXA.

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