Regulation of Mitochondrial Apoptotic Events by p53-mediated Disruption of Complexes between Antiapoptotic Bcl-2 Members and Bim*§

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Multiple mechanisms have been proposed for the mitochondrial function of p53 that are either dependent on or independent of its transcriptional activity. However, none of these mechanisms involves Bim functioning downstream of p53 mitochondrial translocation. Utilizing a p53 nuclear localization signal mutant, whose nuclear import is completely abrogated, we demonstrate that its apoptotic activity at the outer mitochondrial membrane, which involves conformational changes in Bax and Bak, is mediated by Bim. We further demonstrate an inverse correlation between the binding levels of p53 and Bim to Mcl-1. Thus, enhanced binding of p53 to Mcl-1 involves the disruption of existing complexes between Mcl-1 and Bim. We propose that mitochondrial p53 functions as a Bim derepressor by releasing Bim from sequestrating complexes with Mcl-1, Bcl-2, and Bcl-XL, and allowing its engagement in Bak/Bax activation.

The powerful apoptotic function of p53 is central to its tumor suppressor activity (1). p53 can act as a transcription factor that regulates the expression of proapoptotic Bcl-2 family genes that reside or act at the mitochondria, including NOXA, Puma, and Bax (2–6). In addition to the well-established transcription-dependent mechanism of p53 apoptotic function, evidence has also accumulated for a p53 transcription-independent apoptotic role at the mitochondria (7–19). In those studies, p53-dependent transcription was nullified by targeting p53 directly to the mitochondria in p53-deficient tumor cells or by using protein synthesis inhibitors, wheat germ agglutinin as a nuclear import inhibitor, enucleated cytoplast preparations, or transcription-deficient p53 mutants (11, 13, 19). It was proposed that such transcription-independent cell death was mediated by a BH3-like activity of p53 that upon its mitochondrial translocation can bind Bcl-2, Bcl-XL, and Bak (12, 19, 20). Several potential mechanisms were considered for the BH3-like activity of p53: (i) neutralization of antiapoptotic activity of Bcl-2 and Bcl-XL (19); (ii) conversion of Bcl-2 activity from antiapoptotic to proapoptotic (21); (iii) displacement of Bak from Mcl-1 sequestration (20); (iv) direct activation of Bax (12, 13); and (v) competition with PUMA for Bcl-XL binding (11). Thus, p53 may function as a direct Bax activator or as a derepressor that relieves Bak or BH3-only proteins from repression by anti-apoptotic Bcl-2 proteins (13).

Our recent studies have elucidated a central regulatory role for the mitochondrial Mcl-1-Bim complex in the apoptotic response to granzyme B, TRAIL, and certain cytotoxic drugs (22–24). In the current study, we investigated the involvement of the Mcl-1-Bim complex in the p53-mediated mitochondrial apoptotic cascade. We demonstrate that Mcl-1 contributes significantly to the regulation of the mitochondrial response to p53 and that Bim is directly involved in the execution of this response upstream of Bax and Bak. In particular, induced expression of p53 results in disruption of complexes between Bim and Mcl-1, Bcl-2, or Bcl-XL. Such a disruption may be mediated by de novo binding between antiapoptotic Bcl-2 proteins and p53 itself as well as by a selective binding of the p53 transcriptional targets, NOXA and Puma, to their respective antiapoptotic Bcl-2 protein partners. Utilizing a p53 nuclear localization signal (NLS) mutant, we demonstrate the independence of p53 from the de novo transcription of Bax, NOXA, and Puma genes in its disruption of existing complexes between Bim and Mcl-1 and in its mediation of Bim-dependent activation of existing Bax and Bak pools. These findings extend previously proposed models for mitochondrial p53 function to include a novel mechanism where p53 derepresses Bim from the repressive effects of Mcl-1, Bcl-2, and Bcl-XL. This p53 derepressing activity sheds new light on the unexplained resistance of Bim−/− cells to γ-irradiation (25, 26).

EXPERIMENTAL PROCEDURES

Reagents—Anti-human Mcl-1 Abs were from Oncogene (Boston, MA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-actin mAb (clone AC-15) was from Sigma; anti-Cox IV mAb was from Molecular Probes (Eugene, OR); anti-Bax mAb 6A7 was from BD Biosciences, and the N-20 Ab was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Bcl-2 mAb was from BD Biosciences; anti-Bcl-XL mAb was from Abcam (Cambridge, MA); the 3H-[3,4,5,6-tetraethylbenzamidazolocarbocyanin iodide; WT, wild-type; KD, knockdown.

3 The abbreviations used are: NLS, nuclear localization signal; Ab, antibody; mAb, monoclonal antibody; 5-FU, fluorouracil; CHAPS, 3-[3-cholamido
dimethylammonio]-1-propanesulfonic acid; ORF, open reading frame; RNAi, RNA interference; siRNA, small interfering RNA; MIB, mitochondrial buffer; MOPS, 4-morpholinepropanesulfonic acid; JC-1, 22473
from Santa Cruz Biotechnology, Inc.; Rabbit anti-Bak Abs were from Upstate Biotechnology, Inc. (Lake Placid, NY), Santa Cruz Biotechnology, Inc., and Oncogene (AB1); Rabbit anti-Bim and anti-Puma Abs were from ProSci (Poway, CA); an additional Bim-specific mAb was from Apoptech (San Diego, CA); anti-NOXA Ab was from ImageX (San Diego, CA); Abs to Bcl-2, Bcl-XL, p53 (clone DO-1), SMAC, and anti-NOXA Ab was from Imgenex (San Diego, CA); Abs to Bak-specific rat mAb was from Apoptech (San Diego, CA); Rabbit anti-Bak Abs were from Santa Cruz Biotechnology, Inc.; Anti-Xpress mouse mAb for lacZ-encoded protein was from Invitrogen; Protein A-Sepharose beads and Protein G-Sepharose beads were from Amersham Biosciences. TrueBlot reagent was from eBio-Science (San Diego, CA), and fluorouracil (5-FU) was from Amersham Biosciences. TrueBlot reagent was from eBio-Science (San Diego, CA), and fluorouracil (5-FU) was from Amersham Biosciences. TrueBlot reagent was from eBio-Science (San Diego, CA), and fluorouracil (5-FU) was from Amersham Biosciences. TrueBlot reagent was from eBio-Science (San Diego, CA), and fluorouracil (5-FU) was from Amersham Biosciences.

Cell Lines, Cell Lysates, and Cell Extracts—All cell lines utilized in this study were obtained from ATCC, apart from Hct116 cells, which were generous gifts from Dr. Bert Vogelstein (The Johns Hopkins University). Jurkat T leukemic cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM-glutamine, and 100 units/ml each of penicillin and streptomycin. Hct116, T-REx-293, MCF7, MDA-MB-231, and RPMI-8266 cell lines were grown in Dulbecco’s modified Eagle’s medium containing 15% fetal calf serum, 2 mM-glutamine, and 100 units/ml each of penicillin and streptomycin. Transfected T-REx-293 clonal cell lines were maintained in the presence of blasticidin (5 μg/ml) and zeocin (20 μg/ml). Cell lysates were prepared with 1% CHAPS, 50 mM Tris-HCl, pH 7.4, 110 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. To prepare cell extracts, cultured cells were washed twice with phosphate-buffered saline and then resuspended in ice-cold buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose, and protease inhibitors). After incubation on ice for 20 min, cells (2.5 × 10⁶/0.5 ml) were disrupted by Dounce homogenization. Nuclei were removed by centrifugation at 650 × g for 10 min at 4 °C. Cellular extracts were obtained as the supernatants resulting from centrifugation at 14,000 × g at 4 °C for 30 min.

Molecular Cloning of Tet-inducible p53 Expression Plasmid—Total RNA was isolated from Hct116 cells using RNA STAT-60 Reagent (Tel-Test “B”, Inc.). Reverse transcription was carried out with 5 μg of total RNA using an oligo(dT)₁₂–₁₈ primer and SuperScript II RNase H⁻ Reverse Transcriptase (Invitrogen). PCR was performed using the Expand Long Template PCR System kit (Roche Applied Sciences). A p53 amplicon containing its open reading frame (ORF) was generated with the following primer pair (forward and reverse): 5’-CGCGGATCCATCGCAGCTGGAGAGCCG-3’ (which extends 6 nucleotides into the 5’-untranslated region (UTR)) and 5’-ACCGGCGCTGACTCAGTCTGACTCAGGCCGCC-3’ (complementary to the C terminus of ORF). The putative p53 amplicon was size-selected using a 1% agarose gel, and DNA was purified with the QIAquick gel extraction kit (Qiagen). The purified amplicon was digested with the restriction enzymes BamHI and SalI and size-selected, expanded, and harvested. Induction of p53 and lacZ encoded protein 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/T0 vector was obtained by reverse transcription-PCR of their total RNA. We utilized a primer pair that delimits the zeocin resistance gene ORF in pcDNA4/T0 (forward and reverse): 5’-ACCATGGCGAATTCAGCGCCAG-3’ (complementary to nucleotides 2247–2267) and 5’-GAAATTCGCTAGACCCAGTC-3’ (complementary to nucleotides 2622–2642) (data not shown).

T-REx-293 cells stably transfected with inducible p53 were transfected with XmnI linearized Mcl-1 plasmid or pCR3.1 vector using the GenePorter Transfection Reagent as described above. Genetin-resistant cell lines were grown in the presence of G418 (1500 μg/ml). Genetin-resistant clonal cell lines either expressing Mcl-1 or harboring the vector control (see below) were generated by dakocytomation (1 cell/well) utilizing a MOFLO high speed cell sorter and Summit Software.
mation of stable clones containing the pCR3.1 vector was obtained by reverse transcription-PCR using a primer pair that delimits the neomycin resistance gene ORF in pCR3.1 (forward and reverse): 5’-GCCATGATTGAACAAGATGGA-3’ (complementary to nucleotides 3162–3142) and 5’-TCGCTTGGTCGGTCAATT-3’ (corresponding to nucleotides 2335–2352). All experiments were repeated with at least three individual clones or with a mixture of individual clones that had been confirmed to express the transfected gene of interest.

Transient transfections were carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions using non-linearized plasmids. Cells were split in 24-well plates at densities of 2–8 × 10^4 cells/well 24 h prior to the transfection. The cells were harvested and assessed 24–48 h after the transfection.

RNAi—NOXA, Mcl-1 and Bim siRNAs were obtained as duplexes in purified and desalted form (Option C) from Dharmacon/Thermo Fisher Scientific. These siRNAs had the following sense strand sequences: NOXA, 5’-GUCGAGUGUGCUACUACACUdTdT-3’; Mcl-1, 5’-GAAACGGGUAUUGCGACACUdTdT-3’; Bim, 5’-GACCGAAGAGUGUAUUGdTdT-3’. Also obtained from Dharmacon/Thermo Fisher Scientific as siGENOME SMARTpool reagents were Bak (M-003305-01), Bax (M-003308-00), Mcl-1 (M-004501-04), NOXA (M-005275-01), and Puma (M-004380-01) siRNAs. The non-targeting siRNA control used in our RNAi experiments with the Dharmacon siRNAs above is the siCONTROL non-targeting siRNA 1 (D-001210-01). Bim siRNA was also obtained from Invitrogen as Stealth Select siRNA (HSS145411,412,413) and Stealth RNAsi negative control Med GC (12935-300). T-REx-293 cells (2.5 × 10^5) were transfected with 10–50 nM siRNA in Opti-MEM transfection. After 4 h, fetal calf serum was added to a final concentration of 10%. At 40 h, the medium over the cells was made in 100 μl of lysis buffer (20 mM Tris, 137 mM NaCl, 2 mM Na_2HPO_4, 1% Nonidet P-40, 10% glycerol, and 1% protein inhibitors: aprotinin, leupeptin, phenylmethylsulfonyl fluoride). The lysates were precleared with Protein A- or G-Sepharose beads and incubated with the immunoprecipitating Abs (at 1:100–1:1000 dilution) at 4 °C for 4 h. The immune complexes were then precipitated with Protein A- or G-Sepharose beads at 4 °C overnight. The pellets were washed four times with the appropriate lysis buffer and boiled for 5 min in SDS sample buffer.

Confocal Microscopy—Cells were grown and treated on LabTek II chamber slides. After treatment, cells were fixed with 4% paraformaldehyde and permeabilized with the immunoprecipitating Abs (at 1:100–1:1000 dilution) at 4 °C for 4 h. The immune complexes were then precipitated with Protein A- or G-Sepharose beads at 4 °C overnight. The pellets were washed four times with the appropriate lysis buffer and boiled for 5 min in SDS sample buffer.

RESULTS

De Novo Generation of Complexes between Mcl-1 and Tet-induced p53 Is Associated with Disruption of Mcl-1-Bim Complexes—A role for Bim in p53-mediated cell death has been suggested by studies performed with Bim knock-out lymphocytes, which were somewhat refractory to γ-irradiation (25, 26). However, the mechanism underlying the involvement of Bim in the p53-mediated mitochondrial apoptotic cascade has not yet been elucidated. The binding of p53 to Bcl-2 and Bcl-XL has been well established, but the possibility of consequential Bim-mediated mitochondrial changes has not yet been investigated.
A Role for Bim in p53-mediated Mitochondrial Apoptosis

Because Bim constitutively binds antiapoptotic Bcl-2 family members, particularly Mcl-1, we investigated the possibility that such complexes are disrupted by induced expression of p53. Co-precipitation of endogenous p53 with Mcl-1 was observed in multiple tumor cell types that express WT p53, including colon carcinoma Hct116, multiple myeloma RPMI-8266, and breast cancer MDA-MB-231 and MCF7 cells (Fig. 1). Because under quiescent conditions, the expression level of endogenous p53 is low, we also assessed its Mcl-1 binding capability following treatments known to induce its physiologic expression level (30, 31). Thus, in Hct116 cells treated with 5-FU (Fig. 1, D and E) or in UV-irradiated MCF7 cells (Fig. 1F), the expression of endogenous p53 was significantly up-regulated, and a significant part of it co-immunoprecipitated with Mcl-1. The ability of endogenous Mcl-1 to bind p53 was further demonstrated in p53−/− Hct116 cells ectopically transfected with p53 (supplemental Fig. S1).

Endogenous levels of Mcl-1 are highly responsive to cellular stress and may be up-regulated or down-regulated in cell type- and treatment-dependent manners (32). Thus, in 5-FU-treated Hct116 cells, Mcl-1 is up-regulated (Fig. 1E, lane 4 versus lane 1), whereas in UV-treated MCF7 cells, Mcl-1 is down-regulated (Fig. 1F, lane 2 versus lane 1). Therefore, for further analysis of the relationship between Mcl-1-Bim and Mcl-1-p53 complexes, we utilized Tet-inducible p53 T-REx-293 cells, where the induction of p53 does not significantly impact the expression level of Mcl-1. T-REx-293 cells stably transfected with p53 were treated with tetracycline for 6, 12, or 24 h and then subjected to Mcl-1 immunoprecipitation. Induced expression of p53 (Fig. 2A, lanes 2–4 versus lane 1) did not significantly alter the expression levels of either Mcl-1 or Bim. However, the levels of Mcl-1-bound p53 were directly related to the levels of p53 in the original extracts (Fig. 2A, lanes 10–12 versus lanes 2–4). Thus, the unchanged levels of endogenous Mcl-1 were sufficient to accommodate the binding of increasing doses of p53. We and others have previously reported that Mcl-1 is localized mainly to the outer mitochondrial membrane, where it sequesters Bim (22, 33, 34). The binding of p53 to Mcl-1 was associated with a reduction in the levels of Mcl-1-Bim complexes (Fig. 2, lanes 10–12 versus lane 9) and increased levels of Mcl-1-free Bim in the post-immunoprecipitation supernatants of cells expressing induced p53 (Fig. 2A, lanes 6–8 versus lane 5). The inverse correlation between Mcl-1-p53 and Mcl-1-Bim complexes may indicate a direct disruption by p53 of the Mcl-1-Bim complexes. However, an increased level of Mcl-1-free Bim may also result from the de novo generation of complexes between p53-induced NOXA or p53-induced Puma and Mcl-1. Increased expression of p53 was associated with increased levels of NOXA (Fig. 2A, lanes 2–4 versus lane 1). Furthermore, after 24 h of the Tet induction of p53, an increased level of the NOXA-Mcl-1 complex was detected (Fig. 2A, lane 12). Puma induction was also detected in cells with Tet-induced p53 (Fig. 2A, lanes 2–4 versus lane 1), but neither NOXA-Mcl-1 nor Puma-Mcl-1 complexes (Fig. 2A, lanes 9–12) exhibited an inverse correlation with p53-Mcl-1 complexes. Previous studies have established associations between mitochondria-translocated p53 and Bcl-2 or Bcl-XL (19). Because these Bcl-2 family members also form complexes with Bim under non-apoptotic conditions (35–37), we examined whether induction of p53 results in increased levels of free Bim displaced from Bcl-2 and Bcl-XL. To this end, we immunoprecipitated endogenous Bcl-2 (Fig. 2B) and Bcl-XL (Fig. 2C) from Tet-induced p53 cells. Similar to observations made for Mcl-1, both Bcl-2 and Bcl-XL demonstrated increased binding to p53 in a p53 dose-dependent manner (Fig. 2, B and C, lanes 7–9, top). Interestingly, the levels of Bcl-2-Bim were inversely related to the levels of Bcl-2-bound p53 (Fig. 2B, lanes 8 and 9). Likewise, the levels of Bcl-XL-Bim were inversely related to the levels of Bcl-XL-bound p53 (Fig. 2C, lanes 8 and 9). Quantitation of all of the protein bands of Fig. 2 is shown in the supplemental material (supplemental Fig. S2). These results suggest that the binding of p53 to Mcl-1, Bcl-2, and Bcl-XL results in an increased level of Bim freed from sequestration by these antiapoptotic Bcl-2 proteins.
Involvement of Mcl-1, Bim, NOXA, and Puma in Mitochondrial Depolarization Mediated by WT p53—To investigate a role for Mcl-1 in protection against p53-mediated mitochondrial changes, we utilized T-REx-293 clonal cell lines stably transfected with both Mcl-1 (constitutive) and Tet-inducible p53. p53-mediated mitochondrial depolarization was assessed by flow cytometry of the mitochondrial membrane potential dye JC-1 (Fig. 3A). JC-1 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. As assessed by changes in JC-1 red fluorescence, Tet induction of p53, but not of lacZ-encoded protein, resulted in a significant loss in the mitochondrial membrane potential, which was largely abrogated by concomitant overexpression of Mcl-1 (Fig. 3, A and B).

To investigate the involvement of Mcl-1 binding partners in p53-mediated mitochondrial depolarization, we assessed the effects of their knockdown (KD) on mitochondrial JC-1 staining (Fig. 3, C–F). Whereas Mcl-1 KD enhanced the p53-mediated loss of mitochondrial ΔΨm, Bim KD inhibited the induced depolarization. The inhibitory effect of Bim RNAi was more pronounced than that of either Bax or Bak RNAi, potentially due to the ability of Bax and Bak to substitute for each other (38). NOXA and Puma also function upstream of Bak and Bax in p53-mediated mitochondrial apoptotic events (26, 39).

Indeed, knockdown of either Bim, NOXA, or Puma significantly inhibited p53-mediated mitochondrial depolarization (Fig. 3, E and F). However, knockdown of Bim appeared to mediate an additive inhibitory effect to that observed with siRNA targeting of NOXA, Puma, or a combination of NOXA and Puma (Fig. 3E) (data not shown). These results suggest that in p53-mediated mitochondrial depolarization, Bim functions, at least in part, independently of NOXA and Puma.

Induction of Mitochondrial Apoptotic Events by p53 NLS Mutant—To further distinguish between the contribution of Bim (which is not a transcriptional target of p53) (26) and the p53 transcriptional targets NOXA and PUMA in the observed mitochondrial apoptotic events, we generated a p53 NLS mutant. p53 contains a bipartite NLS consisting of two basic amino acid groups, Lys305-Arg306 and Lys319-Lys320-Lys321, separated by a spacer of 12 amino acid residues. Because each part of the bipartite NLS sequences can function individually as a weaker NLS (40), we generated an NLS mutant in which all five amino acids were replaced with Ala. Tetracycline treat-

expression of p53 is associated with enhanced expression of NOXA and Puma (lanes 2–4 versus lane 1). Neither NOXA nor Puma exhibits an inverse relationship to p53 in Mcl-1 binding. Tet treatment of control T-REx-293 cells stably transfected with Tet-inducible lacZ is shown in Fig. 3B. B and C, co-immunoprecipitation of Tet-induced p53 with endogenous Bcl-2 or Bcl-XL, T-REX-293 clonal cells stably transfected with Tet-inducible p53 were treated with tetracycline for 6 or 16 h and then subjected to immunoprecipitation of Bcl-2 (B) and Bcl-XL (C), as described for Mcl-1 in A. The asterisks indicate unidentified protein bands. Quantitation of the protein bands for this figure is shown in supplemental Fig. S2. Similar results were obtained in at least three independent experiments.
FIGURE 3. p53-mediated mitochondrial depolarization is inhibited by overexpression of Mcl-1 or KD of Bim, NOXA, or Puma. A and B, p53-mediated loss in Δψm is regulated by Mcl-1. Tet-inducible p53 T-REx-293 clonal cell line, Tet-inducible lacZ control T-REx-293 cell line, and Tet-inducible p53 T-REx-293 clonal cell line that is also stably transfected with constitutively expressed Mcl-1 were treated with tetracycline (1 μg/ml) for the indicated time periods and assessed for emission of red JC-1 by flow cytometry. Treatment by carboxyl cyanide m-chlorophenyl hydrazone (CCCP) served as a positive control for mitochondrial depolarization. The expression levels of Mcl-1 and p53 of the utilized cells are shown in B.

C and D, p53-mediated mitochondrial depolarization is enhanced by Mcl-1 KD and inhibited by Bim KD. Tet-inducible lacZ control and Tet-inducible p53 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 16 h and assessed for JC-1 staining by flow cytometry. Immunoblot analyses for these cells are shown in D. E and F, Bim RNAi increases the inhibitory effects of NOXA and Puma RNAi on p53-mediated mitochondrial depolarization. Tet-inducible p53 cells were treated with various siRNAs and combinations of siRNAs, including Bim, NOXA, Puma, NOXA + Puma, NOXA + Bim, Puma + Bim, and NOXA + Puma + Bim, as described in C. Controls included Tet-inducible p53 cells transfected with the various siRNA combinations without tetracycline treatment and Tet-inducible lacZ cells transfected with the various siRNA combinations with or without tetracycline treatment. These controls are not shown because no changes in JC-1 staining were detected (all negative controls were similar in staining to Tet-inducible lacZ cells treated with tetracycline and non-targeting siRNA shown on the left, bottom). Immunoblot analyses of Tet-induced p53 cells treated with NOXA, Puma, Bim, or non-targeting siRNAs that were utilized in this experiment are shown in F. To avoid redundancy, immunoblot analyses of control Tet-inducible lacZ cells (treated with siRNAs), as shown in B, are not included for this experiment. The same membrane was successively probed with the indicated Abs. β-Actin serves as an equal loading control. The asterisks indicate unidentified protein bands. Similar results were obtained in at least three independent experiments.
A Role for Bim in p53-mediated Mitochondrial Apoptosis

Differential subcellular localization of WT p53 and p53 NLS mutant. A and B, WT p53 co-localizes with nuclei, whereas p53 NLS mutant surrounds nuclei with no co-localization. T-REx-293 clonal cell lines stably transfect with Tet-inducible WT p53 (left panels) or with Tet-inducible p53 NLS mutant (right panels) were treated with tetracycline (1 μg/ml, 16 h) and assessed by confocal microscopy for co-localization of p53 (green) with either mitochondria (red) or nuclei (blue). Scale bar, 40 μm (A) and 10 μm (B). Both NLS mutant and WT p53 partly co-localize with mitochondria; p53 NLS mutant exclusively surrounds the nucleus, whereas WT p53 primarily co-localizes with the nucleus. C and D, subcellular localization of Tet-induced WT p53 and Tet-induced p53 NLS mutant as assessed by biochemical cellular fractionation. T-REx-293 clonal cell lines stably transfected with Tet-inducible WT p53 (C) or Tet-inducible p53 NLS mutant (D) were treated with tetracycline (1 μg/ml, 16 h). Control and treated cells were Dounce homogenized and, after removal of nuclei, were subjected to subcellular fractionation to obtain extracts, S-100, and mitochondria. The proteins were resolved by SDS-PAGE and detected by immunoblotting. Expression of β-actin and Cox IV are included as markers of the respective subcellular fractions. Cyt C, cytochrome c; DAPI, 4′,6-diamidino-2-phenylindole.

FIGURE 4. Differential subcellular localization of WT p53 and p53 NLS mutant. A and B, WT p53 co-localizes with nuclei, whereas p53 NLS mutant surrounds nuclei with no co-localization. T-REx-293 clonal cell lines stably transfected with Tet-inducible WT p53 (left panels) or with Tet-inducible p53 NLS mutant (right panels) were treated with tetracycline (1 μg/ml, 16 h) and assessed by confocal microscopy for co-localization of p53 (green) with either mitochondria (red) or nuclei (blue). Scale bar, 40 μm (A) and 10 μm (B). Both NLS mutant and WT p53 partly co-localize with mitochondria; p53 NLS mutant exclusively surrounds the nucleus, whereas WT p53 primarily co-localizes with the nucleus. C and D, subcellular localization of Tet-induced WT p53 and Tet-induced p53 NLS mutant as assessed by biochemical cellular fractionation. T-REx-293 clonal cell lines stably transfected with Tet-inducible WT p53 (C) or Tet-inducible p53 NLS mutant (D) were treated with tetracycline (1 μg/ml, 16 h). Control and treated cells were Dounce homogenized and, after removal of nuclei, were subjected to subcellular fractionation to obtain extracts, S-100, and mitochondria. The proteins were resolved by SDS-PAGE and detected by immunoblotting. Expression of β-actin and Cox IV are included as markers of the respective subcellular fractions. Cyt C, cytochrome c; DAPI, 4′,6-diamidino-2-phenylindole.

Involvement of Bim in the Mitochondrial Apoptotic Activity of p53—To confirm that the disruption of Mcl-1-Bim complexes is mediated by p53 itself, independent of NOXA and Puma, we immunoprecipitated Mcl-1 from Tet-inducible p53 NLS mutant T-REx-293 cells treated with tetracycline for 24 or 48 h. Similar to results obtained with WT p53 (Fig. 2A), the level of p53 NLS mutant that co-precipitated with Mcl-1 was inversely correlated with the level of co-precipitated Bim (Fig. 6A). Quantitation of these protein bands is shown in the supplemental material (supplemental Fig. S5). These results show that the inverse relationship is directly related to the expression levels of p53 independent of NOXA and Puma. Also, the induced p53 NLS mutant mediated mitochondrial depolarization that was Bim-dependent; it was significantly inhibited by Bim KD and significantly enhanced by Mcl-1 KD (Fig. 6, B and C). To further investigate a role for Bim upstream of p53-mediated Bax and Bak conformational changes, we assessed the effect of Bim knockdown (Fig. 6, D–G). Indeed, Bim knockdown (evident by its lost expression) was associated with a loss in the up-regulated expression of 6A7-detectable Bax and of AB1-detectable Bak, when mediated by either WT or the p53 NLS mutant. These findings provide further evidence for placing Bim upstream of Bax and Bak in the execution of mitochondrial apoptotic events mediated by p53.

DISCUSSION

A role for Bim in p53-mediated apoptosis has been indicated by the partial resistance to γ-irradiation acquired by Bim knock-out lymphocytes (25, 26). The exact mechanism for Bim involvement remained unresolved despite significant advances in the current understanding of the mitochondrial apoptotic function of p53. In this study, we demonstrate a direct involvement of Bim in the mitochondrial response to p53 and provide evidence for disruption of complexes between Bim and anti-apoptotic Bcl-2 proteins in a p53-dependent manner.

Progress in elucidating the p53 mitochondrial apoptotic cascade has been achieved through the discovery of NOXA and PUMA as p53 transcriptional targets (3–5, 26) and the recent identification of complexes between mitochondria-translocated p53 and Bcl-2 or Bcl-XL (11, 19, 44). The current study has identified a heretofore-unexplored association between p53 and Mcl-1. Like other antiapoptotic Bcl-2 proteins, Mcl-1 prevents the permeabilization of the mitochondrial outer membrane, which requires the oligomerization of either Bax or Bak (38). Although it has been established that activation of Bax and Bak is mediated by certain BH3-only proteins, the exact mechanisms involved remain controversial (45–47). Two principal mechanisms have been proposed, focusing on the sequestration by antiapoptotic Bcl-2 proteins of either Bax and Bak or BH3-only proteins. According to the neutralization model, upon receipt of an apoptotic signal, activated BH3-only pro-
peptides remove Bax and Bak from the constraints of prosurvival Bcl-2 proteins (45). According to the alternative model, activator BH3-only proteins, including Bid, Bim, and Puma, are capable of direct activation of Bax and Bak unless kept in check by prosurvival proteins (39, 48–50). In contrast to direct activator BH3-only proteins, derepressor/sensitizer BH3-only proteins (e.g. Bad) function indirectly by displacing the sequestered activators from their complex with Bcl-2, Bcl-XL, or Mcl-1. The first biophysical evidence for a direct activation of Bax by Bim has only recently been reported, utilizing NMR to prove the binding between stapled Bim BH3 peptide and Bax (51). The recently identified complexes between p53 and antiapoptotic Bcl-2 proteins expand the current perspectives on p53 mitochondrial function to include several models that are similar in

FIGURE 5. Assessment of mitochondrial apoptotic events in T-REx-293 cells overexpressing WT or NLS mutant p53. A, WT p53 exhibits accelerated kinetics of cell death in comparison with p53 NLS mutant. Cell viability was assessed by the CellTiterGlo luminescent kit, and the results are mean ± S.D. of triplicate determinations in one of at least five experiments with similar results. B, Tet-induced expression of either WT p53 or p53 NLS mutant results in mitochondrial release of cytochrome c (Cyt c) and SMAC, but the release kinetics for the p53 NLS mutant are slower. T-REx-293 clonal cell lines stably transfected with Tet-inducible WT p53 (left lanes) or with Tet-inducible p53 NLS mutant (right lanes) were treated with tetracycline for the indicated time periods. Control and treated cells were Dounce homogenized and fractionated to heavy membrane and cytosolic fractions. The cytosolic fractions were assessed by immunoblotting for the expression of the indicated proteins. C–E, confocal microscopic evidence for conformational and expression level changes in Bax and Bak following induction of WT or p53 NLS mutant. Scale bars, 40 μm. C, increased expression of cellular Bax (N20 Ab) is detected mainly following Tet induction of WT p53. D, increased levels of conformationally altered Bax (6A7 Ab) are detected in cells with induced expression of either WT p53 or p53 NLS mutant. E, increased detectability of Bak is mediated by WT p53 and to a lesser extent by p53 NLS mutant. F, flow cytometry evidence for conformational and expression level changes in Bax and Bak following induction of WT or p53 NLS mutant. T-REx-293 clonal cell lines stably transfected with Tet-inducible WT p53 (left lanes) or with Tet-inducible p53 NLS mutant (right lanes) were treated with tetracycline for 16 h and assessed by flow cytometry for changes in mean fluorescence intensity (MFI) of Bax (N20 Ab), active Bax (6A7 Ab), and active Bak (AB1 Ab). Whereas induction of WT p53 results in marked increase in MFI of Bax (N20), Bax (6A7), and Bak (AB1), induction of NLS mutant p53 results in milder increase in MFI of Bax (6A7) and Bak (AB1) but no increase in Bax (N20). Similar results were obtained in at least three independent experiments. DAPI, 4′,6-diamidino-2-phenylindole.
FIGURE 6. **Functional relationship between p53 NLS mutant and Bim.**

**A**, inverse relationship between p53 NLS mutant and Bim binding to Mcl-1. Increased Mcl-1 binding to p53 NLS mutant is associated with reduced Mcl-1 binding to Bim. Tet-inducible p53 NLS mutant T-REx-293 cells were treated with tetracycline for the indicated time periods. The cells were then lysed and subjected to Mcl-1 immunoprecipitation (IP). The left panels of cell lysate (lanes 1–3) and depleted supernatant (lanes 4–6) represent 25% of input, and the corresponding films were exposed for 20 s; the immunoprecipitated (IP) pellet (lanes 7–9) represents 75% of input, and the corresponding films were exposed for 5 s. The asterisks indicate unidentified protein bands. Quantitation of the protein bands of this figure is shown in supplemental Fig. S5.

**B and C**, mitochondrial depolarization mediated by the p53 NLS mutant is enhanced by Mcl-1 KD and inhibited by Bim KD. Tet-inducible p53 NLS mutant T-REx-293 cells were treated with non-target control, Mcl-1, or Bim siRNAs for 40 h. The cells were then left untreated (control) or treated with tetracycline for 24 h. Mitochondrial depolarization was assessed by flow cytometry of JC-1-stained cells. Efficacy of Bim and Mcl-1 RNAi is shown in C.

**D–G**, changes in patterns of expression of Bax (D and E) or Bak (F and G) following induction of WT or NLS mutant p53s are inhibited by Bim KD. Scale bars, 40 μm. Tet-inducible WT p53 (D and F) and Tet-inducible p53 NLS mutant (E and G) T-REx-293 cells were treated with the indicated siRNAs and tetracycline as described above. The cells were assessed by confocal microscopy for expression of Bim (red), conformationally altered Bax (6A7 Ab; green), conformationally altered Bak (AB1 Ab; green), and 4′,6-diamidino-2-phenylindole (DAPI) (blue). The increased expression of conformationally altered Bax and Bak by either WT p53 or p53 NLS mutant is blocked in cells treated with Bim siRNA.
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In summary, the current study demonstrates the involvement of Bim in the execution of p53-mediated mitochondrial apoptotic events and proposes the possibility that the release of Bim from sequestration by antiapoptotic Bcl-2 family members serves as the underlying mechanism for such involvement.

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