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BOK and *NOXA* Are Essential Mediators of p53-dependent Apoptosis*

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Cellular stress leads to DNA damage and activation of the intrinsic apoptotic pathway in which translocation of mitochondrial cytochrome *c* to the cytosol plays a critical role. Previous studies have suggested alternative mechanisms responsible for this process. We examined initiation mechanisms of the intrinsic apoptotic pathway using human neuroblastoma and breast cancer cells. Results indicated that translocation of cytochrome *c* does not require prior activation of caspases but rather depends on activation of specific BCL-2 family members, depending upon the type of death signal. Thus, DNA damage-induced apoptosis requires new protein synthesis, accumulation of p53 tumor suppressor protein, and p53-dependent induction of *BOK* and *NOXA* genes, while a role for *BAX* in this pathway is not essential. In contrast, apoptosis induced by staurosporine does not require protein synthesis but is characterized by translocation of BAX. Based on these findings, we propose a model of the intrinsic apoptotic cascade induced by DNA damage where proapoptotic BOK substitutes for a function of BAX.

Apoptosis plays an important role in normal development as well as in diverse human disorders including cancer, autoimmunity, and neurodegeneration. It has often been characterized as an active process that depends on activation of "death" genes and new protein synthesis (1, 2); however, other reports support the idea of constitutively expressed apoptotic machinery (3). The difference in requirements of gene activation and protein synthesis for cell death has been clarified in part with the discovery of alternative apoptotic pathways (4–6). It has become clear that the extrinsic pathway regulated by extracellular death factors does not typically require gene activation, whereas the intrinsic or mitochondrial pathway may depend on it (7).

The intrinsic pathway of apoptosis is initiated by the release of mitochondrial cytochrome *c* (Cyto-c)[1] to the cytoplasm (5). In the presence of ATP or dATP, Cyto-c binds to APAF-1 leading to activation of the initiator caspase-9, which in turn activates executioner caspases (5). Previous studies have suggested that Cyto-c release can be induced by specific caspases (8, 9). Of particular interest are recent studies that suggest a role for caspase-2 (9, 10). However, other studies demonstrate that initiation of Cyto-c release occurs prior to caspase activation (11) where BCL-2 family proteins play a major role.

The BCL-2 family includes antiapoptotic proteins BCL-2, BCL-xL, BCL-w, A1, and MCL-1; proapoptotic BAX, BAK, and BOK; and BH3-only members (12). Initiation of Cyto-c release depends on oligomerization of multidomain BAK and BAX on mitochondrial membranes (13). Members of the prosurvival BCL-2 group inhibit cell death induced by a variety of cytotoxic stimuli. It appears that different members of this group can specifically protect different cell types (12). A role for individual BH3-only proteins in controlling apoptosis also appears to be cell type-specific (12). These proteins are believed to bind to and neutralize BCL-2-like family members (14). Induction of BH3-only genes can trigger activation of proapoptotic BAK and BAX (15).

An essential role in apoptosis induced by BH3-only proteins has been attributed to p53 tumor suppressor protein (7). p53 is activated in response to DNA damage. Recent reports demonstrate that DNA damage leads to rapid accumulation of this transcription factor, which can regulate transcription of proapoptotic *BAK*, *BAX*, *NOXA*, and *PUMA* genes (16–19). The extent of p53-dependent induction of these genes varies significantly in different cell types, and a role for individual proapoptotic members of BCL-2 family in DNA damage-induced p53-dependent apoptosis remains to be clarified. In this study, we examined a role for caspases and new protein synthesis in induction of the intrinsic pathway of apoptosis and identified an essential role for BOK and NOXA proteins in p53-dependent cell death induced by DNA damage.

EXPERIMENTAL PROCEDURES

Cell Culture—SH-SY5Y and MCF7 cells were obtained from the Cancer Cell Line Repository at Georgetown University Lombardi Cancer Center (Washington, D. C.). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) in a humidified atmosphere of 5% CO_2 in air at 37 °C. The cultures were maintained in a logarithmic phase by passage every 2–3 days.

Transfection experiments were performed using Mirus TransIT-100 reagent (Panvera). To obtain stably transfected cells, selection was performed during 2–3 weeks in the presence of 1 mg/ml G418 (Invitrogen). In control experiments, cells were transfected with an empty corresponding expression vector. Apoptosis was induced in cultured cells by incubation with 0.5 μM staurosporine or 50 μM etoposide in the absence or presence of 10 μg/ml cycloheximide (CHX).

Assessment of Cell Viability—Cell viability was measured by retention and deesterification of calcein AM (20). In brief, culture media in 96-well plates were replaced with 5 μM calcein AM (Molecular Probes,

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[1] The abbreviations used are: Cyto-c, cytochrome *c*; BH, BCL-2 homology; CHX, cycloheximide; AM, acetoxymethyl ester; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; AMC, aminomethylcoumarin; RT, reverse transcription; TAFE, transverse alternating field electrophoresis; PBS, phosphate-buffered saline; DN, dominant negative; siRNA, small interfering RNA.

Eugene, OR) in Locke's buffer containing 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.6 mM glucose, and 5 mM Hepes, pH 7.4. After incubation at 37 °C for 30 min, fluorescence was measured using a CytoFluor 4000 fluorometer (PerSeptive Biosystems) at 485 nm excitation and 560 nm emission wavelengths. In some experiments, cell viability was measured by direct counting of apoptotic cells after staining with Hoechst 33258. At least 100 cells were counted in each of three independent experiments.

Apoptosome Activation Assay—Pellets of control or treated cells were homogenized in a Dounce homogenizer in 20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 250 mM sucrose. Homogenates were centrifuged at 13,000 $\times$ *g* for 30 min. Supernatants were transferred to new tubes and stored at −80 °C until used. Protein concentration was estimated using the Bradford reagent (Bio-Rad) according to recommendations by the manufacturer. Twenty-microgram aliquots of the cytosolic extracts were incubated in the absence or presence of 1 mM dATP and 10 μg/ml Cyto-c at 37 °C for 1 h in a final volume of 20 μl of caspase activation buffer (10 mM Hepes, pH 7.4, 5 mM EGTA, 2 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 mM phosphocreatinine, 150 μg/ml creatine kinase). At the end of the incubation, aliquots of reaction mixtures (20 μg of protein in 100 μl of caspase activity assay buffer consisting of 50 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, and 10% glycerol) were mixed with equal volumes of 40 μM fluorescent tetrapeptide substrate (Ac-DEVD-AMC, Bachem) in the same buffer solution. Caspase activity was measured using a CytoFluor 4000 fluorometer (Applied Biosystems) as described below.

Caspase Activity Assay—Aliquots of cytosolic extracts (20 μg of protein in 100 μl of caspase assay buffer) were mixed with equal volumes of 40 μM Ac-DEVD-AMC in the same buffer. Free aminomethylcoumarin (AMC) accumulation, which resulted from cleavage of the aspartate-AMC bond, was monitored continuously in each sample over 30 min in 96-well microtiter plates using a CytoFluor 4000 fluorometer at 360 nm excitation and 460 emission wavelengths. The emission from each well was plotted against time. Linear regression analysis of the initial velocity (slope) of each curve yielded an activity for each sample.

Immunoblotting—Cells were harvested and washed once with ice-cold phosphate-buffered saline. To analyze a total protein content, cells were lysed on ice in a solution containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 0.25% SDS, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. To obtain cytosolic fractions, cells were lysed for 5 min on ice in a solution consisting of 20 mM Hepes, pH 7.2, 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM pepstatin, 250 mM sucrose, and 200 μg/ml digitonin. After removal of cell debris by centrifugation at 20,000 $\times$ *g* for 20 min, a portion of the lysate (30–50 μg of protein) was then fractionated by SDS-PAGE, and the separated proteins were transferred to a nitrocellulose filter. The filter was stained with Ponceau S to confirm equal loading and transfer of samples and was then probed with specific antibodies. Immune complexes were detected with appropriate secondary antibodies and chemiluminescence reagents (Pierce). A polyclonal rabbit antibody to active caspase-3 was obtained from Cell Signaling Technology (New England Biolabs, 9661); a monoclonal mouse antibody to caspase-9 was from Medical and Biological Laboratories (MBL, clone 5B4); a monoclonal rat antibody to APAF-1 was from Alexis Biochemicals (804-348-C100); a monoclonal mouse antibody to β-actin was from Sigma (A5441), polyclonal rabbit antibodies to BAK and BAX were from Upstate Biotechnology (06-536 and 06-499); polyclonal rabbit antibodies to procaspase-3, BCL-2, and BCL-xL were from Santa Cruz Biotechnology (sc-7148, sc-783, and sc-1690); a polyclonal rabbit antibody to BOK was from Abgent (AP1310a); a mouse monoclonal antibody to Cyto-c was from BD Biosciences (556433); a mouse monoclonal antibody to NOXA was from Imgenex (IMG-349); a mouse monoclonal antibody to p53 was from Oncogene Research Products (OP43); rabbit polyclonal antibodies to PUMA were from Oncogene Research Products (PC686) and Axxora (PSC-3043-C100); and a monoclonal rat antibody to caspase-2 was from Apotech (APO-20A-053-C100).

Reverse Transcription-PCR—Abundance of mRNAs encoding proapoptotic members of the BCL-2 family was examined by reverse transcription (RT)-PCR. Total cellular RNA was isolated by acidic phenol extraction (50) and treated with DNase I. RT was performed using 10 μg of total RNA in a 20-μl Moloney murine leukemia virus reverse transcription reaction (Invitrogen). One-tenth of the resulting cDNA was amplified by PCR. PCR primers were as follows (sense and antisense, respectively): BAD, 5′-GGCGATGAGCTGGAGATGAT-3′ and 5′-ACATTTGGTAGTGAGCACGG-3′; BAK, 5′-AGAGCTGTCTGAACTCACGT-3′ and 5′-TTACACTGTGCCAGAGCCAT-3′; BAX, 5′-AAGAAGCTGAGCGAGTGT-3′ and 5′-GGAGGAAGTCCAATGTC-3′; BID, 5′-ACCCTAGAGACATGGAGAAG-3′ and 5′-AGCTATCTTCCAGCCTGTCT-3′; BIK, 5′-TCATGGACGGTTTCACCACA-3′ and 5′-CAGTGTTCCAGCACTATCTC-3′; BIM, 5′-ATGAGAAGATCCTCCCTGCT-3′ and 5′-AATGCATTCTCCACACCAGG-3′; BOK, 5′-GGCGATGAGCTGGAGATGAT-3′ and 5′-ACACTTGAGGACATCAGTCC-3′; HRK, 5′-CAGGCGGAACTTGTAGGAAC-3′ and 5′-GCTGGATTTCCAAAGGGCTT-3′; NOXA, 5′-GTGCCCTTGGAAACGGAAGA-3′ and 5′-CCAGCCGCCCAGTCTAATCA-3′; PUMA, 5′-CAGACTGTGAATCCTGTGCT-3′ and 5′-ACAGTATCTTACAGGCTGGG-3′; and human glyceraldehyde-3-phosphate dehydrogenase, 5′-TCTGCCCCCTCTGCTGATGC-3′ and 5′-CCACCACCCTGTTGCTGTAG-3′. Amplification profiles included denaturation for 30 s at 94 °C, annealing for 15 s at 55 °C, and primer extension for 60 s at 72 °C. Numbers of cycles were estimated to be optimal to provide a linear relationship between the amount of input template and the amount of PCR product generated over a wide concentration range from 1 to 20 μg of total RNA as described in detail previously (21). The amplification products were analyzed by electrophoresis in 2% agarose gels in the presence of 0.5 μg/ml ethidium bromide. The identity of specific PCR products was confirmed by subcloning in the pCR2.1 TA cloning vector (Invitrogen) and sequencing of the inserts.

Real Time Multiplex PCR—RT was performed as described above. Fluorophore-labeled LUX forward and unlabeled reverse PCR primers were as follows: BAX, 5′-CACAGGTGGTGCTCAAGGCCCTG5G-3′ and 5′-GGGTGAGGAGGCTTGAGGAGT-3′; BOK, 5′-CACGTTCAGCGTCTACCGCAACG5G-3′ and 5′-CGCATACAGGGACACCACCT-3′; NOXA, 5′-GACGCTTCGTGTTCAGCTCGCG5C-3′ and 5′-CTCGGTTGA GCGTTCTTGC-3′; PUMA, 5′-CACCTTGGACTCAGCATCGGAAGG5G-3′ and 5′-GCACCAGCACAACAGCCTTT-3′; and glyceraldehyde-3-phosphate dehydrogenase, 5′-CACCCATTTGGCTACAGCAA CAG5G-3′ and 5′-GGACTGAGTGTGGCAGGGACT-3′. Multiplex PCRs combined pairs of primers to amplify each experimental and glyceraldehyde-3-phosphate dehydrogenase cDNA. Fifty-microliter reactions contained 5 μl of cDNA, a 200 nM concentration of each gene-specific primer, 1× Platinum Quantitative PCR SuperMix-UDG (Invitrogen), and 1× ROX reference dye. PCR was conducted in a 96-well spectrofluorometric thermal cycler (ABI PRISM 7700 sequence detector system, Applied Biosystems). Reactions were incubated at 25 °C for 2 min and then at 95 °C for 2 min and subjected to 50 cycles of amplification. Amplification profiles included denaturation for 15 s at 95 °C, annealing for 15 s at 55 °C, and primer extension for 30 s at 72 °C. Fluorescence was automatically monitored during every PCR cycle and during the post-PCR temperature ramp. Changes in gene expression were calculated according to the manufacturer's recommendations (Invitrogen) and previous reports (22).

Transverse Alternating Field Electrophoresis (TAFE)—Cells were washed once with ice-cold phosphate-buffered saline, resuspended in 100 μl of lysis buffer (100 mM EDTA, pH 8.0, 20 mM NaCl, 10 mM Tris, pH 8.0), mixed with 150 μl of agarose solution (1% agarose in lysis buffer kept at 42 °C), and poured into a plug mold. After solidification, plugs were incubated twice for 24 h each in 5 volumes of lysis buffer supplemented with 1 mg/ml Proteinase K (Sigma) and 1% sodium lauroyl sarcosinate (Sigma) at 50 °C. Plugs were then incubated for at least 24 h in 100 volumes of Tris-EDTA buffer, pH 7.4, at 4 °C with at least two changes of buffer and then stored at 4 °C until usage. DNA was subjected to TAFE in 1% agarose in 1× TAFE buffer (20× TAFE: 0.2 M Tris, 7.8 mM EDTA, and 0.5% glacial acetic acid) at 170 V for 30 min with 4-s pulses followed by 150 V for 18 h with 35-s pulses. This program allowed for resolution of DNA molecules up to 1000 kb. λ DNA ladders (50–1000 kb) were used as standards. DNA fragments were visualized by staining with 0.5 μg/ml ethidium bromide.

Confocal Laser Scanning Fluorescence Microscopy—Cells were plated on glass coverslips (1943-10012, Bellco Glass Inc., Vineland, NJ) in 24-well plates. Control and etoposide-treated cell cultures were fixed by incubating with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.5) for 10 min at room temperature. The coverslips were washed three times for 3 min each time with PBS followed by incubation for 1 h at room temperature in blocking buffer (10% normal goat serum (S-1000, Vector Laboratories, Burlingame CA), 0.1%Triton X-100 in PBS). The coverslips were incubated overnight at 4 °C with the primary antibodies diluted in blocking buffer. A polyclonal rabbit antibody to BAX was obtained from Upstate Biotechnology (06-499), a polyclonal rabbit antibody to BOK was from Oncogene Research Products (PC627), and a mouse monoclonal antibody to NOXA was from

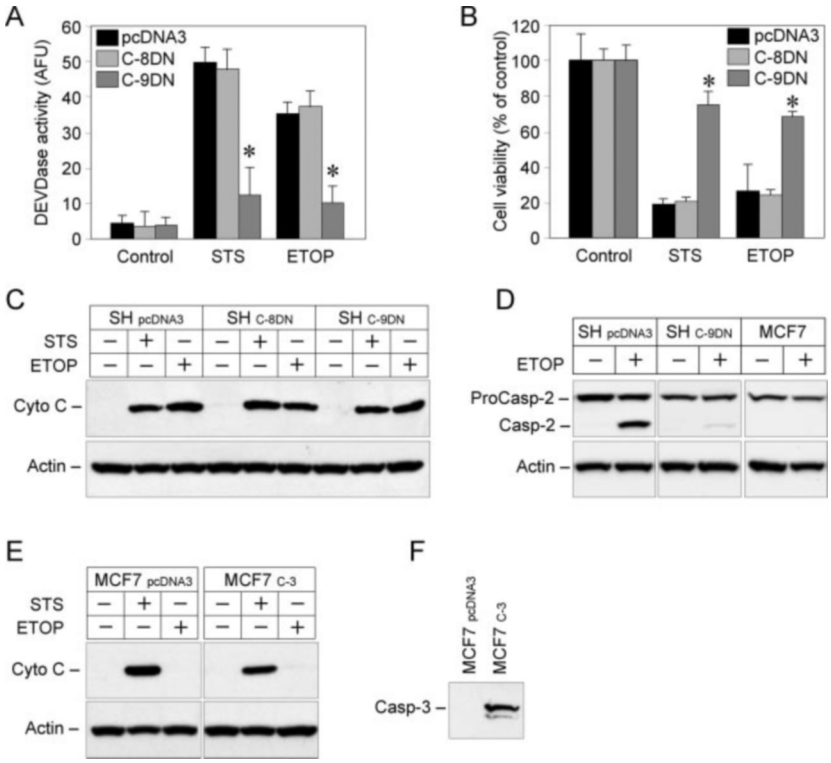

FIG. 1. **Induction of Cyto-c release does not require activity of caspase-2, -3, -8, and -9.** *A*, pooled stably mock-transfected SH-SY5Y cells (*pcDNA3.1*) and stable clones expressing caspase-8 DN (*C-8DN*) or caspase-9 DN (*C-9DN*) were treated with 50 μM etoposide (*ETOP*) or 0.5 μM staurosporine (*STS*) for 6 h. Untreated cultures (*Control*) served as negative controls. Caspase-3-like (DEVDase) activity in cytosolic extracts from treated or control cells was assayed fluorometrically. Protease activity is expressed in arbitrary fluorescence units (*AFU*) ± S.D. ($n = 4$). *, $p < 0.01$ compared with DEVDase activity in treated mock-transfected cells by analysis of variance and Dunnett's test. *B*, the cultures were treated with etoposide for 24 h, and cell viability was analyzed by measurement of calcein AM fluorescence. Data are expressed as a percentage of the value for control cultures not exposed to etoposide ± S.D. ($n = 4$). *, $p < 0.01$ compared with viability of mock-transfected cells by analysis of variance and Dunnett's test. *C* and *D*, samples of cytosolic protein fractions from SH-SY5Y (*SH*) or MCF7 cells treated for 6 h with etoposide or staurosporine were separated by SDS-PAGE followed by staining with anti-Cyto-c, anti-caspase-2, and anti-β-actin antibodies. *E*, MCF7 cells stably transfected with pcDNA3 or the same vector encoding human caspase-3 (*C-3*) were treated as described above followed by immunoblotting of cytosolic protein fractions using anti-Cyto-c and anti-β-actin antibodies. *F*, MCF7 cells were treated with 30 ng/ml tumor necrosis factor α and 10 μg/ml CHX followed by immunoblotting using anti-active caspase-3 antibodies. *Casp*, caspase.

Imgenex (IMG-349). To establish subcellular localization we used antibodies against mitochondrial markers such as cytochrome *c* oxidase subunit I (A-6403, Molecular Probes) and superoxide dismutase 2 (SOD-110, Stressgen). The coverslips were washed three times for 3 min each time with PBS and incubated for 1 h at room temperature with the secondary antibodies diluted in blocking buffer solution (a combination of goat anti-rabbit Alexa Fluor 488 (2 μg/ml, A-11034, Molecular Probes) with goat anti-mouse Alexa Fluor 546 (2 μg/ml, A-11030, Molecular Probes)). The coverslips were washed two times for 3 min each time with PBS followed by mounting on microscope slides with the ProLong Antifade kit (P7481, Molecular Probes). The imaging was performed using a Zeiss 510 Meta confocal laser scanning microscope (LSM 510 META). The visualization of the fluorophores was achieved using the argon ion laser emitting at 488 nm and helium-neon laser emitting at 543 nm, respectively.

RNA Interference—Complementary hairpin oligonucleotides were designed using the "siRNA Converter" Internet tool (Ambion),[2] annealed, and cloned between BamHI and HindIII restriction sites in pSilencer™ 3.1-H1/neo (Ambion) according to the manufacturer's recommendations. The oligonucleotide sequences were as follows (sense and antisense, respectively): human BAX, 5′-GATCCCGCGCATCGGGGACGAACTGTTCAAGAGACAGTTCGTCCCCGATGCGCTTTTTTGGAAA-3′ and 5′-AGCTTTTTCCAAAAAAGCGCATCGGGGACGAACTGTCTCTTGAACAGTTCGTCCCCGATGCGCGG-3′; human BOK, 5′-GATCCCGGTGGTGTCCCTGTATGCGTTCAAGAGACGCATACAGGGACACCACCTTTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAAGGTGGTGTCCCTGTATGCGTCTCTTGAACGCATACAGGGACACCACCGG-3′; and human NOXA, 5′-GATCCCGTCGAGTGTGCTACTCAACTTCAAGAGAGTTGAGTAGCACACTCGACTTTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAAGTCGAGTGTGCTACTCAACTCTCTTGAAGTTGAGTAGCACACTCGACGG-3′. Inhibitory effects of RNA interference on expression of specific genes were tested in transiently transfected MCF7 cells by Western blotting or RT-PCR.

[2] See www.ambion.com/techlib/misc/siRNA_finder.html.

RESULTS

Induction of Apoptosis by Etoposide and Staurosporine—Treatment of SH-SY5Y human neuroblastoma cells with etoposide or staurosporine caused a rapid increase in caspase-3-like activity and cell death (Fig. 1, *A* and *B*). To evaluate specific pathways of apoptosis induced by these drugs, we used SH-SY5Y cells stably transfected with the dominant negative (DN) forms of human procaspase-8 or procaspase-9. Cells transfected with the empty vector (pcDNA3.1) served as a transfection control. Previous studies have shown that expression of these DN procaspases specifically inhibits activation of their endogenous counterparts (23). Expression of the recombinant caspases in pooled clones was confirmed using the antibodies that recognized a FLAG epitope fused at the C termini of the DN mutants (data not shown). In accord with previous reports (24, 25), we found that overexpression of procaspase-9 DN inhibited DEVDase activation and cell death after treatment with etoposide or staurosporine. Overexpression of procaspase-8 DN mutant did not affect caspase activation or cell death induced by these drugs (Fig. 1, *A* and *B*).

Inhibition of caspase-8 or -9 by their DN forms did not inhibit the release of Cyto-c to the cytosol as shown by results of immunoblotting analysis (Fig. 1*C*). Western immunostaining of protein extracts from etoposide-treated SH-SY5Y cells demonstrated specific activation of caspase-2 (Fig. 1*D*). In contrast, cleavage of procaspase-2 was almost completely inhibited in

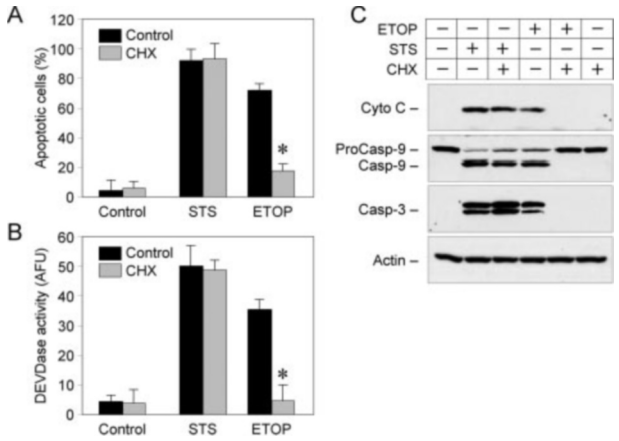

FIG. 2. **Etoposide-induced apoptosis depends on new protein synthesis.** *A*, SH-SY5Y cells were treated with etoposide or staurosporine for 5 h in the presence or absence of CHX. Untreated cells served as a control. Cell viability was analyzed by counting apoptotic cells after staining with Hoechst 33258. At least 250 cells were counted in each of three independent experiments. Data are expressed as a percentage of the value for control cells ± S.D. *, $p < 0.05$. *B*, caspase-3-like activity was assayed fluorometrically and expressed in arbitrary fluorescence units (*AFU*) ± S.D. ($n = 5$). *, $p < 0.05$ compared with control cells. *C*, cytosol from control and treated SH-SY5Y cells was subjected to Western blotting and probed with the antibodies against Cyto-c, procaspase-9, active caspase-3, and β-actin. *STS*, staurosporine; *ETOP*, etoposide, *Casp*, caspase.

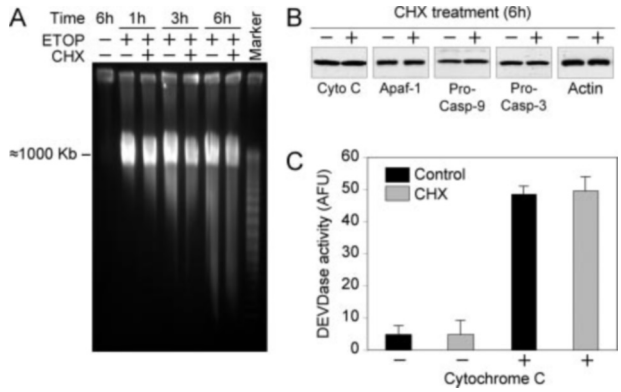

FIG. 3. **Treatment with CHX does not decrease apoptotic potential.** *A*, SH-SY5Y cells were treated with etoposide for the indicated times in the absence or presence of CHX. Nuclear DNA was then analyzed by TAFE. *B*, total protein fractions from cells treated for 6 h were analyzed for expression of the indicated proteins. *C*, 50-μg aliquots of cytosolic protein extracts isolated from control SH-SY5Y cells or cells treated with CHX for 6 h were incubated in the presence or absence of Cyto-c and dATP in the caspase activation buffer as described under "Experimental Procedures." Caspase-3-like activity in treated and control extracts was assayed fluorometrically. Protease activity is expressed in arbitrary fluorescence units (*AFU*) ($n = 5$). *ETOP*, etoposide; *Casp*, caspase.

SH-SY5Y cells overexpressing procaspase-9 DN and was not detected in MCF7 cells that do not express procaspase-3 (26) (Fig. 1*D*). Induction of apoptosis by staurosporine in this MCF7 cell line and MCF7 cells stably transfected with human procaspase-3 resulted in cytosolic accumulation of Cyto-c with no significant difference between the cell lines (Fig. 1*E*). In contrast, treatment with etoposide failed to induce the release of Cyto-c in nontransfected or transfected MCF7 cells (Fig. 1*E*). An ability of recombinant procaspase-3 to undergo activation in MCF7 cells was demonstrated after treatment with 30 ng/ml tumor necrosis factor α and 10 μg/ml CHX (Fig. 1*F*).

Requirement of Protein Synthesis for Etoposide-induced Apoptosis—A role for new protein synthesis in initiation of apoptosis was addressed by treatment of SH-SY5Y cells with staurosporine or etoposide in the absence or presence of 10 μg/ml CHX. The extent of cell death was measured by direct counting of cells with apoptotic nuclear morphology 24 h after treatment (Fig. 2*A*). Caspase activity was assessed using the fluorogenic substrate assay (Fig. 2*B*). Specific cleavage of procaspase-3 and procaspase-9 was examined by Western blot analysis (Fig. 2*C*). In the absence of CHX, treatment with each of the drugs resulted in activation of caspase-9 and -3 followed by manifestation of apoptotic morphology in ~95% of cells. Inhibition of protein synthesis by CHX did not affect the extent of apoptosis induced by staurosporine but markedly inhibited activation of caspase-9 and -3 as well as chromatin condensation in cells treated with etoposide (Fig. 2*C*).

Progression of caspase-9-dependent apoptosis is mediated by the release of Cyto-c from mitochondria to cell cytosol. Therefore, we examined whether pretreatment with CHX inhibited this process, and levels of cytosolic Cyto-c were analyzed in SH-SY5Y cells treated with etoposide or staurosporine in the absence or presence of CHX. Cyto-c content in cell cytosol was examined by immunoblotting. Results showed that both drugs induced the release of Cyto-c within 5 h. CHX blocked this effect of etoposide but did not inhibit Cyto-c release induced by staurosporine (Fig. 2*C*).

Inhibition of protein synthesis could potentially weaken proapoptotic effects of etoposide by decreasing expression levels of topoisomerase 2, thus leading to inhibition of DNA damage. Alternatively it could decrease apoptotic potential by reducing expression levels of essential proapoptotic proteins. To test these possibilities, we examined effects of CHX on the extent of DNA damage and expression levels of cytochrome *c*, APAF-1, procaspase-9, and procaspase-3. DNA damage was assessed using transverse alternating field electrophoresis. This method detected an appearance of 1000-kb DNA fragments as early as 1 h in etoposide-treated but not in control cells. Inhibition of protein synthesis did not affect the extent of DNA degradation at least during the first 6 h of treatment (Fig. 3*A*). Results of Western analysis showed that protein levels of none of the examined components of the apoptosome changed significantly in the presence of CHX (Fig. 3*B*). Furthermore cytosolic extracts isolated from control or CHX-treated SH-SY5Y cells were incubated in the presence of Cyto-c and dATP to evaluate an overall apoptosome potential. In these experiments caspase-3-like activity was measured using the fluorogenic substrate assay. Results demonstrated that inhibition of protein synthesis during 6 h had no effect on apoptosome-mediated caspase-3 activation (Fig. 3*C*).

Requirement of p53 Induction for Etoposide-induced Apoptosis—DNA damage leads to stabilization and activation of p53 tumor suppressor protein and results in Cyto-c release (7); however, the actual connection between p53 activation and initiation of apoptosis remains to be clarified.

Western blot analysis revealed that treatment of SH-SY5Y cells with etoposide resulted in a marked increase in p53 protein level. Treatment with staurosporine had a much weaker effect. Accumulation of p53 was blocked in the presence of CHX (Fig. 4*A*).

To assess a potential link between induction of p53, Cyto-c release, and consequent caspase activation, we inhibited endogenous p53 by overexpression of its DN mutant (R175H) (27, 28) in SH-SY5Y cells. R175H-mediated inhibition of p53 blocked the release of Cyto-c induced by etoposide; however, it did not inhibit this process after treatment with staurosporine (Fig. 4*A*). Overexpression of this p53 DN mutant also inhibited etoposide-induced activation of caspase-3 but did not affect activation of this caspase in cells treated with staurosporine (Fig. 4*B*). Furthermore inactivation of p53 significantly in-

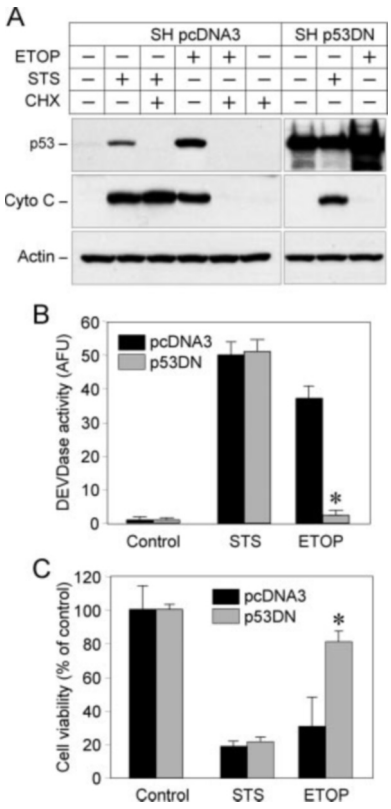

FIG. 4. **Requirement of p53 activity for induction of apoptosis.** *A*, mock-transfected SH-SY5Y (*SH*) cells and those transfected with the p53 DN construct were treated with etoposide (*ETOP*) or staurosporine (*STS*) for 5 h. Total protein extracts were probed with the antibodies against p53. Expression levels of Cyto-c and β-actin were evaluated in cytosolic fractions. *B*, caspase-3-like activity was assayed fluorometrically. Protease activity is expressed in arbitrary fluorescence units (*AFU*) ± S.D. ($n = 3$). *, $p < 0.01$ compared with wild type cells. *C*, cell viability was analyzed using calcein AM fluorescence assay. Data are expressed as a percentage of the value for control cells ± S.D. ($n = 3$). *, $p < 0.01$ compared with wild type cells.

creased viability of SH-SY5Y cells after treatment with etoposide but not after treatment with staurosporine (Fig. 4*C*).

p53-dependent Expression Profiles of Proapoptotic BCL-2 Family Members—p53 is an essential transcription factor that regulates gene expression in response to DNA damage. Consensus p53 response elements have been found in promoter regions of proapoptotic *BAX*, *NOXA*, and *PUMA* genes able to induce Cyto-c release and caspase activation (17). We examined expression of these and other proapoptotic genes of the BCL-2 family using semiquantitative and real time RT-PCR approaches. Because etoposide-induced apoptosis in SH-SY5Y cells depends on p53 function and this process is altered in MCF7 cells, gene expression analysis was performed in etoposide-treated SH-SY5Y cells, SH-SY5Y cells stably transfected with the DN p53 mutant, and MCF7 cells. This experimental design was expected to allow identification of candidate genes that potentially mediate p53-dependent apoptosis in SH-SY5Y cells.

Results of semiquantitative RT-PCR revealed marked p53-dependent induction of *BOK* and *NOXA* mRNA expression in SH-SY5Y but not in MCF7 cells (Fig. 5*A*). *BAX* mRNA levels appeared to be only slightly increased in SH-SY5Y and MCF7 cell lines but not in SH-SY5Y expressing the p53 DN mutant. Expression of *BAK*, *BAD*, *BID*, and *BIM* mRNA was not affected by treatment or inhibition of p53 function. *BIK* and *Harakiri* (*HRK*) mRNA were not detected under our experimental conditions. Expression of the *PUMA* gene was low in all tested cell lines and was somewhat induced by etoposide only in SH-SY5Y cells expressing active p53.

Induction of *BAX*, *BOK*, *NOXA*, and *PUMA* mRNA was further quantified by real time PCR analysis (Fig. 5*B*). A 6-fold increase in *BOK* mRNA content was observed in SH-SY5Y cells after 2.5 h of etoposide treatment and reached an ~10-fold induction level after 5 h. *NOXA* mRNA levels began to increase after 2.5 h of treatment and reached ~4.5-fold of the control level after 5 h. Increases in *BAX* and *PUMA* mRNA were significantly lower (~0.6 and ~1.5-fold after 5 h, respectively). *BOK*, *NOXA*, and *PUMA* genes were not induced in SH-SY5Y cells transfected with the p53 DN mutant and in MCF7 cells. In contrast, *BAX* expression was induced to a similar low extent (~0.7-fold) in both wild type SH-SY5Y and MCF7 but not in R175H-transfected SH-SY5Y cultures.

Western analysis revealed increased protein expression of BOK and NOXA in SH-SY5Y after treatment with etoposide. Timing of induction of these proteins preceded Cyto-c release (Fig. 6*A*). Expression of PUMA protein was not detected using two different antibodies (data not shown). Total cellular contents of BAX, BAK, and BCL-xL proteins were not changed after the treatment (Fig. 6, *A* and *B*).

Expression of BOK and NOXA but Not BAX Is Essential for Apoptosis Induced by DNA Damage—We examined intracellular localization of BAX, BOK, and NOXA proteins in control and etoposide-treated SH-SY5Y cells using confocal microscopy and found that BAX was evenly distributed in the cytosol and cell nuclei. The expression level of this protein and its localization did not change significantly in cells treated with etoposide for 5 h (Fig. 7*A*). In contrast, the confocal imaging clearly demonstrated increased expression of BOK and NOXA proteins as well as their association with mitochondria in cells treated with etoposide. These data confirm previous reports on mitochondrial localization of NOXA and the BOK ortholog (29, 30) and suggest that BAX does not translocate to mitochondria in this cell line.

Hence cellular localization of BAX was further examined using Western immunoblotting. Treatment of SH-SY5Y and MCF7 cells with staurosporine resulted in the depletion of cytosolic BAX indicative of its translocation to mitochondria. This was accompanied by the release of Cyto-c to the cytosol. However, treatment with etoposide, which caused a significant increase in p53 protein content and Cyto-c release in SH-SY5Y, did not change the amount of BAX in the cytosol (Fig. 7*B*).

Results of expression profiling of proapoptotic BCL-2 family members suggested a role for *BOK* and *NOXA* gene induction in the initiation of p53-dependent apoptosis caused by etoposide-induced DNA damage. We tested the ability of BOK, NOXA, and BAX to induce Cyto-c release by transfecting the corresponding genes into MCF7 cells, which normally do not demonstrate induction of these genes and Cyto-c release after treatment with etoposide. Western blot analysis of cytosolic extracts isolated 24 h after transfection showed that expression of each gene induced the release of Cyto-c (Fig. 7*C*).

A contribution of BAX, BOK, and NOXA in etoposide-induced apoptosis was further assessed using RNA interference. To estimate the potency of gene silencing, MCF7 cells were transfected with recombinant plasmids encoding BAX, BOK, or NOXA in the absence or presence of corresponding pSilencer vectors followed by analysis of protein expression. Results demonstrated that the siRNAs were able to substantially knock down expression of these target genes (Fig. 7*D*). Plasmids encoding BAX, BOK, or NOXA siRNA were then transiently transfected into SH-SY5Y cells in combination with the green fluorescent protein expression vector. Forty-eight hours after transfection cells were treated with etoposide, and cell viability was measured 24 h later by direct counting of green viable cells under a UV microscope. Results showed that siRNA-mediated repression of BOK and

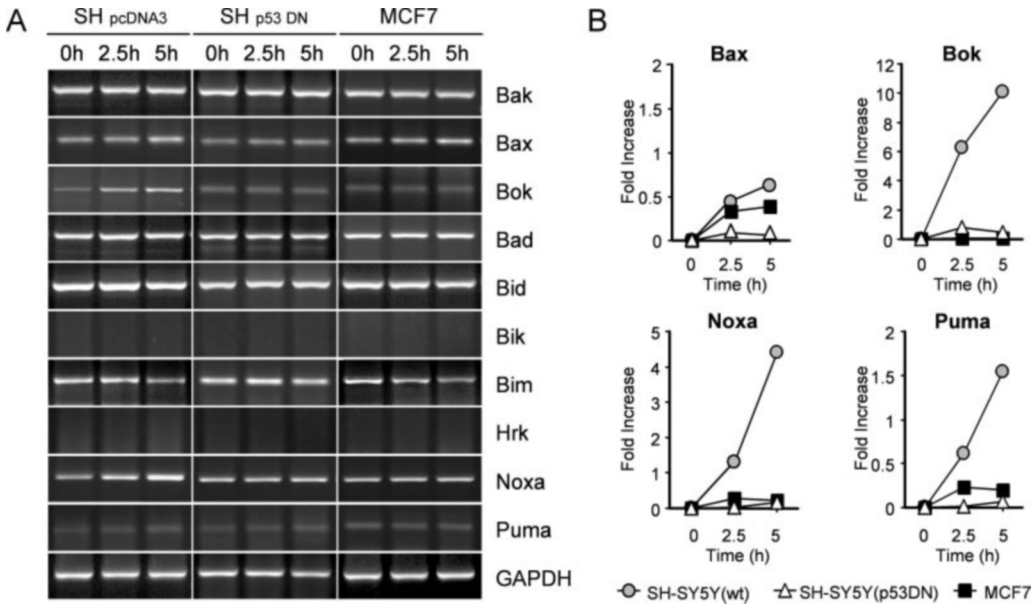

FIG. 5. **mRNA expression profiles of proapoptotic BCL-2 family members.** *A*, cell cultures were treated with etoposide for the indicated times. RT-PCR analysis of the abundance of mRNAs encoding specified members of the BCL-2 family in wild type, p53 DN-transfected, and MCF7 cells. Total cellular RNA was subjected to RT and PCR with gene-specific primers or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers. PCR products were analyzed by agarose gel electrophoresis and staining with ethidium bromide. *B*, results of real time multiplex RT-PCR of selected mRNA demonstrate minor changes in *BAX* and *PUMA* expression but marked induction of *BOK* and *NOXA* genes. Data present mean values of three independent experiments. *SH*, SH-SY5Y.

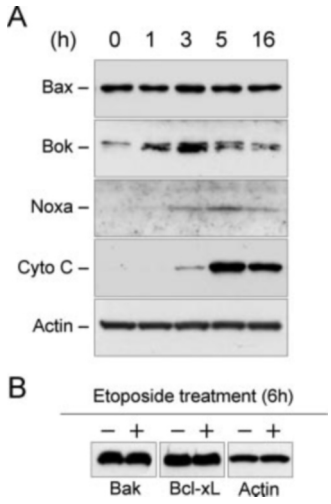

FIG. 6. **BAX, BOK, and NOXA protein expression profiling.** *A*, SH-SY5Y cells were treated with etoposide for the indicated times. Total protein extracts were probed with the antibodies against BAX, BOK, NOXA, and β-actin. The release of Cyto-c was evaluated in cytosolic fractions and shown for comparison with BOK and NOXA expression. *B*, expression levels of BAK, BCL-xL, and β-actin proteins were analyzed in total protein fractions from control and etoposide-treated SH-SY5Y cells.

NOXA but not BAX expression significantly increased viability of the etoposide-treated cells (Fig. 7*E*).

DISCUSSION

Recent studies have presented conflicting conclusion regarding initiation mechanisms for the intrinsic apoptotic pathway. To further address this issue, we used SH-SY5Y cells stably transfected with DN forms of human procaspase-8 or -9 and confirmed that both etoposide and staurosporine caused caspase-9-dependent cell death. However, the mechanisms by which these drugs induce apoptosis differ markedly both with regard to the requirement for new protein synthesis and upstream regulatory pathways. Thus, treatment with CHX blocked etoposide-induced apoptosis in SH-SY5Y cells by preventing the release of Cyto-c and consequent activation of caspase-9 and -3. In contrast, CHX had no effect on apoptosis induced by staurosporine.

The release of Cyto-c from mitochondria is a critical event in activation of caspase-9, and a number of studies have suggested that the release of Cyto-c depends upon activation of caspases, such as caspase-2 or caspase-8 (8–10, 31, 32). Consistent with other reports (7, 11, 33), we found that Cyto-c release is mediated by proapoptotic BCL-2 family members and does not require caspase activity. Thus, using overexpression of procaspase-8 and -9 DN forms, we showed that the release of Cyto-c induced by etoposide and staurosporine did not depend on activation of caspase-8 or -9. A role for caspase-3 activation was examined in MCF7 cells that normally do not express this caspase (26). Treatment of cells with staurosporine resulted in the release of Cyto-c regardless of caspase-3 expression. Surprisingly etoposide failed to induce Cyto-c release or activation of procaspase-9 in MCF7 cells suggesting an alteration in upstream mechanisms. Furthermore etoposide-induced activation of caspase-2 in SH-SY5Y cells was blocked by overexpression of a procaspase-9 DN mutant, and it was undetectable in etoposide-treated MCF7 cells. These data support a previous study showing that activation of procaspase-2 occurs downstream from activation of procaspase-9 and -3 (34) and, therefore, downstream from the release of Cyto-c.

An inhibitory effect of CHX on etoposide-induced cell death has been demonstrated previously; however, precise mechanisms were not delineated (25, 35–37). Etoposide inhibits topoisomerases, leading to DNA damage and consequent induction of p53 (25). In turn, p53-dependent caspase activation requires the release of Cyto-c from mitochondria (7, 11). Therefore, we hypothesized that inhibition of DNA damage-induced apoptosis, by blocking new protein synthesis, might occur at the level of p53. Using treatment with CHX and overexpression of the p53 DN mutant, we found that induction of protein synthesis-dependent apoptosis was mediated by p53.

Suggested mechanisms of p53-dependent initiation of the intrinsic apoptotic cascade include transcriptional activation of proapoptotic genes of the BCL-2 family such as *BAX*, *NOXA*, and *PUMA* (7, 11, 17–19). For example, induction of cytosolic

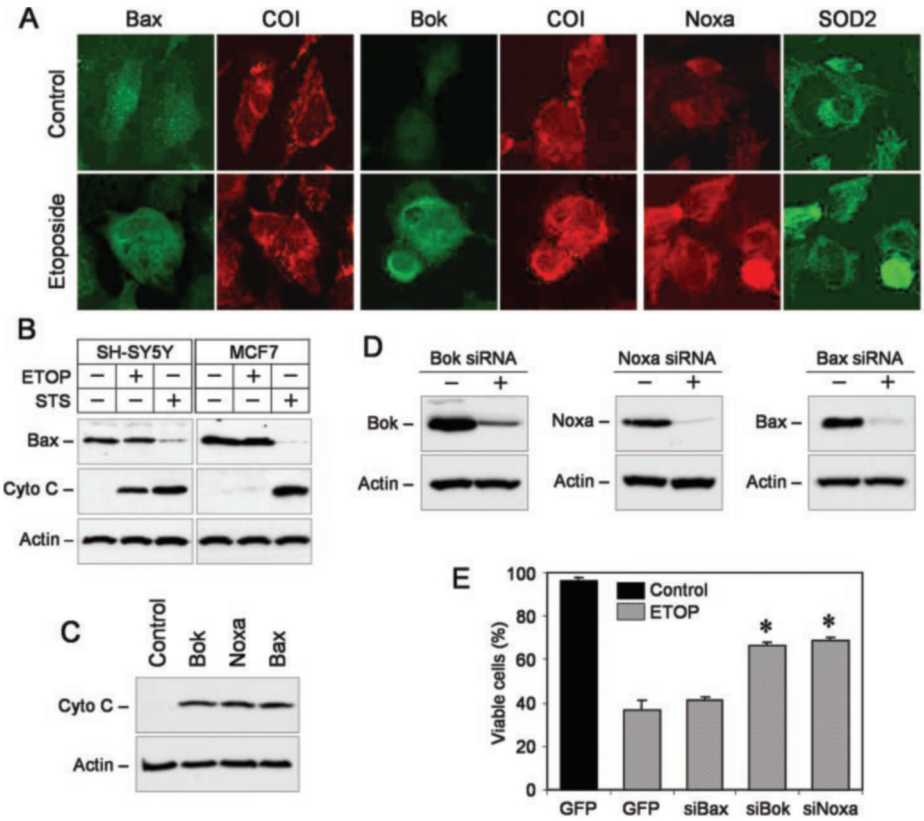

FIG. 7. **BOK and NOXA are essential mediators of DNA damage-induced apoptosis.** *A*, immunostaining for BAX, BOK, NOXA, and mitochondrial markers cytochrome. *B*, SH-SY5Y and MCF7 cells were treated with 50 μM etoposide (*ETOP*) or 0.5 μM staurosporine (*STS*) for 5 h. Cytosolic protein fractions were probed with the antibodies against BAX, Cyto-c, and β-actin. *C*, MCF7 cells were transiently transfected with BAX, BOK, or NOXA expression plasmids or the empty pcDNA3.1 vector (*Control*). The release of Cyto-c was evaluated in cytosolic extracts 18 h after transfection. *D*, MCF7 cells were transiently co-transfected with the expression vectors encoding BAX, BOK, or NOXA and the empty pSilencer 3.1-H1 (−) or the pSilencer vector expressing the indicated siRNAs. Expression of the indicated proteins was analyzed in total protein extracts by immunoblotting 48 h after transfection. Immunostaining of β-actin served as a loading control. *E*, SH-SY5Y cells were co-transfected with green fluorescent protein (*GFP*) expression vector and each of the indicated siRNA (*si*)-expressing constructs or the empty pSilencer 3.1-H1. Forty-eight hours after transfection cells were treated with etoposide for 18 h. Untreated green fluorescent protein cells served as a control. Cell viability was analyzed by counting viable green fluorescent cells. At least 100 cells were counted in each of three independent experiments. Data are expressed as a percentage of the value for etoposide-treated green fluorescent protein-transfected (control) cells ± S.D. *, $p < 0.05$ damage-induced apoptosis. *A*, SH-SY5Y cells were treated with 50 μM etoposide for 5 h; untreated cells served as a control.

BAX protein expression may contribute to its activation and translocation to mitochondria (7). Once translocated, BAX triggers loss of the mitochondrial membrane potential and the release of Cyto-c (38–41). Induction of BH3-only BCL-2 family members, such as NOXA and PUMA, may contribute to the release of BAX from complexes with the antiapoptotic factors BCL-xL or BCL-2. In this study, however, we did not observe significant p53-dependent induction of BAX after etoposide administration or its translocation to mitochondria. In contrast, p53-independent depletion of cytosolic BAX was clearly detected after treatment with staurosporine. Moreover inhibition of BAX expression in SH-SY5Y cells by RNA interference did not affect cell death induced by etoposide. Taken together, these observations suggest that induction of BAX expression and its translocation to mitochondria is dispensable in apoptosis induced in SH-SY5Y cells by etoposide.

Results of mRNA and protein expression analyses of proapoptotic BCL-2 family members demonstrated that treatment of SH-SY5Y cells with etoposide resulted in marked p53-dependent induction of *BOK* and *NOXA* genes. Induction of BOK and NOXA proteins preceded or coincided with Cyto-c release. In contrast, we did not detect induction of *BOK* or *NOXA* or the release of Cyto-c in etoposide-treated MCF-7 cells, although these cells have been reported to express wild type p53 protein (42). However, transient transfection-mediated expression of BOK, NOXA, or BAX in MCF7 cells resulted in the release of Cyto-c to the cytosol even in the absence of treatment. Furthermore, siRNA-mediated inhibition of BOK and NOXA induction in SH-SY5Y cells markedly decreased cell death. Taken together, our findings support a role for p53-dependent induction of *NOXA* in etoposide-induced apoptosis as suggested previously (33, 43) but also indicate that p53 participates in the regulation of *BOK* expression. The latter conclusion is consistent with the study (44) showing induction of *BOK* mRNA expression by E2F1 transcription factor, which is able to induce accumulation of p53 protein (45).

Based on the presence of various BH domains, the BCL-2 family has been divided into the antiapoptotic BCL-2, the proapoptotic BAX, and the BH3-only subfamilies (46). BOK belongs to a subfamily of BAX-related proteins. Like BAX and BAK, it contains conserved BH domains 1, 2, and 3 and the C-terminal transmembrane sequence (47). However, unlike BAX or BAK, it interacts only with MCL-1, BHRF1, and BFL-1 but not other antiapoptotic or proapoptotic family members (47). In this study, we demonstrated that overexpression of human BOK triggers the release of Cyto-c in SH-SY5Y and MCF7 cells.

NOXA is a BH3-only member of the BCL-2 family. Previous studies have suggested that the proapoptotic function of NOXA is independent of BAX translocation and induction of NOXA expression does not change cellular distribution of BAX (18). Moreover NOXA does not bind BAX, but it selectively interacts

with antiapoptotic BCL-xL, BCL-2, and MCL-1 (18). Thus, the antiapoptotic MCL-1 protein may represent a "binding link" between BOK and NOXA. MCL-1 can form heterodimers with other members of the BCL-2 family including BAX and BAK (47). BOK, NOXA, and MCL-1 have common localization in mitochondria (18, 29, 48), suggesting possible interaction during initiation of apoptosis. In contrast, BAX is normally localized in the cytosol. Thus, induction of BOK and NOXA may cooperate in the release of BAK from a complex with MCL-1, leading to the release of Cyto-c. Alternatively BOK itself may contribute to formation of mitochondrial pores and Cyto-c release, whereas NOXA may potentiate this function of BOK by neutralizing its antiapoptotic counterpart, MCL-1. This hypothesis is supported by recent findings showing specific down-regulation of MCL-1 expression in apoptosis induced by UV irradiation (49). Further studies will be required to clarify which mechanism is operative during induction of p53-dependent apoptosis in SH-SY5Y cells.

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