

Daxx Represses Expression of a Subset of Antiapoptotic Genes Regulated by Nuclear Factor- κ B

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Abstract

Daxx is a nuclear protein that localizes to PML oncogenic domains, sensitizes cells to apoptosis, and functions as a transcriptional repressor. We found that Daxx represses the expression of several antiapoptotic genes regulated by nuclear factor- κ B, including *cIAP2*, in human tumor cell lines. Daxx interacts with RelB and inhibits RelB-mediated transcriptional activation of the human *cIAP2* gene promoter. Daxx also forms complexes with RelB while bound to its target sites in the *cIAP2* promoter, as shown by electrophoretic mobility shift assays and chromatin immunoprecipitation experiments. Using cells from *daxx*^{-/-} mouse embryos, we observed that levels of the corresponding murine c-IAP mRNA and protein are increased in cells lacking Daxx. Conversely, c-IAP mRNA and protein levels were reduced in *relB*^{-/-} cells. Taken together, these observations provide a mechanism that links two previously ascribed functions of Daxx: transcriptional repression and sensitization to apoptosis. (Cancer Res 2006; 66(18): 9026-35)

Introduction

Daxx is an apoptosis-modulating protein with various reported cellular and biochemical functions (1–7). Although controversial, most studies have shown that Daxx resides in the nuclei (8), typically within discrete subnuclear compartments called PML oncogenic domains (2, 9, 10). Indeed, Daxx has been shown to bind the PML protein, and colocalization of Daxx with PML is dynamically regulated by IFN- γ and arsenicals in concert with changes in apoptosis (3, 11).

Daxx plays a role in transcription, with several studies demonstrating that Daxx functions as a transcriptional repressor. In this regard, Daxx has been shown to repress the activity of several transcription factors, including Ets-1, Pax-3, Pax-5, and the glucocorticoid receptor (12–14). Consistent with a role in transcriptional regulation, Daxx binds histone deacetylases (15, 16), DNA methyltransferases and their associated proteins (17, 18), and the chromatin-modifying protein α -thalassemia syndrome protein (19). Here, we provide a link between two ascribed functions of Daxx transcriptional repression and sensitization to apoptosis. We show that Daxx binds to and inhibits the activity of RelB, a transcription factor controlling the expression of several apoptosis-regulatory genes.

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Materials and Methods

Antibodies. Immunoblot analyses were done using horseradish peroxidase-conjugated monoclonal antibodies recognizing hemagglutinin (HA), MYC (Santa Cruz Biotechnology, Santa Cruz, CA), and FLAG (Sigma, St. Louis, MO) epitope tags. Immunoprecipitations were done using monoclonal antibodies recognizing HA (Covance, Berkeley, CA), FLAG (Sigma), and MYC (Santa Cruz Biotechnology) epitope tags. Additional antibodies used in these studies include anti-cIAP2 (R&D Systems, Minneapolis, MN); anti-Bcl-XL (20); anti- α -tubulin (Sigma); anti-FLIP (Alexis, San Diego, CA); anti-c-RelB, anti-c-p50, anti-c-p65-cRel (Santa Cruz Biotechnology); anti- β -actin (Sigma); and polyclonal rabbit antisera specific for Bcl-XL, Daxx, and cIAP2 previously described by our laboratory.

Plasmids. The following *cIAP2* promoter constructs were gifts from H. Lee (Yonsei University, Seoul, South Korea): -1400-cIAP2-pGL2, -900-cIAP2-pGL2, and -247-cIAP2-pGL2 (21). Plasmids -194-cIAP2-pGL2 and -120-cIAP2-pGL2 were created by PCR amplification from the -247-cIAP2-pGL2 using the oligonucleotide primers -194cIAP2 (*Xho*I)-F 5'-CCCCC-TCGAGGTTTGCCAGGCCACTGAT-3'; -120-cIAP2 (*Xho*I)-F 5'-CCTAA-CTCGAGAGCACCAGTGCACATGCA-3'; +7-cIAP2 (*Hind*III)-R 5'-GAC-ACAAGCTTCTGGCCTTTCCGTGCC-3'. PCR products were digested with *Xho*I and *Hind*III and subcloned into pGL2-Basic. The human *BCL-X* promoter plasmid (Bcl-X-pGL2) and HA-Daxx-pcDNA3 expression plasmid and deletion mutants have been described (2, 22). The p50-CMV, c-Rel pcDNA3, and Rel-B pcDNA3 plasmids were gifts from M. Karin and A. Hoffmann (University of California, San Diego, La Jolla, CA), and N. Orlshaw (Moffitt Cancer Center, Tampa, FL). The myc-RelB-pcDNA3 expression plasmid was created by subcloning RelB in frame (*Eco*RI/*Xho*I digestion) into myc-pcDNA3.

Cell culture. HT1080, OVCAR3, HEK293T, and MCF7 cells were cultured as described (2, 3). Mouse embryonic cells, RelB (+/+), RelB (-/-), Daxx (+/+), and Daxx (-/-) (17, 23) were cultured in DMEM containing 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin plus L-glutamine and β -mercaptoethanol. HT1080-Neo, HT1080-Daxx-Flag, OVCAR3-Neo, OVCAR3-HA-Daxx, MCF7-Neo, and MCF7-HA-Daxx stable cell lines have been previously described (2) and were cultured in the presence of 400 μ g/mL G418 (Invitrogen, Carlsbad, CA).

For cell death assays, mouse embryo fibroblasts (MEF) were seeded at 100,000 cells per well in 24-well plates. After 12 to 16 hours, cells were treated with various concentrations of mouse tumor necrosis factor (TNF)- α (R&D Systems), staurosporine, Jo-2 anti-Fas antibody (PharMingen, La Jolla, CA), or etoposide (VP-16). Adherent and detached cells were recovered, pooled, suspended in DMEM/15% FBS, and placed into 96-well, clear, flat-bottomed tissue culture dishes at 100 μ L per well. The Via-Count Flex Reagent (Guava Technologies, Hayward, CA) was added to each sample (100 μ L) and the percentage of viable cells was determined using the Guava mini-fluorescence-activated cell sorting cell counter, performing determinations in triplicate.

Microarray analyses. All experiments were done following the minimal information about microarray experiments guidelines.¹ Briefly, total RNA was extracted using the RNeasy Maxi kit (Qiagen, Valencia, CA). Poly-dT (18-mer) oligonucleotide (2 μ g) was hybridized to each RNA sample (75 μ g)

¹ <http://www.mged.org/Workgroups/MIAME/miame.html>.

and then reverse transcribed using SuperScriptII (Invitrogen) containing Cy-conjugated dUTP. Pairs of RNA samples (Neo versus Daxx) were reverse transcribed in the presence of either Cy3-dUTP or Cy5-dUTP. The resulting cDNAs were purified (QIAquick PCR Purification kit; Qiagen) and pooled before hybridization to cDNA microarrays. Apoptosis gene-specific cDNA microarrays were prepared on glass slides and were spotted with ~260 apoptosis and apoptosis-related genes. cDNA sequences were 200 to 500 bp in length and were selected to hybridize well at 42°C with bias toward the 3' untranslated region of the corresponding mRNA. cDNA sequences were chosen for optimum specificity within gene families to avoid cross-hybridization among related gene family members. Before hybridization, slides were incubated for 45 minutes at 42°C in prehybridization buffer (5× SSC, 0.1% SDS, 1% bovine serum albumin). Results were visualized using a ScanArray4000 scanner and accompanying software (version 3.1; GSI Lumonics/Perkin-Elmer, Wellesley, MA). Data were analyzed using ImaGene (version 4.2) and GeneSightLite (version 1.0) software packages. Four independent data sets were obtained for each pair of cell lines (Neo versus Daxx).

Reverse transcription-PCR. Semiquantitative reverse transcription-PCR (RT-PCR) was done using total RNA and gene-specific primers designed using PrimerSelect (Lasergene, Madison, WI). Oligonucleotides were purchased from IDT (Coralville, IA). Primers included are the following:

MAIL forward primer	5'-GGGCCCGATTCTGTTGTCTGATGGA-3'
MAIL reverse primer	5'-AAGCGGGGACTGGGAGGTATGG-3'
Bok forward primer	5'-GGTGTCTGCGCGCTCCTCGGTCT-3'
Bok reverse primer	5'-CACGCCAGGAACGCATCGGTCAC-3'
cFLIP forward primer	5'-TACATGGGCCGAGGCAAGATAA-3'
cFLIP reverse primer	5'-ATAGCCAGGGAAGTGAAGGTGTC-3'
cIAP2 forward primer	5'-TGCTCGCGCTGGTTTCTATTA-3'
cIAP2 reverse primer	5'-AAAGCAAGCCACTCTGTCTCCA-3'
Survivin forward primer	5'-GCCTGGCAGCCCTTCTCAA-3'
Survivin reverse primer	5'-CTCGATGGCAGCGCGCATCTTCT-3'
DAPK3 forward primer	5'-ATCCGGCACCCCAACATCATCA-3'
DAPK3 reverse primer	5'-CCAGCGGCTCATAGTTCACAATC-3'
Daxx forward primer	5'-CGGCGCTGCAGAAAAGGAGT-3'
Daxx reverse primer	5'-GCGCCGGCCAAACACAGGA-3'
Bcl-XL forward primer	5'-GGAGGCAGGCGACGAGTTTGAA-3'
Bcl-XL reverse primer	5'-AAGGGGTGGGAGGGTAGAGTGG-3'
cIAP1 forward primer	5'-TTCCAGGTCCTCGTATCAAAA-3'
cIAP1 reverse primer	5'-TGGAGAAAGGCTGGAGTAAGAACC-3'
XIAP forward primer	5'-TGGCACGAGCAGGGTTTCTTIA-3'
XIAP reverse primer	5'-TGGGGTTAGGTGAGCATAGTCTGG-3'
murine <i>miap1</i>	5'-TGCTGTGGCTGATGTTGGATA-3'
forward primer	
murine <i>miap1</i>	5'-GAAATGTCTGCGGTGCTCTGA-3'
reverse primer	

Primers used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Clontech (Mountain View, CA; GAPDH 5' and 3' Amplimer set). RT-PCR was done using the SuperScript One-Step RT-PCR kit (Invitrogen) using 100 ng total RNA as template. The thermocycler was programmed as follows: 50°C for 30 minutes, 94°C for 2 minutes, 25 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 45 seconds, 72°C for 10 minutes, hold at 4°C. The primer pairs for all genes were specifically selected such that all reactions could be accomplished simultaneously using a 54°C annealing temperature. RT-PCR products were analyzed via 1.0% agarose gel electrophoresis and stained with ethidium bromide for UV visualization.

Luciferase assays. HT1080 cells were transfected with various plasmids using LipofectAMINE 2000 reagent (Invitrogen) in six-well plates and 4 μg DNA per well. Cells were incubated for 48 hours posttransfection, and Luciferase assays were done according to the protocol of the manufacturer using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase values were normalized to renilla luciferase values. All experiments were done three or more times.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSA) were done essentially as described (24, 25). DNA oligonucleotide sequences used for EMSA probes [*cIAP2* nuclear factor-κB (NF-κB) sites 1, 2, and 3] have been described (21). Competitor oligonucleotides (NF-κB consensus and mutant sequences) were purchased from Santa Cruz Biotechnology.

Chromatin immunoprecipitation assays. Neo- and Daxx-expressing HT1080 cells were subjected to formaldehyde cross-linking (23). Cross-linking reactions were quenched by PBS containing 0.125 mol/L glycine. Chromatin samples were sonicated to average length 500 bp immunoprecipitation; chromatin from 3×10^6 cells was adjusted to a 1.0 mL volume of radioimmunoprecipitation assay buffer and precleared with protein A-Sepharose for 30 minutes at 4°C. FLAG-tagged Daxx was immunoprecipitated overnight with a rabbit polyclonal antibody directed against the FLAG epitope tag (Affinity Bioreagents, Golden, CO), or a rabbit polyclonal antibody against RelB (Santa Cruz Biotechnology). Following immunoprecipitation and reversal of cross-links, the samples were analyzed by PCR amplification using primers specific for the *cIAP2* promoter: 5'-CCCCCTCGAGTTGCCAGGCCACTGAT-3' and 5'-GACACAAGCTCTCGGCCCTTTCCGTGCC-3'. Control amplifications were accomplished using primers specific for the *cyclophilin A* gene: 5'-CTCCTTTGAGCTGTTGCAG-3' and 5'-CACCACATGCTTGCATCC-3'. Amplifications were done using the following variables: 95°C/4 minutes hot start, followed by 26 cycles of 95°C/45 seconds, 60°C/30 seconds, and 72°C/1 minute. PCR products were analyzed in 2% agarose gels and stained with ethidium bromide, followed by UV visualization.

Coimmunoprecipitation and immunoblot analyses. Cells were lysed in immunoprecipitation buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% Igepal, 1× protease inhibitor cocktail, and 1 μmol/L phenylmethylsulfonyl fluoride. Lysates then underwent three cycles of freeze/thaw to lyse nuclei. Total cell lysates (800 μg protein) were incubated with 2 μg of either anti-Flag or anti-HA antibody. Proteins associated with HA- or FLAG-Daxx were precipitated using sheep anti-mouse IgG Dynabeads (Dyna, Carlsbad, CA), or protein G beads. Immunocomplexes were analyzed by SDS-PAGE and associated proteins were detected by immunoblotting using the enhanced chemiluminescence protein detection system (Amersham, Piscataway, NJ).

Results

DNA microarray analysis reveals Daxx-responsive gene targets. To identify potential targets of Daxx-mediated transcriptional activity relevant to apoptosis, we prepared a 260-member array containing cDNAs for apoptosis-relevant genes, including the following: (a) 15 members of the Bcl-2 family; (b) seven caspases; and (c) six members of the IAP family including NAIP, cIAP1, cIAP2, XIAP, survivin, and apollon. Pairs of stable transfectants of human tumor cell lines were prepared that contained either a control plasmid ("neo") or Daxx-expressing plasmid. Immunoblot analysis indicated that the Daxx protein was overexpressed ~3-fold compared with endogenous Daxx (Fig. 1). We compared two distinctly different tumor cell lines with the intent of taking the intersection of these two data sets (e.g., HT1080 fibrosarcoma and MCF7 breast cancer cells). Additionally, these stable cell lines contained different tagged versions of Daxx (Flag or HA) to avoid bias from the heterologous tag. Increased sensitivity to Fas-induced apoptosis was confirmed for the Daxx-overexpressing member of both of these pairs of stable transfectants (data not shown), consistent with our prior observations (2).

RNA from pairs of Neo and Daxx transfectants was reverse transcribed into cDNA using Cy3 and Cy5 dye-labeled UTP and hybridized to cDNA arrays. Each sample was hybridized in quadruplicate and the experiment was repeated with an

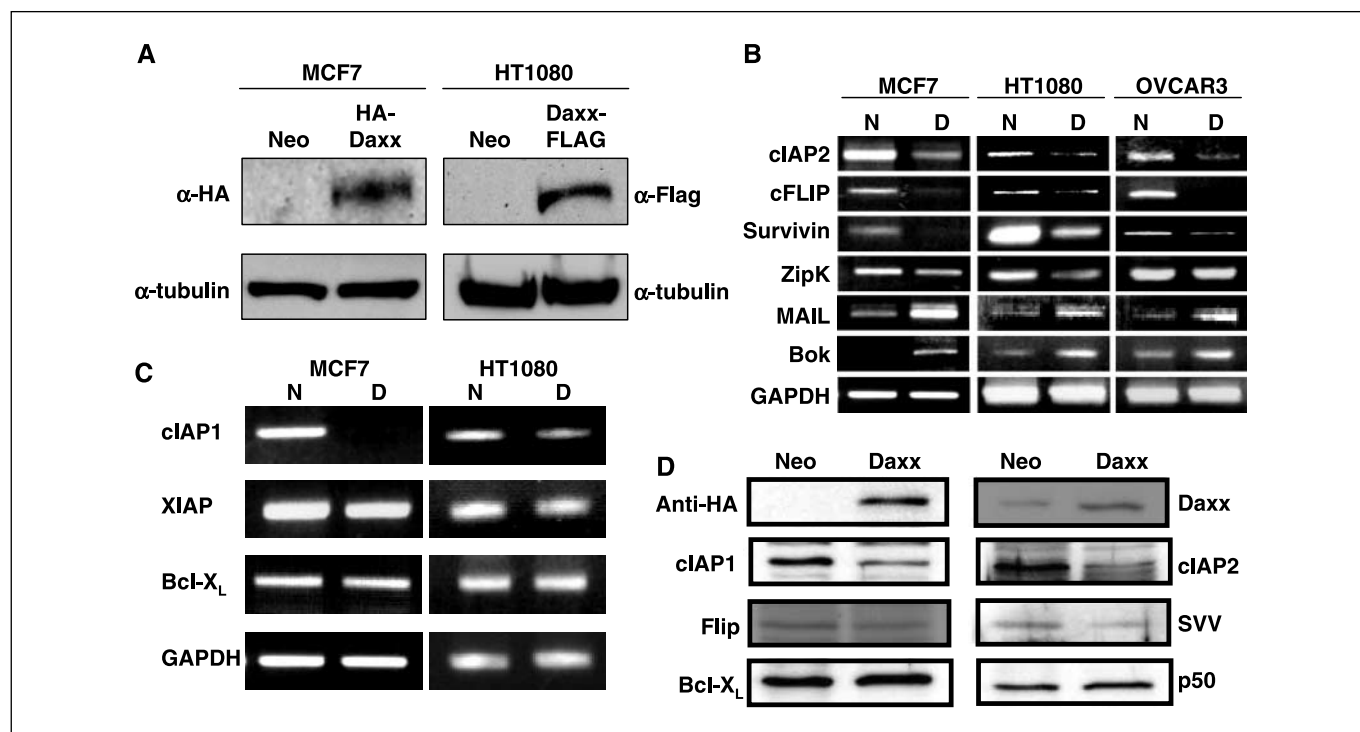


Figure 1. Analysis of Daxx effects on gene expression. *A*, stable expression of Daxx in human tumor cell lines. Total cell lysates (100 μ g) from stably transfected MCF7 and HT1080 cell lines containing pcDNA3 control plasmid (Neo) or Daxx-expressing plasmids were separated by SDS-PAGE, blotted, and probed with antibodies directed against either the HA or Flag epitopes (*top*) or α -tubulin (*bottom*). *B*, RT-PCR confirmation of DNA microarray results. Total RNA was prepared from MCF7, HT1080, and OVCAR3 (N, Neo-control; D, Daxx expressing) cells, and 100 ng aliquots were analyzed by RT-PCR (25 cycles) using gene-specific primers. *C*, effects of Daxx on other IAP family members. Total RNA was prepared from MCF7 and HT1080 cells, and analyzed by RT-PCR as above. *D*, analysis of apoptosis proteins in Daxx-expressing cells. Lysates were prepared from MCF7-Neo and MCF7-Daxx cells, normalized for protein content (50 μ g), and analyzed by SDS-PAGE/immunoblotting using antibodies specific for HA epitope, Daxx, *cIAP1*, *cIAP2*, *Survivin*, *c-FLIP*, *Bcl-X_L*, and *p50-NF- κ B*.

independent preparation of RNA, so that eight array data sets were available (four data sets per cell line). Those genes where the expression showed consistent increases or decreases on at least four of eight replica arrays were further considered.

From the DNA microarray analysis of the MCF7 cells (Neo versus HA-Daxx), a total of 17 genes were found to be regulated by Daxx, with 6 genes up-regulated and 11 genes down-regulated in response to Daxx overexpression. In HT1080 cells (Neo versus Daxx-Flag), 18 genes showed changes in gene expression, where nine genes were up-regulated and nine genes were down-regulated. When the lists of affected genes from the two cell lines were compared, six genes showed a common pattern of regulation (Table 1).

Among the down-regulated genes were caspase antagonists *cIAP2*, *c-FLIP*, and *SURVIVIN*. Expression of the gene encoding the Daxx-interacting kinase, *ZipK*, was also reduced in Daxx-expressing cells. Among the candidate Daxx target genes that were up-regulated upon Daxx overexpression were *MAIL* (also known as $\text{I}\kappa\text{B-}\zeta$), an ankyrin-repeat containing protein that resembles members of the $\text{I}\kappa\text{B}$ -family of $\text{NF-}\kappa\text{B}$ inhibitors (26, 27), and *Bok*, a proapoptotic member of the *Bcl-2* family (28).

Verification of microarray findings. To confirm the results obtained from the DNA microarray experiments, semiquantitative RT-PCR was done to compare mRNA levels of the Daxx-responsive genes in three pairs of Neo- and Daxx-expressing cell lines, including the original lines used for microarrays (MCF7, HT1080) and an additional line (OVCAR3, an ovarian cancer cell line; Fig. 1B). The genes whose expression was up-regulated upon Daxx

overexpression in DNA microarray analyses were found to be up-regulated by RT-PCR (i.e., *MAIL* and *Bok*) in three of three pairs of cell lines. Similarly, RT-PCR analysis also confirmed reduced mRNA expression of the *cIAP2*, *cFLIP*, and *SURVIVIN* genes in all three Daxx-overexpressing cell lines. Expression of *ZipK* mRNA was reduced in the two tumor lines used for the DNA microarray studies (HT1080, MCF7) but not in OVCAR3 cells.

An analysis of the genomic region located immediately upstream of the transcription start site of each candidate Daxx-regulated gene was done using TRASER.² Common DNA regulatory elements among these Daxx-responsive genes were identified using TFSEARCH version 1.3.³ Notably, each of these Daxx-responsive genes contained at least one putative $\text{NF-}\kappa\text{B}$ -binding site when analyzed by TFSEARCH using the vertebrate matrix at the high-stringency default threshold of 85.0.

Because we observed the down-regulation of two IAP family members upon Daxx expression (*cIAP2* and *survivin*), we extended the RT-PCR analysis to some other well-characterized members of this family. In addition to *cIAP2* and *survivin*, the levels of *cIAP1* mRNA were also dramatically repressed by Daxx overexpression, whereas *XIAP* mRNA levels were unaffected (Fig. 1C). Levels of *Bcl-XL* mRNA, a $\text{NF-}\kappa\text{B}$ -inducible, antiapoptotic member of the *Bcl-2* family, were unaffected by Daxx overexpression, suggesting

² <http://genome-www6.stanford.edu/cgi-bin/Traser/>.

³ <http://www.cbrc.jp/research/db/TFSEARCH.html>.

Table 1. Microarray analysis of endogenous Daxx gene targets

Gene ID	Gene name	Total representation	Average fold induction
R76514	<i>MAIL (molecule possessing ankyrin repeats induced by LPS)</i>	6/8	2.00
AA78794	<i>Bok (Bcl-2-related ovarian killer protein)</i>	4/8	2.37
AA002126	<i>cIAP2 (baculoviral IAP repeat-containing 3)</i>	5/8	0.78
AA453766	<i>c-FLIP (Casp8 and FADD-like apoptosis regulator)</i>	5/8	0.57
AA460685	<i>Survivin (baculoviral IAP repeat-containing 5)</i>	4/8	0.55
AA973730	<i>ZipK (death-associated protein kinase 3, DAPK3)</i>	4/8	0.59

NOTE: Four DNA microarray data sets were generated for each pair of cell lines (HT1080 Neo versus HT1080 Daxx-Flag; MCF7 Neo versus MCF7 HA-Daxx). "Total representation" refers to the number of data sets (of eight) in which the particular gene was found to be up-regulated or down-regulated in Daxx-expressing cells. Average fold increase or decrease compared to Neo-control cells is indicated.

that not all NF- κ B-regulated, antiapoptotic genes are repressed by Daxx. Immunoblot analysis confirmed reduced levels of cIAP1, cIAP2, and Survivin proteins in Daxx-overexpressing cells and showed a slight reduction in cFLIP protein, whereas levels of several other proteins, including Bcl-XL, p50 NF- κ B, and tubulin, were not altered, serving as specificity controls (Fig. 1D and data not shown).

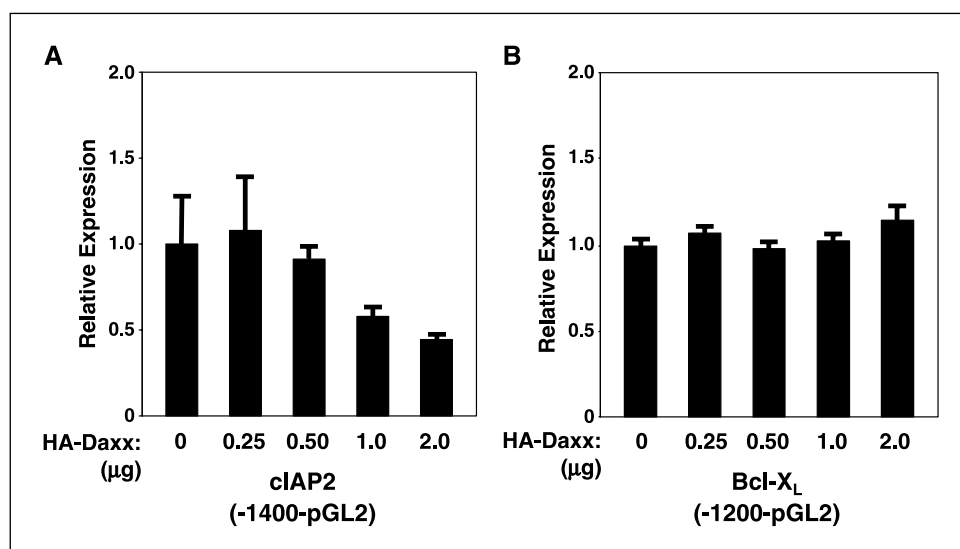
Daxx affects a subset of NF- κ B-regulated genes. Of the genes identified by cDNA microarray as Daxx-responsive, only *cIAP2* has been well characterized at the transcriptional level (21). We used reporter gene assays to test whether Daxx can affect *cIAP2* promoter activity, making a comparison with another NF- κ B-regulated gene whose expression was not reduced by Daxx, namely, *BCL-X* (21, 22). Daxx repressed the *cIAP2* promoter in a concentration-dependent manner, whereas the *BCL-X* promoter was unaffected even at the highest doses of HA-Daxx plasmid transfected (Fig. 2). Although not all members of the NF- κ B family were investigated, immunoblot analysis indicated that Daxx did not alter the levels of endogenous RelB or p50 proteins (data not shown), thus excluding reductions in prominent members of the NF- κ B family as a trivial explanation for the results.

RelB-mediated activation and Daxx-mediated repression of the *cIAP2* promoter. Although the *BCL-X* promoter is regulated by NF- κ B (22), one member of the NF- κ B family (i.e., RelB) is

reportedly unable to bind the NF- κ B-binding site within this promoter (29). This finding, coupled with the fact that Daxx did not affect *BCL-X* promoter activity or alter endogenous levels of *BCL-X* expression, led us to hypothesize that Daxx may regulate NF- κ B activity via RelB. To this end, we tested the effects of overexpressing RelB (in combination with p50) on the activity of a luciferase reporter gene plasmid containing a 1.4 kbp region of the human *cIAP2* promoter (Fig. 3A). Indeed, RelB (in combination with p50) increased activity of the *cIAP2* promoter by ~7-fold (Fig. 3B). In contrast, when Daxx was cotransfected with RelB, the activity of the *cIAP2* promoter was suppressed (Fig. 3B).

To identify the region(s) of the *cIAP2* promoter that responds to RelB and Daxx, several truncated versions of the promoter were tested via luciferase-based reporter gene assays. The putative transcription factor binding sites within these constructs were identified by TFSEARCH or have been experimentally determined (21), including two Ets-, two AP1-, and three NF- κ B-binding sites (Fig. 3A). RelB increased (and Daxx suppressed) the activity of a fragment of the *cIAP2* promoter corresponding -247 to +1 bp relative to the transcription start site, but not a fragment corresponding to -194 to +1 bp (Fig. 3B). The -247/+1 bp fragment contains all three NF- κ B-binding sites previously identified in the *cIAP2* promoter (21), whereas the -194/+1 bp fragment contains only the two most proximal NF- κ B sites

Figure 2. Daxx represses *cIAP2* gene promoter. HT1080 cells in 100-mm dishes were transfected with 1.0 μ g luciferase reporter gene plasmids -1400-*cIAP2*-pGL2 (A) or -1200-Bcl-XL-pGL2 (B), 200 ng renilla luciferase reporter (pRL-TK), and increasing concentrations of pcDNA3-HA-Daxx, as indicated, normalizing total DNA to 4.0 μ g by addition of pcDNA3, as needed. Luciferase assays were done at 48 hours posttransfection. Normalized data were compared with results from control-transfected cells (1.0). Columns, mean ($n > 3$); bars, SD.



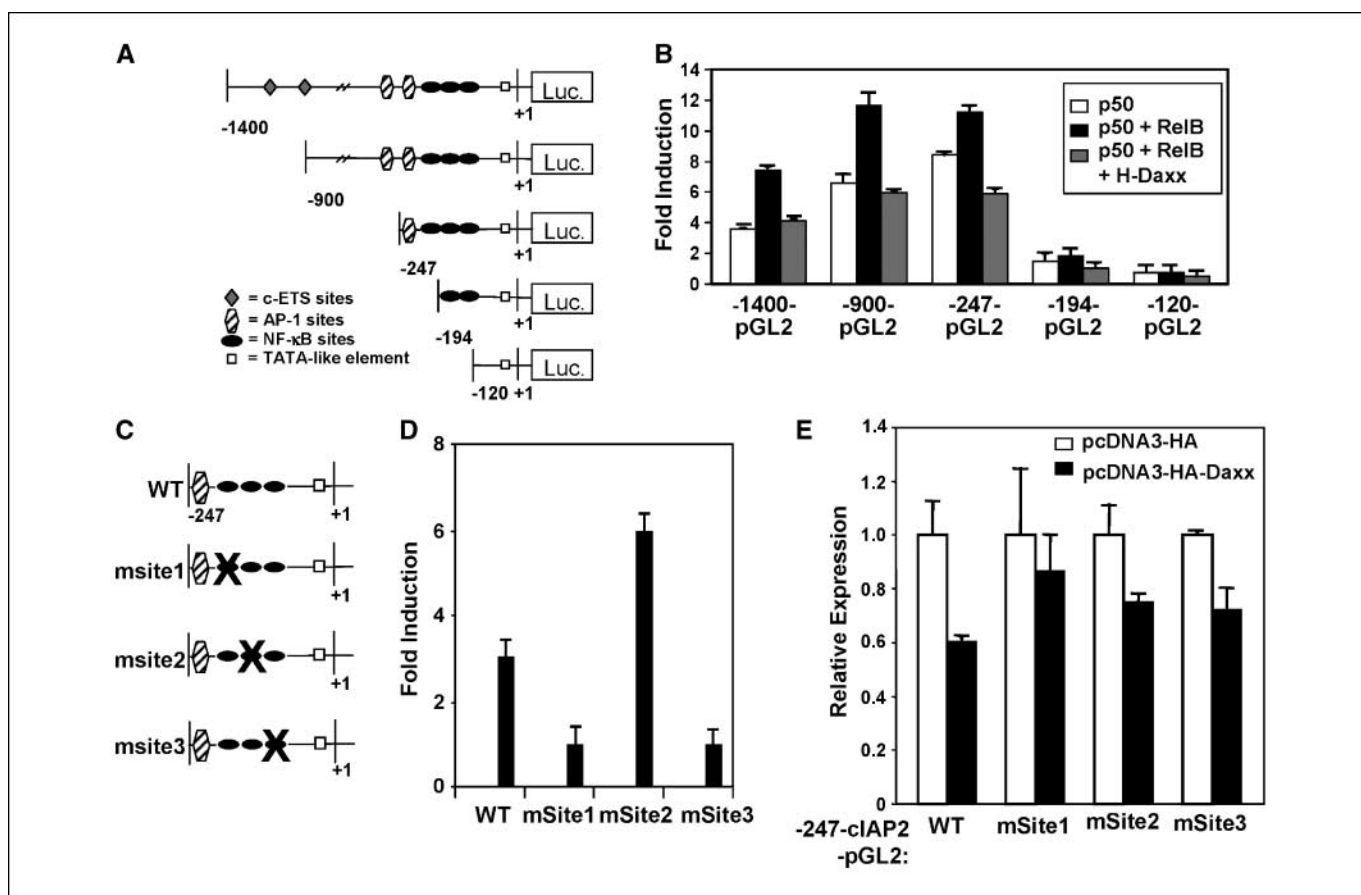


Figure 3. Functional analysis of effects of RelB and Daxx on *cIAP2* promoter. **A**, schematic diagram of the putative transcription factor binding sites within the human *cIAP2* promoter. Transcription factor binding sites are indicated at their approximate positions relative to the transcription start site. Two putative c-Ets sites, two putative activator protein-1 (AP-1) sites, and three putative NF- κ B sites are present in the longest promoter fragment analyzed (-1400 -*cIAP2*). All *cIAP2* promoter constructs displayed were cloned into pGL2-Basic for luciferase assays. **B**, mapping of RelB- and Daxx-responsive regions in human *cIAP2* promoter. HT1080 cells in 100-mm dishes were transfected with 1.0 μ g of each *cIAP2* promoter-luciferase plasmid; 200 ng of renilla luciferase reporter (pRL-TK); and 50 ng p50-CMV, 50 ng RelBpcDNA3, and 2.0 μ g of HA-Daxx-pcDNA3, in various combinations as indicated, normalizing total DNA to 4 μ g. Luciferase assays were done at 48 hours. Normalized data are expressed as fold induction compared with transfections lacking p50, RelB, or Daxx plasmids (control = 1.0). Columns, mean ($n > 3$); bars, SD. **C**, mutational analysis of NF- κ B sites in human *cIAP2* promoter. A schematic representation is provided of the wild-type (WT) and mutant *cIAP2* promoter constructs generated for reporter gene assays. Each NF- κ B site was individually disrupted by point mutation within the given NF- κ B-binding site as indicated by an "X". **D**, HT1080 cells were transfected as above with $-247/+1$ wild-type and mutant *cIAP2* promoter-luciferase plasmids, and renilla luciferase plasmid, with either pcDNA3 control or p50/RelB-encoding plasmids, normalizing for total DNA. Data are expressed as fold induction, comparing control (1.0) to p50/RelB-transfected cells. Columns, mean ($n = 3$); bars, SD. **E**, HT1080 cells in 100-mm dishes were transfected with 1.0 μ g of each mutant *cIAP2* promoter-luciferase construct, 200 ng of renilla luciferase reporter (pRL-TK), and 2.0 μ g of either pcDNA3-HA (white columns) or pcDNA3-HA-Daxx (black columns), normalizing total DNA to 4 μ g. Luciferase assays were done at 48 hours. Normalized data are expressed relative to cells transfected with WT *cIAP2* promoter without Daxx (1.0). Columns, mean ($n \geq 3$); bars, SD. *, $P \leq 0.05$.

(Fig. 3A). Thus, the 5'-most NF- κ B-binding site seems to be important for RelB-mediated transactivation of the human *cIAP2* promoter.

To further characterize the importance of each of the three NF- κ B sites within the *cIAP2* gene promoter, mutant versions of the *cIAP2* promoter (in which the NF- κ B sites were each disrupted by point mutation; ref. 21) were tested by luciferase-based reporter gene assays (Fig. 3C). Disruption of the first (NF- κ B site 1) and third (NF- κ B site 3) NF- κ B-binding sites greatly reduced RelB-mediated transactivation of the *cIAP2* gene promoter (Fig. 3D). When Daxx was cotransfected with RelB, expression of the NF- κ B sites 2 and 3 mutant *cIAP2* promoter constructs was still significantly repressed ($P < 0.05$), whereas the mutant NF- κ B site 1 was not significantly repressed by Daxx (Fig. 3E). Interestingly, mutation of site 2 actually increased RelB responsiveness in these experiments, suggesting removal of a repressive influence. The extent of Daxx-mediated repression was less for all of these

NF- κ B site mutants compared with the wild-type promoter construct, suggesting they may each make contributions to Daxx-mediated repression. We conclude, therefore, that RelB induces the *cIAP2* promoter, requiring the NF- κ B site 1 for optimal activity, and that Daxx also requires this site for optimal repression of RelB. However, at least two of the three NF- κ B elements make contributions to RelB-mediated induction and all three may make contributions to Daxx-mediated repression.

Analysis of Daxx effects on RelB DNA-binding activity. Because reporter gene assays suggested that one or more of the candidate NF- κ B-binding sites in the *cIAP2* promoter might be required for RelB-mediated transactivation and Daxx-mediated repression, we focused on these sites, through EMSAs, using the three NF- κ B-binding elements as oligonucleotide probes to further interrogate the binding of RelB and Daxx to these DNA elements (Fig. 4A). EMSAs showed that the NF- κ B site 1 of the *cIAP2* promoter interacts with proteins present in nuclear extracts from

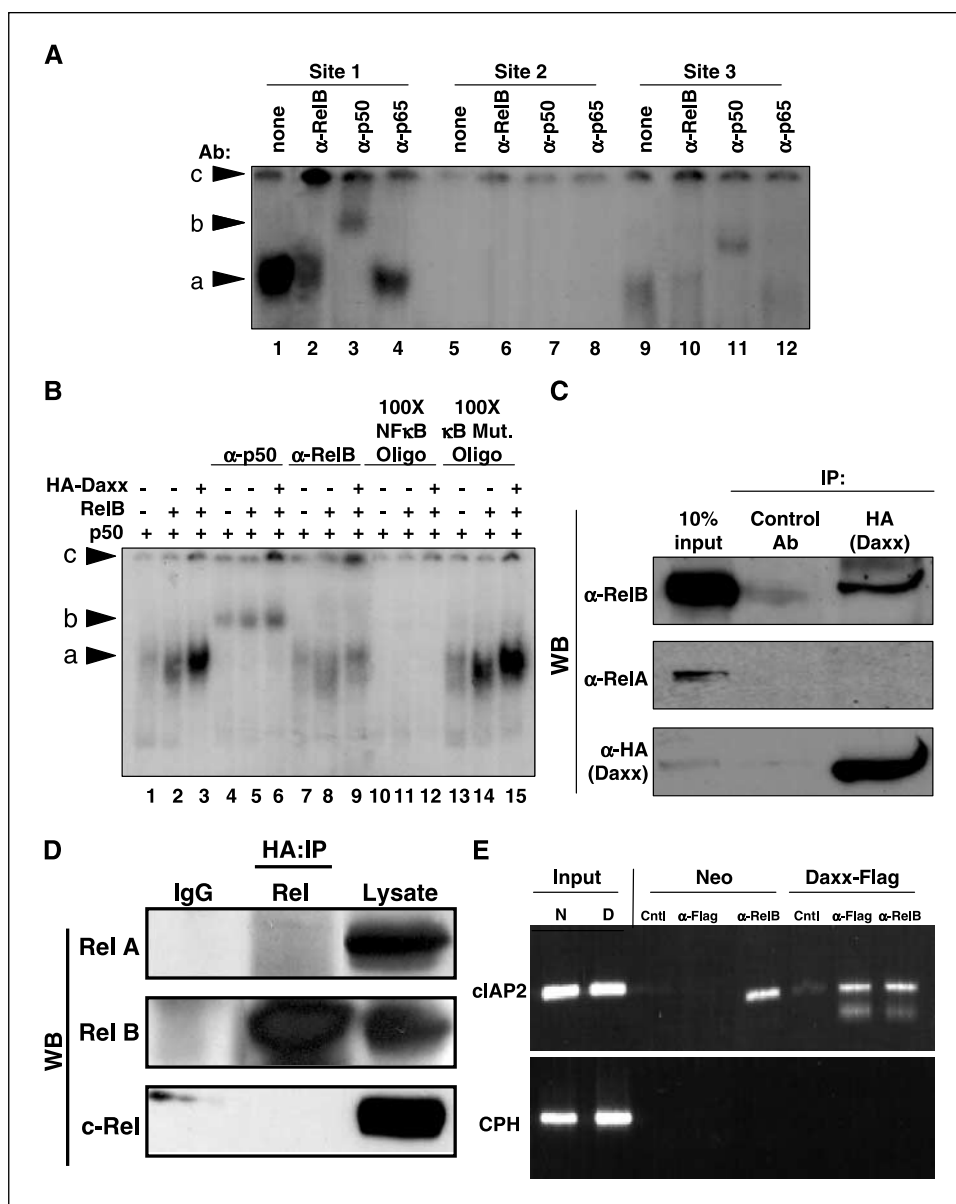


Figure 4. Analysis of Daxx effects on RelB DNA-binding activity. *A*, characterization of NF- κ B factors binding *cIAP2* promoter elements. HT1080 cells in 100-mm dishes were transfected with 10 μ g each of RelB-pcDNA3 and p50-CMV. Lysates were prepared after 48 hours and EMSAs were done using 32 P-labeled oligonucleotide probes corresponding to each of the putative NF- κ B binding sites within the *cIAP2* promoter. Antibody supershift experiments were done by adding 2 μ g of specific antibody to completed binding reactions. *Arrows*, positions of the major protein/DNA complex (*a*) and supershifted complexes formed upon addition of anti-p50 (*b*) or anti-RelB (*c*) antibodies. Note that the anti-RelB supershifted complex is retained at the top of the gel (*c*). *B*, Daxx does not inhibit RelB binding to NF- κ B site 1 of *cIAP2* promoter. EMSAs and antibody supershift experiments were done as above, except that 5 μ g of p50-CMV, RelB-pcDNA3, or HA-Daxx-pcDNA3 plasmids were used for transfection. [32 P]NF- κ B binding site 1 probe was used for all samples. Oligonucleotide competition experiments were done using 100-fold molar excess of unlabeled oligonucleotides representing either the consensus NF- κ B-binding sequence or a mutated NF- κ B control oligomer. *C*, Daxx associates with endogenous RelB. Total cell lysates (800 μ g) from MCF7 cells stably expressing HA-Daxx were immunoprecipitated with 5 μ g anti-HA or anti-FLAG (control antibody), followed by SDS-PAGE/immunoblot (WB) analysis of the immunocomplexes using antibodies specific for RelB (*top*), RelA (*middle*), or HA (*bottom*). *D*, HEK293T cells were transiently transfected with plasmids encoding HA-Daxx and plasmids encoding either RelA (*top*), RelB (*middle*), or c-Rel (*bottom*). Lysates were subjected to immunoprecipitation with anti-HA antibody or control IgG and the resulting immunocomplexes were analyzed by SDS-PAGE/immunoblotting with antibodies specific for various Rel family proteins. As a control, lysates were also analyzed directly. Reprobing blots with anti-HA confirmed effective immunoprecipitation of HA-Daxx for all samples (data not shown). *E*, Daxx associates with the *cIAP2* promoter *in vivo*. ChIP assays were done using Neo control and Daxx-Flag-expressing HT1080 cells. Cross-linked lysates were immunoprecipitated with antibodies specific for either RelB, Daxx-Flag, or a control (*Cntl*) antibody (anti-HA) followed by PCR amplification using primers spanning the NF- κ B binding sites (-247 to +7) of the human *cIAP2* promoter or primers for the cyclophilin A (*CPH*) promoter region, used as a negative control. Input of equivalent amounts of chromatin was confirmed by PCR analysis of a portion of the extract equivalent to the amount used for immunoprecipitations.

HT1080 cells, and that incubating these complexes with antibodies recognizing RelB or p50 led to markedly retarded mobility of the protein/DNA complexes in gels (supershift), thus confirming the presence of these proteins. Anti-p65 failed to produce a clear

supershift, but seemed to reduce the abundance of the site 1 DNA/protein complex (Fig. 4A), suggesting it may also participate to some extent (but less prominently than RelB). Thus, the NF- κ B site 1 displays high affinity for RelB and p50. In contrast, the NF- κ B site

2 failed to interact with proteins in HT0080 extracts, whereas the NF- κ B site 3 showed only weak formation of complexes with nuclear proteins. The site 3 probe complexes, however, were supershifted by antibodies to RelB and p50, and disappeared in the presence of antibodies to p65, suggesting that these NF- κ B family members bind this DNA sequence *in vitro*.

We therefore used the NF- κ B site 1 DNA probe for antibody supershift experiments to determine what effect Daxx overexpression has on the binding of RelB to NF- κ B site 1 *in vitro*, comparing extracts prepared from cells transfected with RelB/p50 alone versus RelB/p50 plus Daxx. As expected, extracts from HT1080 cells cotransfected with p50 and RelB formed complexes with the NF- κ B site 1 oligonucleotide probe (Fig. 4B). Interestingly, the amount of these DNA/protein complexes was increased in extracts from HT1080 cells transfected with p50/RelB plus Daxx, suggesting that Daxx does not interfere with protein binding to the NF- κ B site DNA probe. Moreover, the NF- κ B site 1 probe migrated slightly slower when using extracts from Daxx-transfected cells, suggesting either that a larger protein complex may form on this probe when Daxx is abundant or that a conformational change in the DNA-binding protein is induced by Daxx. (Fig. 4B). Antibody supershift experiments confirmed the presence of p50 and RelB in these DNA-binding complexes (Fig. 4B), indicating that Daxx does not appear to disrupt RelB association with this DNA element. Competition experiments using unlabeled wild-type and mutant oligonucleotides confirmed the specificity of these results (Fig. 4B, lanes 10-15). Experiments done with extracts containing other NF- κ B family members (p50/RelA and p50/cRel), expressed alone or in combination with HA-Daxx, showed no detectable effect of Daxx with the NF- κ B site 1 probe (data not shown), consistent with the preferential interaction of RelB with this DNA sequence derived from the human *cIAP2* gene promoter.

Daxx associates with endogenous RelB and binds the *cIAP2* promoter *in vivo*. Coimmunoprecipitation studies were undertaken to determine whether Daxx associates with RelB. For these experiments, lysates from stably transfected MCF7-HA-Daxx cells (which have 3- to 5-fold greater Daxx expression than control cells) were incubated with an anti-HA antibody to immunoprecipitate HA-Daxx-containing complexes. Poor quality of available anti-Daxx antibodies precluded evaluation of endogenous Daxx, necessitating the use of epitope-tagged Daxx. Immunoblot analysis (Fig. 4C) showed that endogenous RelB associated with Daxx, whereas no association could be detected between Daxx and the more commonly studied NF- κ B family member, RelA (p65). It should be noted, however, that endogenous RelA levels were lower than endogenous RelB in these cells. Note that c-Rel protein could not be detected in MCF7 cells (data not shown), which implies that c-Rel is not required for Daxx-mediated gene regulation given that Daxx represses the expression of antiapoptotic genes in these cells (Table 1; Fig. 1).

To further evaluate the selectivity of Daxx for RelB, we transiently overexpressed RelA, RelB, or c-Rel together with Daxx in HEK293T cells and repeated the coimmunoprecipitation studies. Again, we observed a strong interaction of RelB with Daxx (Fig. 4D). In contrast, little or no interaction of RelA and c-Rel with Daxx was detected by coimmunoprecipitation.

Next, chromatin immunoprecipitation (ChIP) experiments were done to assess whether RelB and Daxx associate with the endogenous *cIAP2* gene promoter in intact cells. For these experiments, nuclei from Neo- versus Daxx-expressing cell lines were subjected to chemical cross-linking, followed by immuno-

precipitation of RelB or Daxx and analysis of associated DNA by PCR using gene-specific primers. Using these CHIP assays, both endogenous RelB and epitope-tagged Daxx were found in association with the endogenous *cIAP2* promoter (Fig. 4E). Moreover, RelB binding to the endogenous *cIAP2* promoter was not inhibited by expression of Daxx-Flag protein, confirming that although Daxx can bind RelB, it does not interfere with binding of RelB to DNA. The immunoprecipitation of the *cIAP2* gene promoter with Daxx was shown to be specific, given that *cIAP2* promoter DNA was not recovered in immunoprecipitations from the Neo-control cells, although ample *cIAP2* promoter DNA was present in the samples, as indicated in the input lanes. PCR amplification of an unrelated DNA sequence from the ChIP samples (i.e., cyclophilin A promoter) further verified the specificity of the interaction, in that this DNA fragment was not detected by PCR analysis of either RelB or Daxx immunocomplexes. We conclude, therefore, that RelB and Daxx associate with the endogenous *cIAP2* promoter in intact cells.

Ablation of *daxx* gene in mouse cells increases expression of the murine orthologue of *cIAP2*. We used MEFs in which either the *daxx* or *relB* gene had been ablated by homologous recombination to determine the relevance of the endogenous *daxx* gene to expression of *c-iap* genes (17, 23). Note that the mouse orthologue of human *cIAP2* (Locus Link NM001165) has been termed *miap-1* (Locus Link NM_007465); thus, the nomenclature for mouse and human is reversed with respect to the *c-iap* genes. Semiquantitative RT-PCR was used to measure relative levels of mRNA encoding the murine orthologue of *cIAP2*. As shown in Fig. 5A, levels of this mRNA levels were increased in Daxx-deficient cells but reduced in RelB-deficient cells.

Using antibodies specific for the corresponding mouse cIAP protein (corresponding to human cIAP2), we did immunoblot analysis of cell lysates from the *daxx*^{+/+} and *daxx*^{-/-} MEFs, and the *relB*^{+/+} and *relB*^{-/-} MEFs. Levels of this murine cIAP protein were strikingly higher in *daxx*^{-/-} compared with *daxx*^{+/+} cells (Fig. 5A), whereas levels of mouse c-IAP protein were reduced in *relB*^{-/-} cells (Fig. 5B and C). In contrast, levels of RelB protein were similar in *daxx*^{-/-} and *daxx*^{+/+} cells and levels of Daxx protein were similar in *relB*^{-/-} and *relB*^{+/+} cells, excluding a trivial explanation for the results. These data therefore support the hypothesis that Daxx is an endogenous regulator of the murine gene corresponding to the human *cIAP2* gene.

In addition to a difference in expression of the gene encoding the murine counterpart of human cIAP2, we also observed that levels of c-FLIP protein were higher in *relB*^{+/+} compared with *relB*^{-/-} cells (Fig. 5C). However, a clear difference in c-FLIP protein levels was not detected in *daxx*^{-/-} compared with *daxx*^{+/+} MEFs (data not shown).

Ablation of *daxx* decreases sensitivity to some apoptotic stimuli. Finally, we correlated the alterations in *c-iap* expression (Fig. 5) with sensitivity of MEFs to apoptotic stimuli, using MTS assays to monitor cell viability. The *relB*^{-/-} cells were more sensitive, whereas the *daxx*^{-/-} cells were more resistant to cytotoxicity induced by TNF- α (Fig. 6A and B) and by anti-Fas antibody (data not shown). Because it is necessary to use blockers of gene expression (such as cycloheximide) to sensitize non-transformed MEFs to TNF- α -induced apoptosis, we presume that basal changes in expression of apoptosis-regulatory genes, such as murine *c-iap1* (before TNF- α stimulation), affect thresholds for apoptosis. However, for this reason, we also examined other types of apoptotic stimuli, finding that *relB*^{-/-} cells are more sensitive

to staurosporine but not VP-16, whereas the *daxx*^{-/-} cells are slightly more resistant to VP-16 but not staurosporine (Fig. 6C and D; data not shown). Taken together, these observations indicate that *relB* and *daxx* control cellular sensitivity to apoptotic stimuli, but suggest complex interactions of these genes with apoptosis pathways, which may depend on the specific cell death stimulus used and the cell type investigated.

Discussion

The results from this study integrate two functions of Daxx transcriptional repression and sensitization to apoptosis. Our data are consistent with a model in which Daxx is recruited to RelB-regulated genes and subsequently neutralizes or represses RelB-mediated transcription at these promoter sites. Daxx associates with RelB but does not prevent RelB from binding target sites in DNA. Rather, we speculate that Daxx may recruit histone deacetylases and/or DNA methyltransferases to the promoters of RelB target genes, thereby reducing their expression. Because several antiapoptotic genes are among the targets of NF- κ B, we surmise that Daxx-mediated repression of these genes explains the apoptosis-sensitive state produced by Daxx (2–4, 30). It should be noted, however, that because of the pleiotropic effects of RelB, the net effects of Daxx on cellular behaviors may be complex, and may not always correlate with apoptosis sensitivity (8, 31, 32). Moreover, our data do not exclude the possibility that Daxx has additional

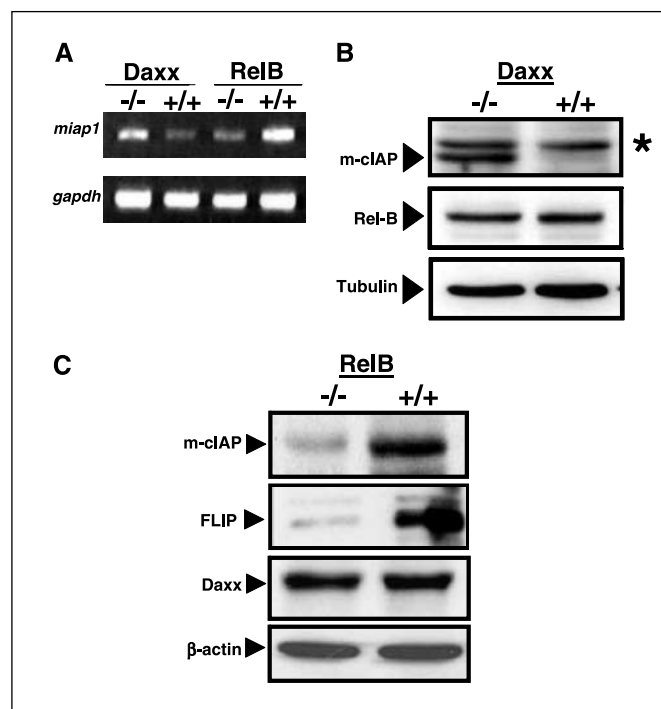


Figure 5. Ablation of *daxx* gene in mouse cells increases expression of endogenous *miap1* gene. **A**, analysis of *miap-1* mRNA levels. Total RNA was prepared from *daxx*^{-/-} or *daxx*^{+/+} MEFs or from *relB*^{-/-} and *relB*^{+/+} MEFs and semiquantitative RT-PCR was done using primers specific for the murine *miap-1* and *GAPDH* genes (25 cycles). DNA products were analyzed by gel electrophoresis with ethidium bromide staining. **B**, analysis of murine orthologue of human cIAP2 (*m-clAP*) protein in MEFs. Lysates from *daxx*^{-/-} and *daxx*^{+/+} MEFs were normalized for protein content (50 μ g) and analyzed by SDS-PAGE/immunoblotting using antibodies specific for mouse cIAP, RelB, and tubulin. *, nonspecific band. Arrow, mouse cIAP protein. **C**, lysates from *relB*^{-/-} and *relB*^{+/+} MEFs were normalized for total protein content and analyzed by immunoblotting using anti-mouse cIAP, c-FLIP, Daxx, and β -actin antibodies.

transcription factor targets besides RelB that may contribute to the apoptosis-sensitizing function of Daxx.

Although RelB may regulate the transcription of several genes relevant to apoptosis (33), we focused on the *cIAP2* gene because its promoter has been previously characterized. We found that Daxx-mediated repression of the *cIAP2* promoter required the presence of NF- κ B-binding sites to which RelB is capable of binding *in vitro*, and these DNA-binding properties correlated with the ability of RelB to induce and Daxx to suppress activity of the *cIAP2* promoter in reporter gene assays. Moreover, RelB and Daxx were associated with the endogenous *cIAP2* promoter, as determined by ChIP assays. In addition, experiments using *daxx*^{-/-} MEFs provided direct support for a role for Daxx in suppressing the expression of the endogenous *miap1* gene in mouse cells (corresponding to the human *cIAP2* gene).

The relative importance of the various apoptosis-regulating genes controlled by Daxx probably varies depending on the cell type and apoptotic stimulus applied. In addition to cIAP2, we observed that levels of mRNAs encoding the IAP family proteins cIAP1 and Survivin were also consistently reduced in human tumor cell lines stably overexpressing Daxx. Because they oppose various caspases, Daxx-mediated repression of genes encoding IAP family proteins could result in broad sensitization to apoptotic stimuli. Conversely, Daxx-mediated repression of the *cFLIP* gene, another known target of NF- κ B (34), would be expected to preferentially affect sensitivity of cells to apoptosis induced by TNF family death ligands and receptors (extrinsic pathway), because cFLIP directly binds caspase-8 and caspase-10 (35). Finally, the observation that Daxx overexpression may induce *BOK* expression suggests a mechanism for potentially sensitizing cells to mitochondria-dependent apoptosis (intrinsic pathway), because the Bok protein represents one of three multidomain proapoptotic members of the Bcl-2/Bax family involved in controlling the release of cytochrome *c* and other proteins from the mitochondria (28, 36). Of note, all of the genes that showed consistent changes in their expression based on DNA microarray studies contain at least one copy of a putative NF- κ B binding site within 1 kbp of their transcriptional start sites. Also, consistent with our data, it has been observed that suppression of Daxx expression can increase expression of an artificial promoter containing NF- κ B-responsive elements (32).

Our data suggesting that Daxx associates with RelB but not RelA or c-Rel are consistent with several experimental observations. First, RelA (but not RelB) has been shown to regulate the *BCL-X* and *XIAP* gene promoters (29, 37). Consistent with differential effects of RelA and RelB on these NF- κ B-inducible genes, we found that Daxx had no effect on expression of *BCL-X* and *XIAP*, unlike other NF- κ B-target genes, such as *cIAP1*, *cIAP2*, and *cFLIP*. Second, RelB differs from RelA and cRel in that it is the only NF- κ B family member that possesses a putative Daxx-interaction domain (12), which may explain the differential interaction of Rel family members with Daxx. Third, RelA entry into nuclei requires stimulation (38), whereas RelB is constitutively found in both the nuclear and cytoplasmic fractions and may be responsible for basal levels of transcriptional activity of NF- κ B-regulated genes (39). Consistent with these different roles for RelA and RelB, suppression of NF- κ B target genes by Daxx was evident in unstimulated cells, not requiring treatment with TNF or other inducers of NF- κ B. Thus, we propose that Daxx selectively inhibits RelB among the NF- κ B family members. Further studies will define the mechanisms by which Daxx represses RelB target genes and its relevance to apoptosis regulation.

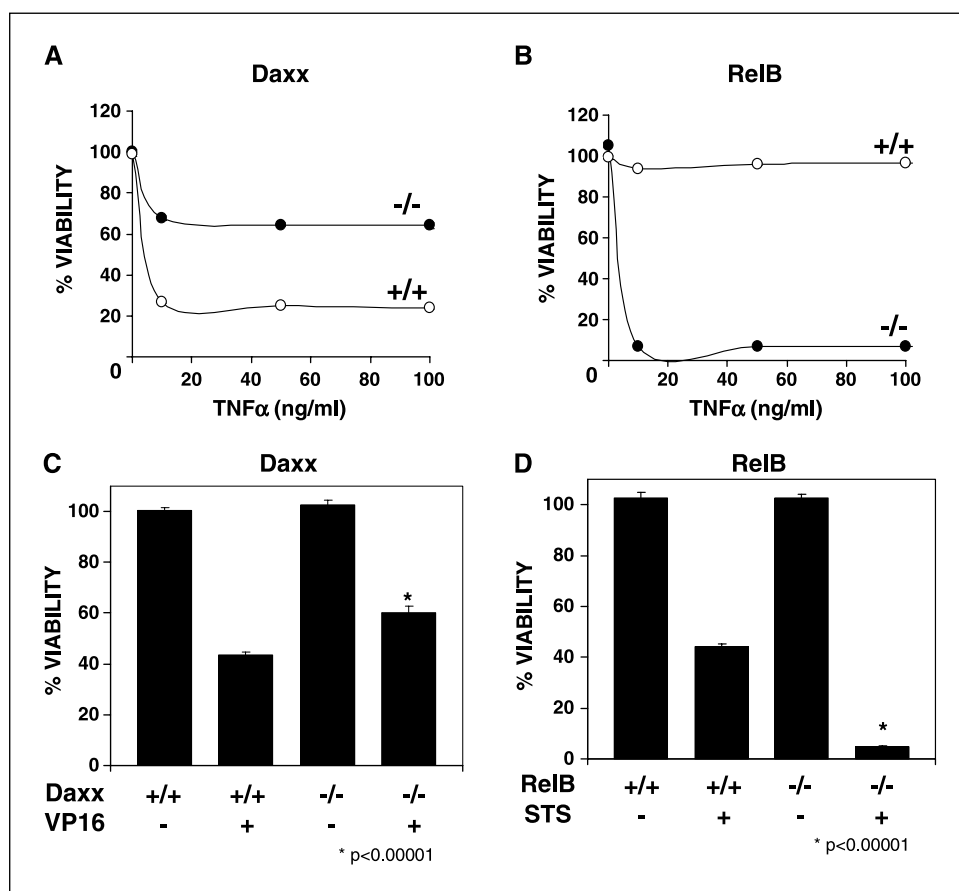


Figure 6. Ablation of *daxx* or *relB* genes alters sensitivity of MEFs to apoptosis. *A* and *B*, MEFs from *daxx*^{-/-} and *daxx*^{+/+} (*A*) or from *relB*^{-/-} and *relB*^{+/+} (*B*) embryos were cultured for 1 day with or without various concentrations of TNF- α and 10 μ M cycloheximide. Relative numbers of viable cells were measured by dye exclusion assay, expressing data as a percentage relative to cell cultures treated with cycloheximide alone. Points, mean ($n = 3$); bars, SE (<10% for each data point). *C* and *D*, *daxx*^{-/-} and *daxx*^{+/+} MEFs (*C*) or *relB*^{-/-} and *relB*^{+/+} MEFs (*D*) were cultured for 2 days with 50 μ M VP-16 (*C*) or 2 μ M staurosporine (*D*), and cell viability was determined by dye exclusion assay, expressing data as above. Columns, mean ($n = 3$); bars, SD. Statistical significance was determined by unpaired *t* test, comparing staurosporine/VP-16-treated ^{-/-} and ^{+/+} cells. *, $P < 0.0001$.

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