Abstract

The c-Myc oncoprotein is a transcription factor involved in cellular transformation as well as apoptotic cell death. We show here that overexpression of c-Myc delivered by an adenovirus vector up-regulates endogenous proapoptotic bax mRNA and protein expression in human cells. In contrast, the cytotoxic tumor necrosis factor-related apoptosis-inducing ligand induces cell death without up-regulating bax expression. c-Myc/C-Myc Max heterodimers bind to canonical E-box elements located in the bax promoter region as demonstrated by electrophoretic mobility shift analysis and DNaseI foot-printing assays. Analysis of bax regulatory region mutants suggests a model involving myc-dependent activation as well as relief of repression through distinct E-box elements. c-Myc-null cells are deficient in bax-promoter activation as compared with wild-type c-Myc expressing cells. Overexpression of c-Myc in serum-starved human or mouse embryonic cells leads to apoptosis which is significantly reduced in the presence of growth factor-containing serum. c-Myc-induced apoptosis appears to be deficient in bax-null as compared with bax-wild-type mouse embryonic fibroblasts. The results suggest that the cell death-promoting gene bax is directly downstream of c-Myc in a pathway leading to apoptosis.

Introduction

Previous studies show that the overexpression of c-Myc may lead to cellular proliferation or apoptotic cell death (1). c-Myc-induced apoptosis occurs when c-Myc is overexpressed in serum- or growth factor-deprived cells. It has been shown that the domains of c-Myc responsible for its function as a transcription factor are necessary for its ability to induce apoptosis (2). At least two c-Myc target genes have been identified that may contribute to the apoptotic-inducing function of c-Myc. ODC\(^4\), a well-characterized c-Myc transcriptional target, can induce apoptosis when overexpressed in growth factor-deprived cells (3). However, specific inhibition of ODC did not prevent c-Myc-induced apoptosis, suggesting that ODC activation by c-Myc may not be required for its apoptosis-inducing function (4). Another c-Myc transcriptional target gene, Cdc25A, which encodes a protein phosphatase, can induce apoptosis when overexpressed in serum-deprived cells and may be required for c-Myc-induced apoptosis (5). A number of genes have been identified recently by cDNA microarray screening using mRNAs collected from c-Myc-induced apoptosis in interleukin-3-deprived myeloid cells, but whether these genes are direct c-Myc targets and are relevant to the activation of apoptosis has yet to be determined (6).

Attempts have been made to characterize the cell death machinery involved in c-Myc-induced apoptosis, and it seems that the release of cytochrome c is necessary for c-Myc-induced cell death (7). Cytochrome c activates the APAF-1/caspase-9 apoptotic pathway (8). Likewise, caspase-9 and APAF-1 were shown to be necessary for c-Myc-induced apoptosis (9). Cytochrome c release from the mitochondria is regulated by the binding of Bcl-2 family members to the mitochondria (10, 11). The overexpression of antiapoptotic Bcl-2 blocks the mitochondrial release of cytochrome c (12). Bcl-2 is able to protect c-Myc-overexpressing cells from either serum or glucose deprivation-induced apoptosis (13–15). Taken together, these results establish a pathway for c-Myc-induced cell killing from the release of cytochrome c to the activation of caspase activity. However, a mechanism by which c-Myc induces the release of cytochrome c has not been established.

Bax is a proapoptotic bcl-2 family member whose apoptotic function is antagonized by bcl-2 expression (16). Bax insertions into mitochondrial membranes and forms channels for the release of cytochrome c (8, 17). These results link bax to the same apoptotic pathway as c-Myc. Indeed, when the human bax gene promoter was first cloned, it was shown to be a p53 primary response gene, but it was also noted to be potentially c-Myc-responsive based on the presence of 4 E-box sequences downstream of the transcriptional start site in the 5׳ untranslated region (18). In the present studies, we investigated whether bax is involved in the c-Myc apoptotic pathway. We show here that bax is a direct transcriptional target of c-Myc and contributes to c-Myc-induced apoptosis.

Materials and Methods

Cell Lines and Culture Conditions. SkOV3 human ovarian carcinoma cells, SKBr3 human breast cancer cells, and HEK-293 human embryonic kidney cells were obtained from American Type Culture Collection (Manassas, VA) and cultured under the recommended conditions. The human non-small cell lung cancer cell line H460 was provided by S. B. Baylin (Johns Hopkins University, Baltimore, MD). SW480 human colon carcinoma cells were obtained from the Cell Center at the University of Pennsylvania (Philadelphia, PA). c-Myc-null and -wild-type Rat1A fibroblasts (19) were obtained from G. Prendergast (The Wistar Institute, Philadelphia, PA) with permission from J. Sedivy (Brown University, Providence, RI). The bax-null and -wild-type mouse embryonic fibroblasts (20) were provided by S. Korsmeyer (Dana-Farber Cancer Institute, Boston, MA). For serum deprivation experiments, the indicated cells were incubated in serum-free media for 48 h before adenovirus infection, as described in the figure legends, and harvested for analysis at the times indicated postinfection.

Adenovirus Infections. The human c-Myc-expressing adenovirus was generated as described previously (21). Adenovirus titers and infections were carried out as described previously (22).

References

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4 The abbreviations used are: ODC, ornithine decarboxylase; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyl transferase; APAF-1, apoptotic protease activating factor-1; PARP, poly(ADP-ribose) polymerase; Ad-c-Myc, adenovirus vector expressing c-Myc; Ad-LucZ, adenovirus vector expressing β-galactosidase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PI, propidium iodide; MOI, multiplicity of infection; MEF, mouse embryonic fibroblast.
**Bax-Promoter Reporter Plasmids.** Serial E-box deletion fragments from pTM604-4 (18) were subcloned into the HindIII site of the promoterless CAT plasmid PUCSVOCAT (18) by blunt-end ligation. This procedure produced the following plasmids: pTM657-3 (HindIII, −318 bp to −687 bp); pMYH426-282 (bax, −172 bp to −687 bp); pTM780-10 (bax, −113 bp to −687 bp); pMYH426-272 (bax, −80 to −687 bp); and pTM668-1 (bax, −61 bp to −687 bp). The luciferase reporter construct pGL3-668-1 was generated by releasing the bax promoter sequence from pTM668-1 using BamHI and HindIII and inserting it into the BglII/HindIII sites of the promoterless firefly luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI). Reporter plasmids containing mutants of each E-box were generated by replacing each E-box sequence (CAGCTG) with GTGCAC. PCR amplification products of bax-promoter sequences using one of the following primers were used to generate the luciferase reporter mutants using the QuickChange Site-Directed Mutagenesis Kit (Stratagene): E-box 1, CACTTGGAGACTGTA-CA-CGGGACCAACCTC; E-box 2, GAGGGTGGGCTGGTGGCATCTC-CCGGCGCCGCCTGC; E-box 3, TTTTGCGCGGCGCGCGCCAGAC-AGCGCAGGTTCA; and E-box 4, TTCAGCGGCTCTCTACAC-CGGCGGCGGTCGC.

**Transfections.** Transfections were performed as described previously (21), with the following modifications. The Lipofectamine Plus reagent (Life Technologies, Inc., Bethesda Research Laboratory, Bethesda, MD) was used to transfect the bax promoter-CAT reporter constructs as recommended by the manufacturer. CAT assays were performed as described previously (23). Quantification of CAT activity was performed using Imagequant densitometer software (Molecular Dynamics). Lipofectin (Life Technologies, Inc.) was used for transfections of SW480 cells plated at a density of 5 × 10^6 cells/well using 1.8 μg of bax promoter-luciferase reporter plasmid and 0.2 μg of a constitutively expressing β-galactosidase plasmid in six-well plates. Extracts were prepared 36 h after transfection, and luciferase activity was assayed using the Luciferase Assay System (Promega).

**RNA Isolation and Northern Blot Analysis.** Total RNA was prepared as described (24) by CsCl gradient ultracentrifugation of guanidine isothiocyanate-lysed cells. RNA was separated on 10% formaldehyde agarose gels, transferred to Zeta-Probe GT membranes (Bio-Rad), and detected after hybridization to 32 P-labeled probes specific for bax using methods described previously (25). The full-length human bax cDNA probe (1.3 kb) was generated by digestion of the pMV10-bax plasmid DNA (21) with HindIII and XmnI.

**Western Analysis.** Protein lysate preparation and immunoblot analysis were performed as described previously (21). Antihuman PARP polyclonal antibody (VIC5) was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). The polyclonal antibody specific for cleaved caspase-9 (D315) was obtained from Cell Signalling Technology/New England Biolabs (Beverly, MA). Anti-actin (C-2) and anti-bax (P-19) antibodies were obtained from Cell Signaling Technology/New England Biolabs (Beverly, MA). Anti-actin (C-2) and anti-bax (P-19) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Immunoprecipitation of the Bax Promoter.** Immunoprecipitation of the bax promoter and human c-Myc or Max proteins was carried out as described (26). A PCR-amplified fragment of the bax-promoter containing the three E-box sequences shown in Fig. 3 was subcloned into the pSP64 vector (Promega). These plasmids were added, with the appropriate DNA polymerase, to rabbit reticulocyte lysates using the TNT-Coupled transcription and translation kit (Promega). To prepare the DNA, pTM668-1 was digested with HindIII, and the linear plasmid was end-labeled with T4 polynucleotide kinase and [γ-32P] ATP. The kinase was inactivated and the DNA ethanol-precipitated. The end-labeled DNA was digested further with SacI to obtain the portion of the bax promoter containing all four E-boxes (−318 to −58) radio-labeled on the 3′-end. Five μl of each in vitro translated c-Myc and Max reticulocyte lysate were incubated with 100,000 cpm of radio-labeled DNA, and the DNA-protein complex was immunoprecipitated with anti-c-Myc antibody. Immunoprecipitated DNA was purified as described (26), digested with DNaI, and the fragmented DNA was separated on a 6% denaturing polyacrylamide gel.

**Electrophoretic Mobility Gel Shift.** Electrophoretic mobility shift of each E-box and corresponding mutants by c-Myc and Max was carried out using methodology described previously (27). One μl of reticulocyte lysate containing in vitro translated c-Myc or Max was added to the binding reaction (2 μg of salmon sperm DNA; 7.1 mM HEPES (pH 7.5); 3.6 mM MgCl2; 100 mM KCl; 5.7% glycerol; and 0.03% NP40), incubated at room temperature for 10 min, and then the indicated bax promoter sequence was added (19 μl, final volume) with incubation for an additional 20 min. E-box-containing sequences added to the binding reactions were the oligonucleotides described for the generation of the bax luciferase reporter plasmids, annealed to their complimentary sequences. The annealed 35 bp oligomers were end-labeled using T4 polynucleotide kinase and [γ-32P] ATP and approximately 100,000 cpm were added to each binding reaction. Proteins were separated on nondenaturing polyacrylamide gels, dried, and autoradiographed.

**Flow Cytometric Analysis.** Preparation of cells for fluorescence activated cell sorting was performed as described (28, 29), with the following modifications: cell sorting was performed on a Coulter Epics Elite counter, and Annexin V assays (Chontec, Palo Alto, CA) were performed according to the manufacturer’s instructions.

**X-gal Staining.** X-gal staining was performed as described previously (21). Briefly, cells were fixed in a solution containing 2% paraformaldehyde and 0.05 M sodium phosphate (pH 7.3) for 5 min. Cells were then washed twice with PBS and incubated at 37°C in the presence of 0.1 M sodium phosphate (pH 7.3), 3 mM KFe(CN)6, 3 mM K3Fe(CN)6, 1.3 mM MgCl2, and 1 mg/ml X-gal for 12 h.

**Results**

**Up-Regulation of Bax mRNA Expression after c-Myc Overexpression.** To investigate the pathway of c-Myc-induced apoptosis in human cells, we used a c-Myc-overexpressing adenovirus (Ad-cMyc) to infect human cells (21). As an approach to isolating potential c-Myc transcriptional targets, we generated a low-density cDNA array containing genes involved in known apoptotic signaling pathways. In preliminary experiments, we screened the array using labeled cDNA probes isolated from Ad-cMyc versus Ad-LacZ-infected SKBr3 breast cancer cells and found that bax mRNA levels were increased specifically in c-Myc-overexpressing cells (data not shown). The mRNA levels of caspase-6, −7, and −10, Fas-associated death domain, and p53 were not increased by c-Myc overexpression (data not shown). To verify that the bax gene is positively regulated by c-Myc, we performed Northern blot analysis comparing Ad-cMyc and Ad-LacZ infections of various human cancer cell lines (Fig. 1a). The results revealed that levels of the 1.5 kb bax transcript are up-regulated after overexpression of c-Myc in human breast (SKBr3), lung (H460), ovarian (SkOV3), and colon (SW480) cancer cell lines.

Because it is possible that bax up-regulation may be a consequence of, rather than a contributing factor to, the death of cells overexpressing c-Myc, we investigated the kinetics of bax gene induction resulting from c-Myc overexpression. Using the lung and colon cancer cell lines after Ad-cMyc versus Ad-LacZ infection (Fig. 1b), we found that bax mRNA steady-state levels were increased as early as 6 h after Ad-cMyc infection. Furthermore, this induction was sustained for at least 30 h postinfection in H460 cells and for at least 45 h postinfection in SW480 cells. Because H460 cells express wild-type p53, whereas SW480, SKBr3, and SKOV3 cells express mutant p53, it seems that, in human cells, the c-Myc-dependent bax induction may occur in the absence of wild-type p53 (30). Thus, the early induction of bax expression in response to c-Myc is consistent with a role for bax as an effector rather than a consequence of c-Myc-induced apoptosis.

**Up-Regulation of Bax Protein Expression and Induction of Apoptosis by c-Myc Overexpression.** Because we observed a sustained increase in bax mRNA levels after c-Myc overexpression in...
H460 and SW480 cancer cell lines, we examined bax protein expression levels after Ad-cMyc infection of the same cells under serum-deprived conditions (Fig. 2a). It is well established that c-Myc overexpression in serum- or growth factor-deprived cells results in the apoptotic response (2, 7, 31). Immunoblot analysis showed a significant induction of bax protein expression after Ad-cMyc infection as compared with Ad-LacZ infection in H460 and SW480 cells. The overexpression of bax has been shown previously to accelerate apoptotic cell death after a death signal (16). To investigate the significance of the regulation of bax expression by c-Myc, we examined the association of bax up-regulation with c-Myc-induced apoptosis. Using caspase-dependent processing of PARP as a marker of apoptosis, immunoblot analysis showed increased PARP cleavage after the infection of serum-starved H460 and SW480 cells by Ad-cMyc at 64 h postinfection (Fig. 2b). PARP cleavage seemed to be significantly enhanced in both H460 and SW480 cells by c-Myc in the absence of serum, consistent with the possibility that c-Myc is mediating the induction of apoptosis. Similar correlations were made between c-Myc overexpression and the activity of the bax effector, caspase-9. As expected, in serum-deprived cells, c-Myc expression resulted in an increase in caspase-9 cleavage (Fig. 2c). Thus, not only is the increased expression of bax associated with the induction of apoptosis in human cells overexpressing c-Myc protein, but it is also associated with the cleavage of caspase-9, which has been shown to be activated after the release of cytochrome c from the mitochondrial membrane (7).

As another test to rule out the possibility that up-regulation of bax could be a consequence of c-Myc-induced apoptosis, we used TRAIL, a potent inducer of cell death (32) to show that another inducer of apoptosis does not up-regulate bax expression. Exposure of human SW480 cells to a lethal dose of TRAIL led, within 4 h, to massive apoptosis, as evidenced by PARP cleavage, without concomitant bax protein expression (Fig. 2d). Thus, the increase observed in bax expression after c-Myc overexpression seems to be attributable to the regulation of bax by c-Myc and not simply a consequence of cell death induction.

**Fig. 1.** c-Myc overexpression leads to up-regulation of bax mRNA expression. a, Northern analysis for bax mRNA levels was performed after infection of human breast, lung, ovarian, and colon cancer cells using a MOI of 25 with either Ad-LacZ or Ad-cMyc (+, as indicated). After 48 h incubation in complete media (10% serum) after infection, the cells were harvested for analysis. b, Northern analysis of bax mRNA levels was performed using total RNA derived from SW480 colon (upper panels) or H460 lung (lower panels) cancer cells after the indicated time (h) postinfection using a MOI of 25 with either Ad-LacZ or Ad-cMyc. Cells were incubated in complete media after infection for 48 h and then harvested for analysis. The mRNA was stained with ethidium bromide to verify equivalent RNA loading (10 µg/lane) in a and b.

**Fig. 2.** c-Myc overexpression leads to the induction of apoptosis in human cancer cells and the up-regulation of bax protein expression. a, lung (H460) and colon (SW480) cancer cells were cultured without serum for 48 h and then infected with either Ad-LacZ (L) or Ad-cMyc (M) at a MOI of 50. After infection the cells were incubated without serum, and at 48 h postinfection the cells were collected in SDS loading buffer and analyzed for bax protein expression. The detection of actin is used as a loading control. b, H460 and SW480 cells were cultured with or without serum for 48 h and then infected with either Ad-LacZ (L) or Ad-cMyc (M) at a MOI of 25. After infection the cells were incubated in media supplemented with either 10% or 0% serum. At 64 h postinfection, the cells were harvested and PARP cleavage was detected by immunoblotting. c, analysis of caspase-9 cleavage was done as described in b, except that the cells were infected with a MOI of 50 and analyzed 48 h postinfection. d, SW480 cells were treated with or without TRAIL (100ng/ml) for 4 h in 10% serum-containing medium and analyzed for PARP, bax, and actin expression as described above.
c-Myc-dependent Transcriptional Control of the Human bax Promoter. The bax promoter has been cloned previously and found to be p53-responsive (18). It was recognized that the bax promoter contains 4 E-boxes which represent canonical c-Myc binding sites. To determine the significance of the E-boxes in the regulation of bax transcription by c-Myc, we tested bax promoter-reporter constructs in transient transfection assays. For these assays we used CAT reporter constructs containing serial deletions of the endogenous E-box elements of bax (Fig. 3a). Following transient transfection of SW480 cells with the deletion series of CAT reporters, we found a striking increase in CAT activity with the three- and four-E-box-containing constructs (Fig. 3b). Thus, endogenous c-Myc, which is known to be overexpressed in the SW480 colon cancer cells (33), presumably transactivates the bax promoter, and the expression of bax is upregulated significantly when at least three of the four endogenous E-boxes are present in the reporter construct.

To determine whether c-Myc is directly responsible for the E-box-dependent regulation of the bax promoter, we transfected c-Myc null Rat-1A fibroblasts (19, 34) with the CAT reporters used in Fig. 3b and compared the CAT activity in these cells with levels observed in c-Myc wild-type Rat-1A fibroblasts (Fig. 3c, upper panel). The results show a 3- to 4-fold increase in CAT activity with the three- and four-E-box-containing bax-promoter constructs occurring in the Rat1A c-Myc-null cells (Fig. 3c, lower panel). These results suggest that the bax promoter is regulated by the c-Myc protein.

Physical Interaction of the E-box-containing bax Promoter Region with the c-Myc Protein. We next investigated a possible physical interaction between c-Myc protein and the E-box-containing region of the bax promoter using an immunoprecipitation assay (26) in which the human bax promoter region was recovered in association with human c-Myc or Max proteins. The results show that the E-box-containing bax promoter region was specifically immunoprecipitated with anti-c-Myc antibodies or anti-Max antibodies (Fig. 4a). To delineate whether c-Myc binds one or more of the four E-boxes, we performed a DNaseI protection assay using the bax promoter region containing all four E-boxes. c-Myc/Max dimers offered some protection to E-boxes 1, 2, and 3 but appeared to bind E-box 3 most strongly (Fig. 4b). To verify whether c-Myc preferentially binds E-box 3, we used an EMSA of the individual E-boxes. Max/Max dimers strongly bound all four wild-type but not mutant E-box sequences, whereas c-Myc/Max alone bound E-box 3 with an affinity comparable with Max/Max binding (Fig. 4c). This binding pattern is in agreement with the observation that E-box 3 confers c-Myc responsiveness to the CAT reporter constructs used in Fig. 3. This pattern is also consistent with DNA site-selection data which indicates that the nucleotides flanking either side of the E-box confer binding specificity by c-Myc/Max dimers, whereas Max/Max dimers are less discriminatory (35, 36). E-boxes 1, 2, and 4, which each have an A or T at both the 5' and 3' flanking positions, would be predicted not to bind c-Myc/Max. E-box 3 is flanked by a 5' A, which is permissive for c-Myc/Max binding. Therefore, E-boxes 1, 2, and 4 would be predicted to be transcriptionally silent with respect to c-Myc (35). To test this, we generated luciferase reporter constructs containing bax promoter mutants shown not to bind either c-Myc/Max or Max/Max dimers by DNA gel shift (Fig. 4c). We observed that the level of bax promoter activity was significantly reduced only when E-box 3 is mutated (Fig. 4d). Unexpectedly, bax promoter activity was enhanced when any of the other E-boxes were mutated, suggesting that these sites may act as repressor sites. Other proteins known to bind E-boxes and act as transcriptional repressors, such as Mnt, may also be involved in bax regulation (37). We propose a complex yet realistic scenario wherein the contribution of the E-boxes to bax activation may result from a combination of c-Myc binding E-box 3 plus c-Myc sequestration of Max, thereby reducing its availability as a repressor. We are currently...
investigating whether other factors that associate with E-boxes are involved in bax regulation.

The Contribution of bax to c-Myc-induced Apoptosis. Up-regulation of bax following various stimuli has been associated with apoptotic cell death (38). To gain insight into whether or not bax was necessary for c-Myc-induced apoptosis, we measured PI staining after infection of serum-starved bax-null and bax-wild-type mouse embryonic fibroblasts (20, 39) with Ad-cMyc (Fig. 5). Quantification of sub-G, PI-stained cells showed that c-Myc induces apoptosis in both bax+/+ and bax−/− cells as compared with overexpression of LacZ, but significantly less apoptosis was induced in bax−/− versus bax+/+ cells observed on either day 2 or day 5 after Ad-cMyc infection (Fig. 5b). The infectivity of bax-null and wild-type MEFs by adenovirus were very similar (data not shown). Three observations are noted in the results shown in Fig. 5, a and b: (a) Ad-cMyc infection resulted in a greater level of apoptosis in bax+/+ as compared with bax−/− MEFs on days 2 and 5. These results reveal that the presence of bax contributes to c-Myc-induced apoptosis, or may be required for a full apoptotic response after c-Myc overexpression; (b) there was evidence of toxicity attributable to adenovirus infection, and this was more evident on the day-5 as compared with the day-2 Ad-LacZ-infected cells; and (c) Ad-cMyc infection led to a greater extent of apoptosis over time in bax−/− cells consistent with the existence of bax-independent c-Myc effectors of apoptosis. These results suggest that bax is only partially required for c-Myc-induced apoptosis, especially at later time points.

To further examine the requirement for bax in c-Myc-induced apoptosis, we evaluated Annexin V binding to bax+/+ and bax−/− cells at a relatively early time point after Ad-cMyc infection. The results in Fig. 6a revealed two points: (a) as in the previous experiments in Fig. 5, Ad-cMyc induced apoptosis to a much greater extent in bax+/+ as compared with bax−/− cells. These results confirm that the expression of bax is required for an efficient apoptotic response after c-Myc overexpression; and (b) in the bax−/− MEFs at the 36 h time point, there was no evidence for greater death in Ad-cMyc versus Ad-LacZ-infected cells. These results suggest that early on during c-Myc-induced apoptosis, the bax-dependent mechanism may predominate (Fig. 6a), whereas later the bax-independent effects contribute significantly to c-Myc-induced death (Fig. 5a and b).

As a further measure of c-Myc specificity in the cell death observed, we compared the induction of apoptosis in the absence of serum with that seen in the presence of serum (Fig. 6b). As expected, the induction of apoptosis was inhibited in the presence of serum. These results are consistent with the interpretation that although bax is partially required for c-Myc-induced apoptosis, its up-regulation is not sufficient. Taken together, we conclude that c-Myc expression in cells deprived of survival factors will engage the cell-death machinery, and this effect is significantly enhanced by the activation of bax. We conclude that the direct transcriptional up-regulation of bax plays a significant role in the c-Myc-dependent apoptotic response, though bax-independent mechanisms are also involved.

Discussion

Although it has been shown that c-Myc induces apoptosis under certain conditions, the mechanism of cell death induction remains obscure. Because of the requirement of a functional transactivation
domain and a basic helix-loop-helix/leucine zipper domain of c-Myc to induce apoptosis, it is expected that c-Myc target genes participate in c-Myc-mediated apoptosis (2, 40–42). By using the Ad-cMyc reagent for c-Myc overexpression, we demonstrate that bax is partially required for the induction of apoptosis by deregulated expression of c-Myc in human or mouse cells. Although past studies show that bax is regulated by p53, the following considerations lead us to suggest that bax is also a physiologically relevant target of c-Myc. First, bax mRNA and protein levels are increased after overexpression of c-Myc (Figs. 1 and 2). The absence of wild-type p53 did not inhibit c-Myc-dependent up-regulation of bax gene expression. Second, the bax gene contains four Myc/Max binding sites within the promoter region (18). c-Myc is able to drive reporter activity from bax promoter-reporter constructs containing at least three of the four E-boxes (Fig. 3). Moreover, c-Myc binds directly the E-box-containing region of the bax promoter (Fig. 4). Finally, the induction of bax mRNA was associated with c-Myc-mediated apoptosis (Fig. 2).

Until now, the regulation of bax expression has been considered to be completely under the control of the p53 tumor suppressor. Miyashita and Reed (18) cloned and performed functional analysis of the bax gene promoter, which indicated that it was a direct target of p53. It was hypothesized that c-Myc might be an additional transactivator of bax, based on the presence of four CACGTG motifs located within the 5’ UTR of the bax gene. Deletion analysis of the bax promoter indicated that E-box 3 is critical for c-Myc transcriptional activation (Fig. 3). Inspection of the sequences flanking the four E-boxes revealed that E-box 3 differed from the other three E-boxes in that it alone possessed a 5’C, whereas the other E-boxes possessed either a 5’A or T. Because previous reports have shown that the presence of a 5’C flanking the E-box confers specificity of c-Myc binding (35, 36), we tested whether c-Myc preferentially binds E-box 3. Our results using DNase1 protection analysis and EMSA confirmed this hypothesis and showed that c-Myc binds most strongly to E-box 3 (Fig. 4, b and c). When we mutated E-box 3, as expected, we observed reduced transcriptional activation by c-Myc (Fig. 4d). However, c-Myc increased transactivation of the bax promoter when E-boxes 1, 2, or 4 were mutated, which suggests that these E-boxes may be regulated by other proteins that can act as transcriptional repressors. Other proteins that heterodimerize with Max and bind to E-box sequences, such as those of the Mad/Mxi1 family and Mnt/Rox1, can act as powerful antagonists of Myc-induced transactivation, attributable in part to their ability to recruit the Sin3 protein and its associated transcriptional corepressors (37, 43). In support of this hypothesis, a recent report showed that c-Myc-induced apoptosis was inhibited in fibroblasts that had been micro-injected with a Mad1-expressing plasmid (44). We are currently investigating whether other Max-binding partners may be involved in bax regulation.

In the present studies, we used an adenovirus vector overexpressing c-Myc protein to investigate the mechanism of c-Myc-induced apoptosis. This strategy has some unique features, strengths as well as limitations. Strengths of this approach include the ability to overexpress the c-Myc protein in multiple human cell lines where its effects can be studied using overexpression of the LacZ gene also delivered by an adenovirus as a control. Unlike other systems such as the myc-ER system (45), where the c-Myc protein is preformed, one unique feature of the system is that immediate early gene expression is delayed probably for at least two hours because of the need to first express the c-Myc protein from the adenovirus vector. Our results showing that bax mRNA expression is detectably up-regulated as early as 6 h after Ad-cMyc infection argues strongly that it is a very early event which precedes apoptotic death in the cells examined. Taken together with the physical association between c-Myc and bax promoter elements, our interpretation is that there is a direct link between c-Myc protein and the regulation of bax gene expression. The adenovirus approach has been used previously to investigate the effects of c-Myc on cell cycle regulation (21). Repression of expression of the cell cycle inhibitor p21 and transcriptional up-regulation of the cyclin-dependent kinase CDK4 by c-Myc have been discovered using adenovirus-based systems (21, 46). Of note, CDK4 up-regulation was not observed with the more traditional myc-ER system, suggesting most likely variable levels of c-Myc protein or perhaps cell type-specific differences in the extent to which particular targets may be controlled (46). Because adenovirus vectors are widely used to investigate protein function including transcription factors, we believe
the system provides a valid approach to investigating c-Myc targets and should be of use in future studies in conjunction with other approaches. In the present studies, we provide other supportive evidence for the regulation of bax expression by c-Myc not relying on overexpression using the adenovirus vector. We observed a striking difference in bax-promoter CAT-reporter gene expression between c-Myc+/+ and c-Myc−/− Rat1A fibroblasts (Fig. 3). Thus, the presence or absence of endogenous c-Myc expression appears to correlate with transcriptional control of the bax gene through at least one E-box element, which c-Myc can bind directly.

We observed that Ad-cMyc overexpression in serum-deprived cells results in apoptosis, and the presence of bax significantly enhances the apoptotic response (Figs. 5 and 6). The data presented using PI staining show that c-Myc overexpression causes more cell death than LacZ in both bax-null and bax-wild-type cells, and that c-Myc causes greater cell death in the presence of bax. The apparent toxicity of adenooviral infection after long infection times led us to assess c-Myc-induced apoptosis at an earlier time point after infection. Using Annexin V staining, it is clear that bax contributes to c-Myc-induced apoptosis (Fig. 6a), but that at longer infection times using PI staining, bax-independent mechanisms contribute to c-Myc induced apoptosis (Fig. 5, a and b). Therefore, it is very likely that other c-Myc target genes or other unknown mechanisms contribute to the apoptotic response.

It was well established that c-Myc-induced apoptosis can be rescued by growth factors or Bcl-2 overexpression. The present studies reveal a direct link between c-Myc and bax gene expression, which correlates with greater apoptosis in serum-free conditions. The rescue of c-Myc-induced apoptosis by serum is consistent with a model wherein c-Myc activates a signal transduction pathway that results in a rapid translocation of Bcl-2 from the cytosol to the mitochondria, thereby inhibiting the release of cytochrome c (10). Our data extend these studies by showing that c-Myc induces a rapid and sustained decrease in the expression of the proapoptotic protein bax.

Future studies will explore these models, which are not mutually exclusive.

In conclusion, our experiments reveal a direct link between c-Myc and bax leading to apoptosis. Specifically, we observed that overexpression of c-Myc increased bax mRNA and protein expression. We show that c-Myc strongly binds one of the E-boxes present in the bax promoter region, contributing to bax expression. We also show that bax is required for a complete death response after c-Myc overexpression, and that survival factors present in serum may attenuate this response. Our results are in agreement with recent reports which suggest that downstream effectors of bax, APAF-1 and caspase-9, are required for c-Myc-induced apoptosis (9) and show the involvement of mitochondrial cytochrome c release in c-Myc-induced apoptosis (7). Furthermore, it is well known that the bax-interacting prosurvival protein Bcl-2 can inhibit c-Myc-induced apoptosis (12). Taken together, these results provide a pathway for c-Myc-induced apoptosis, which occurs through the direct control of bax gene expression.

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References


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